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Fatty Acid Composition of Depot Fat, in Relation to High Fish Consumption in Japan

KUNITARO IMAICHI, KAORU OYAMA, JUN-ICHI FUKUDA, MUTSUKO YAMAZAKI AND TOMIKO HIROSE

Fujikoshi Hospital, Toyama
Toyama Prefectural Central Hospital, Toyama

Plasma lipid lowering effect of highly unsaturated fatty acids which are contained particularly in large amounts in fish oil has been well documented. Japan is one of the few countries where fish consumption is exceptionally high, and it was speculated that high fish consumption was one of the major factors in the low incidence of atherosclerotic disease in Japan. This paper describes the general pattern of depot fatty acids of inhabitants of Toyama Prefecture after examination of 210 cases with particular attention paid to the content of highly unsaturated fatty acids.

A LARGE amount of information was obtained on the relation between dietary fat and depot fat from an extensive study performed by HIRSCH and coworkers[1]. It was well documented that the depot fatty acid composition resembled that of dietary fat after an ingestion of sufficient amounts of oil over a period of years. In our study of long term ingestion of safflower oil, the linoleic acid content in depot fat reached 56 per cent in one case[2]. Therefore the pattern of depot fatty acid is regarded as a reflection of the dietary fat ingested during preceding years. The effect of dietary fat on the plasma lipid levels has been extensively studied in relation to atherosclerotic disease. The plasma lipid lowering effect of highly unsaturated fatty acids which are contained particularly in large amounts in fish oil has been described by several investigators[1,3-5], and the effect seems to be equal to linoleate or more than linoleate.

Japan is one of the few countries where fish consumption is exceptionally high, and it was speculated that high fish consumption was one of the major factors in the low incidence of atherosclerotic disease in Japan. Our statistical study indicated that the plasma lipid levels in subjects who were consuming large amounts of fish were considerably lower[6].

This paper describes the general pattern of depot fatty acids of inhabitants of Toyama Prefecture after examination of 210 cases with particular attention paid to the content of highly unsaturated fatty acids, and also of subjects who have been consuming exceptionally large amounts of fish over many years.

MATERIALS AND METHODS

Depot fat samples were obtained from 210 randomly selected cases, whose distribution covered almost all of Toyama Prefecture. Specimens were taken from abdominal or thoracic wall during surgery. Ten cases out of the 210 were fishermen, members of their family or inhabitants of fishing ports who had a diet similar to that of fisherman during most of their lives. Their fish oil consumption was exceptionally high, possibly several times higher than an average person, based on our investigation in the previous study[6].

Experimental procedures were as follows:

1) Tissues other than adipose tissue were carefully avoided.

2) Fats of adipose tissue were extracted with methanol-acetone (1 : 1), refluxing on a boiling water bath for thirty minutes.

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3) Since fats of adipose tissue consisted almost entirely of triglyceride[1], extracted fats were directly converted to methyl esters by alcoholysis, refluxing in 2% H_2SO_4-methanol on a boiling water bath for three hours. The result of transesterification was confirmed by thin layer chromatography[7].

4) Gas-liquid chromatography was performed with Shimadzu GC-1B and hydrogen flame ionization detector HFD-1. Liquid phase was diethyleneglycol succinate polyester and the carrier gas was nitrogen. The column was maintained at 180°C. Particular effort was made to obtain sufficient stability of the instrument at high sensitivity since our main purpose was to examine highly unsaturated acids which were rather minor components among the depot fatty acids. Roughly 400 micrograms of methyl ester sample was needed for injection to obtain calculable peaks of highly unsaturated acids at sensitivity 100, range 0.1V in the instrument, which was the maximum used. Since we used relatively young phase of column, the retention times of C18-3 and C20-1 and of C20-4 and C22-1 were almost the same. Thirteen specimens were reexamined using a more aged column for the separation of the above four acids[4].

5) Calculation of fatty acid composition from the GLC chart was performed applying the hypothesis of TANDY and coworkers [9] with an electronic computer.

6) Hydrogenation of methyl esters was performed with an instrument designed by LINDGREN with palladium black as a catalyst[10].

RESULTS

Table I shows the results of statistical analysis on the depot fatty acid compositions for a total of 210 cases; 70 males and 140 females. As subjects were selected randomly from various parts of the prefecture, and the sample number was large enough, the statistical values are thought to represent the general pattern of inhabitants of Toyama Prefecture.

Oleic acid which was the largest component, having a coefficient of variation of 0.06, was a quite constant component. As the standard deviation was 2.3%, 68% of the subjects had oleic acid con-

TABLE I DEPOT FATTY ACID COMPOSITION OF A TOTAL 210 CASES; 70 MALES AND 140 FEMALES

		12	14	14=1	16	16=1	18	18=1	18=2	20	18=3, 20=1	18=4	20=2	20=3	20=4, 22=1	20=5	22=5	22=6
Total n=210	Mean	.3%	3.0%	.8%	21.5%	10.3%	3.0%	37.2%	14.8%	.4%	2.8% 1.2%* 1.6%*	.4%	.5%	.1%	1.1% .4%* .7%*	.3%	.5%	1.1%
	Standard deviation	.2	.5	.2	2.4	2.5	1.1	2.3	3.1	.1	.7	.2	.1	.1	.5	.1	.2	.5
	Coefficient of variation	.52	.18	.29	.11	.25	.37	.06	.21	.32	.24	.46	.26	.31	.42	.37	.41	.49
Male n=70	Mean	.3	3.1	.7	21.3	8.8	3.7	37.6	14.7	.5	2.9	.5	.5	.3	1.3	.3	.5	1.3
	Standard deviation	.2	.4	.2	2.3	2.2	1.0	2.7	3.1	.2	.7	.2	.1	.1	.6	.1	.2	.6
	Coefficient of variation	.54	.14	.33	.11	.25	.26	.07	.21	.35	.26	.49	.28	.35	.44	.35	.41	.49
Female n=140	Mean	.3	2.9	.8	21.7	11.0	2.6	37.0	14.9	.4	2.7	.4	.5	.3	1.1	.3	.5	1.0
	Standard deviation	.2	.6	.2	2.5	2.4	1.0	2.1	3.1	.1	.6	.2	.1	.1	.4	.1	.2	.5
	Coefficient of variation	.51	.19	.27	.11	.22	.37	.06	.21	.27	.23	.41	.23	.29	.39	.38	.42	.46

Specimens were obtained from abdominal wall or thoracic wall.
12 = lauric, 14 = myristic, 14=1 = myristoleic, 16 = palmitic, 16=1 = palmitoleic, 18 = stearic, 18=1 = oleic, 18=2 = linoleic, 20 = arachidic, 18=3 = linolenic, 20=1 = eicosenoic, 18=4 = octadecatetraenoic, 20=2 = eicosadienoic, 20=3 = eicosatrienoic, 20=4 = arachidonic, 22=1 = docosenoic, 20=5 = eicosapentaenoic, 22=5 = docosapentaenoic, and 22=6 = docosahexaenoic. Other minor fatty acids omitted.
* Divided values of 18=3 and 20=1 and of 20=4 and 22=1 are those estimated from the values of reexamined 13 cases as mentioned previously.

tent between 34.9% and 39.5%, and 95% of the subjects had oleate content between 32.6% and 41.8% statistically. The second component was palmitic acid and had a mean of 21.6%. The coefficient of variation being 0.11, palmitate was also a constant component. The linoleic acid content was 14.8% on the average and this value was significantly higher than values seen in previous reports in the United States[1][2][3][4]. The content of palmitoleic acid which was fourth component was quite labile, having a standard deviation of 2.5% to a mean of 10.3%. Similarly the stearic and myristoleic acid contents were labile, even though the amount of these acids was relatively small. In the contents of the above three acids, there existed close correlation as shown in Table II. Correlation coefficient being 0.78, palmitoleic and myristoleic acids increased or decreased closely in parallel and stearate showed clearly inverse correlation with other two acids.

Table III shows the statistical values in ten cases in which large amounts of fish were consumed. Means of C20=5, C22=5 and C22=6 were 0.5%, 0.8% and 2.2% respectively, and these values were roughly twice of the values in other 200 cases. Fig. 1 is an example of the GLC chart of a fisherman who revealed very large peaks of C20=5, C22=5 and C22=6 and the values of these acids were 0.8%, 0.8% and 3.8% respectively. Large peaks of C20 and C22 observed after hydrogenation were impressive.

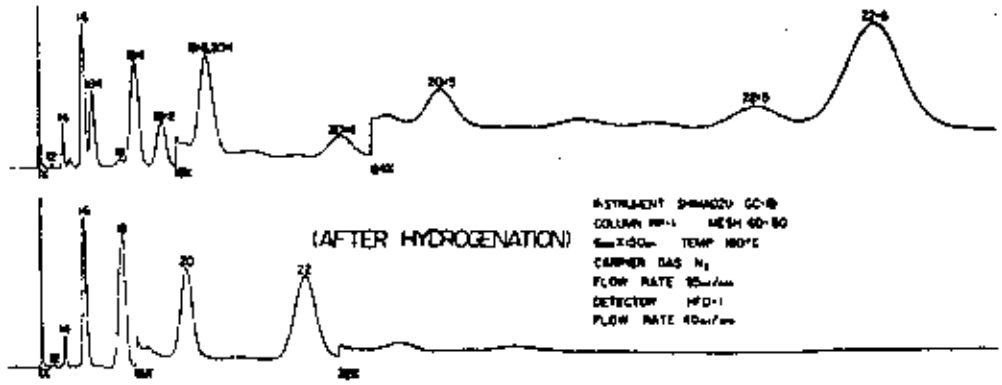
Table IV shows the fatty acid pattern divided by age groups. There was no significant difference in the content of major component fatty acids according to age. There seemed, however, to exist a tendency of an increase in the content of highly unsaturated acids as age increased.

Table V shows the fatty acid composition examined after hydrogenation of methyl esters in 25 randomly selected cases. C18 and C16 were the most prominent components and the sum of them reached 88.1%. Also C14, C20 and C22 were fairly constant components. C15, C17, C19 and C21 were observed in all cases and the sum of fatty acids having odd carbon number was 1.6% on the average. Most of these odd number acids were not clearly detected on the original GLC chart without hydrogenation.

Table II Correlations Between Myristoleate, Palmitoleate and Stearate Contents in Depot Fat.

	Regression equation	Correlation coefficient	t test
Myristoleate (X) & Palmitoleate (Y)	$Y(\%)=3.564\%+8.637X$ $X(\%)=0.057\%+0.070Y$	0.775^{**}	$t_{XY}=17.70$ $t(0.01,\infty)=2.58$
Palmitoleate (Y) & Stearate (Z)	$Z(\%)=6.476\%-0.339Y$ $Y(\%)=15.804\%-1.849Z$	-0.796^{**}	$t_{YZ}=-18.95$ $t(0.01,\infty)=2.58$
Myristoleate (X) & Stearate (Z)	$X(\%)=1.138\%-0.120Z$ $Z(\%)=5.102\%-2.726X$	-0.585^{**}	$t_{XZ}=-10.39$ $t(0.01,\infty)=2.58$

Fig. 1 Gas-Liquid Chromatogram of Depot Fatty Acids



The case is a 65 y/o fisherman.

Discussion

There have been numerous studies on the fatty acid composition of depot fat, mostly in the United States, and the content of highly unsaturated acids in depot fat has been described as being only a trace or as being negligible[1,2,3,11,12,13]. Almost all the cases examined in this study revealed calculable peaks of C20=5, C22=5 and C22=6 on GLC chart, and the mean values were 0.3%, 0.5% and 1.1% respectively. Naturally this difference in the content of highly unsaturated fatty acids in depot fat between Japan and the United States is due to the considerably higher consumption of fish in Japan, since these acids are contained in fish oil in particularly large amounts. The content of highly unsaturated acids in depot fat of subjects who were persistently consuming large amounts of fish over many years was roughly twice that of average subjects. However, the amount of ingested fish oil in these subjects was considered to be easily several times more than the average person, based on our investigation in the previous study [4]. Therefore we speculate that the content of highly unsaturated fatty acids in depot fat does not increase simply proportionally to the amount ingested, as is the case with plasma lipid fatty acids during high fish oil ingestion[5].

The linoleate content, its mean being 14.8%, was considerably higher than that which appeared in previous studies performed in the United States[2,3,11,12]. Shigeta and Hosii[14], Takahashi and Tanaka[15], Goto and Nakamura[16], Aizawa and Nakamura[17] published similar results, so that the difference seems to be definite. Shigeta and Hosii[14] speculated that the difference was due to greater linoleate ingestion in Japan in proportion to the amount of total fat ingested, even though the absolute amount of linoleate consumption was clearly lower in Japan. We feel this is a very logical reason for the difference.

Table III Depot Fatty Acid Composition of Subjects Having a High Fish Diet

		12	14	14−1	16	16−1	18	18−1	18−2	20	18−3, 20−1	18−4	20−2	20−3	20−4, 22−1	20−5	22−5	22−6
High fish group n=10	Mean	.3%	3.1%	.8%	21.3%	10.3%	3.0%	35.1%	15.1%	.4%	3.1%	.5%	.4%	.3%	1.4%	.5%	.8%	2.2%
	Standard deviation	.2	.4	.3	2.8	3.1	1.4	3.8	2.5	.1	.6	.3	.1	.1	.6	.1	.2	.8
Others n=200	Mean	.3	3.0	.8	21.6	10.3	3.0	37.3	14.8	.4	2.7	.4	.5	.3	1.1	.3	.5	1.1
	Standard deviation	.2	.5	.2	2.4	2.5	1.1	2.2	3.1	.1	.7	.2	.1	.1	.5	.1	.2	.5

Other minor components are omitted.

Table IV Depot Fatty Acid Compositions Divided by Age Groups

Age	12	14	14−1	16	16−1	18	18−1	18−2	20	18−3, 20−1	18−4	20−2	20−3	20−4, 22−1	20−5	22−5	22−6	n
0–20	.4%	2.9%	.7%	21.3%	9.7%	3.4%	37.9%	14.8%	.4%	2.8%	.5%	.5%	.3%	1.3%	.3%	.4%	1.0%	22
21–30	.4	3.1	.8	21.7	9.9	3.1	36.7	15.0	.4	2.7	.4	.5	.3	1.0	.3	.4	1.1	63
31–40	.3	3.0	.8	21.9	10.7	2.8	36.7	14.9	.4	2.6	.4	.5	.3	1.0	.3	.5	1.0	47
41–50	.3	2.9	.8	21.4	10.6	2.9	37.5	14.7	.4	2.8	.4	.5	.3	1.1	.3	.5	1.1	40
51–60	.2	2.8	.8	21.2	10.9	2.7	38.0	13.7	.5	2.8	.5	.5	.4	1.3	.4	.6	1.2	24
61–	.3	2.9	.8	20.9	9.8	3.2	37.1	13.6	.6	3.0	.6	.5	.4	1.5	.4	.7	1.8	12

Expressed by means. Other minor components are omitted.

TABLE V DEPOT FATTY ACID COMPOSITION EXAMINED AFTER HYDROGENATIN

	12	14	15	16	17	18	19	20	21	22	23	24
Mean	.3%	3.3%	.3%	31.4%	.2%	56.7%	.1%	3.6%	.3%	2.8%	.1%	.2%
Standard deviation	.2%	.6%	.2%	2.8%	.3%	2.2%	.1%	1.1%	.3%	1.0%	.1%	.2%

25 randomly selected cases examined.

There was no significant difference in the content of major fatty acids in various age groups as shown in Table IV. However, the content of highly unsaturated acids tended to increase as age increased. We think this due to greater fish oil ingestion during preceding years in aged people than was the case with younger generations, especially in proportion to total fat ingested, since the consumption of animal fat and vegetable oil has been steadily increasing in Japan.

C14, C14 1, C16, C16 1, C18, C18 1 and C18 2 are the fatty acids which are discussed as common ones in depot fat in most occasions. Among of them, C18 1, C16 and C18 2 contents were fairly constant. C16 1, C14 1 and C18 contents were labile and there were considerable individual differences. However, there existed notable regularities in the relation of the content of these three acids. As shown in Table II, the palmitoleate and myristoleate contents were closely and directly correlated and the correlation coefficient reached 0.775. Contrarily the stearate content correlated inversely with that of palmitoleate and myristoleate. All of these correlations were highly significant statistically. On the other hand, greater contents of palmitoleate and myristoleate and smaller content of stearate are the most prominent characteristics of the peripheral adipose tissue as compared with the adipose tissue of the central part of the body, as documented in our previous study[8]. These facts indicate that there is an individual difference in depot fatty acid composition, similar to the difference between the body center and periphery, though not to such a great extent. There existed differences in the above three fatty acid contents in depot fat between male and female and the differences were highly significant statistically (p 0.01). Therefore we may say that the female has a somewhat peripheral type of fatty acid pattern of depot fat in comparison with the male.

SUMMARY

1) Fatty acid compositions of depot fat from 210 randomly selected cases living in Toyama Prefecture were examined. The results were considered representative of the pattern of all inhabitants of the prefecture. Also we believe the results must be essentially the same as the pattern of all Japanese people.

2) Highly unsaturated fatty acids which were contained in particularly large amounts in fish oil were observed in depot fat of almost all cases studied in calculable amounts. The means of C20 5, C22 5 and C22 6 content were 0.3% 0.5% and 1.1% respectively.

3) The content of highly unsaturated acids in depot fat in subjects who had been ingesting exceptionally large amounts of fish was roughly twice that of average subjects and it seemed that the contents of highly unsaturated fatty acids in depot fat were not simply proportional to the amount in dietary fat.

4) There was no significant difference in the content of major fatty acids in depot fat according to age groups.

5) Individual differences in the content of palmitoleate, myristoleate and stearate were relatively great compared to that of other major components, and there existed close correlations either directly or inversely between these three acid contents.

6) There was a highly significant differences in the contents of palmitoleate, myristoleate and stearate between male and female.

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Chemical Reactions Involved in the Catalytic Hydrogenation of Oils. III. Further Identification of Volatile By-Products[1,2,3]

TSUKASA KAWADA,[4] B. D. MOOKHERJEE[5] and STEPHEN S. CHANG,
Department of Food Science, Rutgers, The State University, New Brunswick, New Jersey

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Abstract

Two distinct types of hydrogenation flavor may originate from catalytic hydrogenation of fats and oils. One is the characteristic flavor developed during hydrogenation. After it is removed by deodorization, the second type of hydrogenation flavor may develop during the storage of the bland deodorized hydrogenated fat.

The precursors of the characteristic flavor developed during catalytic hydrogenation were demonstrated as the hydroperoxides of the unsaturated fatty esters. A total of 37 compounds was chemically identified by the combination of infrared and mass spectrometry as the major components of the volatile by-products developed during catalytic hydrogenation of soybean oil with nickel as catalyst. Out of the 37 compounds, 14 were hydrocarbons, eight were alcohols, one was an ester, four were aldehydes, eight were ketones, and two were lactones. The seven largest peaks of the gas chromatogram of the volatile by-products were all alcohols and hydrocarbons. It was concluded that the characteristic flavor developed during catalytic hydrogenation appears to be due to the higher members of the aldehydes, ketones, and alcohols and the lactones.

Introduction

THE UNDESIRABLE FLAVORS originating from catalytic hydrogenation may be divided into two distinct types. One is the characteristic flavor developed during the catalytic hydrogenation of an oil. After the removal of this flavor by deodorization, the second type of hydrogenation flavor may develop during the storage of the bland deodorized hydrogenated fat. The first type of the hydrogenation flavor was isolated by Chang et al. (1) by vacuum steam distillation of a slightly autoxidized soybean oil hydrogenated to an iodine value of 71. Silveira, Masuda, and Chang (2) identified the ten major components of this isolated flavor as four hydrocarbons, three alcohols, two aldehydes, and one ketone. The second type of hydrogenation flavor was isolated by Keppler et al. (3) by autoxidizing a deodorized hydrogenated linseed oil and then isolating the volatile flavor compounds produced by stripping the oil under vacuum with a current of nitrogen. They concluded that 6-*cis*-nonenal and 6-*trans*-nonenal, and particularly the latter, are primarily responsible for the hydrogenation flavor developed in bland deodorized hydrogenated oils through autoxidation.

The present paper is an attempt both to determine the precursors of the volatile flavor compounds developed during catalytic hydrogenation and to systematically characterize these compounds.

Experimental

Oil Used for Hydrogenation

The oil used for this experiment was a commercially refined and bleached soybean oil with an iodine value of 131. It had a peroxide number of 10.5 meq/kg, which was developed during processing and during the storage in an oil tank in the plant. This type of oil therefore is sometimes used in industry for catalytic hydrogenation. It should be mentioned that most of the oils used for commerical hydrogenation in this country have peroxide value below 5, usually between 0.5–2.0 meq/kg.

Catalytic Hydrogenation of Soybean Oil

The procedures used were the same as reported previously (1).

Isolation of Volatile By-Products Developed During Hydrogenation

The semicontinuous counter current vacuum steam distillation method of Chang (1) was used. The hydrogenated oil was deodorized at 120C under a vacuum of 0.05 mm Hg with 3% of water. The isolated volatile by-products were separated into acidic and nonacidic compounds by dissolving them in ethyl ether and then extracting with 10% aqueous sodium carbonate solution.

Gas Chromatography

The ethyl ether solution of the volatile nonacidic by-products was fractionated with a Wilkens Model A-90-P gas chromatograph. The temperature was nonlinearly programmed from 55–200C in 45 min. An 8 ft aluminum column, ¼ in. I.D. packed with 15% Ucon Polar 50 HB 280 X on 80/100 mesh Chromosorb W, was used at a helium flow rate of 80 ml/min.

For rechromatography, an 8 ft aluminum column, ¼ in. I.D. packed with 20% of methyl silicone gum SE-30 on 70/80 mesh Anakrom ABS was used. Each fraction was rechromatographed isothermally at a temperature most suitable for that fraction, which varied between 60–180C.

The isolated volatile acidic by-products were converted into their methyl esters with the use of diazomethane (6) and then gas chromatographed with DEGS as stationary phase.

Identification of Gas Chromatographic Fractions

The nonacidic compounds of the isolated volatile by-products developed during hydrogenation were gas chromatographed with Ucon Polar as stationary phase. The eluent represented by each of the gas chromatographic peaks was collected with an acc-

[1] This investigation was supported in whole by Public Health Service Research Grant HE-06411, from the National Heart Institute.
[2] Presented at the AOCS Meeting, Houston, April 1965.
[3] Paper of the Journal Series, New Jersey Agricultural Experiment Station, Rutgers, The State University of New Jersey, New Brunswick.
[4] Present address: Central Research Laboratories, Kao Soap Co., Ltd., Tokyo, Japan.
[5] Present address: International Flavors and Fragrances, Inc., Union Beach, N.J.

TABLE I

Compounds Identified as Volatile By-Products of Catalytic Hydrogenation

Peak No. in gas chromatograms	Size of peak	Identified as
1. Saturated hydrocarbons		
3-A	Small	n-Hexane
7-A	Medium	n-Octane
13-A	Medium	n-Nonane
19-A	Medium	n-Decane
24-B	Small	n-Undecane
29-A	Small	n-Dodecane
40-A	Medium	n-Tetradecane
42-A	Small	n-Hexadecane
44-A	Medium	n-Heptadecane
2. Unsaturated hydrocarbons		
8-A	Small	*trans*-Octene
10-A	Small	*cis*-2-Octene
14-A	Small	*trans*-Nonene
20-A	Small	*trans*-Decene
31-C	Small	*trans*-Dodecene
3. Saturated alcohols		
12-A	Small	n-Propanol
18-A	Small	n-Butanol
23-A	Small	n-Pentanol
28-A	Large	n-Hexanol
33-A	Large	n-Heptanol
36-A	Medium	n-Octanol
39-A	Medium	n-Nonanol
41-A	Large	n-Decanol
4. Esters		
6-A	Medium	Ethyl acetate
5. Carbonyl compounds		
11-A	Small	n-Butanal
17-A	Small	n-Hexanal
34-A	Small	n-Nonanal
38-A	Small	n-Decanal
22-A	Medium	2-Heptanone
27-A	Small	2-Octanone
32-A	Small	2-Nonanone
15-A	Small	3-Hexanone
26-A	Medium	3-Octanone
31-B	Medium	3-Nonanone
25-A	Small	4-Octanone
30-C	Small	4-Nonanone
6. Lactones		
37-A	Small	γ-Hexalactone
43-A	Small	γ-Nonalactone

Numerals indicate the number of gas chromatographic peaks with Ucon Polar as stationary phase (same as the numbers in Fig. 1).
Letters indicate the number of gas chromatographic peak when rechromatographed with SE 30 as stationary phase.

Gas chromatography of the methyl esters of the volatile acidic by-products showed no peak of significant size. The largest peak was that of methyl hexanoate which was only of negligible size. However, if the oil used for hydrogenation was not previously deodorized to remove the volatile compounds in the crude oil, considerable amounts of fatty acids were observed. It seems, therefore, that no significant amount of free fatty acids was produced during catalytic hydrogenation using nickel as catalyst.

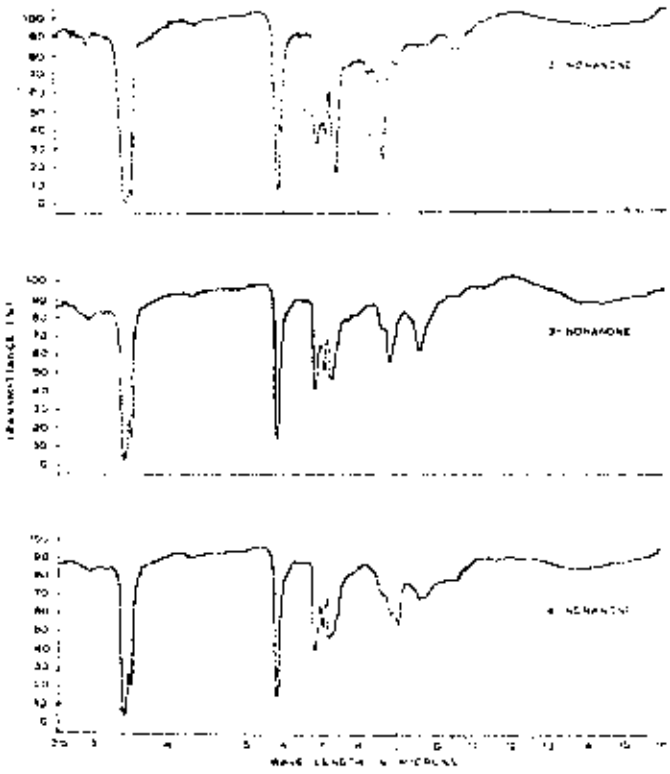


FIG. 2. Infrared spectra of 2-nonanone, 3-nonanone, and 4-nonanone.

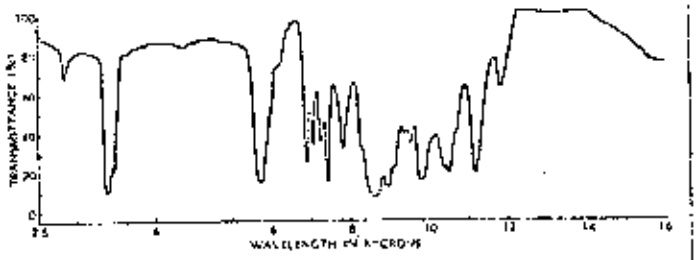


FIG. 3. Infrared spectrum of the fraction 43-A identified as γ-hexalactone.

Gas chromatography of the nonacidic volatile by-products developed during hydrogenation yielded 45 peaks, of which peak No. 2 was the solvent ethyl ether (Fig. 1). This gas chromatogram was significantly different from that of the volatile decomposition products developed during hydrogenation of a slightly autoxidized soybean oil which was not deodorized previous to the hydrogenation in order to remove any volatile compounds already present (2).

A total of 37 compounds was identified as shown in Table I. Nine were a homologous series of normal saturated hydrocarbons from C_6 to C_{17} with the exception of C_7, C_{13} and C_{15}. Five other small gas chromatographic peaks were identified as unsaturated hydrocarbons. The position of the double bond could not be exactly located by the use of infrared and mass spectrometry. It is interesting to note that all of them except one were *trans* isomers.

A complete homologous series of normal saturated primary alcohols from C_3 to C_{10} were identified. Three of these alcohols were the largest peaks of the gas chromatograms. A total of 12 carbonyl compounds was identified, four of which were normal saturated aldehydes, three methyl ketones, three ethyl ketones and two propyl ketones. The infrared spectra of these ketones showed significant differences (Fig. 2). The characteristic absorption band of methyl ketone at 7.39 μ shifted to 7.25 and 7.3 μ and became broader and less intense in ethyl and propyl ketones. The strong absorption of methyl ketone at 8.6 μ was shifted to higher wavelengths with decreased intensity for the ethyl and propyl ketones.

Two γ-lactones of C_6 and C_9 were identified. The infrared spectrum of the rechromatographed fraction 43A (Fig. 3) showed a carbonyl absorption band and a broad band at 8.5 μ, which suggested that it

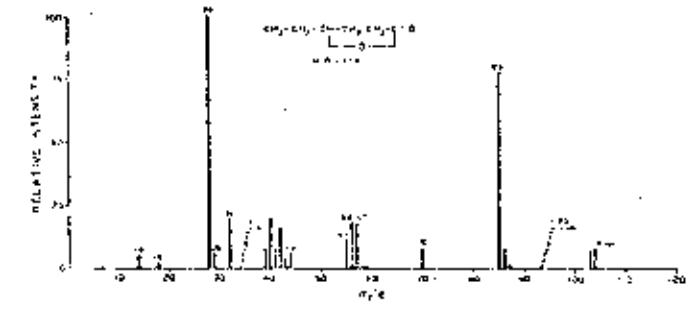


FIG. 4. Mass spectrum of the fraction 43-A identified as γ-hexalactone.

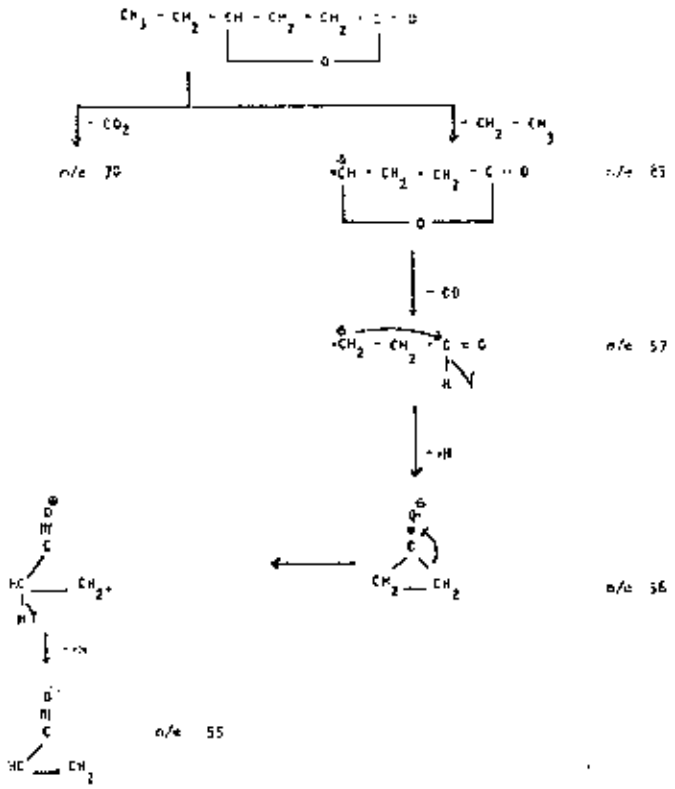
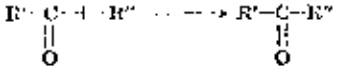


FIG. 5. Interpretation of the mass spectrum of the gas chromatographic fraction identified as γ-hexalactone.

was an ester. The location of the carbonyl band at 5.70 μ indicated the possibility of a saturated γ-lactone. The mass spectrum of this gas chromatographic fraction had a molecular ion of m/e 114 (Fig. 4). A loss of carbon dioxide yielded the peak m/e 70. A loss of ethyl group could produce the peak m/e 85. The latter may then lose a CO to yield the peak m/e 57, which may then undergo rearrangement to produce peaks m/e 56 and 55 (Fig. 5). It was therefore concluded that this fraction was γ-hexalactone. This conclusion was then confirmed by comparison of the retention times of this fraction with those of the authentic compound with the use of two different stationary phases, Ucon Polar and methyl silicone gum.

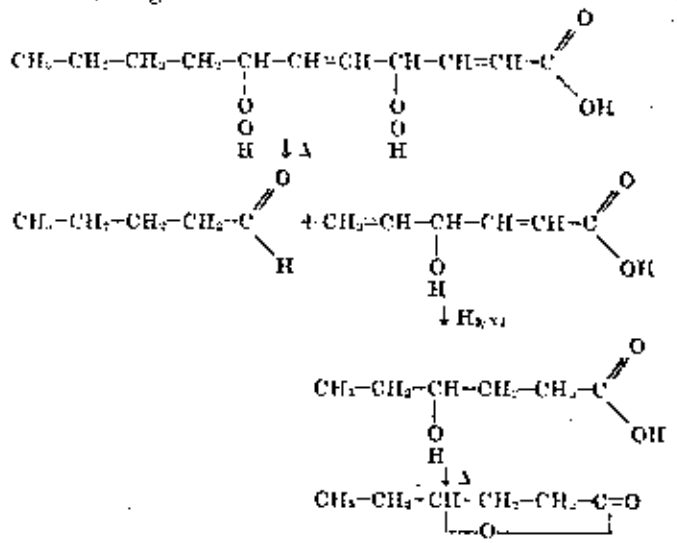
The odor and flavor characteristics of the compounds identified indicated that the characteristic flavor developed during the catalytic hydrogenation is unlikely to be due to one or two compounds. Chang et al. (1) reported that saturated aldehydes and ketones may contribute to the hydrogenation flavor. The present results appear to substantiate their observations. The higher members of the aldehydes and ketones, as well as the higher alcohols and the lactones had flavor notes all of which could contribute to the total characteristic flavor developed during hydrogenation.

The mechanisms for the formation of hydrocarbons, alcohols, aldehydes, and ketones through the decomposition of the precursors of the volatile by-products of hydrogenation, that is hydroperoxides, have been discussed in a previous paper (2). In addition, the 2-alkanones, 3-alkanones, and 4-alkanones may be produced through the coupling of alkyl free radical with methyl, ethyl, and propyl free radicals, respectively (8).

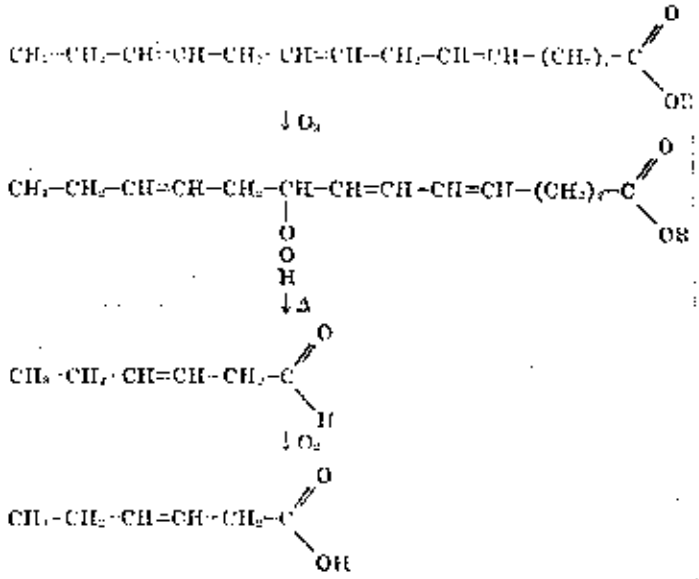


Under the conditions of catalytic hydrogenation, alcohols may also be formed through the reduction of carbonyl compounds with the simultaneous formation of a small amount of hydrocarbon through carbonylation. Studies with model systems indicated that, under the conditions used in this experiment for catalytic hydrogenation of soybean oil, n-nonanal was reduced to 85% n-nonanol, and 5% octane with 7% remained unchanged. The octane was evidently produced through decarbonylation. Under the hydrogenation conditions, 2-pentanone was reduced to only 26% of 2-pentanol with 73% remained unchanged. This explains why relatively large amounts of alcohols and ketones and relatively small amounts of aldehydes were identified.

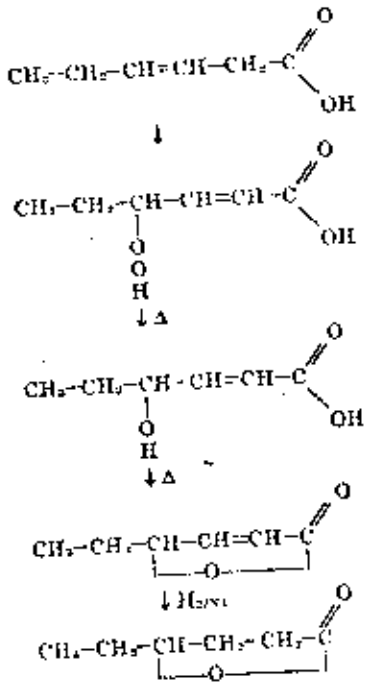
The lactones could only be formed through hydroxy acids. A mechanism for the formation of γ-hexalactone from the dihydroperoxide of a free fatty acid which may originate from linoleate is postulated as following:



The dihydroperoxide has a high boiling point and may therefore remain in the oil during the deodorization which precedes hydrogenation. A second mechanism involves the secondary autoxidation of 3-*cis*-hexenoic acid is also possible. This acid may be produced by the autoxidation of linolenate as follows:



This acid may form a hydroperoxide which is relatively nonvolatile and may remain in the oil during the deodorization previous to hydrogenation.



It should be noted that there were significant qualitative as well as quantitative differences between the volatile decomposition products of hydroperoxides produced under autoxidative and under hydrogenation conditions. Lea and Swoboda (9) distilled autoxidized sunflower oil at 75 and 210C and identified 16 aldehydes, 3 hydrocarbons, 2 ketones, and 1 alcohol. The present investigation, on the other hand, identified 4 aldehydes, 14 hydrocarbons, 8 ketones, 8 alcohols, and 2 lactones. Furthermore, the three largest gas chromatographic peaks were all normal saturated alcohols, and the next five largest peaks were two alcohols, two hydrocarbons and one methyl ketone.

ACKNOWLEDGMENT

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Components of the Hardening Flavor Present in Hardened Linseed Oil and Soybean Oil

J. G. KEPPLER, J. A. SCHOLS,[1] W. H. FEENSTRA and P. W. MEIJBOOM, Unilever Research Laboratory, Vlaardingen, The Netherlands

Abstract

The characteristic hardening flavor which develops in hardened linseed and soybean oils during storage has been coned from hardened linseed oil by stripping with nitrogen. After separating the volatile substances by adsorption chromatography on silica, the fraction containing the hardening flavor has been converted into 2,4-dinitrophenylhydrazones (DNPHs) and separated by means of partition chromatography. On regeneration of the fractions of DNPSs obtained, the characteristic hardening flavor was observed in one specific band. Both by hydrogenation and by oxidation of the free carbonyl the carrier of the flavor was found to be an unsaturated aldehyde; however, not of the α-β unsaturated type. Further separation of the regenerated carbonyls by means of gas-liquid chromatography (GLC) points to a C_9-aldehyde. After synthesis of the 4-,5- and 6-*cis*- and *trans*-nonenals, comparison made it probable that the carrier of the hardening flavor is a mixture of 6-*cis* and 6-*trans*-nonenal, the latter of which has the greatest share in the hardening flavor.

In order to confirm the location of the double bond in the carrier of the hardening flavor a recent isolation technique was applied. The volatile substances from hardened linseed oil were first separated via GLC. After conversion of the carbonyls in question into their DNPHs, the latter have been separated by means of thin-layer chromatography (TLC). By means of IR-analysis and oxidation with osmium tetroxide of the pure derivative, the principal carrier of the hardening flavor has been identified as 6-*trans*-nonenal.

Introduction

FOR MANY YEARS investigations have been carried out to identify the hardening flavor which is observed in hardened linseed oil and soybean oil (1–3). Lemon (4–6) has established that on hardening oils containing linolenic acid, "*iso*-linoleic acid" is formed from which the off-flavor(s) develops. In particular 9,15-octadecadienoic acid is considered to be the precursor. In later experiments, Lemon could no longer demonstrate a marked correlation between the amt of *iso*-linoleic acid formed and the intensity of the hardening flavor. Therefore he put forward the hypothesis that in addition an interaction must take place in the hardened oil between the *iso*-linoleic acid and another component (7).

Other research workers, e.g. Daubert et al. (8,9) and Mattill (1) have found that *iso*-linoleic acid is also formed during the hardening of soybean oil. In their opinion the conclusion must be drawn that a group of *iso*-linoleic acids is responsible for the occurrence of the off-flavors in hardened soybean oil and linseed oil.

For some time, the difference in off-flavor between hardened and unhardened soybean oil and linseed oil was not sufficiently distinguished.

Chang (10), however, has made a clear distinction between the two flavors and carried out investigations into the hardening flavor of hardened soybean oil. By means of a degassing technique Chang (11), obtained a flavor cone from hardened soybean oil which cone then was separated into fractions. He found that the characteristic hardening flavor is present in the fractions of the saturated aldehydes and ketones (12).

For a number of years the problem of the hardening flavor has been investigated in our laboratory and it has been established that the hardening flavor is present in the mixture of aldehydes which can be coned from hardened soybean oil and linseed oil.

Procedure and Discussion

Since both hardened soybean and linseed oils develop hardening flavors on storage, it was first investigated what unsaturated fatty acid is responsible for this flavor. Elias (13) has found that on hardening ethyllinolenate using a nicked catalyst, a strong hardening flavor developed in the hardened product. However, this flavor was not observed in hardened ethyllinoleate or ethyloleate. Since both soybean and linseed oil contain linolenic acid and both oils display the hardening flavor, it can be assumed—in accordance with the findings of other research workers (4,8)—that linolenic acid during hardening may be converted into another unsaturated acid from which the hardening flavor in question will be formed by oxidation. As it is known that hardened linseed oil very often displays a more intensive hardening flavor than hardened soybean oil, it was decided to choose hardened linseed oil as starting material. Linteris (14) pointed out that in hardened soybean oil the hardening flavor—in addition to many other flavors—is present in a stronger degree when air is sucked through at 100–130C.

Concentration of the Volatile Material from Hardened Linseed Oil. The above method was applied to our starting material. Air was sucked through portions of 5–10 kg hardened linseed oil (I.V. 110) for 2–3 hr at 130C. The strongly smelling oil was degassed at 200C by passing it slowly through a current of nitrogen at a vacuum of ca. 0.1 mm Hg. Via a spray trap, the entrained substances were passed through four cold traps. The first trap was kept at −10C in order to collect any fatty material carried along, in the second and third (at −80C) the flavor cone was collected, whereas the fourth trap (at −80C) served as a security and did not contain any condensate.

Preliminary Separation of the Flavor Concentrate. Since in the accelerated reversion procedure a great number of other volatile substances are produced, a preliminary separation of the flavor cone was carried

[1] Present address: R & D Laboratory, DOW Chemical of Canada Ltd., Sarnia, Ontario, Can.

out on an adsorption column of silicagel prepared according to Gordon et al. (15). In order to prevent the flavor components from being destroyed at the surface, the silicagel was moistened with 1-3% H_2O. As eluent light petroleum (bp 40–60C) with gradually increasing percentages of diethyl ether (0,0.5,1,2,5%) was used. In the fractions with 0.5–1% diethyl ether, the characteristic hardening flavor was perceived.

Investigation into the Carbonyl Function. The fractions displaying the hardening flavor were collected and their volume reduced to 10–20 ml by means of careful evaporation of the solvent at low temp. After diluting the solution with diethyl ether, the carbonyls were converted into their DNPHs by refluxing with 2,4-dinitrophenylhydrazine, using acetic acid as a catalyst. After termination of the reaction, the hardening flavor had disappeared. The fact that after careful regeneration of the carbonyls from part of the hydrazones this flavor could be perceived again, confirmed the hypothesis that the carrier of the hardening flavor has a carbonyl function. For this regeneration the following method was used: 1–5 mg DNPHs was dissolved in 1–2 ml 50% acetic acid and then ca. 0.5 ml 40% formaldehyde solution was added. The mixture was refluxed for 5 min, cooled, diluted with 10 ml water and extracted with 1–2 ml light petroleum. The formaldehyde-DNPH formed is practically insoluble in light petroleum. The light petroleum contains the desired carbonyl compound(s), but also some of the excess formaldehyde and acetic acid. Therefore, the light petroleum solution was washed once or twice with 15 ml water. The carbonyl compound can now be recognized in the light petroleum solution by its typical odor.

Partition Chromatography of the DNPHs. The chromatographic separation according to Kramer and Van Duin (16) by which eight colored zones were obtained showed that the first separation over the silica-column had been incomplete. Portions of each zone were regenerated. On the basis of the smell of the liberated carbonyls, it could be established that the carrier of the hardening flavor was present in the hydrazone band which had a retention volume (R_v) of 112 ml.

In experiments with model substances, it appeared that the hydrazones of *n*-heptanal and 2-octenal had the same R_v values. However, the flavors of these aldehydes are entirely different from those of the hardening flavor in question.

Investigation into the Nature of the Flavor Carrier. The following experiments were carried out with the regenerated carbonyls from part of the DNPHs having a R_v value of 112 ml:

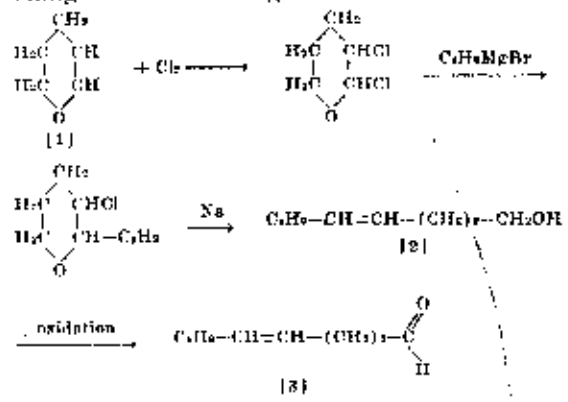
a) A small portion was oxidized with moist silver oxide for 1 hr at 60C (17,18) after which the hardening flavor disappeared completely. From this finding the conclusion was drawn that an aldehyde was present which is even very likely to have the following group $R-CH_2-CH_2-CH_2-\overset{O}{\overset{\|}{C}}-H$, since by this method 2-enals and 2,4-dienals cannot so easily be converted into acids, and ketones such as pentyl-vinylketone and pentyl-ethylketone are not sensitive to this type of oxidation.

b) Another portion of the above carbonyls was hydrogenated for 1–2 min at 25C in a solution of light petroleum (bp 40–60C) using palladium as a catalyst. It may be mentioned that under these mild conditions only carbon-carbon double bonds will be saturated and aldehyde groups will remain unaffected (19). The fact that the hardening flavor disappeared indicates that the aldehyde must have an unsaturated character.

c) A larger amt of the fraction of DNPHs with R_v = 112 ml (56 mg) were regenerated by means of the method mentioned before and coned to a small volume (0.5 ml) by freeze drying. This conc was then analyzed by means of GLC, and ca. 90% heptanal and 9% 2-octenal were found. Approx at the place of nonanal, a very small peak was collected which displayed a strong hardening odor. However, the odor of both nonanal and 2-nonenal are quite different from the hardening flavor in question so that these compounds can be ruled out.

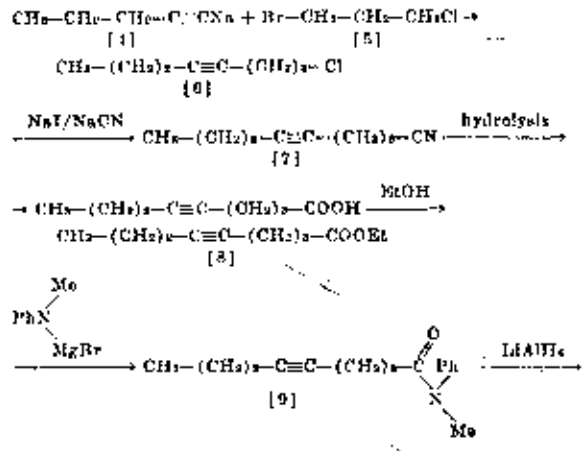
Therefore, it was assumed that an unsaturated C_9-aldehyde was present, having a double bond which is further removed from the carbonyl group than in the case of 2-nonenal. By synthesis of 4-,5- and 6-nonenals and comparing their flavor, flavor potency and R_v of their DNPHs, it was found that 6-*cis* and 6-*trans*-nonenal are most likely to be the carriers of the typical hardening flavor.

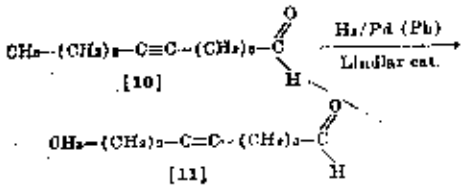
Synthesis of 4-,5- and 6-Nonenals. 4-Nonenal was synthesized starting from dihydropyran (I) (20) according to the following reaction scheme:



A mixture of *cis*- and *trans*-4-nonenols [2] is formed. In our case we could not confirm the findings of Crombie and Harper (21) concerning the exclusive formation of the *trans* compound. The oxidation of the unsaturated alcohol [2] to the unsaturated aldehyde [3] was carried out (i) by slow oxidation with a mixture of bichromate and acetic acid (22), (ii) by rapid oxidation with bichromate-H_2SO_4 (23,24), (iii) by oxidation with tertiary butylchromate (25–27). Although none of these methods was satisfactory, the method mentioned under (i) gave the highest yield (ca. 20%). R_v 4-nonenal-DNPH = 112 ml.

5-cis-Nonenal was prepared from sodium pentyne (4) following the reaction scheme:

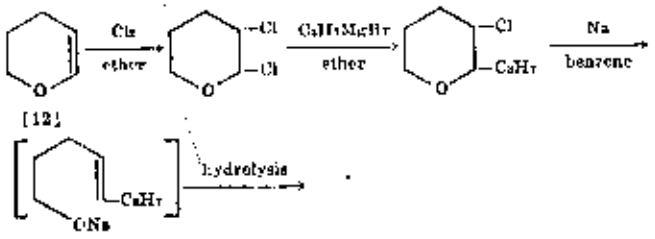




1-Chloro-3-bromopropane [5] was prepared from allyl chloride with HBr under the influence of UV radiation (28). 1-Chloro-4-octyne [6] was converted into the cyanide [7] via the iodide, and the cyanide [7] hydrolyzed and esterified with ethanol. To prevent formation of a *trans* double bond in the final product [11], the ester [8] was first converted into the corresponding anilide [9] with the Grignard compound of mono-methyl anilide (29). The anilide [9] was reduced with lithium tetrahydride aluminate (23,24) to 5-nonynal [10], which after purification via the DNPH was hydrogenated with a Lindlar catalyst. In this way exclusively 5-*cis*-nonenal [11] with a yield of 50.7% was formed. R_f 5-nonynal-DNPH = 250 ml, R_f 5-nonenal-DNPH = 112 ml.

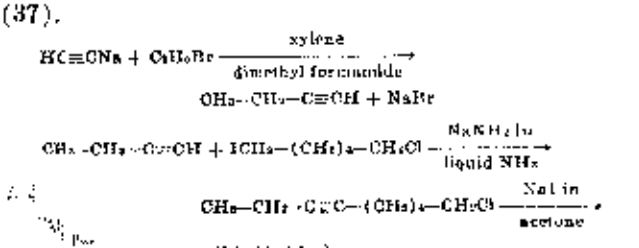
In an attempt to hydrogenate [7] directly to [10] by means of lithium tetrahydride aluminate, no results were obtained.

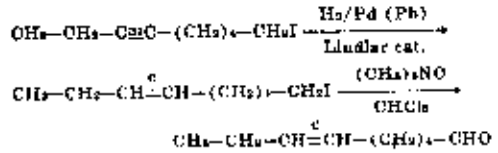
5-trans-Nonenal was prepared in accordance with the following reaction scheme:



First 4-*trans*-octen-1-ol [13] was prepared from 2,3-dihydropyran [12] using the method of Crombie and Harper (30). The corresponding bromide [14] was synthesized according to another procedure also described by Crombie (31). In accordance with the procedure of Smith and Nichols (32), the 1-bromo-4-*trans*-octene [14] formed was coupled via the corresponding Grignard compound to ethyl orthoformate. The 5-*trans*-nonenal diethyl acetal [15] obtained was hydrolyzed by the method of Winter (33) into 5-*trans*-nonenal in 74% yield.

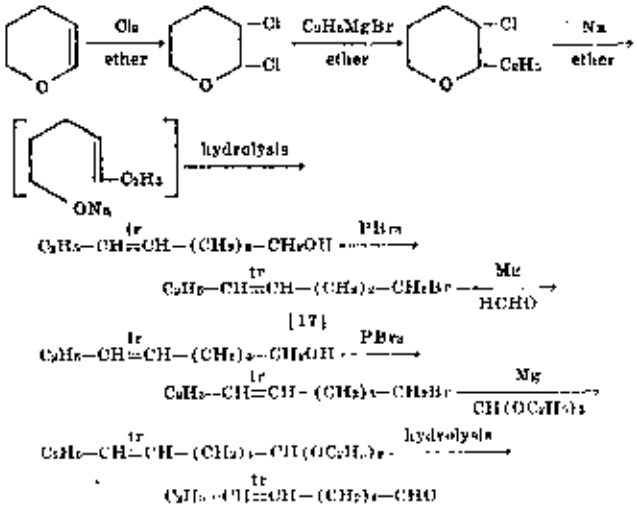
6-cis-Nonenal was prepared according to the following scheme (34–36). The last stage in the synthesis was carried out by the method of Franzen and Otto (37).





The yield was 35%: ca. 90% of the *cis*-form and ca. 10% of the *trans*-form.

6-trans-Nonenal was synthesized as follows:



The synthesis was carried out in a manner analogous to that for 5-*trans*-compound with the exception of the intermediate stage to extend the 1-bromo-4-*trans*-heptene [17] by one carbon atom (38) to 5-*trans*-octenol, which now proceeded in the normal way. The yield of 6-*trans*-nonenal was 70%, purity being 99%.

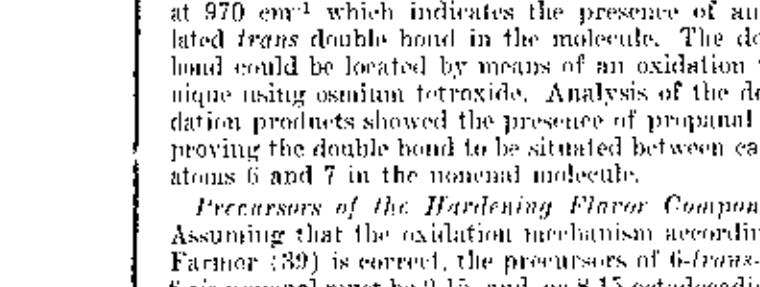
Both 6-*cis*- and 6-*trans*-nonenal have a typical flavor reminiscent of green melon. The flavor potency of the *trans*-form dissolved in paraffin oil is ca. 10 times stronger than that of the synthesized *cis*-form (to be published). Although the type of flavor of 5-*trans*-nonenal is identical with that of 6-nonenal, its flavor potency in paraffin oil is lower than that of 6-*trans*-nonenal by a factor of 100–200 (to be published).

Location of the Double Bond. In order to verify the results obtained so far and to obtain additional proof of the location of the double bond, we applied a modification of the technique of De Jong et al. (40) using TLC with silica impregnated with silver nitrate (41) for the separation of unsaturated DNPHs into various classes. A conc of the hardening flavor was obtained by degassing 50 kg of hardened linseed oil (I.V. 87.6) and by preparative GLC (apparatus F and M 770, temp 170C, press. 20 lbs/sq in, column length 4.80 m) using polyethylene glycol adipate (20%) as immobile phase. The fraction with the typical hardening flavor and with the same retention time as synthetic 6-*trans*-nonenal (carbon number 9.6) (42) was trapped in a condenser which was cooled with liquid nitrogen. The volatile material was taken up in carbonyl-free light petroleum (bp 40–60C) and the carbonyls converted into their DNPHs (43). The DNPHs were purified by passing over a neutral alumina column using a mixture of light petroleum and an increasing amt of diethyl ether as eluent and then separated on chromatoplates using silica impregnated with silver nitrate as adsorbent (to be published). From these plates two fractions were recovered with the same elution rates as those of 6-*trans*- and 6-*cis*-nonenal. Samples of both fractions displayed the typical hardening flavor after regeneration, whereas in the other fractions this flavor was not present.

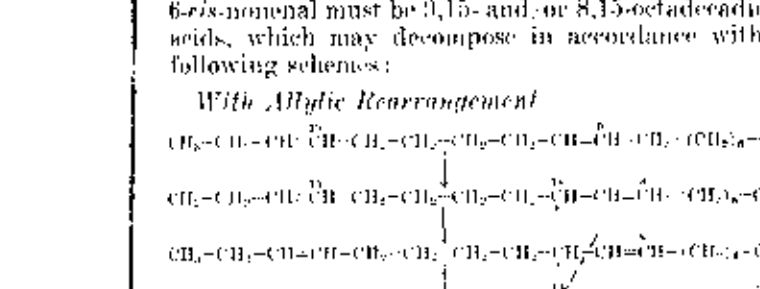
Subsequently, the two DNPH fractions were separated by TLC using silicagel G (ex Merck) (44), and the spots having the same elution rates as the model substances were recovered. After regeneration of the carbonyls from part of the DNPHs, the hardening flavor in question could be perceived again. Finally, both fractions were further purified by passing over an alumina column as described before. For both cases the UV-spectrum showed an absorption maximum at 358 mμ measured in chloroform solution. The absorption at this wave-length is typical of DNPHs of aldehydes with a CH_2-group at the α-position. The amt of material of the fraction showing a chromatographic behaviour similar to that of 6-*cis*-nonenal was too small for further investigation. The IR-spectrum of the *trans* fraction showed an absorption maximum at 970 cm^{-1} which indicates the presence of an isolated *trans* double bond in the molecule. The double bond could be located by means of an oxidation technique using osmium tetroxide. Analysis of the degradation products showed the presence of propanal thus proving the double bond to be situated between carbon atoms 6 and 7 in the nonenal molecule.

Precursors of the Hardening Flavor Components. Assuming that the oxidation mechanism according to Farmer (39) is correct, the precursors of 6-*trans*- and 6-*cis*-nonenal must be 9,15- and/or 8,15-octadecadienoic acids, which may decompose in accordance with the following schemes:

With Allylic Rearrangement

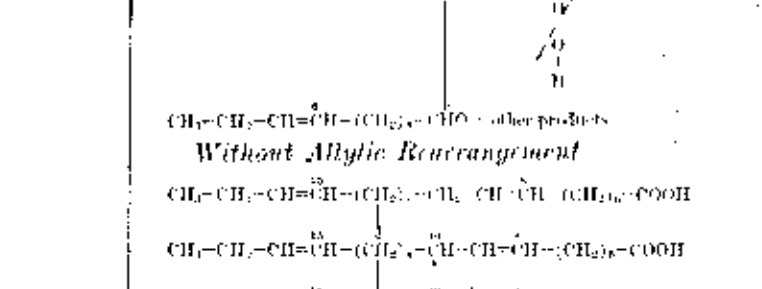


Without Allylic Rearrangement

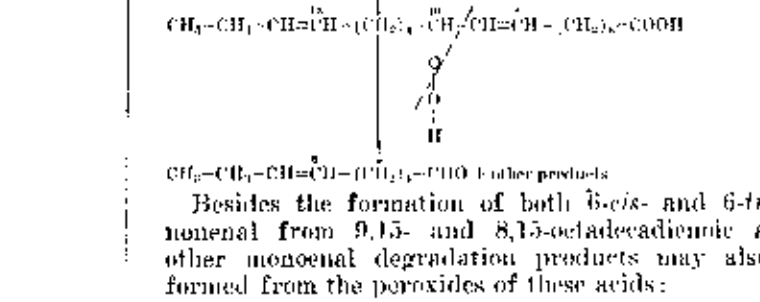


Besides the formation of both 6-*cis*- and 6-*trans*-nonenal from 9,15- and 8,15-octadecadienoic acids other monoenal degradation products may also be formed from the peroxides of these acids:

With Allylic Rearrangement



Without Allylic Rearrangement



By synthesizing 7,15-, 8,15- and 9,15-octadecadienoic acids we could indeed confirm that the 6-nonenals are produced in the very early stages of oxidation. This subject will be dealt with in a future publication.

ACKNOWLEDGMENT

Synthesis of various aldehydes by S. Sparreboom, A. S. M. van der Zijden and G. Lardelli; chromatography of the DNPHs isomer mixture by Miss P. Haverkamp Begemann; osmium tetroxide analysis by K. de Jong and K. Mostert; GLC-analysis by J. H. Reeuwt.

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Gangliosides and Glycerophospholipids in Multiple Sclerosis White Matter

YASUO KISHIMOTO, PhD; NORMAN S. RADIN, PhD; WALLACE W. TOURTELLOTTE, PhD, MD; JULIUS A. PARKER, MA; AND HIDEO H. ITABASHI, MD, ANN ARBOR, MICH

HISTOLOGICALLY, multiple sclerosis is characterized by focal necrotic foci of white matter with degeneration of myelin sheaths and, usually, a high degree of sparing of axons.[1] Chemically, the foci of demyelination show loss of glycerophospholipids, cholesterol, and cerebrosides,[5-6] as well as selective loss of particular fatty acids.[7-8] The chemical changes are consistent with the histological demonstration of myelin loss but are not sufficiently specific to indicate whether the axons passing through the plaques are normal in composition.

The lipid most characteristic of gray matter, gangliosides, has not been studied with modern methods in multiple sclerosis plaques or normal-appearing white matter (NAWM). Probably the main reason for this is the difficulty of adapting the analytical techniques to determining the relatively low concentration of gangliosides in white matter. Lowden and Wolfe,[9] in a detailed study of the distribution of lipid-bound sialic acid, concluded that gangliosides occur in white matter in neuronal processes (not axons) rather than in glia or myelin. Sialic acid occurs in gangliosides as monosialogangliosides, disialogangliosides, and trisialogangliosides; and Svennerholm[10] has shown that the gangliosides of white matter are relatively rich in the *tri*sialogangliosides and poor in the *tri*sialogangliosides. Thus, if the gangliosides of white matter arise by movement from neuronal cell bodies[11-12] or synaptic vesicles[13] through the neuronal processes it would appear that partial hydrolysis of these lipids occurs during their migration. Isotopic experiments in rats have shown[14] that gangliosides do indeed undergo considerable degradation and resynthesis, and evidence has been offered for the presence of appropriate hydrolytic enzymes.[15-16] It is therefore plausible that in multiple sclerosis, a disease in which lipid hydrolyzing processes probably are at work to an excessive degree, abnormalities might be found in the ganglioside composition and concentration.

This paper reports an examination of the gangliosides of multiple sclerosis plaques and NAWM and of white matter from control brains showing no histopathological changes. An additional case showing severe diffuse demyelination was also studied. Samples were taken from the specimens to be analyzed and these were processed with Cajal's silver stain to estimate the axon density. The ganglioside concentration was compared with an axonal density index in the different types of samples.

Materials and Methods

Patient Material.—At autopsy the left cerebral hemisphere was dissected and sliced at room temperature by an electric meat slicer into 3-mm coronal sections. Each section was placed in a plastic bag and stored at −90°C.

Case MS 1.—A 55-year-old woman had relapsing and remitting multiple sclerosis at first, then a chronic progressive course over a period of 21 years. Her neurological status prior to death was as fol-

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From the Mental Health Research Institute and Department of Neurology, University of Michigan, Ann Arbor, Mich.

Reprint requests to Mental Health Research Institute, University of Michigan, Ann Arbor, Mich 48104 (Dr. Radin).

bus; moderately depressed; up in a chair several hours each day; moderate bitemporal pallor of optic disks with small, central scotoma in each eye; moderate upward gaze paresis, mild nystagmus on lateral gaze; moderate to severe spastic quadraparesis (legs > arms) with moderate muscle spasms; moderate intention tremor of upper extremities, pallanesthesia, and incontinence (catheter). She committed suicide with secobarbital (Seconal). The brain showed typical plaques of demyelination. The brain slices were frozen at 16 hours after death.

Case MS 2.—A 31-year-old woman had a similar course as MS 1, except the duration was nine years. Her neurological status prior to death was as follows: mental state, good; confined to bed; optic atrophy with blindness; rotatory nystagmus; scanning speech; spastic quadraparesis (legs > arms) with contractures; intention tremor; paresthesias, pallanesthesia, and incontinence (catheter). She died with carcinoma (26.6 Gm./100 ml.), aspiration pneumonia, severe chronic pyelonephritis without uremia but with anemia (6.6 gm. of hemoglobin per 100 ml.), and large sacral decubiti. The brain showed numerous plaques of demyelination grossly and microscopically. The brain slices were frozen about 24 hours postmortem.

Case MS 3.—This 37-year-old woman showed the typical symptoms, signs, and course of multiple sclerosis over a period of five years. She had chlorpromazine (Thorazine) 1 gm. daily. Her neurological status prior to death was as follows: confined to bed; moderate dementia with flat affect; bitemporal pallor with blindness; ptosis; vertical and dissociated nystagmus; scanning speech; dysphagia (feeding tube in stomach); intention tremor (face, head, and extremities); spastic quadraparesis (legs > arms); pallanesthesia; and incontinence (catheter). She died with a fever of 44.4 C (111.9 F), mild pneumonitis, congestion and edema, fecal chronic pelonephritis without uremia, and sacral decubitus ulcer. At autopsy there was a diffuse mottled appearance of most of the white matter due to a multitude of plaques of demyelination. Because of the diffuseness of the demyelination, no NAWM could be obtained. The brain slices were frozen 24 hours after death.

Control 1.—A 59-year-old woman died with the following abnormalities: rheumatoid arthritis, congestive heart failure secondary to coronary arteriosclerotic heart disease and cardiac dysrhythmia. She also had an infected gangrenous right foot due to arteriosclerosis. The brain, which appeared normal grossly and microscopically, was sliced and frozen by 28 hours postmortem.

Control 2.—A 56-year-old woman died with bronchogenic carcinoma superimposed on an oat cell cancer of the lung which had metastasized to spleen, lymph nodes, and bone marrow. At autopsy her brain appeared normal by gross and microscopic inspection. The slices were frozen 48 hours postmortem.

Control 3.—A 65-year-old woman had at the time of death hypertensive arteriosclerotic cardiovascular disease, mild diabetes mellitus, bronchial asthma, and mild chronic brain syndrome. The brain appeared normal grossly and microscopically. The slices were frozen about 16 hours postmortem.

Tissue Dissection.—Frontal and occipital coronal sections were thawed and dissected grossly. Plaques and NAWM from the first two multiple sclerotic brains were taken from the same coronal sections, and homologous sections were taken from controls 1 and 2. Two plaques were pooled for each analysis.

Diffusely demyelinated white matter from MS 3 was obtained from several serial frontal coronal sections; control 3 consisted of homologous white matter.

Histological Methods.—A 16-gauge needle was used to obtain tissue for histological analysis from the centers of the dissected samples. The specimens were fixed in 10% formaldehyde solution (formalin) and labeled by a code system unknown to neuropathologist. The samples were then embedded in paraffin, sectioned at 8μ, and stained with Cajal's pyridine-silver nitrate method[17] for axons and with the Klüver-Barrera method[18] for myelin. The degree of demyelination was rated 0 to 3, the 3 representing complete demyelination.

The number of axons per unit volume of tissue was assessed in four areas of a single microscopic section from each sample with the aid of an ocular grid. The grid consisted of squares formed by eight horizontal and eight vertical lines (1 mm apart) and was used at a magnification of 970. All axons through the entire depth of section falling on one or more horizontal lines were counted. A single long axon recrossing the same line or another line was counted as many times as it crossed the line. This technique eliminates the problem of identifying individual axons in a field and yields a number ("axonal density index") related to the number of axons per unit volume.

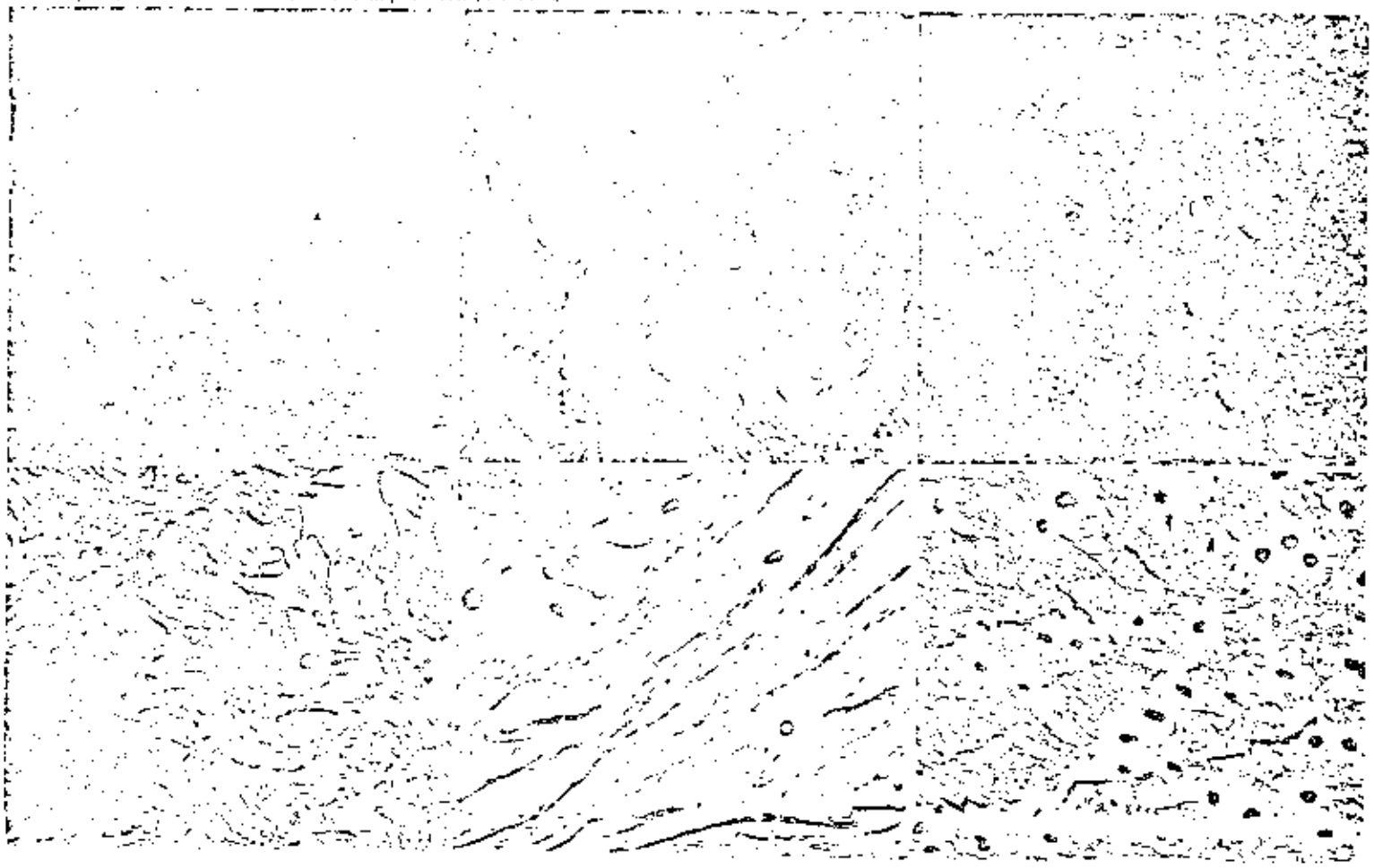
Water and Total Lipid Determination.—The weighed tissue sample was homogenized with 20 volumes of chloroform-methanol 2:1, and an aliquot of the suspension was dried by lyophilization or oven heating. Comparison of the weights of total sample and dried sample yielded the data for water content. In the case of the plaques, an appreciable amount of material formed small sheets during homogenization. Since the sheets could not be taken into the aliquot, they were rinsed off, air-dried, weighed separately, and the weight added to the total dry weight. The parchment-like material amounted to about one-sixth to one-third of the total dry weight.

(The sheets from the plaques, incidentally, do not seem to have been noted by other workers. Possibly, it is denatured proteolipid protein liberated during myelin degeneration or a condensation of fibers derived from proliferated astrocytes.)

The remainder of the tissue homogenate was filtered, and the filtrate was washed three times with aqueous potassium chloride and potassium chloride—containing "Folch upper layer" as described previously.[19] The lower layer, which contains the "total lipids," was evaporated to a small volume with a stream of nitrogen, then lyophilized from benzene and weighed. This weight is a little low because the gangliosides, and a small amount of other lipids enter the upper layers.

Fatty Acids of Glycerophospholipids.—The glycerophospholipids are characterized by possession of an ester linkage between fatty acid and glycerol. This linkage is readily cleaved at room temperature by treatment of the total lipids with sodium hydroxide in chloroform-methanol to form the methyl esters, which are then purified by Unisil chromatography and analyzed by gas chromatography. A

Fig 2.—Photomicrographs from frontal lobe white matter, as in Fig 1. *Right column*, control 2, index=130. *Middle column*, MS 2 NAWM, index=64. Some axons appear thicker than the controls. *Left column*, MS 2 plaque, index=100. Upper photo shows complete demyelination, gliosis, and astrocytic proliferation. Axon cylinders appear well preserved.



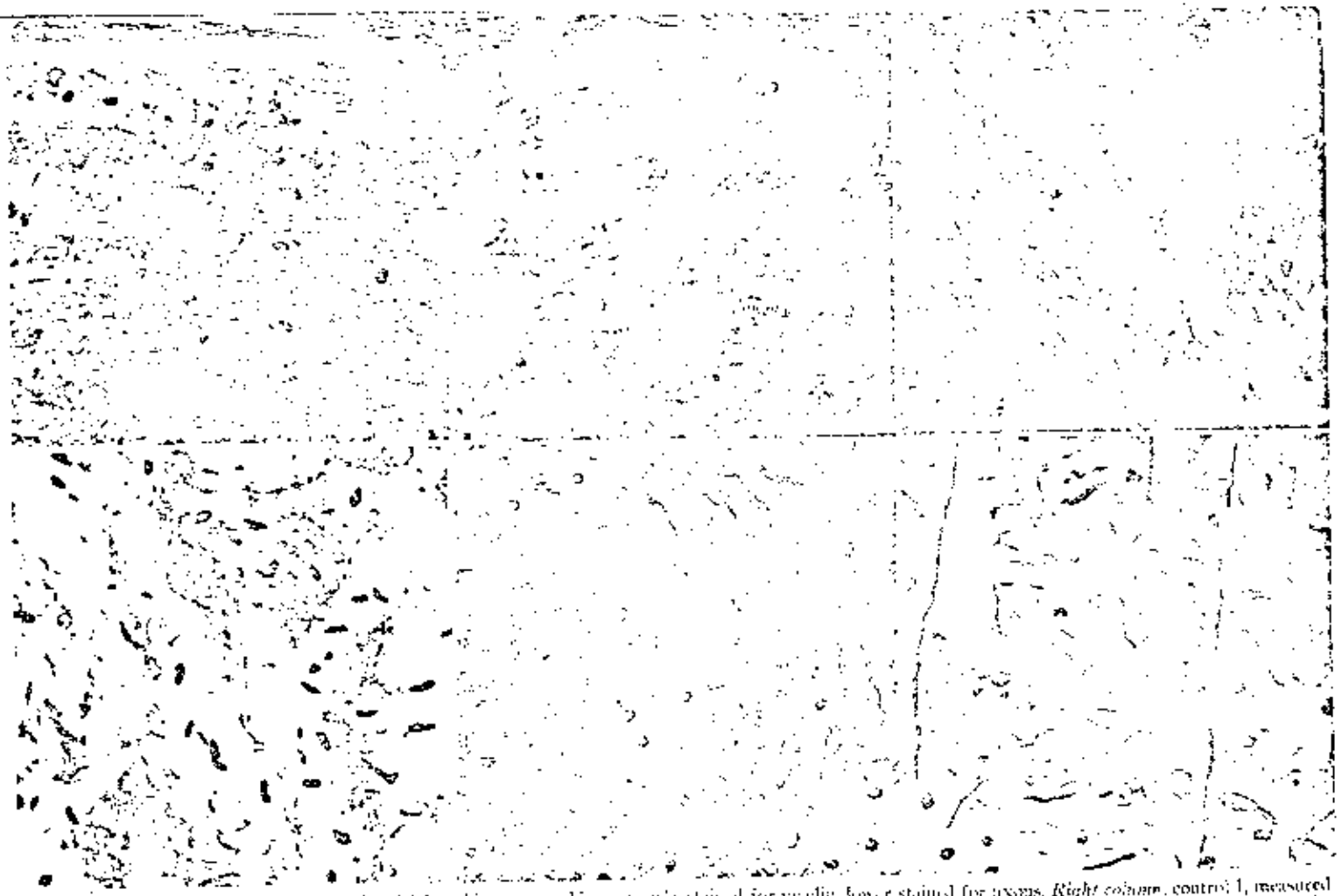


Fig. 3. Photomicrographs from occipital lobe white matter. Upper panels stained for myelin, lower stained for axons. *Right column*, control 1, measured axonal density index [illegible]. *Middle column*, MS 1 NAWM, index=86. *Left column*, MS 1 plaque, index=14. The plaque shows total demyelination. The increased cellularity is due to proliferation of endothelial cells and astrocytes (× 400).

known amount of methyl 23:0 (tricosanoate) is added to the esters before analysis to serve as internal standard. The detailed method has been described previously.[9] The method yields the amount of each fatty acid present in the initial sample, from which the total is calculated.

Ganglioside Determination.—The aqueous layers obtained on washing the lipid extract were analyzed by a previously described method.[9] This involves removal of contaminating lipids by a backwash with "Folch lower phase," methanolysis of the dried residue of the upper layer, and gas chromatographic determination of the ganglioside fatty acids. A mixture of methyl esters of 19:0 and 21:0 was used as the internal standard. It should be noted that the final data represent moles of ganglioside, rather than of sialic acid.

Comparison of Ganglioside Types.—Portions of chloroform-methanol extract corresponding to 250 mg brain were extracted as before, but with omission of the potassium chloride. This yields improved transfer of gangliosides into the upper layer. The pooled upper layers were dried, the residue suspended in chloroform-methanol (3:1), and the suspension added to a column containing 0.5 gm of silica gel. Elution with the same solvent (40 ml total) was followed by elution with 15 ml of chloroform-methanol (1:4) which yielded the gangliosides. GA further elution with methanol did not take out additional gangliosides. The gangliosides were then separated by thin-layer chromatography, using Silica Gel G and propanol-water (7:3).[10] Bromothymol blue, followed by 2-naphthol/sulfuric acid,[11] was used to locate the lipid spots.

Results

Histology.—Figures 1-3 are typical photomicrographs of the samples taken from the grossly dissected white matter. Those areas designated by the dissector as grossly appearing NAWM or plaques were in all cases confirmed by the histopathological analysis.

Water and Lipid Content.—The data, shown in Table 1, reveal the previously noted higher water content in plaques. The average value for plaques was 88% water, while the control samples contained 72% and the NAWM of multiple sclerotic brains contained 71%. The areas in diffuse demyelination (patient MS 3) contained 89% water, the same as that found in plaques.

The differences in lipid contents were also typical. The plaques and diffusely sclerotic areas were very low in lipids. However, the NAWM was not noticeably lower than the controls.

The portions stained for myelin (Fig 1-3) confirmed the gross visual appearance and lipid analyses.

Gangliosides.—Only the data for stearic acid-containing gangliosides are shown in Table 2, calculated on the basis of brain weight and lipid weight. Stearate represents about 85% of the fatty acids in gangliosides, and no notable difference was seen in the relative amounts of the other acids (16:0, 18:1, 20:0).

The most striking finding is that the concentrations of gangliosides in fresh tissue are rather similar in demyelinated, NAWM, and control samples. The P value for the difference between control and plaque concentrations (using the T-test for the averages obtained from each patient) is >0.0. This retention of ganglioside is emphasized by the comparison with the weights of total lipid. Where control and NAWM values range between 600 and 1,670 nanomoles/gm lipids, the demyelinated areas range between 2,540 and 6,890 nanomoles/gm.

Glycerophospholipids.—The concentration of total ester-linked fatty acids is shown in Table 3, where comparison is made between fresh brain weight and total lipid weight. As with the total lipids, there is little clear difference between control and NAWM samples but considerable depletion in demyelinated areas.

When one compares the loss of glycerophospholipid acids with the loss of total lipids in the demyelinated areas, it is apparent that the former are spared more than the other lipids. This is seen in the average value for the normal and NAWM samples: 162 mg fatty acids per gram lipids; the average for the demyelinated tissue is 221 mg/gm.

The relative amounts of the individual ester-linked fatty acids are shown in Table 4. Examining each horizontal line separately, one sees that the NAWM and control white matter are quite similar. However, the demyelinated tissue shows marked differences in every acid. Demyelination results in preferential retention of 16:0, 16:1, arachidonate (20:4), and 22:6. The minor acid, 22:5n3, which is not measurable in normal white matter, is seen as a distinct peak in the chromatograms from plaques. As in the other analyses, the diffusely demyelinated tissue of patient MS 3 is very much like that of the more typical multiple sclerotic patients.

Relation Between Axon Densities and Ganglioside Concentrations.—The seven control samples counted yielded an average axonal density index of 110 (standard error of the mean = 17), while the two NAWM samples examined yielded a lower value, 75 (58 and 87). The density index for seven

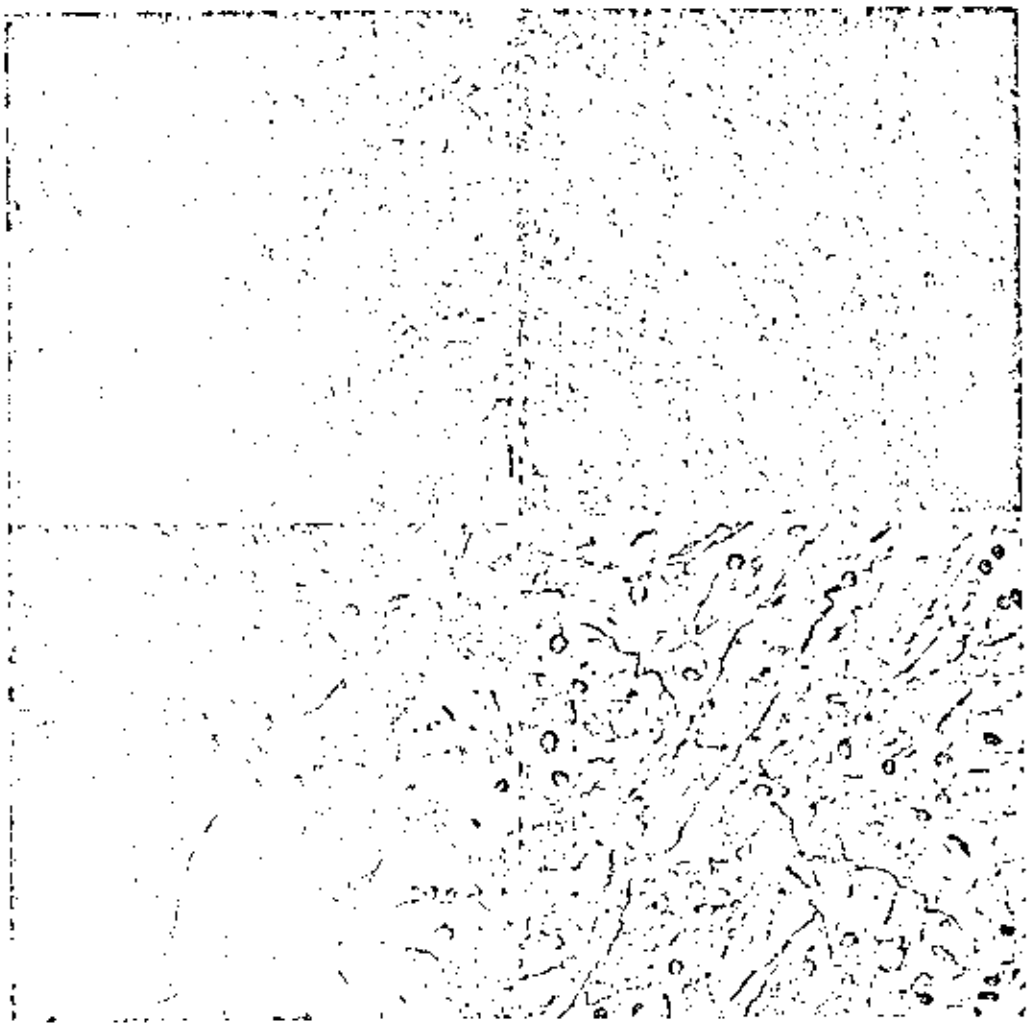


Fig 4.—Frontal lobe white matter, as in Fig 1. *Right column*, control 3, index=75. *Left column*, MS 3, index=19.

plaque samples was 46±12, which is significantly lower than the control values ($P < .01$). It would appear from these data that there is roughly a 60% decrease in axon density.

Since gangliosides are apparently primarily neuronal substances, it is interesting to compare the ratio, ganglioside concentration in whole tissue vs axonal density index. Taking the data from the actual samples counted, the average ratio is 1.4±0.15 in the control samples, 2.7 (2.2 and 3.2) in the NAWM, and 2.5±0.16 in the demyelinated samples. The difference in ratios between control and demyelinated matter is not significant. Even closer agreement is seen in the ratios from the NAWM and plaques of the same patients.

Comparison of Ganglioside Types.—Figure 4 shows a thin-layer chromatogram of the gangliosides from various brain samples. Samples 5 and 6 show that gray and white matter of control brain, taken from adjacent areas, are not greatly different in the distribution of the various types of gangliosides. In agreement with Svennerholm,[10] we find more monosialoganglioside and less trisialoganglioside in white matter.

The NAWM from a multiple sclerosis patient, sample 4, looks very much like normal white matter. The plaque gangliosides from the same patient, sample 3, show a decrease and a changed distribution in the faster moving gangliosides. The more severely degenerated white matter of patient MS 3,

TABLE 1.—*Water and Lipid Content of the Dissected White Matter*

Patient *	Water Content (mg/gm)			Lipid Content (mg/gm)		
	Normal	NAWM	Demyelinated	Normal	NAWM	Demyelinated
MS 1		663	875		176	11
MS 2		734	860		161	24
		740	876		159	25
MS 3			833			13
			853			19
			884			17
			910			30
			918			22
Control 1	670			180		
	728			152		
Control 2	683			210		
	684			202		
	696			210		
	710			187		
Control 3	724			125		
	734			141		
	742			147		
	743			152		
	752			129		
Average	716	712	894	168	165	20

* Areas chosen for dissection were occipital region for MS 1 and control 1, frontal region for the others. Multiple samples analyzed in all but MS 1. Data are based on fresh weight.

TABLE 2.—*Ganglioside Content of the Dissected White Matter*

Patient	mμmoles/gm Fresh Tissue			mμmoles/gm Total Lipid		
	Normal	NAWM	Demyelinated	Normal	NAWM	Demyelinated
MS 1		126	110		720	5000
MS 2		238	158		840	2540
		304	235		1170	6960
MS 3			48			3640
			115			6700
			92			3470
			137			4130
			100			4980
Control 1	118			660		
	93			410		
Control 2	184			830		
	269			1830		
	190			870		
	253			1240		
Control 3	209			1670		
	156			1100		
	212			1430		
	247			1610		
	171			1320		
Average	189	223	120	1150	910	4870

sample 2, resembles the plaque gangliosides, but is more depleted.

Comment

The Subcellular Localization of Gangliosides in White Matter.—Our finding that the ratio, ganglioside concentration/axon density, remains relatively unchanged during demyelination suggests that the gangliosides are localized in the axon rather than in glial cells or myelin. As yet there is no method for isolating axons in quantity or for histological staining for gangliosides, so the question cannot be settled. Preparations of myelin, isolated by centrifugal methods, vary in their ganglioside content, and it may be that the gangliosides appear in the myelin as the result of axonal contamination or of ionic binding of liberated gangliosides.[23] While the axons of peripheral nerves seem to lack gangliosides,[9] this may merely be the reflection of a peculiarity of very long axons. Perhaps the gangliosides diffusing along the axon from the neuronal cell body or from

TABLE 3.—*Glycerophospholipid Fatty Acid Content of the Dissected White Matter*

	mg/gm Fresh Tissue			mg/gm Total Lipids		
Patient	Normal	NAWM	Demyelinated	Normal	NAWM	Demyelinated
MS 1		23.4	5.6		134	250
MS 2		25.7	5.8		160	237
		25.8	6.9		163	294
MS 3			2.1			156
			4.8			250
			3.9			227
			5.0			164
			4.1			187
Control 1	23.2			129		
	21.2			149		
Control 2	30.6			169		
	31.3			170		
	33.9			166		
	34.9			176		
Control 3	21.6			173		
	23.5			160		
	22.7			164		
	26.4			161		
	22.5			174		
Average	27.3	25.0	4.8	162	159	231

Fatty acid values obtained by adding the amounts of all the major individual fatty acids determined by gas-liquid chromatography.

TABLE 4.—*Distribution of Individual Glycerophospholipid Fatty Acids in White Matter Samples*

	NAWM and Normal Tissue					Demyelinated Tissue		
Fatty Acid*	MS 1	MS 2	C 1	C 2	C 3	MS 1	MS 2	MS 3
16:0	17.8	15.0	15.3	14.0	15.3	27.3	23.6	26.2
16:1	2.2	2.1	1.6	1.8	2.0	3.7	4.0	3.1
18:1	21.2	20.6	23.7	20.4	21.4	10.0	14.9	13.4
18:2	43.4	44.0	46.0	42.1	45.4	33.0	35.2	35.8
20:1	6.3	3.8	6.1	7.0	6.3	1.5	1.5	1.8
20:1,6	4.1	5.0	3.7	5.2	4.1	12.0	10.1	10.5
22:5,3	...	...	...	--	...	1.7	2.0	1.5
22:5,6	5.0	7.0	7.6	7.4	5.8	4.1	2.8	2.3
22:6,3	1.1	1.9	1.2	2.2	1.4	6.4	6.3	5.1

* Fatty acids are identified by the total number of carbon atoms, the number of double bonds in the molecule, and, where this is known, by the position of the first double bond, count ng from the methyl end of the molecule. Where the double bond position is not known, the fatty acid is a mixture of isomers differing in the location of the bond.[15]

† Numbers in the table refer to percentages of the total fatty acids in the glycerophospholipid samples. The averages of replicate MS, are shown.

degraded synaptic vesicles undergo hydrolysis before they can proceed more than a few centimeters.

The finding by Svennerholm and ourselves that white matter gangliosides have, on the average, fewer sialic acid residues per ganglioside molecule is consistent with the above. However, recent data from Suzuki[24] on the distribution of individual gangliosides indicates that the actual values differ somewhat with the brain area, some gray areas showing a high proportion of monosialogangliosides. Thus, the nature of the variability requires further study.

Since multiple sclerotic plaques contain other formed elements besides axons, particularly astrocytes, there is the possibility that some of the plaque gangliosides are derived from these cells. Indeed, the abnormal distribution of gangliosides seen in plaques (Fig 4) could reflect such a contribution. However, Lowden and Wolfe,[9] in a study of pathological human cortex exhibiting marked loss of neurons and heavy astrocytic proliferation, found a great decrease in ganglioside concentration. From this it would appear that astrocytes contain little or no gangliosides.

If the gangliosides of white matter are primarily contained in the axoplasm, determination of the ganglioside concentration in white matter may give a value related to the axon density or the state of the axons. A particularly sensitive index of the state of a section

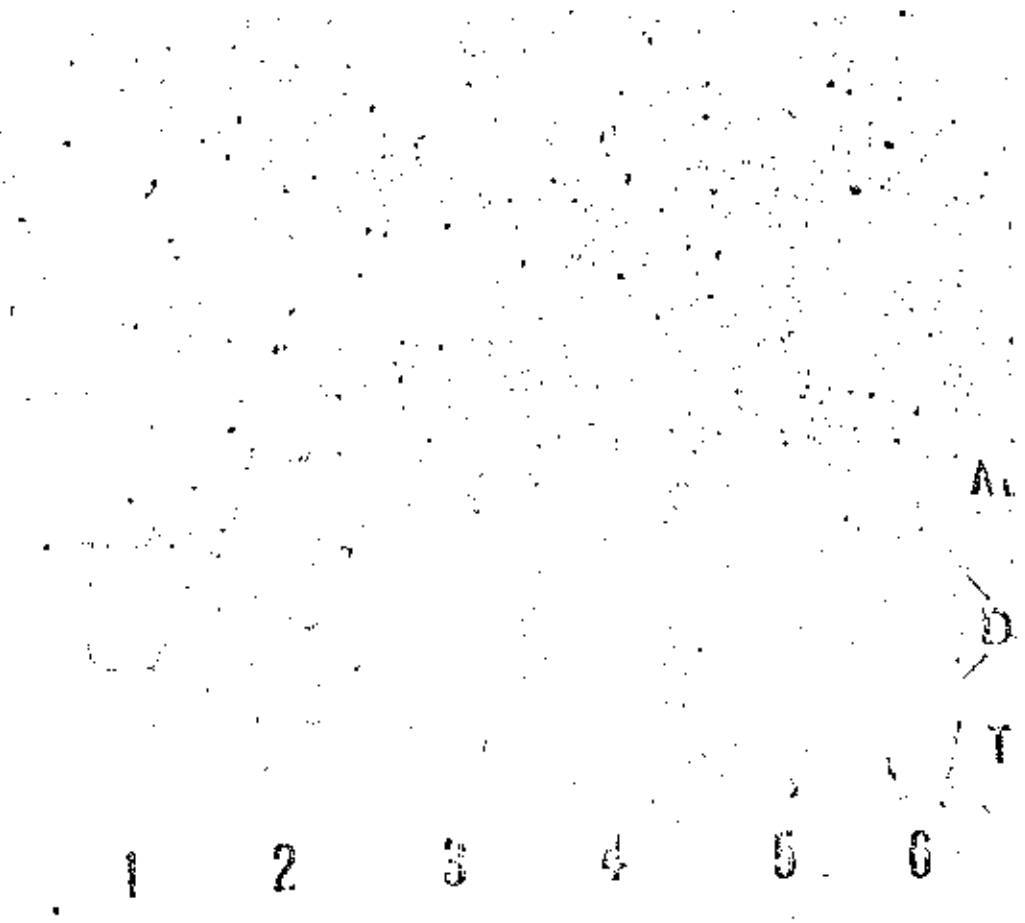


Fig. 4.—Thin-layer chromatogram of gangliosides from different brain areas. *Lane 1*, mixture of standards; *2*, MS 3; *3*, MS 2 plaque; *4*, MS 2 NAWM; *5*, control 1 white matter; *6*, control 1 gray matter. *M* indicates monosialo; *D*, disialo, *T*, trisialogangliosides.

might be obtained if both cerebroside and ganglioside determinations were made. The cerebroside of white matter is probably located mainly in the myelin and is, therefore, a more specific measure of myelin concentration than total lipids, which include the axonal lipids. Such a pair of determinations might aid in distinguishing primary from secondary demyelination.

The Determination of Gangliosides in White Matter.—The method of analysis used in this study, like nearly all other methods for ganglioside determination, is based on previous reports that virtually all the ganglioside is extracted from chloroform-methanol solutions by aqueous potassium chloride. However, recent studies[27,28] have shown that cerebroside sulfate and other lipids tend to hold gangliosides back in the lower layer. The interference is greater with monosialogangliosides, the least polar of the gangliosides. Since white matter extracts are distinctly lighter than brain extracts in total lipids, the extraction method probably yields low values, especially for monosialogangliosides. It is thus very likely that our reported values for white matter are low and that the observed reduction in plaques is even greater than shown in Table 2. This would make the ratio, ganglioside:axonal density index, even more normal in plaques and strengthen our conclusions that the gangliosides of white matter are located in axons and that the axons traversing plaques are relatively normal in lipid composition.

The Fatty Acid Changes.—Comparison of plaque and gray matter composition (Table 5) shows marked similarities. Both have relatively high concentrations of palmitate, 20:4, 22:6, and 22:5ω3, and low concentra-

TABLE 5.—*Comparison of Brain Regions With Respect to the Ester-Linked Fatty Acids*

Fatty Acid	Plaques	Normal * Gray	NAWM	Normal * White
16:0	25.7	23.1	15.4	14.8
16:1	3.6	...	2.2	...
18:0	12.7	22.0	20.9	20.8
18:1	34.7	23.8	43.7	44.0
20:1	1.6	1.1	5.1	4.8
20:4	10.0	8.8	5.0	4.8
22:5	4.0	4.6	6.5	7.7
22:6	5.9	10.7	1.5	3.5

* Values (percentages of total) are averages of data from four control brains, from patients ages 33, 55, 78, and 86 years.[14] Other values are averages taken from Table 4.

tions of 18:1, 20:1, and 22:5ω6. Only the stearate concentration is discordantly low in plaques. However, this discordance may reflect differences in brain samples, and it is relevant to note the considerable variation in the gray:white ratio for stearate in the data of O'Brien and Sampson.[26]

Some of our data can be compared with those of Gerstl et al.[5] who measured the polyunsaturated fatty acids of plaques and other regions by an isomerization ultraviolet absorption method. These workers also found 22:6 to be high in plaques, with values between gray and white matter. Similarly, they found tetraenes (primarily 20:4) to be higher in plaques than in gray matter.

Also in agreement with our findings are the data of Cumings et al.[7] for lecithin fatty acids. The plaques showed an increased proportion of 16:0 and decreased proportions of 18:1 and 20:1; thus, plaque lecithin resembles gray matter lecithin.

Summary

Data are presented for the composition of brain samples from controls and multiple sclerosis patients. Both plaques and adjacent normal appearing white matter from the latter were analyzed. It was found that the ganglioside concentration and axon density in the plaques were below normal, but the decrease was much less than that found for typical myelin lipids. The parallelism in the effect was interpreted to mean that the gangliosides of white matter are primarily in the axons. The fatty acid distribution in the glycerophospholipids of the plaques greatly resembled that of gray matter, rather than white matter. The composition of the normal appearing white matter was close to that of control white matter.

Dr. Samuel P. Hicks and Miss Constance D'Amato assisted in the preparation of the histological sections, and Mrs. Carolyn Seidl assisted in the chemical analyses. Dr. Subhash Basu provided samples of known gangliosides.

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Generic and Trade Names of Drugs

Secobarbital—*Seconal.*
Tolbutamide—*Orinase.*

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BATTLES AND BABIES

In the year 1809, with Napoleon on the march, men's feverish thoughts were on the latest news of the war. And all the while, in their own homes, babies were being born. But who could thing about babies? Everybody was thinking of battles.

In that very year, in the birth lists, were written the names of Gladstone and Tennyson and Oliver Wendell Holmes and Darwin and Abraham Lincoln and Mendelssohn and Samuel Morley. But nobody thought of babies. Everybody was thinking of battles.

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Selective Hydrogenation of Soybean Oil. III. Copper-Exchanged Molecular Sieves and Other Supported Catalysts[1]

SAMBASIVARAO KORITALA, Northern Regional Research Laboratory,[2] Peoria, Illinois 61604

Abstract

Copper-chromium catalysts promote selective reduction of linolenyl groups in soybean oil. Since commercially available catalysts possess only moderate activity, more active catalysts were sought. Copper was dispersed on high-surface-area supports, such as silica, alumina, and molecular sieves. These catalysts had varying activities. Precipitation of copper on Cab-O-Sil, a pure form of silica with a large external surface area, gave the most active catalyst. Selectivity ratios (K_{L_3}/K_{L_2}) for the hydrogenation of soybean oil with these catalysts varied from 4 to 16; a copper-on-Cab-O-Sil catalyst exhibited the greatest selectivity. Improved selectivity and activity were observed when some supports were treated with hydrochloric acid. For example, with a copper-on-Celite catalyst, soybean oil was hydrogenated in 165 min and gave a selectivity ratio of 5.9. Hydrochloric acid treatment of Celite improved the selectivity to 9.9 and reduced hydrogenation time to 54 min. To ensure maximum activity of some of these catalysts, soybean oil should be more thoroughly bleached than is customarily done for nickel hydrogenation. A commercially refined and bleached soybean oil was hydrogenated with a copper-on-Cab-O-Sil catalyst in 18 min. The same oil, re-refined in the laboratory, was reduced in 11.5 min and had the same selectivity ratio of 15.

Introduction

COPPER-CHROMIUM CATALYSTS are useful because of their selectivity for removing linolenyl groups in soybean oil. Since commercial and laboratory-precipitated catalysts exhibit only moderate activity (1), more active catalysts were sought. In the absence of chromium, copper can function as a catalyst if exchanged on a molecular sieve (1). This investigation reports the preparation and performance of copper catalysts prepared on several supports such as silica, alumina, and molecular sieves.

Experimental Procedures

Catalysts were prepared in three different ways. The following examples illustrate the general procedures.

Copper-Exchanged Molecular Sieves. About 3 g of Linde molecular sieve 13X was stirred in 30 ml of an aqueous solution containing 2 g of copper nitrate. After exactly 1 hr the copper-exchanged molecular sieve (Cu:13X) was separated by filtration, washed thoroughly with distilled water, and dried at 100C. Analysis showed that Cu:13X contains 9.4% copper. This catalyst was then treated in two different ways. First, the Cu:13X was suspended in 20 ml of water, and 10 ml of a 5% sodium borohydride solution was added with stirring. The resulting product was separated by filtration, washed with distilled water, and dried (Cu:13X–$NaBH_4$). It was then heated to 350C for 2 hr before use. Alternatively Cu:13X was stirred with 30 ml of a 10% sodium carbonate solution for 0.5 hr. The product was filtered, washed, dried (Cu:13X–Na_2CO_3), and heated to 350C for 2 hr before use. Other catalysts were similarly prepared with 3A, 4A, 5A, 10X, and SK40 (Y) Linde molecular sieves.

Catalysts were also prepared by incorporating copper into sieves according to the procedure of Weisz et al. (2). To 3.7 g of sodium aluminate in 15 ml of water, 2 g of copper nitrate in 10 ml of water was added, followed by 7.2 g of sodium silicate in 35 ml of water. An additional 50 ml of water was added, and the whole mixture was refluxed while stirring for 7 hr. The resulting precipitate was separated by filtration, washed with distilled water, and dried. The catalyst (Cu in sieve $SiO_2/Al_2O_3 = 1.2$) was heated to 350C for 2 hr before use. Another catalyst was similarly prepared with a silica-to-alumina ratio of 2.5. The results of hydrogenation with these catalysts at two different temperatures are given in Tables I and II.

Coprecipitated Catalysts. To a stirred solution of 2 g of copper nitrate in 30 ml of water, 1.76 g of sodium silicate in 15 ml water was added. The resulting blue-green precipitate was separated by filtration, washed with distilled water, and dried. The ground powder was heated to 350C for 2 hr before use. Another catalyst was similarly prepared with sodium aluminate.

Supported Catalysts. These catalysts were prepared by precipitating copper on the support with the addition of stoichiometric amounts of either sodium carbonate or hydroxide. To 50 ml of aqueous solution containing 1 g of copper nitrate, 2.5 g Cab-O-Sil was added. The support was kept in suspension by vigorous stirring while about 439 mg of sodium carbonate in 10 ml of water was slowly added. The precipitate was separated, washed with distilled water, and dried. It was ground to a fine powder and heated to 350C for 2 hr before use. Catalysts were also similarly prepared with Aerosil 2491-380 (Degussa, Kearny, N.J.) and Santocel-62 (Monsanto, St. Louis, Mo.). All other supported catalysts were prepared with 3 g

TABLE I

Hydrogenation of Soybean Oil with Copper-Exchanged Molecular Sieve Catalysts (300 ml oil at 200C and 30 psi)

Expt. No.	Catalyst	Reaction time, min	IV Drop (GLC)	% Linolenate (alkali isomerization)	trans, %	Selectivity (K_{L_3}/K_{L_2})
1	Cu:3A–K_2CO_3	37	15.0	2.1	9.8	5.0
2	Cu:4A–Na_2CO_3	10	10.3	1.4	7.9	10.0
3	Cu:4A–$NaBH_4$	32	14.2	1.5	9.5	7.2
4	Cu:5A–Na_2CO_3	157	12.6	3.1	10.0	4.3
5	Cu:5A–$NaBH_4$	91	14.4	2.7	9.6	4.0
6	Cu:10X–Na_2CO_3	105	14.5	2.5	11.8	4.3
7	Cu:13X–$NaBH_4$ (0.13%)[a]	13	14.7	0.8	8.4	11.0
8	Cu:13X–Na_2CO_3 (0.13%)	34	14.8	1.0	9.9	9.1
9	Cu:SK40–Na_2CO_3	36	10.5	1.7	7.8	9.6

TABLE II

Hydrogenation of Soybean Oil with Copper-Exchanged Molecular Sieve Catalysts (300 ml oil at 170C and 80 psi)

Expt. No.	Catalyst	Reaction time, min	IV Drop (GLC)	% Linolenate (alkali isomerization)	trans, %	Selectivity, (K_{L3}/K_{L2})
10	Cu:4A–Na_2CO_3	60	12.8	0.0	8.8	11.1
11	Cu:13X–Na_2CO_3 (0.26%)[a]	49	13.0	1.4	9.1	8.0
12	4A–Na_2CO_3–$Cu(NO_3)_2$ (0.2%)	22	15.8	1.1	7.2	12.1
13	Cu:SK-10–Na_2CO_3	87	12.2	1.0	9.1	10.7
14	Cu in sieve $\frac{SiO_2}{Al_2O_3} = 1.2$ (0.2%)	12	13.7	0.7	8.7	12.4
15	Cu in sieve $\frac{SiO_2}{Al_2O_3} = 1.2$ (0.1%)	45	13.8	0.8	9.3	12.2
16[b]	Cu in sieve $\frac{SiO_2}{Al_2O_3} = 1.2$ (0.1%)	26	10.7	1.0	6.8	12.1
17	Cu in sieve $\frac{SiO_2}{Al_2O_3} = 2.5$ (0.2%)	15	12.8	1.3	10.1	9.5

[a] Values in parentheses represent catalyst concentration expressed as g of copper oxide per 100 ml of soybean oil.
[b] Re-refined and bleached oil.

of support for 1 g of copper nitrate. These include Celite, Filtercel (Johns-Manville, New York, N.Y.); Silica 923, MS Gel, ID Gel, Syloids 63, 72, and 244 (Davison Chemical Division, W. R. Grace Company, Baltimore, Md.).

Silica and alumina supports were also prepared in the laboratory by bubbling carbon dioxide gas into sodium silicate and sodium aluminate solutions respectively. The resulting gels (SiO_2 or Al_2O_3) were washed with distilled water, dried, and crushed to pass through a 100-mesh screen. Some supports were treated overnight with concentrated hydrochloric acid with occasional stirring. They were then washed thoroughly with distilled water and dried. The analytical results for soybean oil, hydrogenated with coprecipitated and supported catalysts, are given in Tables III and IV.

The commercially refined and bleached oils (FFA 0.04%) contained 7.6 to 8.2% linolenic acid (IV 133 to 137). One batch of oil was re-refined and bleached in the laboratory. The second refining was accomplished by adding to the stirred oil the theoretical amount of 10% sodium hydroxide required to neutralize the free fatty acids (FFA). The formed soap was removed by vacuum filtration over a bed of Celite. By this treatment FFA was reduced to 0.01% or less. The re-refined oil was then bleached at 110–115C with 1% of Super Filtrol-activated carbon mixture (9:1). While the oil was being heated under agitation, nitrogen gas was bubbled through the oil. When the temperature reached 80C, a bleaching earth-carbon mixture was added and heating was continued. Fifteen minutes after reaching the desired temperature, the oil was cooled and filtered under vacuum. The FFA of the bleached oil increased to 0.04%. Bleaching was also performed under vacuum with 0.5% AOCS neutral bleaching earth. FFA did not increase (0.01% or less) with this procedure.

The hydrogenation apparatus and the analytical methods were described previously (1,3). All experiments were performed with 300 ml of oil and with the same speed of agitation of 1,700 rpm. Selectivity ratios (K_{L3}/K_{L2}) were determined with a digital computer, based on a program written by Butterfield and Dutton (4).

Discussion

Selectivity ratios at 200C for the hydrogenation of soybean oil with all copper-exchanged molecular sieves which were tested varied between 4 and 11 (Table I). The most active catalysts made with 4A and 13X (Expt. 2 and 7) were also the most selective for the reduction of linolenate. Treatment with sodium carbonate instead of sodium borohydride gave a more active catalyst with 4A (Expt. 2 and 3) whereas the reverse was true with 13X (Expt. 7 and 8). The structure of molecular sieve 4A was destroyed when copper was exchanged for sodium (5). Also, although triglycerides are not expected to pass into the pores of a 3A molecular sieve, yet hydrogenation did take place with this catalyst (Expt. 1). Copper precipitated on the external surface by adding the metallic salt solution to an alkaline solution containing 4A (Expt. 12, Table II) gave an equally good catalyst. Apparently the catalytic activity of these catalysts (at least 3A and 4A) lay mostly on the external surface. Catalysts with 5A and 10X (Expt. 4–6, Table I) were the least active and selective. Their low activity was probably caused either by the low exchange of

TABLE III

Hydrogenation of Soybean Oil with Supported Copper Catalysts (300 ml oil at 170C and 80 psi; catalyst, 0.1% as copper oxide)

Expt. No.	Support	Precipitating agent	Reaction time, min	IV Drop (GLC)	% Linolenate (alkali isomerization)	trans, %	Selectivity (K_{L3}/K_{L2})
18	Celite	NaOH	105	13.4	2.2	9.3	5.9
19	Washed Celite	NaOH	54	13.1	1.1	8.4	9.9
20	Filtercel	Na_2CO_3	115	7.0	2.7	6.4	7.7
21	Washed Filtercel	Na_2CO_3	40	10.3	2.0	6.1	11.3
22	Silica 923	NaOH	48	13.3	0.9	8.0	10.9
23	Washed Silica 923	NaOH	68	11.1	1.3	8.0	12.3
24	Washed Silica 923[a]	NaOH	54	14.0	0.3	7.7	11.6
25	MS Gel	NaOH	15	12.2	0.7	9.2	13.8
26	Washed MS Gel	NaOH	30	13.1	0.7	10.5	13.1
27	ID Gel	NaOH	30	13.3	0.7	8.7	12.6
28	Washed ID Gel	NaOH	28	12.0	0.0	10.0	13.0
29	Syloid 63	NaOH	28	13.7	0.6	9.3	13.1
30	Syloid 72	NaOH	32	13.8	0.6	9.7	14.3
31	Syloid 244	NaOH	33	13.7	0.8	9.2	13.2
32	Hardrow alumina[b]	Na_2CO_3	57	11.4	1.7	8.5	9.0
33	Al_2O_3	Na_2CO_3	54	13.4	0.6	7.6	14.2
34	SiO_2	NaOH	37	13.8	0.0	9.0	12.1
35	None	$NaAlO_2$[c]	64	13.0	1.3	10.1	9.2
36	None	Na_2SiO_3[c]	40	12.5	1.2	8.9	10.5
37	Raney copper[c]		21	11.0	1.4	7.6	8.6

[a] Treated with 1N hydrochloric acid.
[b] Catalyst concentration 0.2% expressed as g of copper oxide per 100 ml of oil.
[c] Catalyst concentration 1.0%.

TABLE IV

Hydrogenation of Soybean Oil with Supported Copper Catalysts
(300 ml at 170C and 30 psi; catalyst, 0.1% as copper oxide)

Expt. No.	Support	Precipitating agent	Reaction time, min	IV Drop (GLC)	% Linoleate (alkali isomerization)	trans, %	Selectivity (K_{18}/K_{18})
38	Cab-o-Sil M5	Na_2CO_3	13	12.1	0.8	8.8	13.2
39	Catalyst from Expt. 38		25	11.7	0.8	10.0	12.7
40	Cab-o-Sil M5	Na_2CO_3	18	12.0	0.6	9.8	15.5
41[a]	Cab-o-Sil M5	Na_2CO_3	11.6	12.8	0.5	8.6	15.3
42[b]	Cab-o-Sil M5	Na_2CO_3	12.6	12.6	0.5	10.1	16.0
43[c]	Cab-o-Sil M5[c]	Na_2CO_3	16	13.0	0.6	9.1	15.9
44[a]	Cab-o-Sil M5	NaOH	65	11.4	1.4	7.2	11.4
45[a]	Santocel 62	Na_2CO_3	13	13.4	0.8	9.8	14.4
46	Cab-o-Sil EH5	Na_2CO_3	22	15.8	0.6	8.7	12.9
47[a]	Cab-o-Sil EH5	Na_2CO_3	18	12.6	0.6	11.0	15.2
48[b]	Cab-o-Sil EH5	Na_2CO_3	20	13.6	0.5	9.7	16.5
49[a]	Cab-o-Sil M5[d]	Na_2CO_3	18	11.0	1.1	7.9	13.2
50[a]	Aerosil[d]	Na_2CO_3	31	10.0	1.2	7.7	14.9
51[a]	Nickel catalyst[e]		2	16.1	3.8	10.8	2.1

[a] Re-refined and bleached oil.
[b] Vacuum-bleached with AOCS neutral bleach.
[c] 0.75 g of support.
[d] Catalyst concentration 0.05%; 45 psi.
[e] D-15 commercial nickel catalyst (filtrher catalysts, Chemetron Corporation, Louisville, Ky.) 0.04% Ni; 15 psi.

copper or by the presence of calcium which may have inhibited the reaction.

Both 4A and 13X copper catalysts active at 200C showed only moderate activity at 170C (Expt. 10 and 11, Table III). These catalysts probably require prior activation at the higher temperature. Better activity and selectivity were achieved with catalysts made by incorporating copper into molecular sieves while they were being crystallized (Expt. 14–17). The molecular sieve catalyst with the higher silica content gave lower selectivity. Experiments 15 and 16 were performed with two different oils, a commercial refined oil and a laboratory re-refined oil. It can be readily seen that treating the oils in the laboratory improved catalyst activity. This improvement was also true with other catalysts, as will be seen later. The most active catalyst (Expt. 14) promoted the hydrogenation of soybean oil in 12 min when 0.2% copper oxide was used. The most active commercial copper-chromium catalyst under the same conditions required 36 min (1). Thus a copper-in-sieve catalyst was three times more active.

The selectivity achieved with supported copper catalysts varied between 5.9 to 16.5 (Tables III and IV). Washing the supports before precipitating copper improved activity or selectivity, or both (Expt. 18–28). With Celite and Filtercel both selectivity and activity improved markedly. With Silica 923 there was only slight improvement in selectivity but not in activity. With MS and ID Gels, selectivity remained the same, but activity improved. Iron was extracted from the support during acid treatment. The increased activity of the catalysts after acid extraction of the supports was probably attributable to the removal of iron, which may be a catalytic inhibitor.

There does not seem to be any correlation between the activity of the catalyst and the surface area of the support. Among the Davison silica gels (Expt. 22–28), the one with the least surface area (ID Gel) had the greatest activity. The higher activity with the low surface support may have been caused by its large pores, through which triglycerides could diffuse in and out more freely. In spite of the large difference in surface area among the Syloids (300–800 sq m/g), the activity of the catalysts was nearly the same (Expt. 29–31). Laboratory-precipitated silica performed almost as well as the most active commercial silica (Expt. 27 and 34). Laboratory-precipitated alumina had greater selectivity and activity than commercial grade (Expt. 32 and 33).

Raney copper (Expt. 37) catalyzed the hydrogenation of soybean oil even though selectivity and activity were markedly lower than with some of the supported catalysts. This behavior raised the question as to the nature of the active form of copper in the catalysts. Connor et al. (6) maintained that the active agent in copper-chromium catalysts is copper in its divalent state and that, whenever copper is reduced to reddish metallic or monovalent copper, the catalyst is rather inactive. My experience was in accord with theirs, particularly with copper-exchanged molecular sieves 3A, 5A, and 10X and catalysts supported on Celite and Filtercel, which turned red during the reaction. In a recent study (7) it was claimed that the active agent in Cu-Mg-SiO_2 catalyst is in the metallic state. Opposing views on the nature of the active catalyst suggest that further work is required before any definitive conclusions can be reached.

By far the most active and selective catalysts were obtained with fumed silicas (Table IV), which are claimed to be the purest forms of silica available. All their surface area lies on their exterior, and they have no internal voids. Thus there will be no diffusion limitations, and all the copper precipitated on this support is readily available for the triglycerides.

Experiments 38 and 39 were performed with an original batch of oil. Some activity (but no selectivity) was lost during re-use of the catalyst. When a different batch of oil was used (Expt. 40) with the same catalyst, the time of hydrogenation increased considerably. Re-refining and bleaching this oil with either active or neutral bleaching earth (Expt. 41 and 42) improved activity considerably. It was therefore important to bleach the oil more thoroughly than is customarily done for nickel hydrogenation in order to achieve maximum activity with this catalyst. With EH5 Cab-O-Sil however there was no improvement in activity (Expt. 46–48). Even though EH5 Cab-O-Sil and Aerosil has about twice as large a surface area as M5 Cab-O-Sil, their activity was much lower (comparison between Expt. 41 and 48; 49 and 50). As little as 0.75 g of support for 1 g of copper nitrate gave a good catalyst even though the activity was slightly diminished (Expt. 43). Copper supported on Santocel also formed an active and selective catalyst (Expt. 45).

The precipitating agent seems to have a great effect on the activity of the catalyst. When ammonia was used to precipitate copper on Cab-O-Sil, a catalyst with poor activity and selectivity resulted (Expt. 44).

The most active copper catalyst was about nine times less active than a commercial nickel catalyst when the same amount of metal was employed (Expt. 49 and 51). However selectivity of a copper catalyst was much higher than with nickel. The most active copper-chromium catalyst hydrogenated soybean oil in 36 min when 0.2% copper oxide was used (1). The same reaction was complete in 11.5 min with Copper-on-Cab-Sil (Expt. 41) even though much less copper oxide was present.

With all catalysts prepared for and used in this study, about 7–12% *trans* isomers were formed during hydrogenation. Also 1–3% diene conjugation was produced; the higher temperature of hydrogenation favored the formation of conjugation. More than 75% of the original linoleate in soybean oil was retained in the hydrogenated products as measured by alkali isomerization.

ACKNOWLEDGMENT

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CHOLESTEROL VEHICLE IN EXPERIMENTAL ATHEROSCLEROSIS

PART II. PEANUT OIL

D. KRUTCHEVSKY*, SHIRLEY A. TEPPER, DRAGOSLAVA VESSELINOVITCH AND R. W. WISSLER

The Wistar Institute of Anatomy and Biology, Philadelphia, Penn. 19104, and Department of Pathology, Pritzker School of Medicine, University of Chicago, Chicago, Ill. (U.S.A.)

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SUMMARY

In 4 consecutive experiments, young adult, male, Dutch belted rabbits were fed diets, containing 6% of fats of varying fatty acid composition. These fats included coconut oil (CNO), corn oil (CO), peanut oil (PNO), and a special fat (PGF) simulated to resemble peanut oil minus arachidic and behenic acids. All diets contained 2% cholesterol and were fed to the rabbits for 8 weeks. Biochemical and histological findings were then compared.

In the 4 experiments, the following characteristics were generally seen. The rabbits fed corn oil showed a slightly greater weight gain than did the other groups, with the group fed coconut oil exhibiting the least weight gain. The liver weight of the group fed coconut oil was consistently lowest, as was the liver cholesterol content of this group; the liver weight and liver cholesterol levels observed in the other 3 groups were comparable. Serum cholesterol levels were highest in the group fed the special fat and those fed corn oil. Visual grading of the prestained aortas showed the groups fed the special fat or corn oil to have lesions of comparable severity, but less severe than the lesions of the groups fed coconut or peanut oil, which were similar to each other.

The gross visual and microscopic evaluations of the severity of aortic lesions indicated that the rabbits fed coconut or peanut oil had the most extensive, as well as the most frequent and severe, lesions. In contrast, the animals fed the special fat consistently showed much less aortic disease, no matter how the disease was

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* Wistar Professor of Biochemistry, The Wistar Animal Biology, School of Veterinary Medicine, University of Pennsylvania, Philadelphia, Pa. (U.S.A.)

alliated; generally in these animals the aortic involvement was si[illegible]o that seen in the rabbits fed corn oil. The main characteristic of the lesions seen in rabbits fed peanut oil was a prominent intimal proliferation with a high proportion of collagen, whereas in animals fed coconut oil intimal proliferation was over the area of lipid deposition.

The trend in severity and frequency of the coronary artery lesion was similar to that seen in the aorta. Intimal proliferation with lipid deposition was a characteristic finding, regardless of the lipid fed.

These findings support the concept that arachidic and behenic acids, which are present in peanut oil, but not in the special fat, may be responsible, in part, for the unexpectedly greater atherogenic effect of peanut oil. However, our observations suggest that other factors, such as triglyceride structure, may also play an important role in atherogenesis.

Key words: *Arachidic acid – Atherosclerosis – Behenic acid – Cholesterol-fed rabbit – Peanut oil*

INTRODUCTION

Experimental data from several laboratories, including ours, have suggested that the type of fat used in atherogenic diets may affect the gross visual and microscopic character of the experimental lesions in at least 4 different species[1-8]. The atherogenicity of the fat did not necessarily correspond to its degree of unsaturation.

Peanut oil appears to be more atherogenic than would be expected judging by its iodine value alone (95 ± 3). GUESHAM AND HOWARD[2] found that rats developed atherosclerosis when fed a diet containing cholesterol, cholic acid, thiouracil and peanut oil, whereas they exhibited thrombosis when the dietary fat was butter. SCOTT and his coworkers reported similar findings[3]. WISSLER *et al.*[6] have reported that peanut oil was more atherogenic than butter or corn oil when Rhesus monkeys were fed a diet containing 2% cholesterol and 25% fat for 52 weeks.

Peanut oil differs from many other fats in that it contains 5–6% long-chain, saturated fatty acids, principally arachidic (20:0) and behenic (22:0) acids*. To properly assess the contribution of these fatty acids towards the atherogenicity of peanut oil, we estimated the composition of the fat that would result if peanut oil were freed of its arachidic and behenic acids and found that such an oil could be obtained by blending cottonseed (10%), safflower (35%), and olive (55%) oils. We designated this material "PGF". A series of experiments was designed in which rabbits were fed 2% cholesterol plus 6% of different fats. The fats used were coconut

* The trivial names are used instead of eicosanoic (20:0) and docosanoic (22:0) acid

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TABLE 1 [a]

FATTY ACID COMPOSITION OF COCONUT OIL, PEANUT OIL, CORN OIL, AND PGF

Fatty acid	Coconut (CNO)	Corn (CO)	Peanut (PNO)	PGF[b] (calc'd)	PGF[b]
8:0	10.0	—	—	—	—
10:0	6.1	—	—	—	—
12:0	46.7	—	—	—	—
14:0	18.5	—	—	—	0.1
16:0	9.0	11.1	9.7	10.2	9.4
16:1	—	0.2	0.2	0.2	0.7
18:0	2.6	0.8	1.9	2.0	2.5
18:1	5.6	29.5	46.1	48.7	49.1
18:2	0.5	56.0	35.6	37.5	36.3
18:3	—	0.8	1.2	1.3	1.4
20:0	—	0.9	1.6	—	—
22:0	—	—	3.8	—	—

[a] Calc'd for PNO minus 20:0 and 22:0.
[b] 10% cottonseed oil, 55% olive oil, 35% safflower oil.

oil (CNO), corn oil (CO), peanut oil (PNO) and PGF. The experiments with these fats are the basis of this report. The goal was to correlate the presence or absence of lesions with various biochemical measurements made during these studies.

MATERIALS AND METHODS

One hundred-eighty young adult male rabbits of the Dutch belted strain (1700–2000 g) were used in these studies. The rabbits were divided at random into 4 comparable groups in all the experiments. In each experiment the average starting weight of the 4 groups was identical (± 3 g). The diets were prepared by suspending commercial cholesterol in the appropriate oil and thoroughly mixing the suspension with rabbit chow (Purina). The fatty acid composition of these oils is given in Table 1. The final mix contained 2% cholesterol and 6% of the added fat. The rabbits were fed the diets for 8 weeks; venous blood samples were then taken and the rabbits were killed. Complete post-mortem examinations were performed. Livers were removed and weighed, and a sample was taken for cholesterol analysis. The total cholesterol in liver and serum was determined by the method of MANN[9]. The aorta, heart and liver were examined microscopically. The aorta was removed from the animal, cleaned of all adventitial fat, and graded visually for atherosclerotic lesions on a 0–4 scale according to the scheme of DUFF AND MCMILLAN[10]. The parts of each aorta which were to be used for microscopic examination were pinned on wax blocks and fixed for 24 hours in 10% neutral formalin and then stained with Sudan IV according to the method of EGGEN *et al.*[11]. The areas of gross cardiovascular sudanophilia were estimated with the aid of a dissecting microscope and an evaluation of each area was made. The degree of aortic surface area involved was expressed as a percent of the total surface of the aorta. After grading, two blocks of aorta, one from the ascending and the other from the middle part of thoracic aorta, were obtained

TABLE 2

INFLUENCE OF COCONUT OIL (CNO), PEANUT OIL (PNO), CORN OIL (CO) AND PGF ON CHOLESTEROL INDUCED ATHEROSCLEROSIS IN RABBITS[a]

Group	Survival ratio	Weight gain (g)	Liver weight (g)	Cholesterol: liver (g/100 g)	Cholesterol: serum (mg/100 ml)	Atheromata: arch	Atheromata: thoracic	RAE[b]
Expt. 1								
CNO	14/14	335	113.3	3.57 ± 0.29[c]	1743 ± 164	2.9	2.3	1.49
PNO	14/14	214	134.4	5.52 ± 0.42	1844 ± 223	2.4	1.4	1.03
PGF	13/14	213	138.8	4.75 ± 0.37	2193 ± 207	2.5	1.7	0.96
CO	14/14	261	131.3	5.90 ± 0.36	1633 ± 207	2.5	1.7	1.09
Expt. 2								
CNO	10/10	81	112.1	7.38 ± 0.56	1055 ± 99	1.6	1.2	1.33
PNO	10/10	161	132.4	9.35 ± 0.44	961 ± 135	1.6	1.2	1.46
PGF	10/10	117	124.9	9.19 ± 0.68	1424 ± 182	1.3	1.1	0.84
CO	10/10	187	132.5	8.75 ± 0.48	1324 ± 137	1.4	1.2	0.98
Expt. 3								
CNO	10/10	112	100.4	4.15 ± 0.54	696 ± 121	1.7	1.2	2.08
PNO	10/10	239	119.1	6.70 ± 1.08	688 ± 123	2.0	1.3	2.40
PGF	8/10	217	132.5	6.32 ± 0.53	725 ± 139	1.4	0.9	1.59
CO	10/10	329	124.1	3.85 ± 0.53	675 ± 116	1.2	0.7	1.41
Expt. 4								
CNO	11/11	7	98.5	7.70 ± 0.59	1787 ± 258	2.2	1.5	1.04
PNO	11/11	53	105.3	7.69 ± 0.65	1469 ± 226	1.9	1.3	1.09
PGF	11/11	122	119.6	8.47 ± 0.44	1676 ± 218	1.3	1.0	0.69
CO	9/11	141	118.8	8.65 ± 0.76	2017 ± 242	1.5	1.1	0.64

[a] 2% cholesterol in 6% fat fed for 8 weeks.
[b] Relative atherogenic effect.
[c] Standard error.

for microscopic evaluation. When possible, abdominal aorta was also sampled. The sections were stained with oil red O, hematoxylin–eosin, and Gomori trichrome aldehyde fuchsin. Sections of the heart embedded in carbowax were stained similarly.

RESULTS AND DISCUSSION

Results of the 4 experiments are detailed in Table 2. The relative atherogenic effect (RAE) was calculated by dividing the average grade of atheromata by the serum cholesterol level (g/100 ml). This method was found to be effective in correlating the level of cholesterol with the degree of atherosclerotic lesions in previous experiments with a variety of fats[8].

In the first experiment, the CNO fed group exhibited more severe atherosclerotic lesions than did the other 3 dietary groups. In the other experiments, however, it became evident that the atherogenic effect of PNO was similar to that of CNO and that both were considerably more atherogenic than CO or PGF.

TABLE 3

DISTRIBUTION OF ATHEROMATA IN RABBITS FED 2% CHOLESTEROL AND COCONUT OIL (CNO), PEANUT OIL (PNO), SPECIAL FAT (PGF) OR CORN OIL (CO) (AVERAGE OF 4 EXPERIMENTS)

Grade	CNO (44/45)[a]		PNO (45/45)		PGF (42/45)		CO (42/45)	
	arch	thoracic	arch	thoracic	arch	thoracic	arch	thoracic
4.0	5	1	6	1	2	—	3	—
3.5	5	2	—	—	2	1	2	
3.0	2	3	3	1	3	3	3	4
2.5	6	6	7	4	3	2	2	1
2.0	7	5	11	7	10	4	5	2
1.5	9	10	5	4	6	3	13	5
1.0	7	10	7	12	5	15	5	13
0.5	3	7	6	10	11	14	9	17
0.0	—	—	—	1	—	—	—	—
Average	2.15 ± 0.16[b]	1.67 ± 0.14	2.00 ± 0.16	1.31 ± 0.12	1.69 ± 0.16	1.24 ± 0.13	1.71 ± 0.16	1.13 ± 0.12
Average serum cholesterol (mg/100 ml)	1360		1300		1561		1475	
RAE[c]	1.40		1.27		0.94		0.91	

[a] Survival ratio.
[b] Standard error.
[c] Relative atherogenic effect.

The distribution of atherosclerotic lesions in all 4 experiments reflects the results of the individual experiments (Table 3). The order of atherogenicity of the 4 fats, when added to a cholesterol-containing diet, is CNO ≥ PNO > CO = PGF.

There were no definite, consistent differences in weight gain or in liver weight. In general, over the 4 experiments, the average weight gain of the CO-fed group (240 g) was somewhat higher than those of CNO- (152 g), PNO- (168 g), or PGF-fed (168 g) groups. The average liver weight of the CNO-fed rabbits (109.0 g) was slightly lower than in the rabbits fed cholesterol plus PNO (123.4 g), PGF (129.3 g), or CO (130.4 g). The average liver cholesterol levels in the CNO-fed group (5.68 g/100 g) were lower than liver cholesterol levels found in the rabbits fed PNO (7.16 g/100 g), CO (6.82 g/100 g), or PGF (7.08 g/100 g). The average total liver cholesterol levels for each group were: CNO, 6.19 g; PNO, 8.84 g; CO, 8.89 g; and PGF, 9.15 g.

Gross visual and microscopic findings

Table 4 presents findings by gross visualization in aortic lesions before and after staining, as well as a microscopic evaluation of the severity and frequency of aortic lesions. Atherosclerotic lesions mainly affected the aortic arch. Parts of the thoracic and abdominal aorta were also involved, but to a less severe degree.

In animals fed PNO and CNO, 29% and 27% of the intimal surface was involved, respectively, as compared to 21% and 22% in the CO- and PGF-fed rabbits. These are the combined results of all 4 experiments; if each experiment was treated separately, the peanut oil (PNO)-fed rabbits still had the most severe atherosclerosis

TABLE 4

EFFECT OF DIETARY FATS (6%) ON CHOLESTEROL (2%)-INDUCED ATHEROMATA (AVERAGE OF 4 EXPERIMENTS)

Fat	No. of animals	Gross visual		Gross visual stained aorta (area involved %)	Microscopic evaluation of lesions	
		arch	thoracic		frequency (%)	severity[a]
Coconut (CNO)	44	2.18 ± 0.16	1.67 ± 0.14	27 ± 4.1	50	36
Peanut (PNO)	45	2.00 ± 0.16	1.31 ± 0.12	29 ± 4.4	56	37
PGF	42	1.69 ± 0.16	1.24 ± 0.13	22 ± 4.4	38	20
Corn (CO)	42	1.71 ± 0.16	1.13 ± 0.12	21 ± 4.6	35	20

[a] $\frac{\text{Sum of severity}}{\text{Number of animals}} \times 10.$

whereas atherosclerosis was least apparent in the CO-fed animals. Although the gross visual differences in surface area involved between the groups were small, a definite trend toward greater involvement was seen in the aorta of PNO- and CNO-fed animals. Even visualized grossly, the character of the lesions seemed different. The lesions in PNO-fed animals were raised and sometimes very thick, while in the CNO-fed animals the lesions were pale yellow and relatively flat. Although CO-fed rabbits had lesions, they were small, pale and only slightly elevated. Lesions of the PGF-fed group were similar in appearance to those in the PNO-fed group but less severe.

Microscopic examination of the severity and frequency of lesions (Table 4) was carried out on standard aortic sections. The most frequent and severe lesions were observed in PNO- and CNO-fed rabbits. All aortic lesions showed lipid, demonstrable histochemically with oil red O, but the amount varied with the type of fat in the diet. A difference in the characteristics of the atherosclerotic lesions in the aortas of the rabbits fed PNO and CNO was seen consistently.

In the rabbits fed PNO a very prominent intimal proliferation with particularly high proportion of collagen deposition was observed, as compared to the rabbits fed other fats. In Fig. 1, a typical lesion in the thoracic aorta of a PNO-fed rabbit can be seen. It is characterized by predominantly cellular proliferation, composed of a cap of fibro-elastic tissue with a small amount of lipid giving the whole lesion a dense appearance. When Gomori trichrome aldehyde fuchsin stain was used, the aortic lesion in PNO-fed rabbits showed that the smooth muscle cells were separated by the abundant collagen and elastin in which they were embedded.

The animals fed CNO had various sized, proliferative, pale yellow lesions which, on microscopic examination, revealed remarkable intimal proliferation characterized by less collagen and elastin and more intracellular lipid. Much of the stainable lipid in both types of lesions appeared to be diffusely deposited in or on smooth muscle cells of innermost media (Fig. 2). Lipid deposition inside pre-existing medial cells in aorta was present in all of the groups, but was most frequent in CNO-fed animals. Except for these differences, there were histological features common to both the

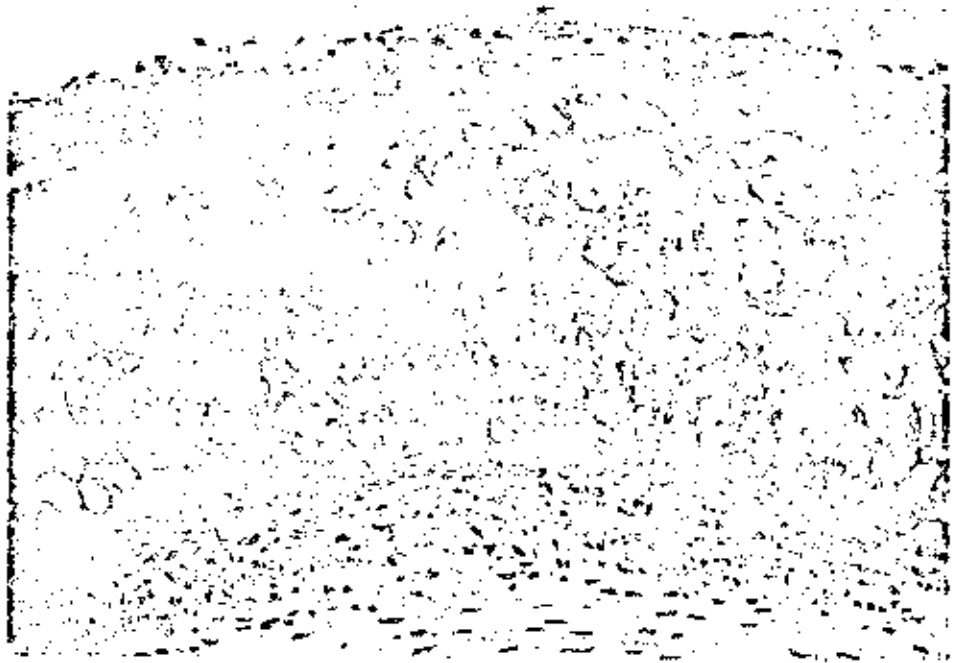

Fig. 1. Photomicrograph of a lesion in the thoracic aorta from a PNO-fed rabbit. The lesion is characterized by pronounced proliferation with abundant intracellular material which is predominantly collagen with some elastin. Oil red O; × 195, reduced in reproduction × 0.75.

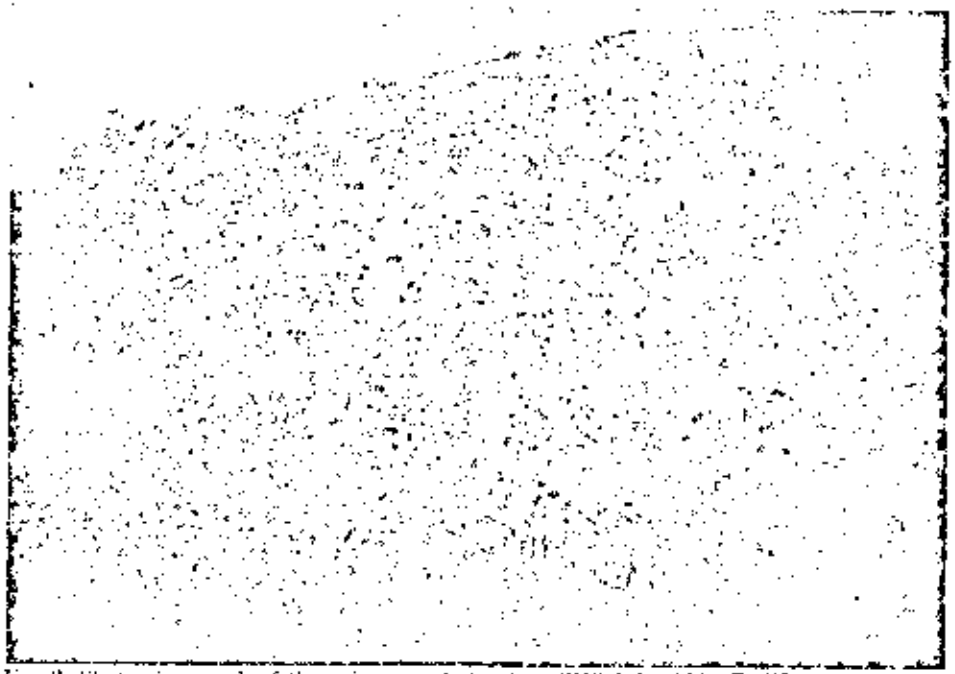

Fig. 2. Photomicrograph of thoracic aortic lesion in a CNO fed rabbit. Proliferation over lipid deposition was a frequent finding in CNO-fed animals. Oil red O; × 195, reduced in reproduction × 0.75.

PNO- and CNO-fed groups. The lesions in PGF-fed animals also had some similarities with those of the PNO-fed rabbits, but they were less severe (Fig. 3). The lesions in the CO-fed rabbits were characterized by less lipid deposition in pre-existing cells and by mild intimal proliferation (Fig. 4).

A bar-graph (Fig. 5) summarizes the results of microscopic grading of several of the histopathological characteristics of the lesions based on standard sections taken from each aorta. A tendency toward collagen deposition was most pronounced in PNO-fed animals. On the other hand, a more consistent increase in lipid deposition inside pre-existing medial cells was seen in aortas of CNO-fed rabbits, giving the appearance of a softer atheroma.

Table 5 presents the frequency and severity evaluated microscopically of the lesions in the coronary arteries. The occurrence of lesions was highest in the group fed PNO and lowest in the group fed CO. The more advanced coronary artery lesions in PNO-fed rabbits were characterized by intimal proliferation and lipid deposition in both the intima and media. Lipid-rich proliferative intima sometimes resulted in severe stenosis of the intramural coronary vessels. Qualitative differences among coronary lesions were not great in spite of the different fats fed.

Myocardial infarction was observed in one rabbit fed PGF. The infarct was located on the endocardial side of the left ventricular wall and involved almost one-third of the wall thickness. The coronary artery in the vicinity of the infarct, although not completely occluded, exhibited severe atherosclerotic lesions. Most of the fibers

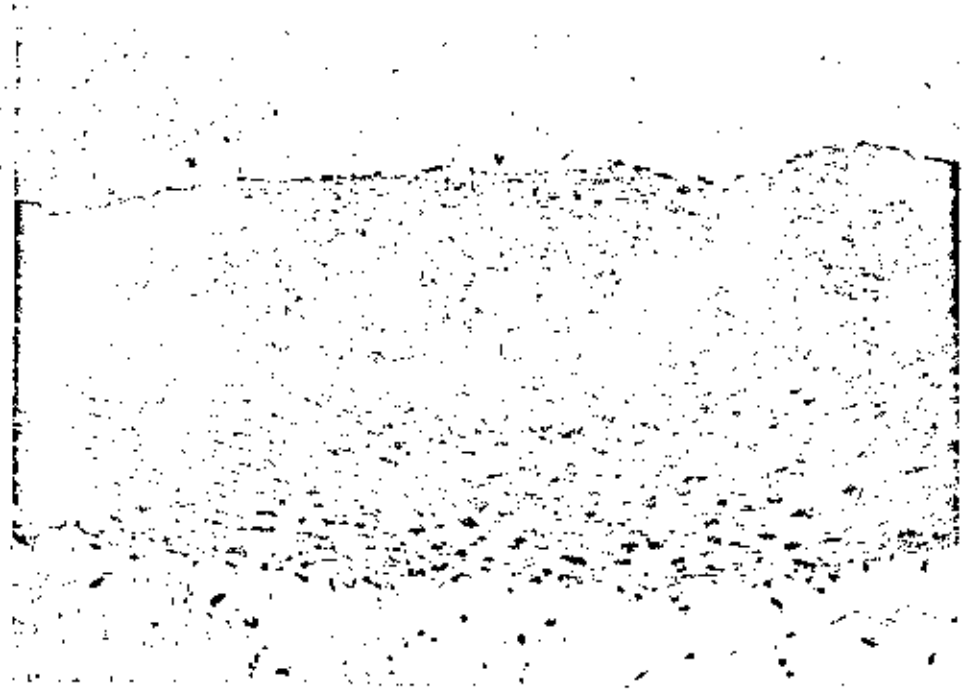

Fig. 3. Photomicrograph of thoracic aortic lesion of a PGF-fed animal. It is similar to the lesions produced by peanut oil, only less severe. Oil red O; × 195, reduced in reproduction × 0.75.

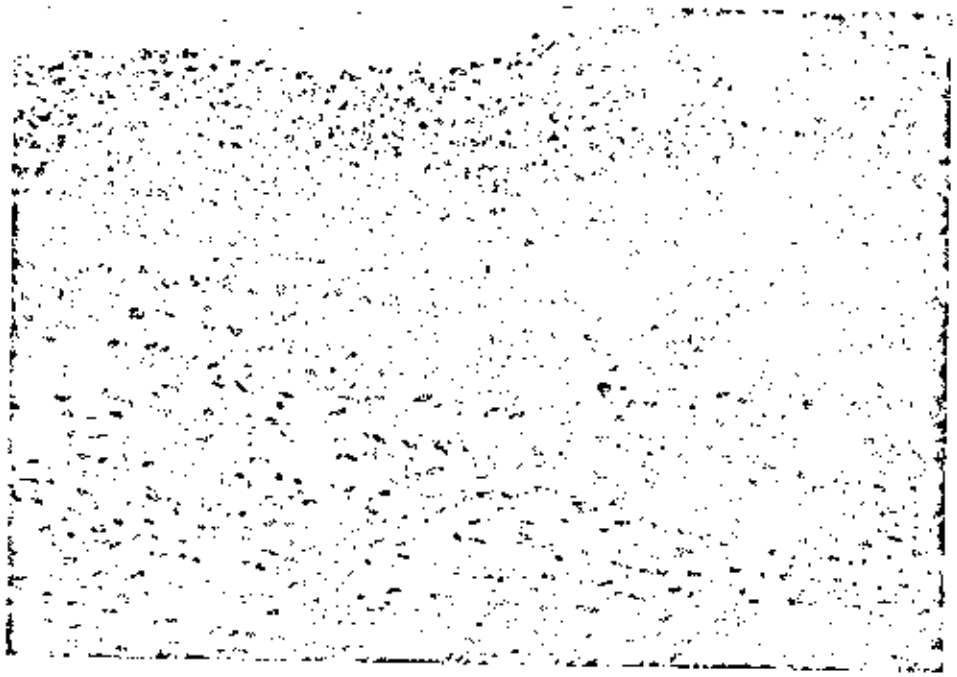

Fig. 4. Photomicrograph of thoracic aortic lesion in a CO-fed rabbit. Deposition of lipid in pre-existing cells and mild intimal proliferation were seen. Oil red O; × 195, reduced in reproduction × 0.75.

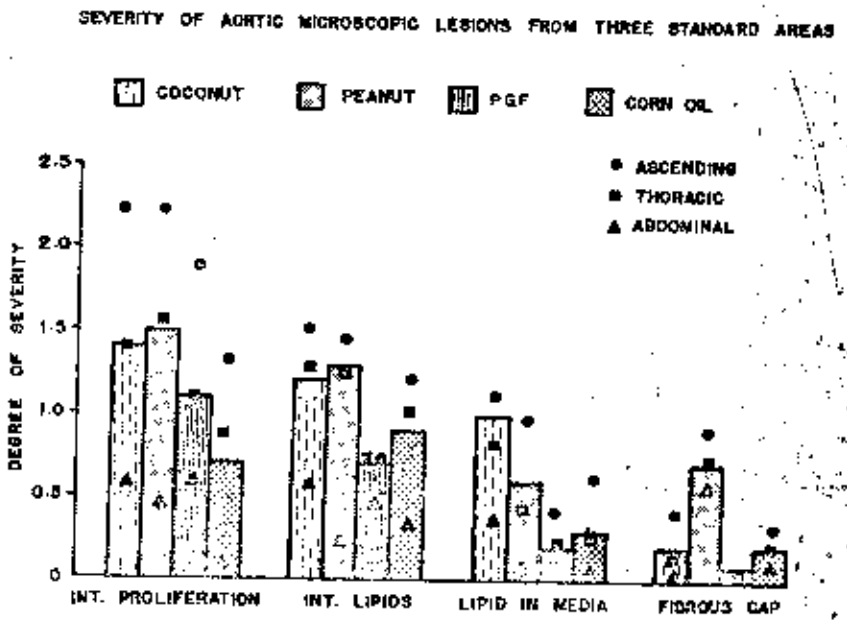

Fig. 5. Bar graph illustrates microscopic grading of several characteristics of the aortic lesions, based on standard sections taken in each aorta.

TABLE 5

MICROSCOPIC LESIONS IN CORONARY ARTERIES

Group	Treatment	Microscopic evaluation of lesions	
		frequency	severity
I	coconut oil	31	13
II	peanut oil	42	28
III	PGF	31	14
IV	corn oil	21	10

were coagulated and their nuclei had disappeared. There was a fairly good demarcation between normal and ischemic tissue. Architecture was visible in some parts as a shadowy outline. Inflammatory exudation infiltrated only partly into the necrotic focus (Fig. 6).

Fatty liver changes were observed in most animals regardless of the diet fed. Although there was no remarkable difference among the dietary groups, still there was a slight tendency for the fatty changes to be more severe in the PGF-fed rabbits and slightly milder in the CO-fed animals.

Pathologists have been puzzled about the factors responsible for the progression of atherosclerosis. It appears that the chemical nature of the lipid deposited in the aortic wall may, at least partly, determine the quantity and quality of atherosclerotic lesions, as well as the rate of progression from fatty streak to fibrous plaque.

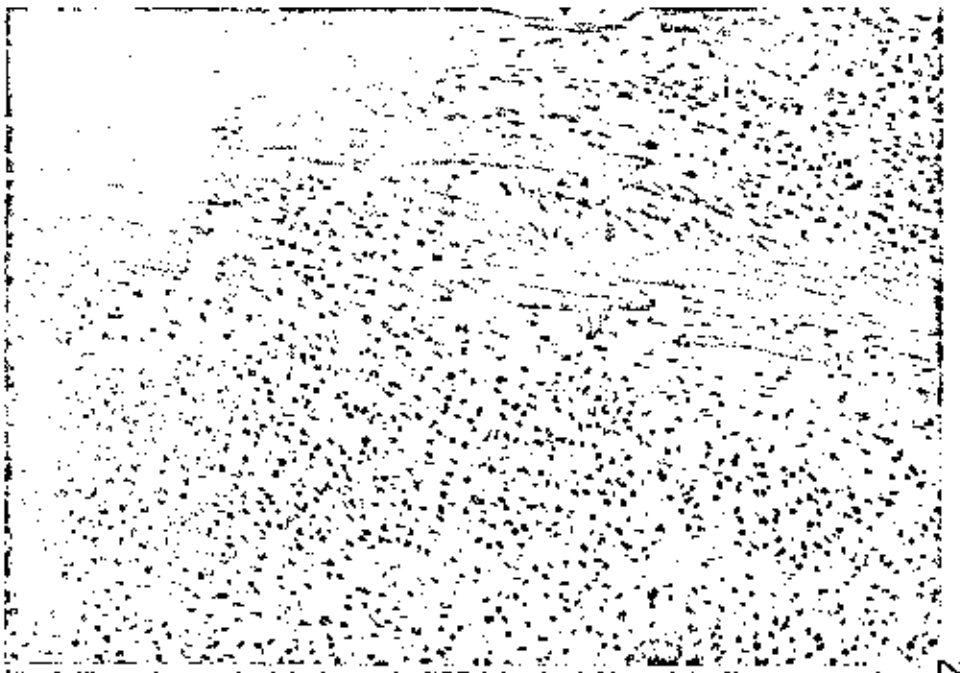

Fig. 6. Photomicrograph of the heart of a PGF-fed animal. Most of the fibers are coagulated; their nuclei have disappeared. H.-E.; × 195, reduced in reproduction × 0.75.

There veral possible ways to explain the pathogenetic patterns of atherosclerosis caused by the various food fats. One possibility is that some types of lipid may be metabolized more effectively by the liver and the artery wall than others. There may be either a greater gastrointestinal absorption of the dietary cholesterol or a relatively retarded catabolism of the exogenous cholesterol associated with the consistent accumulation of stainable aortic lipid in the CNO-fed rabbits. This deposition of stainable lipid in the aortic walls, particularly in the smooth muscle cells of CNO-fed animals, suggests that these arterial cells may be critical in the metabolism of lipid, particularly that brought to them by the blood stream in the form of lipoproteins. This has been suggested before by GEER *et al.*[12] and WISSLER *et al.*[13]. A further possibility is that some types of lipids when deposited within cells of the aortic wall may lead to a more severe metabolic reaction than others. This reaction is probably at least partly responsible for the characteristic morphology of the lesions. Only further experimentation will resolve this question.

The results of the gross visual and microscopic examinations of the aortas of the rabbits fed cholesterol plus the 4 different fats would, at first, appear to uphold the obvious suggestion that the factor that causes peanut oil to exert an atherogenic effect more severe than would be expected from its level of unsaturation is the presence of arachidic and behenic acids. However, several factors suggest a more complex reason for our observations. First is that the lesions of PGF-fed rabbits morphologically resemble the lesions of PNO- rather than CO-fed rabbits. This might be explained on the basis of the level of unsaturation. However, we have previously shown, in a comparison of coconut oil, lard, partially hydrogenated corn oil and corn oil, that atherogenicity may be correlated with iodine value[7,8]. On this basis, PGF should be more atherogenic than CO, whereas, in fact, it is not. The iodine values of the 4 fats used in this experiment were: CNO, 8; PNO, 95; PGF, 105; CO, 126. While PGF has a gross fatty acid composition similar to that of PNO, the structure of the component triglycerides of PGF must be considerably different from PNO since 3 different oils were used for preparation of this fat. Hence, some aspect of triglyceride structure must also be important to cholesterol absorption, transport and deposition. MATTSON AND VOLPENHEIN[14,15] have shown that the percentage of component fatty acids in the oils used in these experiments varies; the percentage of fatty acids in the 2-position of the triglycerides, however, was calculated to be comparable for PNO and PGF.

The primary fatty acids present in the 2-position of the triglycerides of all the fats used were oleic (18:1) and linoleic (18:2). These 2 fatty acids represented about 95% of all the fatty acids present in this position. The ratio of 18:1/18:2 in the 2-position of CO and PNO triglycerides is 0.36 and 0.59, respectively; for PGF it is calculated to be 0.88. Thus there is little difference in the structure of the 2-monoglycerides which arise during digestion and absorption of PNO and PGF. Possibly, then, the structure of the intact triglyceride may be important or the specific free fatty acids that arise on lipolysis may exert an effect.

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Studies on Monoene Fatty Acid Isomers in Hydrogenated Fish Oils[1]

GEORG LAMBERTSEN, HAKON MYKLESTAD and OLAF R. BRAEKKAN
Government Vitamin Laboratory, P.O. Box 187, 5001 Bergen, and Institute of Physiology, University of Bergen, Norway

ABSTRACT

An analytical study of the geometrical and positional isomerisation of the monoenoic acids of partially hydrogenated fish oil is presented. The results showed that the monoene fatty acids of chain lengths 16, 18, 20 and 22 consisted of 75% in the *trans*-form and 25% in the *cis*-form. The double bonds were distributed symmetrically over the chain length, with well defined maxima in position Δ-9 for the fatty acids of chain length 16 and 18, and in position Δ-11 for the fatty acids of chain length 20 and 22. *Trans*- and *cis*-isomers showed the same positional distribution. Geometric as well as positional isomerization seemed to have reached an equilibrium state in the sample investigated.

INTRODUCTION

Fish oils produced by reduction of whole fish of the species herring, mackerel, capelin and some of the smaller species of the cod family from the North Atlantic area are used as raw material for the production of margarine. Lambertsen and Braekkan (1) determined the fatty acid composition of selected types of these fish oils and found a content of 45-65% of monoene fatty acids of the chain lengths 16, 18, 20 and 22 with each acid representing 5% to 20% of the total oil. Similar findings are reported by Ackman et al. (2,3) for species caught off the Canadian east coast. After industrial hydrogenation of these oils to melting points between 30 C and 40 C the percentages remain approximately the same, whereas polyenes decrease and saturates increase proportionally (4). The monoene fatty acids are, however, extensively isomerized as to configuration and position of the double bonds. The theory behind these conversions has been discussed by Dutton (5). The present paper reports results from an analytical study of the content of such isomers in hydrogenated fish oils. The methods are those generally applied for the analysis of hydrogenated vegetable oils.

METHODS

An outline of the analytical procedure is given in Figure 1.

Five grams of a refined and hydrogenated fish oil (melting point 38 C) were saponified, the fatty acids isolated and methylated in methanol containing BF_3. Details of these steps were reported in a previous publication (4). The methyl esters were analysed for fatty acid composition by gas liquid chromatography (GLC) and the results corresponded well with the findings previously reported for this type of fat.

The methyl esters were fractionated into groups by partition chromatography on columns 9 x 1000 mm. The stationary phase was silanized celite (Kieselguhr Merck) saturated with a purified paraffin of boiling point 200 C. The eluent was acetonitril-methanol (85:15) saturated with the paraffin. On this column, 200-400 mg could be separated and 100-200 fractions of 1 ml each were collected in a fraction collector. The fractions were checked by thin layer chromatography (TLC) and corresponding fractions were pooled. In a few cases fractions were rechromatographed to obtain better purity.

The four fractions (Fig. 1) were each further fractionated by preparative argentation TLC. Glass plates (20 x 20 cm) were prepared with Silica Gel G (Merck) containing 25% silver nitrate. The methyl esters were spotted along the base line. Each plate was loaded with 15-20 spots each containing 0.8 to 1 mg methyl esters. The plates were developed with 15% ethyl ether in hexane. After visualizing the spots with 0.01% 5,6-dichlorofluorescein in water, the *trans*- and *cis*-fractions were scraped off and extracted from the silica gel with ethyl ether and purified by chromatography through small columns of silica gel. By this procedure eight fractions weighing from 20 to 135 mg were collected, corresponding to the *trans*- and *cis*-monoenes of the chain lengths 16, 18, 20 and 22 carbon atoms. The total

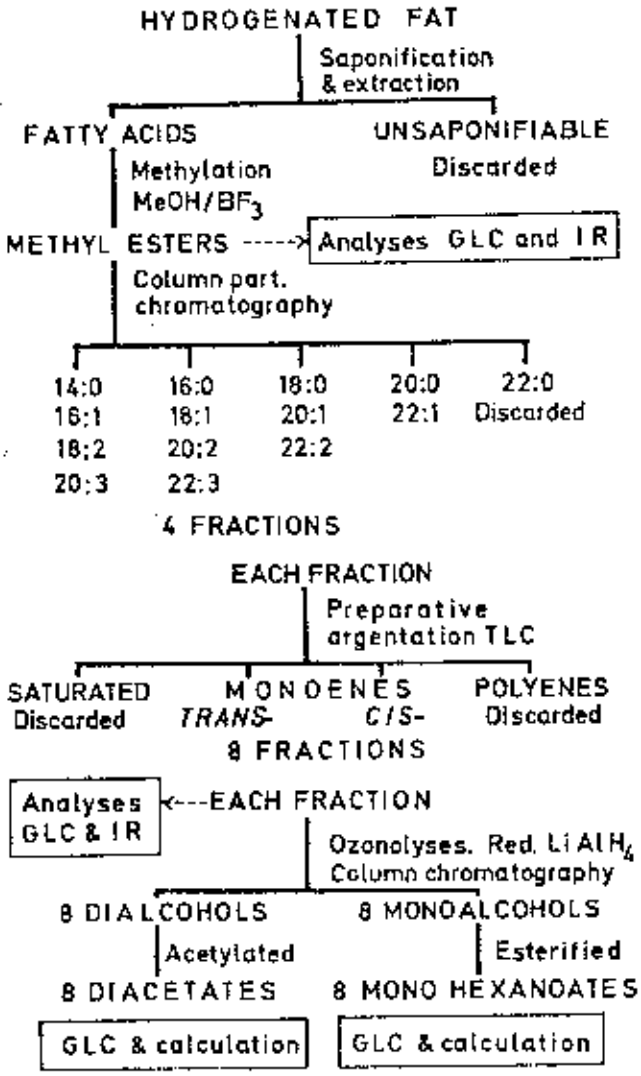

FIG. 1. Analytical procedure.

[1]Presented at the ISF-AOCS World Congress, Chicago, October 1970.

TABLE I

Relative Peak Areas From GLC of Diacetates From Monoenoic Acids in Hydrogenated Fish Oil Cleaved by Reductive Ozonolysis[a]

Chain length of monoenoic acid	Geometric isomer	Chain length of dialcohol									
		7	8	9	10	11	12	13	14	15	16
16	*cis*	15	60	100	80	52	27	16	10		
	trans	10	56	100	90	54	38	21	12		
18	*cis*	18	60	100	94	80	55	36	25		
	trans	12	67	100	98	88	73	45	--		
20	*cis*	14	37	61	78	100	71	42	25	13	8
	trans	8	18	46	73	100	73	47	29	20	13
22	*cis*		20	49	77	100	67	41	20	13	9
	trans		12	30	68	100	76	43	24	16	12

[a]Maximal peak area, 100.

weight of the fractions was 500 mg.

The final fractions, as well as the four fractions before LC, were dissolved in hexane and their *trans* content determined in an Unicam SP 200 G IR-Spectrophotometer. The *trans* peak was measured at 965 cm-1 and calculated compared with the carbonyl peak at 1720 cm-1. Mixtures methyl esters of elaidic acid and oleic acid in graded proportion were used as reference. In a recent publication Allen (6) has applied the same principle. All eight fractions were further analyzed for their contents of monoene fatty acids of each chain length by GLC.

The methyl esters in the eight fractions were cleaved at the double bond by means of reductive ozonolysis. To the fractions in pentane was added a saturated solution of ozone in pentane (7). The ozonides were reduced by adding a small amount of solid lithium aluminium hydride, and the complexes hydrolyzed by careful addition of dilute sulfuric acid. This hydrolysis was carried out at a temperature around 0 C. The mixture was diluted with water and the mono- and dialcohols extracted with ethyl ether. Hexane was added to this extract to give a final mixture of ethyl ether-hexane 20:80, followed by chromatography through a small column of alumina which retained the dialcohols giving a solution of the monoalcohols. The dialcohols were subsequently eluted with pure ethyl ether.

The eight dialcohol fractions were acetylated by acetic anhydride in purified pyridine and dissolved in hexane.

The eight monoalcohol fractions were esterified with hexanoyl chloride in pyridine, and the resulting hexanoates purified and dissolved in hexane.

These final 16 ester fractions were analyzed by GLC in a Perkin Elmer 900 Gas Chromatograph. All separations were carried out on 6 ft 1/8 in. stainless steel columns with 80-100 mesh silanized celite to which was added 15% butanediol succinate polyester as a stationary phase. The temperatures were monitored to obtain a 45 min run on each type of fraction, usually around 170-190 C. All GLC runs were isothermal to give exact "semilog plots" for chain length identification. Weight percentages were calculated from peak areas as height x width at half height.

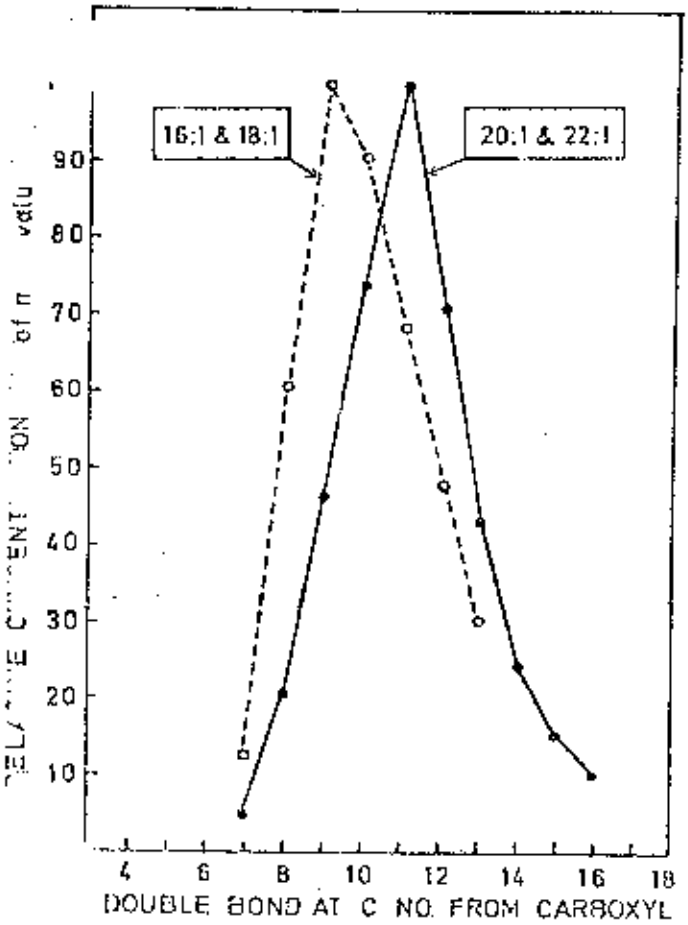

FIG. 2. Double bond positions in monoene acids of hydrogenated fish oils from dialcohol GLC. Mean concentrations for *cis* and *trans*-isomers.

RESULTS AND DISCUSSION

To achieve fractions of sufficient quantities for the subsequent ozonolyses two major chromatographic steps had to be used. The first of these, the column partition chromatography, does not provide a complete separation according to the scheme outlined in Figure 1. In our experience a purity around 80% was achieved but quantities up to 500-1000 mg could be prepared within a few days. The recently introduced high pressure columns may give improved separation if applied to this problem.

The agentation chromatographic step is mostly used to separate according to unsaturation. Our problem was to achieve a separation between *cis*- and *trans*- isomers. Attempt to apply columns to this operation failed to give a good enough separation and TLC was applied. The high load of 25% silver nitrate to the silica gel gave a sufficiently clear separation of *cis*- and *trans*- monoenes (8). As determined by GLC, 80.9% monoenoic acids together with 12.8% saturated acids and 6.3% dienoic acids could be recovered totally. Small amounts of saturated acids of chain lengths 14 and partly 16 had moved with the *trans* acids but were not considered since they did not ozonolyse. A part of the dienoic acids moved with the *cis*-acids and resulted in a high *trans* value for these acids. A calculation of this value taken together with the gas chromatographic results showed that they were *trans-trans* dienes of the next higher chain length. UV absorption of the fraction only showed the presence of minor amounts of conjugated

double bonds. This systematic error could only be observed and taken into consideration. Summing up, the monoenoic acid analyses gave the values. 6.4% 16:1, 17.4% 18:1, 11.5% 20:1 and 11.1% 22:1, sum 46.4%. This result corresponded well with the original GLC analysis of the fat.

The percentage of *trans*-isomers in the monoene fractions could be measured by two different methods. First, the *trans* values were measured by IR spectrophotometry. A value of 75.4 ± 3% was found for the four chain lengths. Secondly, trans values could be calculated from the gas chromatographic data of the eight fractions. By this method a value of 74.4 ± 1.2% was found for the four chain lengths. As these variations are within the analytical accuracy of the methods, it may be concluded that the hydrogenated marine fat studied had 75% of its monoenoic acids as *trans* isomers and 25% as *cis* isomers, regardless of chain length. These results are within the equilibrium values found by previous investigators, from 2:1 for oleic acid-elaidic acid up to 7:1 for hydrogenated vegetable oils (9).

Ozonolysis of the double bond is the preferred method for establishing its position (7). Usually the fragments are determined after reduction to aldehydes. Our working routine and the number of different fractions made it necessary to consider more stable fragments than the aldehydes. Complete reduction with lithium aluminum hydride gives the corresponding alcohols and further a reduction of the ester groups takes place. Thus a homologous set of *n*-1-alcohols and a corresponding homologous set of *n*-1-ω-dialcohols are obtained. These groups were easily separated by column chromatography on alumina followed by esterification to obtain suitable and stable compounds for GLC analysis. The diacetates gave very clear series of peaks. Relative values for the chainlengths of these alcohols are given in Table I. The results for *cis*- and *trans*-isomers were closely related and one set from chain lengths 16 and 18 and one from 20 and 22 was averaged and are shown in Figure 2. The figure shows an even distribution of the double bond position around a maximum at Δ-9 for the two lower chain lengths and at Δ-11 for the two higher chain lengths. This corresponds to the ω-positions 7, 9, 11, 13 for respectively chain lengths 16, 18, 20, and 22. Ackman and Castell (10) have determined the positions of the double bond in monoenoic acids of herring oil, and found the same pattern for the major isomers.

The monoalcohol esters showed some losses and additional peaks in the chromatograms. This made calculations for the monoalcohols less precise but, at least for the chain lengths 20 and 22, the same distribution as for the dialcohols could be established.

The even distribution of the double bond position after hydrogenation corresponds with the results of Scholfield et al. (11) for the *trans* acids. The results with regard to geometric as well as positional isomerization indicate that an equilibrium state has been reached during the hydrogenation of the sample investigated. A recent report by Hølmer and Aaes-Jørgensen (12) gives results from the analysis of partially hydrogenated herring oil. Their values correspond to the present findings with regard to the chain length composition of monoenes, but their *trans* values range from 21 to 49 F%. This may reflect differences in the processing of the oil, resulting in less extensive isomerization.

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CHAPTER 5

Donald C. Malins
and John C. Wekell

Chromatographic Methods

INTRODUCTION

Current interest in the composition of the lipids of fish and other marine animals demands the systematic application of highly refined and discriminating methods of analysis. Over the last few years, a variety of chromatographic techniques were developed that fulfill these requirements. Accordingly, several promising methods of solid-liquid and liquid-liquid chromatography based on adsorption, silver π-complexes, and partition equilibria will be outlined. The techniques are discussed individually, but each is best exploited when used imaginatively in conjunction with complimentary methods such as gas-liquid chromatography (GLC).

ADSORPTION CHROMATOGRAPHY

Adsorption chromatography is used primarily for the resolution of mixtures *into* classes of compounds such as hydrocarbons, steryl esters, and triglycerides. In some cases, however, separations *within* classes can be accomplished by employing special adsorbents (e.g., hydroxyl apatite) or derivative techniques (e.g., mercuric acetate addition compounds).

Class separations of lipids are affected by employing either *column* or *open face* techniques. Column chromatography is used to its best advantage for the preparative isolation of gram-amounts, whereas the open face techniques, paper chromatography and thin-layer chromatography (TLC), can be exploited for both analytical and micro preparative purposes.

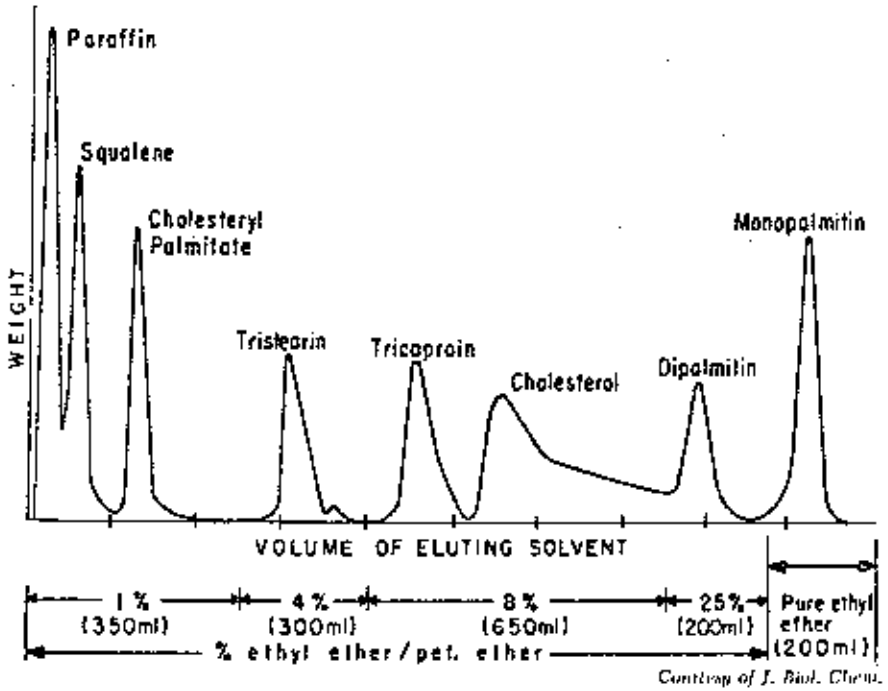
Column chromatography is widely used for the fractionation of complex mixtures of lipids, particularly those of biological origin. Best results with fish oils are generally obtained by employing carefully controlled conditions (e.g., temperature, flow rate, and particle size) and gradient elution techniques.

Hirsch and Ahrens (1958) developed standardized methods of gradient elution chromatography on columns of adsorbents for the fractionation of natural mixtures of lipids. Figure 5 shows a weight curve obtained by these workers after fractionating a mixture containing paraffin, squalene, cholesteryl palmitate, tristearin, tricaproin, cholesterol, dipalmitin, and monopalmitin. The adsorbent and gradient elution system (petroleum ether with increasing amounts of diethyl ether) employed by Hirsch and

Ahrens [illegible] erally well suited to the separation of the neutral lipids (including fatty acids) of fish oils.

The fractionation of phospholipids requires the use of more polar solvent systems. The system described by Silk and DeKoning (1964) for resolving the phospholipids of South African pilchard oil, notably elution of silica gel columns with various proportions of chloroform-methanol (e.g., 7:1 ratio), is recommended for general application to the fractionation of the phospholipids.

There are several noteworthy examples of the fractionation of fish oils by chromatography on adsorption columns. Lovern (1956) employed gradient elution chromatography on silicic acid for the fractionation of the neutral lipids of haddock. Fractions concentrated in hydrocarbons, steryl esters, wax esters, triglycerides, and sterols were isolated. Highly pure steryl esters, triglycerides, and hydrocarbons were obtained by rechromatographing these fractions. Brockerhoff and co-workers (1964), also employing gradient elution chromatography, resolved the neutral, acidic, and basic lipids extracted from cod, trout, and lobster. Fractions consisting of steryl esters; triglycerides and fatty acids; cholesterol and acidic phospho-



Courtesy of J. Biol. Chem.

FIG. 5. ABSORPTION COLUMN CHROMATOGRAPHY OF LIPIDS

lipids; and lecithins were obtained. The lecithin fra[illegible] usually contained small percentages of sphingomyelin and phosphat[illegible] inositol.

Paper chromatography can also be employed for the resolution of micro amounts of neutral, acidic, and basic lipids derived from marine species. Rouser and co-workers (1963) demonstrated the fractionation of the lipids of the sea anemone (*Anthopleura elegantissima*). Neutral lipid fractions containing steryl esters, triglycerides, fatty acids, and cholesterol were well resolved on paper impregnated with silicic acid. For example, the phospholipids of this series were resolved by chromatography on alkaline silicic acid paper into phosphatidyl ethanolamine, phosphatidyl serine, ceramide aminethylphosphonate, and lecithin. The solvent systems described by Rouser and co-workers (1963) for the resolution of the neutral lipids (hexane-diethyl ether, e.g., 95:5 v/v) and phospholipids (various proportions of chloroform-methanol-water) of the sea anemone are generally useful for the chromatography of the lipids of fish on silicic acid impregnated papers.

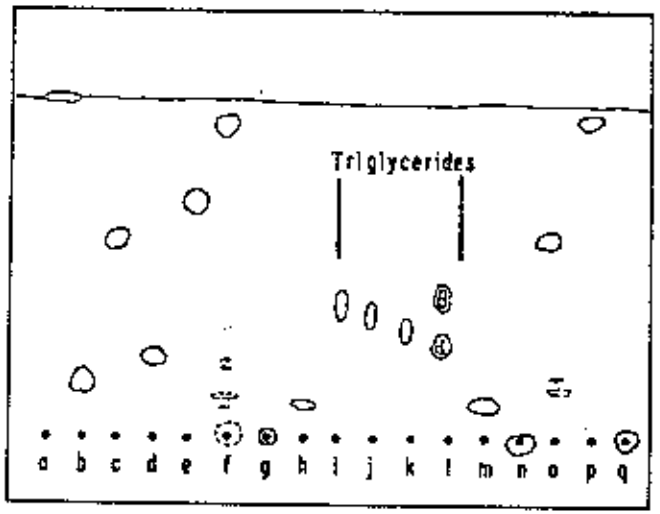


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FIG. 6. THIN-LAYER ADSORPTION CHROMATOGRAPHY OF LIPID CLASSES ON SILICA GEL

Solvent system: 90:10:1 petroleum ether (b.p. 60–70° C.)-diethyl ether-acetic acid, v/v/v. Indicator: 2′, 7′-dichlorofluorescein, 0.2% in ethanol. a, 9-octadecene; b, oleyl alcohol; c, oleylaldehyde; d, oleic acid; e, methyl oleate; f, cholesteryl oleate; g, monoolein; h, diolein; i, triolein; j, trilinolein; k, trilinolenin; l, tributyrin (α) plus tristearin (β); m, cholesterol; n, selachyl alcohol; o, chimyldiolein; p, oleyl oleate; q, dioleoyl lecithin.

Courtesy of Academic Press

FIG. 7. THIN-LAYER CHROMATOGRAPHY OF FISH OILS HAVING VASTLY DIFFERENT CLASS COMPOSITIONS

Solvent systems: 90:10 petroleum ether (b.p. 30–60°C.)-diethyl ether, v/v. Indicator: chromic-sulfuric acid-charring. (a) Coelacanth (Latimeria chalumnae) swim-bladder oil, predominantly wax esters. (b) Ratfish (Chimaera monstrosa) liver oil, predominantly diacyl glyceryl ethers (upper spot) and triglycerides (lower spot). (c) Menhaden oil, predominantly triglycerides.

Lipid mixtures can be separated readily into classes of compounds by TLC on adsorbent layers (Fig. 6). Some useful indicators for detecting these classes on chromatoplates are iodine vapors, chromic-sulfuric acid, and 2′,7′ dichlorofluorescein (Mangold and Malins 1960).

Hydrocarbons, steryl esters, wax esters, diacyl glyceryl ethers, triglycerides, fatty acids, and diglycerides—common constituents of fish oils—are fractionated on thin layers of silicic acid in less than one hour by elution with 90:10:1 petroleum ether-diethyl ether-acetic acid.[1] Glyceryl ethers, monoglycerides, and compounds of comparable polarity can be resolved from these lipids and from phospholipids by elution with 60:40 petroleum ether-diethyl ether.

Mangold and Malins (1960) demonstrated the resolution of the neutral lipids of basking shark, sounfin shark, and fur seal by TLC on silicic acid. Malins and co-workers (1965) resolved diacyl glyceryl ethers from triglycerides of the dogfish (*Squalus acanthias*) on silicic acid plates, and each fraction was isolated for further analysis by GLC. Nevenzel and co-workers (1965) have employed TLC for the analysis of the oil from *Rucettus pretiosus* (castor oil fish) that contains high percentages of wax esters in the muscle. Brockerhoff and co-workers (1964) utilized this type of chromatography for the analysis of fractions obtained by column chromatography of the oils of several species of fish. Hanahan a[illegible]-workers (1963) also employed TLC for investigating the composition of commercial samples of glyceryl ethers derived from shark-liver oils. Figure 7 illustrates the chromatography of marine oils that have vastly different compositions.

[1] Slight modification in the solvent system must be made, depending on the activity of the adsorbent layer; i.e., highly active adsorbents may demand the use of more polar solvent systems.

Hofmann (1964) found hydroxyl apatite, $Ca_{10}(OH)_2(PO_4)_6$, useful for the resolution of certain types of lipids (e.g., phospholipids and isomeric monoglycerides). The usefulness of this adsorbent for the resolution of 1- and 2-monoglycerides in intestinal absorption studies was demonstrated in humans (Hofmann 1962). Predictably, future studies on the intestinal lipids of fish such as those described by Bottino and Brenner (1962) could profitably employ hydroxyl apatite chromatography.

Phospholipids of fish can be fractionated by either one-dimensional or two-dimensional TLC, depending upon the complexity of the sample. The technique of two-dimensional chromatography on thin layers of silica gel described by Skidmore and Entenmann (1962) is recommended for separating complex mixtures containing phosphatidic acid, phosphatidyl serine, phosphatidyl ethanolamine, phosphatidyl inositol, phosphatidyl choline, sphingomyelin, and lysophosphatidyl choline. The solvent systems are, respectively, 60:35:5 and 35:60:5 chloroform-methanol-7N ammonium hydroxide.

Mangold and Kammereck (1961) demonstrated that mono-, di-, and tri-enoic fatty acids can be resolved according to unsaturation by the TLC of their acetoxymercurimethoxy derivatives. The derivatives are prepared by allowing mercuric acetate to react with the unsaturated lipids in methanol.

Recent work (White and Williams 1965) suggests that the C_{20}:4, C_{20}:5, and C_{22}:6 acids common to fish can be similarly resolved by this technique. Wolfe and coworkers (1965) demonstrated the application of this method to the resolution of the fatty acids of crayfish (*Orconectes rusticus*). The fatty acids were separated according to degrees of unsaturation, then each fraction was further analyzed by GLC.

CHROMATOGRAPHY ON ADSORBENTS IMPREGNATED WITH SILVER NITRATE

DeVries (1963) showed that the equilibria between the π electrons of olefins and Ag^+ to form coordination complexes can be employed for the column chromatographic separation of lipids that are inseparable by conventional techniques such as column or thin-layer adsorption chromatography. Chromatography on coating materials impregnated with silver nitrate can be employed for the resolution of complex lipid mixtures (Cubero and Mangold 1965). Either column chromatography or TLC on impregnated adsorbents can be used to fractionate members of *homolog-*

ous series . rding to degrees of unsaturation and, in some cases, geometric and positional isomers can also be resolved.

Coating materials may be conveniently impregnated with silver nitrate by spraying with a solution of the reagent, by adding the complexing agent in the formulation of the slurry, or by allowing an aqueous solution of silver nitrate to ascend the height of the plate. Suitable indicators are 2',7'dichlorofluorescein and chromic-sulfuric acid (Maier and Mangold 1964).

The principal advantage of this form of chromatography over other methods, notably GLC, is that analysis is accomplished under mild conditions without the use of special equipment. In addition, this technique permits the fractionation of compounds that are nonvolatile and unstable at elevated temperatures.

Probably the major advantage that this form of chromatography has for fractionating fish oils and their derivatives is the separation of the saturates, monoenes, and dienes from the polyenoic derivatives. Each unsaturated fraction can be subjected to further analysis by either reverse-phase partition chromatography or GLC. It should be noted that polyenoic fatty acids containing 4, 5, and 6 double bonds are not well resolved by current applications of this technique. Furthermore, the usefulness of this type of chromatography for fractionating isomers present in fish oils has not been evaluated. The reader is referred to the reviews by Morris (1964) and Cubero and Mangold (1965) for a more detailed discussion of the chromatography of lipids employing layers impregnated with silver nitrate.

Duley and Olcott (1965) have shown that triglycerides of sablefish may be resolved into groups on columns of silica gel impregnated with silver

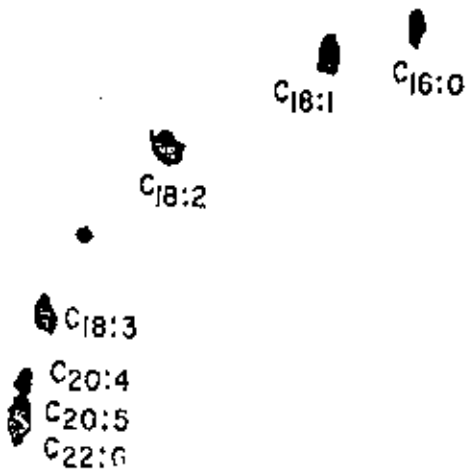

FIG. 8. TWO-DIMENSIONAL SILVER NITRATE TLC SEPARATION OF A MIXTURE OF REFERENCE STANDARDS OF FATTY ACID METHYL ESTERS

Solvent systems: (vertically) 88:12 ethyl acetate-benzene, v/v and (horizontally) benzene.

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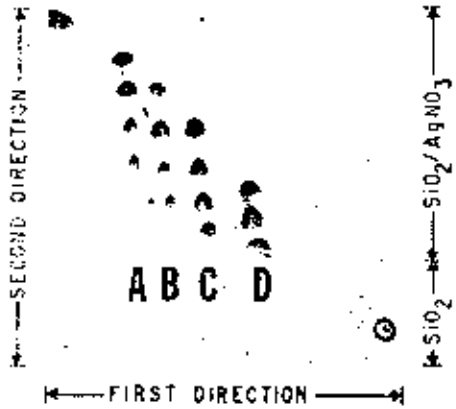


FIG. 9. TWO-DIMENSIONAL TLC EMPLOYING SILICA GEL AND SILVER NITRATE IMPREGNATED SILICA GEL ON A SINGLE PLATE

Solvent systems: 85:15:1, petroleum ether-ethyl ether-acetic acid, v/v/v, run twice in each direction. Indicator: chromic sulfuric acid-charring. A, triethers; B, dialkoxyglycerides; C, alkoxydiglycerides; D, triglycerides. The spots in columns A, B, C, and D (from top to bottom) contain 0, 1, 2, and 3 double bonds.

nitrate as a first step in their structural analysis. Primarily, the group separations were accomplished according to the total unsaturation in each fraction. Insight into the fatty acid composition of selected samples was obtained by GLC. Horning and Vandenheuvel (1964), employing two-dimensional TLC on silver nitrate impregnated silicic acid, resolved the characteristic fatty acids of fish oils according to degrees of unsaturation. These workers fractionated the following methyl esters: 16:0, 18:1, 18:2, 18:3, 20:4, 20:5, and 22:6. Discrete separations were obtained with the 16:0, 18:1, 18:2, and the 18:3 esters; however, the remaining derivatives were less distinctly resolved (Fig. 8).

A novel system of two-dimensional thin-layer chromatography, which has application to fish oils, was developed by Baumann and co-workers (1965). They obtained both *class* and *within class* separations on a single layer of silica gel, part of which was impregnated with silver nitrate. A mixture of triglycerides, and mono-, di-, and tri-ether derivatives of glycerol was first resolved into classes on the silica gel part, then each class was further fractionated according to unsaturation by elution on the silver nitrate impregnated portion in the second dimension (Fig. 9).

REVERSED-PHASE PARTITION CHROMATOGRAPHY

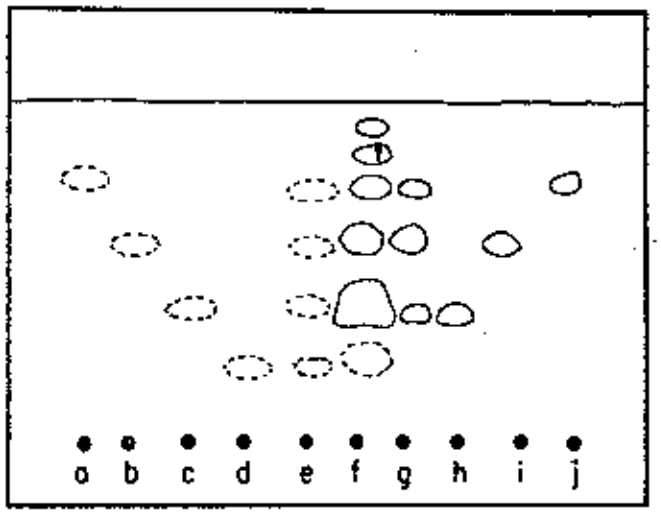
Separations *within* classes can be effected by reversed-phase partition chromatography. In such a liquid-liquid partition system, the stationary phase is a nonpolar liquid (e.g., silicone oil) and the mobile phase is a polar solvent that usually contains water (e.g., acetronitrile-water). As a general rule, reversed-phase partition chromatography is used for the resolution of *lipophilic* substances.

This type of chromatography, which effects separations according to both unsaturation and chain length, is advantageously used for fractionat-

ing fish o. d their derivatives when employed in conjunction with methods such as distillation, chromatography on adsorbents impregnated with silver nitrate, and GLC.

Methyl esters derived from menhaden oil, for example, can be first fractionated into groups according to unsaturation (e.g., saturates, monoenes, dienes, trienes, and C_{20} and C_{22} polyenes) and then each group may be further resolved by reversed-phase partition chromatography, primarily according to chain length. Additional insight into the composition of the C_{20} and C_{22} polyene fractions can be obtained by GLC.

Several examples have been reported that demonstrate the application of reversed-phase partition chromatography to fish oils. Column chromatography on siliconized adsorbents has been employed by Gellerman and Schlenk (1959) for the resolution of odd-chain fatty acids derived from menhaden oil. These workers separated *n*-nona-, *n*-hepta-, *n*-tri-, and *n*-penta-decanoic acids on columns consisting of a 1:1 ratio of celite to silicone oil. The eluent was 75% aqueous acetonitrile. In later work, Sen and Schlenk (1964) similarly employed reversed-phase partition chromatography for the resoluton of the odd and even chain-l acids of the mullet (*Mugil cephalus*). Kayama and co-workers (19.., in studies of the biogenesis of the polyenoic acid in kelp bass (*Paralabrax clathratus*) also utilized this type of chromatography for the isolation and purification of C_{14}-labelled eicosapentaenoic acid and docosahexaenoic acid.



Courtesy of J. Am. Oil Chemists' Soc.

FIG. 10. REVERSED-PHASE PARTITION CHROMATOGRAPHY OF METHYL ESTERS ON A SILICONE IMPREGNATED CHROMATOPLATE

Solvent systems: 70:10:25 acetonitrile-acetic acid-water, v/v/v. Indicators: Iodine (solid lines), followed by α-cyclodextrin, 1% in 30% aqueous ethanol and iodine (dotted lines). a, methyl laurate; b, methyl myristate; c, methyl palmitate; d, methyl stearate; e, mixture of saturated methyl esters; f, fraction derived from menhaden oil; g, mixture of unsaturated C_{18}-esters; h, methyl oleate; i, methyl linoleate; j, methyl linolenate.

Malins and Mangold (1960) demonstrated the application of this form of chromatography on siliconized layers to the resolution of methyl esters derived from fish oils. These workers resolved a C_{18} fraction (obtained by distillation) of methyl esters derived from menhaden oil into methyl stearate, methyl oleate, and four unsaturated components (Fig. 10).

Kaufmann and Wessels (1964) ably demonstrated the value of reversed-phase partition chromatography for the further resolution of triglycerides separated on silver nitrate-silica gel plates. By employing these two chromatographic techniques, the triglycerides of rapeseed oil were separated into over 50 fractions. The combined use of such complementary methods has obvious value for the analysis of the highly complex lipids of marine organisms.

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THE EFFECT OF MELTING POINT OF FAT UPON ITS UTILIZATION BY GUINEA PIGS

C. M. McCAY AND HENRY PAUL
Laboratory of Animal Nutrition, Cornell University, Ithaca

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For 50 years or more it has been recognized that one of the factors which determines the utilization of fat by an animal body is the melting point of this fat. Arnschink (1889) was one of the early workers who classified fats into three groups upon the basis of their melting points and relative utilization within the body. Most of these early studies were made with dogs. Recent studies of Steenbock, Irwin and Weber ('36) indicate that the rate of digestion and absorption of different fats within the body of the rat differs during the first few hours, but after 12 hours the differences are questionable.

A few balance studies have been made with men to determine the relative utilization of fats of different properties. For the most part, however, the periods used in such studies have been very short. Thus in the recent work of Massatsch and Steudel ('35), 3-day periods were employed in comparing the utilization of lard and hydrogenated fat.

Few studies of fat utilization by species other than omnivora or carnivora have been made. In studying the passage of fat from the feed into the milk of goats Gogitidse ('04) concluded that higher melting fats were absorbed with difficulty in the case of goats.

In experiments with brook trout to determine the relative utilization of hard and soft fats, McCay and Tunison ('34) found that oils such as cottonseed or salmon oils were always better utilized than hard fats such as hydrogenated ones. In

the case of brook trout this might be anticipated since the processes of the body operate at a temperature of 8°C. These reactions in the body of the fish have been reviewed recently (McCay, '37).

To study this matter further exploratory experiments were made with rats and guinea pigs. Four adult rats were fed dry skimmed milk for a period of 10 days to establish the intake. In a following period of 10 days approximately 20% of the calories was replaced by cottonseed oil.[1] The last 5 days of this period the feces were collected. During the following

TABLE 1

Utilization of the fat in diets of skim milk milk and cottonseed oil and skim milk and hydrogenated oil by rats

	RAT NO.	FAT INTAKE MILK	(5 DAYS) FAT	FAT IN FECES (5 DAYS)	UTILIZATION
		gm.	gm.	gm.	%
Cottonseed oil	1	0.261	3.310	0.076	97.9
	2	0.194	3.128	0.113	96.6
	3	0.286	3.300	0.123	96.0
	4	0.267	3.152	0.052	98.5
Hydrogenated oil	1	0.260	3.011	0.105	95.0
	2	0.252	3.020	0.144	95.6
	3	0.303	3.470	0.134	96.5
	4	0.187	3.027	0.248	92.2

10 days hydrogenated fat[2] was fed similarly and the feces collected during the latter half of the period. The data from this experiment are summarized in table 1. These results indicate that the rat is able to use both fats quite effectively.

In the case of guinea pigs[3] another technic was employed. A diet low in fat was first prepared by extracting a mixture of grain and ground alfalfa with isopropyl ether. This extracted mixture was then supplemented by the feeding of 10 cc. of tomato juice daily to each animal. Care was used to

[1] Wesson oil was the product used.

[2] Crisco was the product used.

[3] These preliminary studies with guinea pigs were made by Mrs. L. Dudgeon and Miss M. Zoller.

moisten this grain mixture to insure adequate consumption. The experiment was divided into three periods of 11 days each. During the first, the low fat diet was fed. The feces were collected during the last 6 days of the period. During the next period the feed was mixed so as to contain 6% of cottonseed oil and the feces collected again during the last 6 days. In the final period the feed contained hydrogenated oil[4] at the same level instead of the cottonseed oil. Three guinea pigs were used in these experiments.

The feces were preserved in alcohol then dried in vacuo and ground. Samples were then extracted with ether in the

TABLE 2

Relative utilization by guinea pigs of oil before and after hydrogenation

Diet	Lipids in dry feces, per cent
Basal (ctrl.)	0.96
	0.97
	0.80
Basal plus cottonseed	1.09
	1.85
	1.85
Basal plus hydrogenated oil	5.43
	5.86
	5.46

usual manner. Table 2 shows the variations in the fat content of the feces, although the weight of dried feces excreted was but little different in the various periods.

Further calculations indicated that in this trial about 90% of the oil was utilized and only about 60% of the hydrogenated product. These values indicate the differences clearly but are probably much less accurate than later ones that were obtained after a couple of years additional experience in feeding guinea pigs these special diets.

In order to explore this field further, a new series of preliminary trials with a number of different oils was run using essentially the same technics. Details will not be reported since the later experiments were similar and somewhat more

[4] The hydrogenated oil used was Crisco.

accurate. In this second preliminary series were included not only cottonseed oil and its hydrogenated product but also shark liver oil, soybean oil, castor oil, palm kernel oil, hydrogenated cod liver oil, cod liver oil, olive oil, butter, coconut oil and peanut oil. These preliminary trials yielded some surprising results in addition to confirming the first findings, namely, that hydrogenated oils are not utilized to the same degree as lower melting ones. Castor oil not only seemed to have no cathartic action upon the guinea pigs but apparently was utilized as well as such oils as shark liver and peanut.

TABLE 3

Utilization by guinea pigs of various fats as supplements to a low-fat diet

Fat supplement in diet	Fat in feces, per cent	Utilization, per cent
Castor oil	1.25	96.2
Soybean oil	1.51	94.5
Olive oil	1.60	94.5
Coconut oil	1.69	94.0
Salmon oil	2.03	94.0
Cod liver oil	1.65	93.8
Neatsfoot oil	1.64	93.5
Peanut oil	2.33	91.8
Butter	2.58	91.0
Cottonseed oil	3.36	87.4
Corn oil	3.35	86.5
Mutton tallow	5.22	79.8
Lard	6.70	75.2
Hydrogenated cottonseed oil	8.42	73.8
Beef tallow	7.05	72.0

A third experiment was now run taking advantage of earlier experience in feeding the animals. Ten kilograms of the basal diet were first extracted and dried carefully to free it from isopropyl ether. Three healthy guinea pigs were selected that would consume the diets readily. Each lipid to be tested was incorporated to comprise 6% of the diet. This mixture was then fed for a period of 11 days. The feed intake was determined and the feces collected during the last 7 days of each period. Since these animals were maintained upon the diets for a long period of time, they were fed adequate supplements of an A-D concentrate and tomato juice, allowing 8 cc. of the latter per day.

The data from this series are summarized in table 3. The results are very similar to those of the two preceding experiments. The guinea pig seems unable to utilize higher melting fats as effectively as oils. In this series again the castor oil seemed to be well digested and absorbed. No evidence of a cathartic action was found.

No corrections were made for metabolic fat in these studies since the results are evident without such corrections and since calculations involving metabolic fat make the assumption that the excretion of fat into the intestine is the same during periods of low fat and of normal fat ingestion.

TABLE 4

Fecal lipids as determined by the Tidwell-Holt method and by the regular ether extraction procedure

Oil supplement	Tidwell-Holt method, per cent	Ether extract method, per cent
Castor oil	2.31	1.26
Olive oil	2.34	1.60
Cottonseed oil	3.75	3.36
Hydrogenated oil	9.15	8.42

In order to compare the amount of lipid extracted from the feces by ethyl ether and another procedure, a few typical fecal samples were run by the method of Tidwell and Holt ('36). The values of table 4 show the latter method yields somewhat higher values but the differences between the oils and higher melting fats show the same trend for both methods.

SUMMARY

In order to determine if the melting points of a fat in region of body temperature is important in influencing its utilization in Herbivora, a series of balance studies was made chiefly with guinea pigs. The following oils were fed, incorporated at a 6% level in a diet of alfalfa hay and grain which had been extracted previously with isopropyl ether: castor, soybean, olive, cocoanut, salmon, cod liver, neatsfoot, peanut, butter, cottonseed, hydrogenated cottonseed, corn, tallow and lard. The fecal lipid tended to be much higher after feeding

the higher melting fats. The higher melting fats are not so well utilized as the oils by the guinea pig in contrast to the rat. Even castor oil seems well absorbed.

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Relationship Between Molecular Structure and Flavor Perceptibility of Aliphatic Aldehydes

P. W. MEIJBOOM, Unilever Research Laboratory, Vlaardingen, The Netherlands

Abstract

The mean threshold values for odor and taste of some aliphatic aldehydes are determined in solutions of paraffin oil. These threshold values lie at low concn and display a distinct alternating effect in the same homologous series. Moreover it appears from experiments with mixtures of various aldehydes that in the case of certain ratios a masking effect in odor and taste occurs.

Introduction

OILS AND FATS, which are present in our daily diet, may develop during the induction period of the autoxidation the so-called reversion flavors (1,2). These flavor carriers formed from the odor- and tasteless-hydroperoxides (3) are mainly aldehydes. An investigation on odors is difficult, because the odor carriers and their precursors are present in very low concn. Although they can be clearly observed organoleptically, the picture of the composition of the volatile substances can be obtained only with very sensitive gas chromatographic methods (head space analysis).

Since it appears that this volatile part contains a large number of components, the question arises how and to what extent the type of reversion flavors is determined by these components. Does a single component with a "top fragrance" with respect to the other components in the same system play a predominant role? Are there other factors such as synergism and or antagonism (intensifying respectively masking effects) which influence the taste of the fat in question? As aldehydes are important as flavor carriers, an investigation has been initiated into the relationship between the molecular structure and taste and odor of saturated and unsaturated aldehydes.

Results of a similar investigation in the field of perfumes have been published by Beets (4). Lea and Swoboda (5) have determined the flavor threshold value in water, in groundnut oil, and in paraffin oil, of a number of saturated and of two unsaturated aldehydes.

Our experiments were carried out with aldehydes of various homologous series and the mean threshold values for odor and taste in paraffin oil were determined in order to investigate the influence of chain length, of number and position of the double bonds and of stereo-isomerism (*cis-trans*).

Moreover, by means of experiments with mixtures of various aldehydes the mutual influence in some cases was demonstrated.

Experimental

Saturated aldehydes. Saturated unbranched aliphatic aldehydes (C_8-C_{12}) were purified by distilling commercially available products repeatedly; if necessary the purification took place via their sodium bisulphite addition product. The purity (according to the GLC analysis) was increased to 98% or more. The substances dosed gravimetrically were dissolved in paraffin oil with concn of 0.0001, 0.001 etc. up to 1000 ppm inclusive, and to guarantee a homogeneous

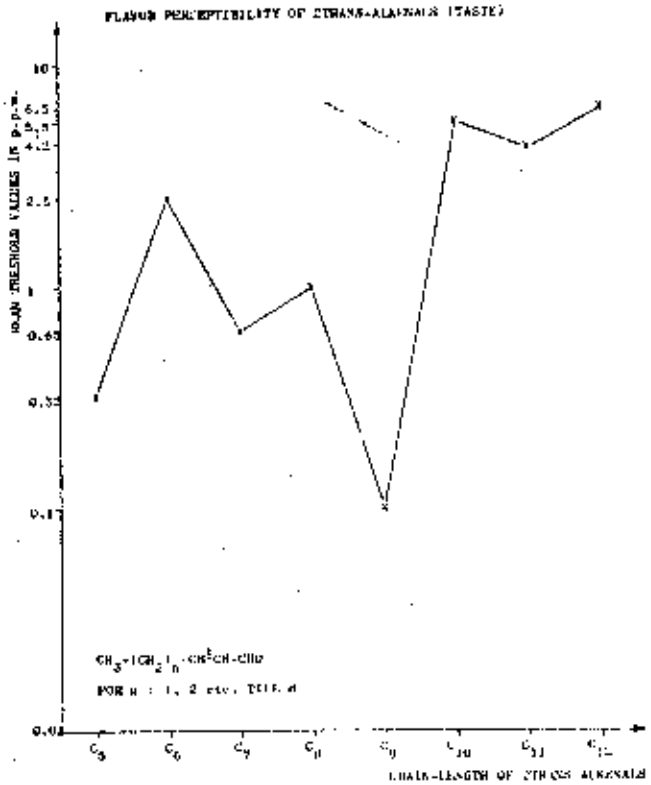

FIG. 1. Flavor perceptibility of 2*trans*-alkenals (taste).

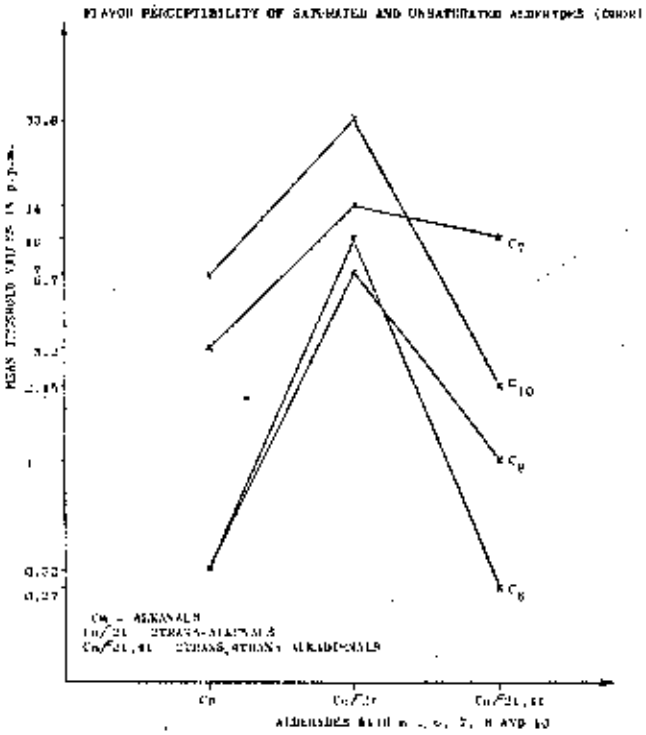

FIG. 2. Flavor perceptibility of saturated and unsaturated aldehydes (odor).

C_n = alkenals.
$C_n/2t$ = 2*trans* alkenals.
$C_n/2t,4t$ = 2*trans*,4*trans*-alkadienals.

double bond between carbon atoms 3 and 4 (terminal position), was investigated. Also here this alternating effect was observed and was again in correspondence with that of the 2 *trans*-alkenals (Table I).

We have found this alternating effect in various series of odor and taste experiments, but an explanation for this phenomenon has not yet been found. However, it is generally known that various homologous series display alternating effects in the case of certain physical properties as for instance the melting points, and heat of crystallization. Such alternating effects usually refer to a physical property of the crystalline state of the substance in question. It is therefore not impossible that during the odor and taste perception molecular orientation plays a part.

Further it appears from Table I that when in the chain of the 2 *trans*-alkenal a *trans*-double bond is introduced in the 4th position (conjugated diene system) the threshold value decreases with respect to the alkenal (Fig. 2).

The fact that also here the position of this second double bond is of great importance appears from the threshold value of the alkadienals having a double bond in the 3-4 position, terminally. The difference in threshold value, is considerable in some cases.

With respect to the *cis-trans* isomerism it appears by comparing the threshold values of:

2 *trans*,4 *cis*-heptadienal
2 *trans*,6 *cis* nonadienal
2 *trans*,7 *cis* decadienal
3 *cis*-hexenal

with those of their respective *trans*-isomers that the *cis*-double bond in these cases yields a higher flavor intensity (8). However, this is no proof at all that we can speak of a general rule.

From Table II it can be deduced that in the presence of larger amounts of decadienal, the 3 *cis*-hexenal in a concentration of 13.2 ppm is only weakly observed whereas the threshold value is 0.1 ppm.

Such an antagonistic effect on the odor of 3 *cis*-hexenal is also displayed by the 2 *trans*-nonenal. At a concentration of 4.5 ppm the 3 *cis*-hexenal is only weakly observed, when 2 *trans*-nonenal is present in a concentration of 2.8 ppm. The latter concn lies even below its threshold value.

On mixing 3 *cis*-hexenal and 2 *trans*,4 *trans*-heptadienal (respectively concentrations of 13.2 and 12.5 ppm) an odorless and almost tasteless mixture likewise was obtained.

From the above experiments with aldehydes it appears that during the reversion extinguishing or masking effects of odor and taste properties may occur. A similar effect was illustrated by Wright (9) with a mixture of nitro-benzene and methyl-salicylate. However, with respect to the reversion flavor, also additive effects may be expected, which assumption is supported by observations made by Lillard et al. (10).

In order to gain a better insight into this complicated interplay of various known and as yet unknown components, a thorough knowledge of the relationship between molecular structure and perception of aldehydes is indispensable.

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Valuable assistance by J. H. Recourt; synthesizing some of the aldehydes by W. J. Bigemann and P. D. Harkes.

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TABLE I

Flavor Potential of Saturated and Unsaturated Aldehydes Dissolved in Paraffin Oil

Substance	Threshold in ppm		Lea and Swoboda (5) (taste)
	Odor	Taste	
Propanal	3.6	1.6	1.0
Butanal	0.15	3.024	0.7
Pentanal	0.24	0.15	...
Hexanal	0.37	0.15	0.6
Heptanal	3.2	0.042	...
Octanal	0.32	0.068	0.6
Nonanal	13.5	0.32	...
Decanal	6.7	1.0	0.7
Undecanal	6.4	0.7	...
Dodecanal	3.0	0.046	0.4
2 *trans*-pentenal	2.3	0.32	...
2 *trans*-hexenal	10.0	2.5	...
2 *trans*-heptenal	14.0	0.03	...
2 *trans*-octenal	7.0	1.0	...
2 *trans*-nonenal	3.2	0.1	0.4
2 *trans*-decenal	33.4	6.5	...
2 *trans*-undecenal	150.0	4.3	...
2 *trans*-dodecenal	30.6	6.3	...
3 *cis*-hexenal	0.11	0.11	...
3 *trans*-hexenal	1.2	1.2	...
2 *trans*,4 *trans*-hexadienal	0.27	0.036	...
2 *trans*,4 *trans*-heptadienal	10.9	0.16	...
2 *trans*,4 *trans*-octadienal	1.6	0.15	...
2 *trans*,4 *trans*-nonadienal	2.5	0.46	...
2 *trans*,4 *trans*-decadienal	2.15	0.28	...
2 *trans*,4 *cis*-heptadienal	3.6	0.035	...
2 *trans*,3 *cis*-octadienal	15.3	0.21	...
2 *trans*,6 *cis*-nonadienal	0.61	0.002	...
2 *trans*,7 *cis*-decadienal	0.648	0.02	...
2 *trans*,6 *trans*-nonadienal	0.21	0.018	...
2 *trans*,7 *trans*-decadienal	1.0	0.32	...

solution, the mixture was stirred magnetically for ca. 20 min in brown, wide mouthed bottles (capacity 50 ml) which had been thoroughly cleaned and steamed.

These solutions were assessed for odor and taste by a test panel of 10 persons who were not specially trained; a sample of pure paraffin oil was used as a blank.

The results were obtained in accordance with the method by Patton (6), by plotting the percentage of positive responses against the logarithm of the conen, after which the mean threshold value (50% positive response) for odor and taste could be read from the graph obtained. The aldehydes investigated show in Table I.

Unsaturated aldehydes. The unsaturated aldehydes investigated were synthesized in our laboratory. (Some of these have not yet been mentioned in the literature and will be published elsewhere). To prepare the solutions known amounts of pure fractions were collected by means of gas chromatography in a column filled with glass beads and a small amount of paraffin oil. Subsequently the contents were transferred with 10 g paraffin oil into a 50-ml brown wide mouthed bottle after which the mixture was stirred magnetically for ca. 20 min. The dilutions of this stock solution were prepared in the same way as stated above and the odor and taste assessments were again carried out by the same test panel.

The determined threshold values show in Table I.

Experiments with mixtures of various aldehydes. Known amounts of 3 *cis*-hexenal, likewise in paraffin oil, were added to a known amount of 2 *trans*,4 *trans*-decadienal in paraffin oil and the mixture assessed for flavor. The results show in Table II.

Results and Discussion

Table I shows the result of the taste and odor experiments with saturated unbranched aldehydes and unsaturated unbranched aldehydes. From propanal up to dodecanal, the conen of the threshold values for the odor lie in the order of 10–0.1 ppm and for the taste of 1–0.01 ppm. Lea and Swoboda (5) have found considerably higher threshold values for some of these aldehydes. Since the authors give no

TABLE II

Antagonistic Effect on the Odor of 3 *cis*-hexenal

Odor assessment	Composition of the mixture in paraffin oil	
	3 *cis*-hexenal (ppm)	2 *trans*, 4 *trans*-decadienal (ppm)
Decadienal	3.3	20.3
Decadienal	6.2	24.7
Decadienal	8.8	23.3
Decadienal	11.1	22.1
Decadienal + weakly green beans	13.3	21.0
Decadienal + weakly green beans	9.2	10.5
Only weakly green beans	1.4	1.6
Weakly green beans	0.1	.....
Green beans	1.0	.....

details about the substances they used, the differences might be attributed to differences in purity of the aldehydes. A definite relation between the threshold value and the chain length of the compound cannot be concluded from values in Table I. This is in correspondence with the work by Lea and Swoboda. Since the bp in the series of investigated aldehydes rise monotonously with increasing chain length ($bp_{1\,atm}$ C_3 : 21C; $bp_{1\,atm}$ C_{12} : 235C) the conclusion might be that at least in the interval C_9–C_{12}, the number of molecules required to stimulate the olfactory receptors decreases with increasing mol wt. In all cases the threshold values for taste are lower than those for odor; this also is found with the unsaturated aldehydes.

In this respect it may be noted that for aldehydes also the gradually more accepted rule applies that the taste assessment takes in fact place on the odor receptors in the nose, and consequently can be qualified as odor.

In the series of the 2 *trans*-alkenals for the odor threshold values at conen ranging between 100–1 ppm and for the taste between 1–0.1 ppm were found. If the separate values are compared with those of the saturated aldehydes with the same chain length, it appears that on introducing a *trans*-double bond in the α-β position the threshold value rises. The C_9-homologue forms an exception to this rule.

The investigation into the influence of the double bond (*cis* or *trans*) in another position in the chain is being carried out. From the threshold value found for the 3 *trans*-hexenal it appears that the position of the double bond in alkenals influences the flavor intensity. If the threshold values for taste of these alkenals are plotted as a function of the chain length it appears that there is an alternating effect (Fig. 1). The aldehydes in this series with an uneven number of carbon atoms have a lower threshold value than the corresponding aldehydes with even numbers.

An extension of the investigation in the series of the 2-alkenals into the influence of the *cis*-double bond in the α-β position cannot be realized; attempts to synthesize the 2 *cis*-alkenals resulted in formation of the stabler *trans*-alkenals (7).

In the series of 2 *trans*,4 *trans*-alkadienals an analogous effect was observed. If the threshold values for the odor (10–0.1 ppm) and for the taste (0.1–0.01 ppm) are plotted against the chain length, lower threshold values are found for the aldehydes with an even number of carbon atoms than for those with uneven number in contrast with the previous series (Table I). The investigation is also here restricted to the 2 *trans*,4 *trans*-alkadienals, since the 2 *cis*,4 *trans* and 2 *cis*, 4 *cis* compounds are no more stable than the 2 *cis*-alkenals.

In addition, a homologous series of alkadienals with the *trans*-double bond in the α-β-position and a *cis*-

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SOYA BEAN OIL COMPOSITIONS

Daniel Melnick, Teaneck, N. J., Chester M. Gooding, Staten Island, N. Y., and Hans W. Vahlteich, Englewood, N. J., assignors to The Best Foods, Inc., New York, N. Y., a corporation of New Jersey

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This invention relates to soya bean oil and more particularly to a hydrogenated and winterized soya bean oil in combination with a vegetable salad oil having an iodine number of less than 120 for use as a salad oil such as that usable in mayonnaise.

The present application is a continuation-in-part of our earlier application Serial No. 216,100, filed March 16, 1951.

Mayonnaise can readily withstand temperatures as low as 50° F. However, on those occasions when the housewife lowers the temperature of the refrigerator for making frozen desserts, for example, oil emulsion failure occurs with ensuing oil separation of mayonnaise stored under such conditions. In the winter months, low temperatures encountered in shipping and storage have frequently been responsible for oil separation. To overcome this defect, corn oil has been used as a substitute for cottonseed or other salad oil in mayonnaise, but aside from the limited available supply of corn oil, corn oil does not possess the desired degree of flavor stability to warrant its extensive use for this purpose, especially during grocery shelf storage at summer temperatures. Refined soya bean oil is sometimes used as a means of avoiding emulsion failure due to freezing, but such soya bean oil has only limited value in this respect and reverts in flavor to render substantially impossible its use in quality mayonnaise.

It is an object of the present invention to provide a salad oil exhibiting unexpected cold resistance by itself and particularly when the salad oil is incorporated into mayonnaise.

It is another object of the invention to provide a salad oil imparting superior emulsion stability to mayonnaise produced from the salad oil composition.

It is a further object of the invention to provide a salad oil composition and a mayonnaise prepared from the said salad oil, both having improved flavor characteristics and stability.

It is a still further object of the invention to provide a mayonnaise having desired color elegance and stability, desired firmness in texture and the ability to absorb added aqueous components without a disruption of the emulsion characteristics.

It will be apparent from the disclosure which follows that the above objects have been accomplished by the present invention. Further objects and advantages of the invention will be apparent and will be explained in connection with the description which follows.

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In accordance with this invention, a salad oil is provided comprising a vegetable salad oil having an iodine number of less than 120 and a hydrogenated and winterized soya bean oil having an iodine number of 100 to 120 and containing less than 5% of conjugated dienoic components, less than 0.2% of conjugated trienoic components and less than 0.02% of conjugated tetraenoic acid components. The preparation, properties, and utility features of the hydrogenated winterized soya bean oils have been disclosed in our copending U. S. patent applications (Vahlteich, Gooding and Melnick, S. N. 216,100 and Gooding, S. N. 216,099). When the oil blends of this invention are used as the oil component of mayonnaise, they produce a mayonnaise which has a marked freeze resistance and emulsion stability, equal to that prepared from corn oil, and better flavor stability than a quality mayonnaise such as that prepared from cottonseed salad oil. The resulting mayonnaise differs from mayonnaise made with corn oil in having markedly superior flavor stability, and from a mayonnaise made with cottonseed oil in having markedly superior emulsion stability. In other words, the oil blends of this invention combine the best properties of the two types of oils, hitherto used on a seasonal basis, so that one oil formulation can now be used successfully all year round in the manufacture of quality mayonnaise.

The salad oil of this invention is an edible glyceridic oil composition comprising desirably 25 to 75% of a vegetable salad oil having an iodine value of less than 120 and 25 to 75% of a hydrogenated winterized soya bean oil having an iodine value of 100 to 120 and preferably 110 to 120. The preferable compositions of the salad oil comprise 40 to 60% of a vegetable salad oil having an iodine value of less than 120 and 40 to 60% of a hydrogenated winterized soya bean oil having an iodine value of 100 to 120 and preferably 110 to 120. Optimum compositions of the salad oil comprise a 50–50% mixture of the soya bean oil and the vegetable salad oil. The hydrogenated and winterized soya bean oil contains less than 5% and preferably less than 3.5% or 1.0% of conjugated dienoic acid components, less than 0.2% and preferably less than 0.1% of conjugated trienoic acid components; and less than 0.02%, and preferably less than 0.015% of conjugated tetraenoic acid components. The most desirable hydrogenated winterized soya bean oils for use according to the invention contain less than 0.8% of conjugated dienoic acid components, less than 0.03% or 0.015% of conjugated trienoic acid

components and less than 0.002% of conjugated tetraenoic acid components. The amounts of conjugated components in the hydrogenated oil are determined by the method according to B. A. Brice et al. in Oil and Soap, XXII, 219 (1945).

The vegetable salad oil may be a mixture of two or more vegetable oils and makes up the difference between the soya bean oil used and the total of the glyceridic oil in the composition.

To best illustrate the patentable features of this invention, examples are given of the new salad oils and mayonnaise products prepared therewith.

The mayonnaise examples were produced from the salad oils of this invention by incorporating such an oil with vinegar, egg, salt and other ingredients in the manner ordinarily employed for this spurpose (e. g. in accordance with standard for mayonnaise of the Federal Security Agency, Federal Register, August 12, 1950, pp. 5227–5232). Preferably, however, incorporation of oxygen in the mayonnaise should be avoided by protecting the product during processing, for example, with nitrogen.

In Table I are listed 6 salad oil examples illustrating the synergistic effects noted in cold tests when vegetable salad oils having an iodine value of less than 120 are blended with hydrogenated winterized soya bean oil having an iodine value of 110 to 120. The cold test values for the salad oils were obtained by the Official Method Cc 11–42 of the American Oil Chemists' Society. The examples in the table show the excellent results which may be obtained using hydrogenated winterized soya bean oils having the preferred limits of conjugated unsaturated acid components.

value) with a well winterized salad oil (one with a high cold test value) in order to up-grade the poorer salad oil. Under such circumstances, the cold test on such oils approaches the cold test value of the oil with the poorer resistance to low holding temperatures. For this reason, it is the routine practice in preparing salad oils to return to the winterizing plant all oils low in cold test values for a repetition of the winterization process using such oils in making a marketable salad oil, a mayonnaise, or related products. However, it will be noted from the examples in Table I that a 50:50 blend of winterized cottonseed oil and hydrogenated winterized soya bean oil exhibits a cold test value not approximating the lower value of the two oils, not approximating the average, but one well above the highest figure for the two oils in the blend. This synergistic up-grading of vegetable salad oils having an iodine value of less than 120 and blended with hydrogenated winterized soya bean oil with an iodine value of 110 to 120 and low in concentration of conjugated fatty acids is routinely noted when the two oil components are blended in 50:50 ratio. Even when blended in a ratio of 75 parts of one oil to 25 parts of the other, this synergistic up-grading of the cold test values is noted. The same occurs with vegetable salad oils other than cottonseed oil but also having an iodine value of less than 120; sesame oil and peanut oil are good examples of these other type oils. In the case of some blends (Example 6 in Table I), the cold test value may not exceed the cold test figure of the highest in the blend, although it will be very much greater than the average weighted cold test value for the blend. That synergistic up-

TABLE I

Synergistic effects noted in cold tests when vegetable salad oils having an iodine value of less than 120 are blended with hydrogenated winterized soya bean oil having an iodine value of 110 to 120

Salad Oil Example	Salad Oil Component	Blend	Iodine Value	Conjugated Fatty Acids of Soya Bean Oil			Cold Test at 32° F.[1]	
				Dienoic	Trienoic	Tetraenoic	Individual Oils	Oil Blend
		Percent		*Percent*	*Percent*	*Percent*	*Hours*	*Hours*
1	Cotton seed	50	113				12	
	Soya Bean	50	117	0.0	0.00	0.00	18	36
2	Cottonseed	25	113				18	
	Soya Bean	75	118	0.4	0.014	0.00	15	30
3	Cottonseed	75	113				12	
	Soya Bean	25	118	0.4	0.014	0.00	15	21
4	Sesame	50	112				20	
	Soya Bean	50	112	0.6	0.012	0.001	6	22
5	Cottonseed	37.5	110				12	
	Sesame	25.0	114				14	
	Soya Bean	37.5	116	0.00	0.00	0.00	8	22
6	Cottonseed	25	115				10	
	Sesame	35	113				20	
	Peanut	16	92				0.25	12
	Soya Bean	25	112	0.9	0.015	0.003	7	

[1] Number of hours required for first appearance of turbidity, i. e. of solid triglycerides.

The oil formulations of the present invention, illustrated by the examples in Table I, have shown the unexpected property of having cold test values far in excess of those anticipated, based upon experiences up to the present time in the preparation of salad oil blends. It is contrary to the teachings of oil technologists to blend a poorly winterized oil (one with a low cold test

grading of the cold test value in such blends also occurs is apparent from the very low cold test values of the oil blend prepared without the hydrogenated winterized soya bean oil component. Thus, in the case of Example 6 prepared without the soya bean oil, a cold test value of less than two hours was obtained.

The salad oil examples, given in Table I, are

those recommended for sale per se through retail outlets to the housewife. When the hydrogenated winterized soya bean oils in these formulations comprise those having an iodine value of 100 to 110 and/or are characterized by a relatively high concentration of conjugated fatty acids, viz. 5% dienoic, 0.2% trienoic and 0.02% tetraenoic fatty acids, the synergistic up-

in Table II. Thus, the salad oils containing the hydrogenated winterized soya bean oils having an iodine value of 100 to 110 with the larger concentrations of conjugated unsaturated fatty acids are no less important than those containing the preferred hydrogenated winterized soya bean oils when the resulting salad oil is used for mayonnaise manufacture. Table II follows:

Table II

Synergistic up-grading of the freeze resistance of mayonnaise samples prepared with vegetable salad oils having an iodine value of less than 120 when blended with hydrogenated winterized soya bean oil having an iodine value of 100 to 120

Mayonnaise Example	Salad Oil Component	Blend	Iodine Value	Conjugated Fatty Acids of Soya Bean Oil			Cold Test of Oil Blend at 32° F.[1]	Emulsion Stability of Mayonnaise[2]	
				Dienoic	Trienoic	Tetraenoic		At 32° F.	At 15° F.
		Percent		*Percent*	*Percent*	*Percent*	*Hours*	*Hours*	*Hours*
1	Cottonseed	100	113				10	10	5
2	Corn	100	127				60	More than 168	64
3	Soya Bean	100	135	4.7	0.12	0.01	0.2	68	35
4	do	100	115	2.2	0.04	0.005	1.5	168	64
5	do	100	109	0.00	0.000	0.00	1	140	60
6	do	100	117	0.00	0.00	0.00	19	More than 168	118
7	Cottonseed	50	114						
	Soya Bean	50	135	4.7	0.12	0.01	3	44	30
8	Cottonseed	50	114						
	Soya Bean	50	115	2.2	0.04	0.005	7	100	40
9	Cottonseed	50	112						
	Soya Bean	50	109	0.00	0.000	0.00	2	90	38
10	Cottonseed	50	113						
	Soya Bean	50	117	0.00	0.00	0.00	30	More than 168	110
11	Cottonseed	50	114						
	Soya Bean	50	111	0.00	0.00	0.00	14	146	60
12	Cottonseed	25	113						
	Soya Bean	75	115	2.2	0.04	0.005	4	120	53
13	Cottonseed	75	113						
	Soya Bean	25	115	2.2	0.04	0.005	10	60	30
14	Sesame	60	112						
	Soya Bean	40	112	0.6	0.012	0.001	22	100	70
15	Cottonseed	37.5	110						
	Sesame	25.0	114				22	More than 168	84
	Soya Bean	37.5	116	0.00	0.00	0.00			
16	Cottonseed	25	115						
	Sesame	35	113				12	80	38
	Peanut	15	92						
	Soya Bean	25	112	0.9	0.018	0.001			

[1] Number of hours required for first appearance of turbidity, i. e. of solid triglycerides.
[2] Number of hours required for the development of an emulsion break, i. e. separation of oleaginous from aqueous components.

grading of cold test values is not noted; the cold test values approximate those of the oil in the system with the lowest cold test value. Thus, oil blends containing such hydrogenated winterized soya bean oils may not be employed in the production of a marketable salad oil for sale through retail outlets. Such oils, when stored in the home-refrigerator would show heavy precipitates of solid triglycerides and would therefore be objectionable to the consumer. For preferred salad oil preparations having optimum freeze test properties, it is desirable to use hydrogenated winterized soya bean oils according to the invention having an iodine number of 110 to 120 and a minimum of conjugated unsaturated fatty acids; less than 1.0% of conjugated dienoic acid components; less than 0.02% and preferably less than 0.01% of conjugated trienoic acid components; and less than 0.002% of conjugated tetraenoic acid components.

The salad oils made with hydrogenated winterized soya bean oils having an iodine value of 100 to 110 and containing the relatively larger amounts of conjugated unsaturated fatty acids may be used as a salad oil by a manufacturer of mayonnaise with striking improvements in the quality of his product or that of the prior art. The improvement in freeze resistance is apparent from the illustrative mayonnaise examples listed

The resistance to emulsion failure is determined by subjecting a plurality of samples of the mayonnaise under test to a low temperature (in these tests the samples were run at two temperatures, 23–27° F., an average of 25° F., and 13–17° F., an average of 15° F.), withdrawing one of the samples at intervals from the low temperature holding cabinet, raising the withdrawn sample to room temperature, and observing whether a definite break between the oleaginous and aqueous component occurs. The minimum time required to effect such an emulsion break is the value appearing in the last two columns of Table II.

The first two examples in Table II demonstrate for reference purposes the emulsion stability of mayonnaise made with either cottonseed oil or corn oil as the sole oil component. It will be noted that the corn oil mayonnaise is far superior to the cottonseed oil mayonnaise in emulsion stability. This observation is confirmatory of the teachings of oil technologists that corn oil is the preferred oil in the manufacture of mayonnaise during the winter months. Mayonnaise Examples 3–6 cover products made with soya bean oils described in the co-pending applications cited earlier in this specification. The results with these oils show that any one of them is far superior to the mayonnaise made with cot-

tonseed oil in resistance to oil separation on freezing. These results were totally unexpected in view of the low cold test values for the oils, particularly those in Examples 3–5. Such low cold test figures would lead an oil technologist to dismiss from consideration use of such oils in the manufacture of mayonnaise.

Mayonnaise Examples 7–11 cover products made with salad oils of this invention. It will be noted that all of these samples are far superior to cottonseed oil mayonnaise in resistance to an emulsion break following low temperature exposure. As the concentration of conjugated fatty acids in the hydrogenated winterized soya bean oil of a given iodine number decreases, greater emulsion stability is obtained. Also, in the case of those oil blends containing hydrogenated winterized soya bean oil of the same content of conjugated fatty acids, but differing in iodine value, improvement in emulsion stability is obtained with the higher iodine value. Whereas the cold tests on the oil blends are of no value in predicting the freeze resistance of the mayonnaise when compared with mayonnaise products made with other types of oils, there is a good correlation between emulsion stability and cold test values of oil blends of this invention, when the same component oils are blended in the same ratio. This point is further emphasized by Mayonnaise Examples 12 and 13. Here the two basic oils are present in a 25:75 ratio; in one example, the cottonseed oil is the minor component, in the other example, the hydrogenated winterized soya bean oil is the minor component. It will be noted that whereas both products are far superior in freeze resistance to cottonseed oil mayonnaise, there is no correlation between results for the mayonnaise products with the cold test values for the oil blends. The mayonnaise example containing the higher proportion of soya bean oil in the oil blend is the superior product in emulsion stability. Decreasing the soya bean oil component to a concentration less than 25% of the oil blend brings the product too close in performance to that of Mayonnaise Example 1 made with cottonseed oil as the sole oil component; such oil blends are of very little value for mayonnaise manufacture, shipping and storage during the winter months. Thus, we have discovered that when the salad oil compositions according to our invention contain 25% or more of the hydrogenated winterized soya bean oil of our copending application S. N. 216,100, there is obtained a composition giving unexpected emulsion stability.

Mayonnaise Examples 14–16 show that when vegetable salad oils, other than cottonseed oil but also having an iodine value of less than 120, are blended with hydrogenated winterized soya bean oil having an iodine value of 100 to 120, highly satisfactory values for emulsion stability are obtained.

The scorings in the cold tests on the salad oil examples and in the freeze tests on the mayonnaise examples covered in Tables I and II were objective and reproducible. The results depicted are representative of hundreds of samples which were subjected to the tests. Only illustrative data shown in Tables I and II have been presented for sake of brevity. They cover adequately the performance of the salad oils of this invention in satisfying one of the two basic requirements of a quality salad oil to be marketed as such or to be used in the manufacture of quality mayonnaise, the requirement being maintenance of the original physical state of the products despite low temperature storage.

The second important requirement of a quality salad oil or of a quality mayonnaise prepared therewith is flavor stability. Flavor scorings were formerly characterized as subjective, since no objective physical or chemical methods are available for this purpose. The importance of flavor to food technologists has necessitated a reappraisal of flavor scoring techniques, with the goal that these be converted to an objective basis. This has led to the development of expert and consumer panels for blind flavor scorings of food products. The results of such tests lend themselves to statistical interpretation with conclusions which are truly objective, despite the use of subjective tool-taste impressions. In the present investigation, objective techniques and interpretations of flavor scorings were applied in evaluating both the salad oils and mayonnaise products of this invention versus the customary salad oils and mayonnaise products regarded hitherto as acceptable when used on a seasonal basis.

The following procedure was used for determining flavor stability of the salad oils and the resulting mayonnaise products. The samples under test were coded and flavored by an expert panel of 8 judges when the samples were fresh and after periodic storage at an elevated temperature of 95° F. and at room temperature of 75° F. Numerical values were assigned to the flavor ratings (5=good; down to 1=very poor), and the average score for the product was plotted periodically on a graph versus the length of storage at each of the temperatures indicated above. From these graphs values were obtained for the test samples covering the two important criteria of flavor stability. Figures for shelf life were obtained representing the number of weeks the samples, at each of the specified temperatures, were scored by the panel as being "fair" or above in flavor. The overall flavor acceptability of each of the test samples was obtained by measuring the area under the curve above the "fair" level. Two products may have the same shelf life but differ appreciably in overall flavor acceptability, and as a corollary, two products may have the same overall flavor acceptability but differ in shelf life.

A statistical analysis of the average flavor ratings of two samples at a given time indicated that a difference of 1.0 unit in score was required for significance, probability of 0.05, i. e. 95 chances out of 100 that the difference noted between the average flavor ratings of a pair of samples was real. However, inasmuch as each stability study involved flavor scorings on the fresh samples, 6 weekly scorings of those held at 95° F., and 6 bi-weekly scorings of the samples held at 75° F., it was found not necessary to obtain at each scoring a difference of one full unit between two samples in a pair for a statistical significance. As the samples aged, the panel of expert judges progressively scored the products lower in the acceptability scale. Even small differences between products, of the order of 0.25 unit, were uniformly maintained week after week to a surprising degree, viz. six out of the seven scoring tests at a given holding temperature. Under such circumstances, a difference of as little as 0.25 unit between two samples in a flavor stability evaluation was statistically significant at a probability level of 0.05 (95 chances out of 100).

More than 80 samples, covering more than

8000 flavor scorings by the expert 8 judges were used in the present investigation. Two consumer surveys, each comprising about 100 housewives, were conducted to check on the reliability of the expert panel in predicting consumer preference. In one consumer survey, mayonnaise made with winterized corn oil as the sole oil component was used as the reference point in evaluating the flavor stability of the mayonnaise prepared with an oil blend covered in this invention. In the other survey, the reference oil was winterized cottonseed oil. The latter survey also covered scorings by the consumer panel of mayonnaise after 1, 2, 3 and 4 months of storage at uncontrolled temperatures blanketing the summer months. Concomitant scorings of the same samples always under code by the expert panel were also made. It was found in these associated scoring studies that the consumer panel had the same preferences as shown in the tests conducted with the expert panel, even though the average consumer tolerated to a greater degree flavor deterioration in the two products submitted at one time for evaluation. Thus, the average expert was found to score the test products below "fair" in one-third the time required by the average consumer. We, therefore, emphasize that the conclusions on flavor drawn from expert panel scorings in the present investigation are valid in reflecting consumer reactions. The more critical scoring by experts is definitely desirable since wide scattering of individual scorings among a consumer panel occurs; an average score of better than "fair" by a consumer panel can be cause for concern to the manufacturer if a significant number, viz. 20 percent, score the product as "poor." The latter was actually noted in the consumer response following the evaluation of the test and reference mayonnaise samples held 4 months at prevailing summer temperatures.

In Table III are presented a number of mayonnaise examples, No. 7–16, illustrating the synergistic flavor improvement of mayonnaise made with hydrogenated winterized soya bean oil having an iodine value of 100 to 120 when blended with vegetable salad oils having an iodine value of less than 120. For a better appreciation of the flavor improvement noted, the results obtained with 4 mayonnaise examples, No. 1–4, are included. The mayonnaise examples, identified by number in Table III, are the same as those of the same number in Table II, so that there is no need to repeat the conjugated fatty acid composition of the soya bean oil fractions. Listed are the shelf lives of each of the mayonnaise examples when stored at 95° F. and at 75° F.; these represent the number of weeks during which the product scored "fair" or above with the panel of experts. Overall acceptabilities of the mayonnaise during the period of satisfactory shelf life at 95° F. and at 75° F. are also listed; these are represented by the areas under the curves plotting degree of acceptability, during which the products scored "fair" or above, versus time. Assigning a flavor score of 100 to each of the performances of the mayonnaise made with the cottonseed salad oil as the sole oil component, an average value for the four categories of flavor performance in the case of each of the other samples was obtained. In this way, it was possible to obtain one figure covering the flavor performance of each product under realistic storage conditions simulating those found in retail outlets. Table III follows:

TABLE III

Synergistic flavor improvement of mayonnaise made with hydrogenated winterized soya bean oil having an iodine value of 100–120 when blended with vegetable salad oils having an iodine value of less than 120

Mayonnaise Example	Salad Oil Component	Blend	Iodine Value	Shelf Life [1] (Weeks) at 95° F.	Shelf Life [1] (Weeks) at 75° F.	Overall Acceptability [2] at 95° F.	Overall Acceptability [2] at 75° F.	Relative Flavor Acceptability [3]
		Percent		*Weeks*	*Weeks*	*sq. mm.*	*sq. mm.*	*Percent*
1	Cottonseed	100	113	6	10	365	675	100
2	Corn	100	127	1½	4½	140	275	38
3	Soya Bean	100	105	4	8	175	400	66
4	Soya Bean	100	115	4	8	165	375	66
7	Cottonseed	50	114					
	Soya Bean	50	105	5	12	465	1,250	129
8	Cottonseed	50	114					
	Soya Bean	50	115	5	10	465	900	111
11	Cottonseed	50	114					
	Soya Bean	50	111	5	12	410	1,050	118
12	Cottonseed	25	113					
	Soya Bean	75	115	5	8½	300	600	85
13	Cottonseed	75	113					
	Soya Bean	25	115	6	12	350	650	103
14	Sesame	60	112					
	Soya Bean	40	112	6	12	390	900	115
15	Cottonseed	37.5	110					
	Sesame	25.0	114	6	13	380	825	112
	Soya Bean	37.5	110					
16	Cottonseed	25	115					
	Sesame	35	113					
	Peanut	15	92	6	12	350	710	108
	Soya Bean	25	112					

[1] Number of weeks during which the product scored "fair" or above.
[2] Shelf life × degree of acceptability; area obtained from graphs on which were plotted the scorings week after week.
[3] Assigning a flavor score of 100 to each of the performances of the mayonnaise made with cottonseed salad oil as the sole component, and calculating an average value for the four categories of flavor performance in the case of each of the other samples.

The values for cottonseed oil mayonnaise (Example No. 1) represent what is generally regarded as the best obtainable up to the present time in

the manufacture of a quality product. The poorer values with corn oil mayonnaise (Example No. 2) indicate the sacrifice a manufacturer must be willing to make in the flavor stability of his mayonnaise product during the winter months in order to obtain satisfactory freeze resistance. Significant improvement in flavor stability over corn oil mayonnaise is obtained, when the mayonnaise (Example No. 3 or No. 4) is made with the hydrogenated winterized soya bean oils of our co-pending applications, supra. However, these products are inferior in flavor performance to a quality mayonnaise made with cottonseed oil. It will be noted that most of the hydrogenated winterized soya bean oils listed in Table III are those characterized by the higher concentrations of conjugated fatty acids. For the sake of brevity, only a few hydrogenated winterized soya bean oils containing the very small concentrations of conjugated fatty acids were included in this tabulation since these routinely are at least equal in flavor performance to those containing the higher concentrations of conjugated fatty acids.

It is the general opinion of oil technologists that when an oil of poorer flavor stability is blended with an oil of better flavor stability, the resulting blend, whether used as such or in mayonnaise manufacture, will have a flavor life characteristic of the poorer oil in the blend. Thus, past attempts have shown that the addition of unhydrogenated freshly-deodorized soya bean oil in very small concentrations, viz. 5 to 10%, to a quality cottonseed salad oil gives a mayonnaise unacceptable in flavor life. Contrary to the teachings of oil technologists and contrary to our own past experiences, we have now discovered that blending the hydrogenated winterized soya bean oil having an iodine value of 100–120 with vegetable salad oils having an iodine value of less than 120 yields mayonnaise characterized by extraordinary good shelf life. Thus, with the 50:50 blends of the hydrogenated winterized soya bean oil with the cottonseed salad oil, Examples 7–11, the flavor stability of the resulting mayonnaise products was not similar to that of mayonnaise made with the soya bean oil fraction alone (Examples 3 and 4) but even superior to that obtained with the mayonnaise made with the cottonseed oil as the sole oil component (Example 1). When one oil is present in the ratio of 25:75 to the other (Examples 12 and 13), the flavor performance is still good, comparable to that of the quality cottonseed oil mayonnaise. The preferred ratio of one oil to the other is as 60 is to 40 (viz. Example 14). Examples 14–16 show the flavor performance in mayonnaise of oil blends of the invention which include vegetable salad oils other than cottonseed oil but also having an iodine value of less than 120.

The examples in Table III cover specific mayonnaise products evaluated in the course of the investigation which resulted in the present invention. Actually, 60 different products of the more important type were evaluated with 104 flavor scorings on each sample by the expert 8 judges, 8 on the fresh product, 48 during the 95° F. holding period and 48 during the 75° F. holding period.

In Table IV is a statistical evaluation of the overall flavor acceptability of the more important mayonnaise products of this invention. For reference purposes, the results obtained with mayonnaise products made with a single oil component, cottonseed salad oil, corn salad oil, or the hydrogenated soya bean oils of our co-pending patent application (supra) are included. For the sake of brevity, the performance of samples held only at 75° F. are given. In Table IV are listed the average values for overall flavor acceptability, the standard deviations and the latter expressed on a percentage basis (frequently called the coefficient of variation). In addition, a "t" value reflecting the significance of the difference in flavor performance between any two types of mayonnaise products was calculated. The "t" value is the difference between the mean values obtained divided by the standard error of the difference between the means. On the bottom of Table IV is shown a comparison of the average flavor performance of one type of mayonnaise product versus the average performance of another type of product. The found "t" values and found probabilities are listed. A probability of .05 means that there are 95 chances out of 100 that the difference between means is real. Statisticians universally hold that this probability must be attained before the found difference can be regarded as significant. A probability value of .01 means that the observed difference is likely to be noted in 99 times out of 100 and is highly significant. That the found "t" values reflect a certain probability that the difference between one type of mayonnaise product and another is real, is established by comparing these found "t" values with the significant "t" values for a given probability taken from statistical tables. The significant "t" values and corresponding probabilities listed in Table IV are those for a specific number of tests on the samples in the pair under comparison, i. e. based upon the number of degrees of freedom.

It will be noted that mayonnaise made with cottonseed oil as the sole oil component (product type A) is definitely superior to mayonnaise made with either the corn oil (product type B) or the hydrogenated winterized soya bean oil having an iodine value of 100–120 (product type C). However, mayonnaise made with salad oils of this invention, wherein the soya bean oil fraction has an iodine value of 100–110 (product type D) is definitely superior in flavor performance to mayonnaise made with cottonseed oil as the sole oil component (product type A). Mayonnaise with this type of salad oil is even superior in flavor performance to that noted when hydrogenated winterized soya bean oil having an iodine value of 110–120 (product type E) is used to prepare the mayonnaise products of this invention. When the latter type oil blend is employed, the superiority of the resulting mayonnaise (product type E) over one made with cottonseed salad oil as the sole oil component (product type A) borders on statistical significance. Such mayonnaise (product type E), however, is definitely superior to mayonnaise made with either corn oil (product type B) or hydrogenated winterized soya bean oil having an iodine value of 100–120 (product type C) as the sole oil component.

With respect to other properties required in a quality mayonnaise—such as satisfactory color, desirable firmness, and ability to absorb added aqueous materials without an emulsion break—the said oils of this invention are equal to those of mayonnaise made with either cottonseed salad oil or corn oil as the sole oil component.

TABLE IV

Statistical evaluation of overall flavor acceptability of mayonnaise samples[1] made with vegetable salad oil having an iodine value of less than 120 and/or with hydrogenated winterized soya bean oil having an iodine value of 100–120

Product Type	Winterized Oil in Mayonnaise	Blend	Iodine Value	Average Overall Flavor Acceptability[2]		
				Value from Graph	Standard— Absolute	Deviation— Percentage
		Percent		*sq. mm.*		
A	Cottonseed	100	110–116	670	17	2.6
B	Corn	100	125–128	351	177	50.5
C	Soya Bean	100	120–129	400	18	4.5
D	Cottonseed	50	110–116			
	Soya Bean	50	100–110	1,110	210	18.9
E	Cottonseed	50	110–116			
	Soya Bean	50	110–120	860	170	19.8

Comparisons	"t" Found Value	Found Probability	Significant "t" Value[3]	Corresponding Probability[3]
A better than B	4.00	.01–.001	2.077	.01.
			4.149	.001.
A better than C	23.5	.001	4.140	.001.
C better than B	0.6	.6 –.5	Not significant	Not significant.
D better than A	4.2	.01–.001	3.055	.01.
			4.318	.001.
D better than B	5.8	.001	4.140	.001.
D better than C	6.4	.001	4.140	.001.
D better than E	2.3	.05–.02	2.018	.05.
			2.467	.02.
E better than A	2.0	.1 –.05	1.701	.1.
			2.048	.05.
E better than B	5.5	.001	3.646	.001.
E better than C	10.0	.001	3.646	.001.

[1] Sixty different samples, with 50 flavor scorings on each sample by the eight judges at the bi-weekly observation periods during the overall test period of 12 weeks, were involved in this portion of the study.
[2] Shelf life × degree of acceptability of samples held at 75° F.; areas obtained from graphs on which were plotted the scorings week after week.
[3] From Statistical Tables for Biological, Agricultural and Medical Research by Fisher, R. A., and Yates, F.; Hafner Publishing Co., N. Y. (1948).

While the soya bean oil blends of this invention have been described with particular reference to their use as a salad oil to be marketed as such or to be used in mayonnaise, these descriptions are illustrative and are not to be construed as a limitation of the invention. Such soya bean oil blends may be used for numerous other purposes, such as a component in other salad dressings, or the liquid fraction of a compounded shortening or margarine oil, or wherever a liquid vegetable oil of good stability is required.

The flavor life of the oil blends of this invention may be improved for certain purposes by incorporating in them a monoester of citric acid as described in U. S. Patent 2,485,631 and 2,485,633 granted on October 25, 1949 to The Best Foods, Inc., as assignee of H. W. Vahlteich, C. M. Gooding and R. H. Neal.

In accordance with the term as used in this application, "vegetable salad oil" is intended to include a salad oil of a glyceridic ester of a fatty acid, derived from vegetable sources having an iodine number of less than 120 and having a history of satisfactory usage in mayonnaise manufacture. Preferred oils are winterized cottonseed oil, peanut oil and sesame oil.

It is intended, in accordance with the invention, that the hydrogenated winterized soya bean oil with prescribed properties make up at least 25% of the glyceridic salad oil content of the composition. The remainder of the glyceridic salad oil content is made up of vegetable salad oil of iodine number less than 120. The vegetable salad oil may be incorporated as a mixture of vegetable salad oils from more than a single vegetable source.

Our invention provides a novel salad oil composition imparting superior emulsion stability to mayonnaise. The novel salad oil and mayonnaise prepared from this salad oil have improved flavor characteristics and stability. The firmness and texture of mayonnaise containing the salad oil composition of the invention is of optimum quality. This mayonnaise also possesses the ability to absorb added aqueous components without disturbing the emulsion characteristics of the mayonnaise.

The terms and expressions which we have employed are used as terms of description and not limitation, and we have no intention in the use of such terms and expressions of excluding any equivalents of the features shown and described or portions thereof, but recognize that various modifications are possible within the scope of the invention claimed.

What is claimed is:

1. An edible glyceridic oil composition comprising a vegetable salad oil having an iodine number of less than 120 and a hydrogenated and winterized soya bean oil having an iodine number of 100 to 120 and containing less than 5% of conjugated dienoic acid components, less than 0.2% of conjugated trienoic acid components and less than 0.02% of conjugated tetraenoic acid components.

2. An edible glyceridic oil composition comprising 25 to 75% of a vegetable salad oil having an iodine number of less than 120 and 25 to 75% of a hydrogenated and winterized soya bean oil having an iodine number of 100 to 120 and containing less than 5% of conjugated dienoic acid components, less than 0.2% of conjugated trienoic acid components and less than 0.02% of conjugated tetraenoic acid components.

3. An edible glyceridic oil composition comprising 40 to 60% of a vegetable salad oil having an iodine number of less than 120 and 40 to 60% of a hydrogenated and winterized soya bean oil having an iodine number of 100 to 120 and containing less than 5% of conjugated dienoic acid components, less than 0.2% of conjugated trienoic acid components and less than 0.02% of conjugated tetraenoic acid components.

4. An edible glyceridic oil composition comprising about 50% of a vegetable salad oil having an iodine number of less than 120 and about 50% of a hydrogenated and winterized soya bean oil having an iodine number of 100 to 120 and containing less than 5% of conjugated dienoic acid components, less than 0.2% of conjugated trienoic acid components, and less than 0.02% of conjugated tetraenoic acid components.

5. An edible glyceridic oil composition comprising 25 to 75% of a vegetable salad oil having an iodine value of less than 120 and 25 to 75% of a hydrogenated and winterized soya bean oil having an iodine number of 110 to 120 and containing less than 3½% of conjugated dienoic acid components, less than 0.1% of conjugated trienoic acid components and less than 0.015% of conjugated tetraenoic acid components.

6. An edible glyceridic oil composition comprising 40 to 60% of a vegetable salad oil having an iodine value of less than 120 and 40 to 60% of a hydrogenated and winterized soya bean oil having an iodine number of 110 to 120 and containing less than 3½% of conjugated dienoic acid components, less than 0.1% of conjugated trienoic acid components and less than 0.015% of conjugated tetraenoic acid components.

7. An edible glyceridic oil composition comprising about 50% of a vegetable salad oil having an iodine value of less than 120 and about 50% of a hydrogenated and winterized soya bean oil having an iodine number of 110 to 120 and containing less than 3½% of conjugated dienoic acid components, less than 0.1% of conjugated trienoic acid components, and less than 0.015% of conjugated tetraenoic acid components.

8. An edible glyceridic oil composition comprising 25 to 75% of a vegetable salad oil having an iodine number of less than 120 and 25 to 75% of a hydrogenated and winterized soya bean oil having an iodine number of 100 to 120 and containing less than 1% of conjugated dienoic acid components, less than 0.02% of conjugated trienoic acid components and less than 0.002% of conjugated tetraenoic acid components.

9. An edible glyceridic oil composition comprising 40 to 60% of a vegetable salad oil having an iodine number of less than 120 and 40 to 60% of a hydrogenated and winterized soya bean oil having an iodine number of 100 to 120 and containing less than 1% of conjugated dienoic acid components, less than 0.02% of conjugated trienoic acid components and less than 0.002% of conjugated tetraenoic acid components.

10. An edible glyceridic oil composition comprising about 50% of a vegetable salad oil having an iodine number of less than 120 and about 50% of a hydrogenated and winterized soya bean oil having an iodine number of 100 to 120 and containing less than 1% of conjugated dienoic acid components, less than 0.02% of conjugated trienoic acid components, and less than 0.002% of conjugated tetraenoic acid components.

11. An edible glyceridic oil composition comprising 25 to 75% of a vegetable salad oil having an iodine value of less than 120 and a hydrogenated and winterized soya bean oil having an iodine number of 110 to 120 and containing less than 0.8% of conjugated dienoic acid components, less than 0.015% of conjugated trienoic acid components and less than 0.002% of conjugated tetraenoic acid components.

12. An edible glyceridic oil composition comprising 40 to 60% of a vegetable salad oil having an iodine value of less than 120 and a hydrogenated and winterized soya bean oil having an iodine number of 110 to 120 and containing less than 0.8% of conjugated dienoic acid components, less than 0.015% of conjugated trienoic acid components and less than 0.002% of conjugated tetraenoic acid components.

13. An edible glyceridic oil composition comprising about 50% of a vegetable salad oil having an iodine value of less than 120 and a hydrogenated and winterized soya bean oil having an iodine number of 110 to 120 and containing less than 0.8% of conjugated dienoic acid components, less than 0.015% of conjugated trienoic acid components, and less than 0.002% of conjugated tetraenoic acid components.

14. An edible glyceridic oil composition comprising about 50% of a cottonseed salad oil having an iodine number of less than 120 and about 50% of a hydrogenated and winterized soya bean oil having an iodine number of 100 to 120 and containing less than 5% of conjugated dienoic acid components, less than 0.2% of conjugated trienoic acid components and less than 0.02% of conjugated tetraenoic acid components.

15. An edible glyceridic oil composition comprising 50% of a cottonseed salad oil having an iodine number of less than 120 and 50% of a hydrogenated and winterized soya bean oil having an iodine number of 110 to 120 and containing less than 1% of conjugated dienoic acid components, less than 0.02% of conjugated trienoic acid components, and less than 0.002% of conjugated tetraenoic acid components.

16. A mayonnaise comprising vinegar, eggs, salt, and a glyceridic oil composition comprising a vegetable salad oil having an iodine value of less than 120 and a hydrogenated and winterized soya bean oil having an iodine number of 100 to 120 and containing less than 5% of conjugated dienoic acid components, less than 0.2% of conjugated trienoic acid components, and less than 0.02% of conjugated tetraenoic acid components.

17. A mayonnaise comprising vinegar, eggs, salt and a glyceridic oil composition comprising 25 to 75% of a vegetable salad oil having an iodine value of less than 120 and 25 to 75% of a hydrogenated and winterized soya bean oil having an iodine number of 100 to 120 and containing less than 5% of conjugated dienoic acid components, less than 0.2% of conjugated trienoic acid components and less than 0.02% of conjugated tetraenoic acid components.

18. A mayonnaise comprising vinegar, eggs, salt and a glyceridic oil composition comprising 40 to 60% of a vegetable salad oil having an iodine value of less than 120 and 40 to 60% of a hydrogenated and winterized soya bean oil having an iodine number of 100 to 120 and containing less than 5% of conjugated dienoic acid components, less than 0.2% of conjugated trienoic acid components and less than 0.02% of conjugated tetraenoic acid components.

19. A mayonnaise comprising vinegar, eggs, salt, and a glyceridic oil composition comprising about 50% of a vegetable salad oil having an iodine value of less than 120 and about 50% of

a hydrogenated and winterized soya bean oil having an iodine number of 100 to 120 and containing less than 5% of conjugated dienoic acid components, less than 0.2% of conjugated trienoic acid components, and less than 0.02% of conjugated tetraenoic acid components.

20. A mayonnaise comprising vinegar, eggs, salt and a glyceridic oil composition comprising 25 to 75% of a vegetable salad oil having an iodine value of less than 120 and 25 to 75% of a hydrogenated and winterized soya bean oil having an iodine number of 110 to 120 and containing less than 3½% of conjugated dienoic acid components, less than 0.1% of conjugated trienoic acid components and less than 0.015% of conjugated tetraenoic acid components.

21. A mayonnaise comprising vinegar, eggs, salt and a glyceridic oil composition comprising 40 to 60% of a vegetable salad oil having an iodine value of less than 120 and 40 to 60% of a hydrogenated and winterized soya bean oil having an iodine number of 110 to 120 and containing less than 3½% of conjugated dienoic acid components, less than 0.1% of conjugated trienoic acid components and less than 0.015% of conjugated tetraenoic acid components.

22. A mayonnaise comprising vinegar, eggs, salt, and a glyceridic oil composition comprising about 50% of a vegetable salad oil having an iodine value of less than 120 and about 50% of a hydrogenated and winterized soya bean oil having an iodine number of 110 to 120 and containing less than 3½% of conjugated dienoic acid components, less than 0.1% of conjugated trienoic acid components, and less than 0.015% of conjugated tetraenoic acid components.

23. A mayonnaise comprising vinegar, eggs, salt and a glyceridic oil composition comprising 25 to 75% of a vegetable salad oil having an iodine value of less than 120 and 25 to 75% of a hydrogenated and winterized soya bean oil having an iodine number of 100 to 120 and containing less than 1% of conjugated dienoic acid components, less than 0.02% of conjugated trienoic acid components and less than 0.002% of conjugated tetraenoic acid components.

24. A mayonnaise comprising vinegar, eggs, salt and a glyceridic oil composition comprising 40 to 60% of a vegetable salad oil having an iodine value of less than 120 and 40 to 60% of a hydrogenated and winterized soya bean oil having an iodine number of 100 to 120 and containing less than 1% of conjugated dienoic acid components, less than 0.02% of conjugated trienoic acid components and less than 0.002% of conjugated tetraenoic acid components.

25. A mayonnaise comprising vinegar, eggs, salt, and a glyceridic oil composition comprising about 50% of a vegetable salad oil having an iodine value of less than 120 and about 50% of a hydrogenated and winterized soya bean oil having an iodine number of 100 to 120 and containing less than 1% of conjugated dienoic acid components, less than 0.02% of conjugated trienoic acid components, and less than 0.002% of conjugated tetraenoic acid components.

26. A mayonnaise comprising vinegar, eggs, salt and a glyceridic oil composition comprising 25 to 75% of a vegetable salad oil having an iodine value of less than 120 and 25 to 75% of a hydrogenated and winterized soya bean oil having an iodine number of 110 to 120 and containing less than 0.8% of conjugated dienoic acid components, less than 0.015% of conjugated trienoic acid components and less than 0.002% of conjugated tetraenoic acid components.

27. A mayonnaise comprising vinegar, eggs, salt and a glyceridic oil composition comprising 40 to 60% of a vegetable salad oil having an iodine value of less than 120 and 40 to 60% of a hydrogenated and winterized soya bean oil having an iodine number of 110 to 120 and containing less than 0.8% of conjugated dienoic acid components, less than 0.015% of conjugated trienoic acid components and less than 0.002% of conjugated tetraenoic acid components.

28. A mayonnaise comprising vinegar, eggs, salt and a glyceridic oil composition comprising about 50% of a cottonseed salad oil having an iodine value of less than 120 and about 50% of a hydrogenated and winterized soya bean oil having an iodine number of 100 to 120 and containing less than 5% of conjugated dienoic acid components, less than 0.2% of conjugated trienoic acid components and less than 0.02% of conjugated tetraenoic acid components.

29. A mayonnaise comprising vinegar, eggs, salt, and a glyceridic oil composition comprising about 50% of a vegetable salad oil having an iodine value of less than 120 and about 50% of a hydrogenated and winterized soya bean oil having an iodine number of 110 to 120 and containing less than 0.8% of conjugated dienoic acid components, less than 0.015% of conjugated trienoic acid components, and less than 0.002% of conjugated tetraenoic acid components.

DANIEL MELNICK.
CHESTER M. GOODING.
HANS W. VAHLTEICH.

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Patented Feb. 26, 1957

1

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DEODORIZATION OF HYDROGENATED FATTY OILS

Dwight R. Merker, Chicago, Ill., assignor to Swift & Company, Chicago, Ill., a corporation of Illinois

No Drawing. Application April 2, 1953, Serial No. 346,528

5 Claims. (Cl. 260—424)

The present invention relates in general to the treatment of hydrogenated fatty oils. More specifically, the invention is directed to the removal of hydrogenation odors from hydrogenated fatty oils.

Hydrogenation of vegetable oils is widely practiced for the purpose of producing a hardened vegetable oil to be used in the production of margarine or shortening. Much difficulty has accompanied this particular treatment inasmuch as hydrogenation odors, which are highly undesirable, are formed. These odors, present in hydrogenated oils, must be removed before the oil may be successfully incorporated in an edible product such as margarine inasmuch as the odors are responsible for undesirable flavor in the product. Many efforts have been made to devise methods for removing these odors, but no substantial success has been realized aside from steam deodorization which is customarily used to remove these odors. Other efforts have been made to chemically treat the oil for the purpose of removing the odor-forming constituents. Some success has been realized by these particular treatments, but such success has been only partial as it has been noted that not all odor-forming constituents were removed.

It is therefore an object of the present invention to treat hydrogenated fatty oils in such a manner as to remove hydrogenation odors therefrom.

It is another object to deodorize hydrogenated fatty oils so as to produce a hardened oil having a desirable flavor.

Still other objects, not specifically set forth, will become apparent from the following detailed description.

Generally, the present invention comprises the steps of adding a quantity of phosphoric acid to a hydrogenated fatty oil, maintaining the mixture at an elevated temperature for a period of time while injecting air therein, adding a neutral bleaching earth to the mixture, terminating the injection of air, raising the temperature of the mixture and maintaining the mixture at this raised temperature for a period of time, and thereafter cooling the mixture and removing the solid constituents therefrom by filtration.

It has been found that the treatment of hardened vegetable oils with phosphoric acid and a neutral bleaching earth in the presence of air and under elevated temperature conditions substantially removes hydrogenation odors from the hydrogenated oil. It has also been found that not only is the oil deodorized but it is substantially stabilized against odor and flavor reversion. Deodorized fatty oils are noted for their tendency to revert back to a condition in which undesirable odors are again present. The reason for this tendency is not completely clear, but it is believed that the hydrogenation odor-forming constituents are not sufficiently modified or removed under customary deodorization techniques but instead are only temporarily inactivated. These odor-forming constituents normally become active again upon subsequent storage. The stabilization that occurs as a result of deodorizing fatty oils in accordance with the teachings of the present invention is

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most desirable. The resultant oil is substantially odorless and is also substantially stabilized against odor and flavor reversion.

In the present process the hydrogenated oil may be treated with a comparatively substantial amount of phosphoric acid (0.5 to 4 percent by weight) which is added to the oil in an open tank while the oil is maintained under constant agitation conditions. The phosphoric acid utilized is preferably a 75 percent solution, but other concentrations may be satisfactorily utilized. Air is injected into the open tank and thoroughly admixed with the mixture of oil and phosphoric acid while the temperature of the mixture is maintained within the range of 60° to 95° C. The aeration and agitation of the mixture is continued under these particular temperature conditions for a period of about ½ to 1 hour. At the end of this particular phase of treatment, the aeration is terminated and from 1 to 6 percent (by weight) of a neutral bleaching earth is added to the heated mixture while agitation of the mixture is continued. At this stage of the treatment it is not necessary to add to the mixture the entire amount of bleaching earth to be used. A portion may be added at this point and the remainder may be introduced while cooling the mixture prior to filtration. Such use of the bleaching earth depends on the circumstances and it may be most profitable either to split the addition of the bleaching earth as set forth above or to utilize its action all at one time.

The temperature of the mixture is then raised to approximately 130° to 140° C., and this particular phase of the treatment is carried out for a period of from about ½ to 1 hour. During this second phase of the treatment, the typical odors of hydrogenation disappear. In most instances the oil so treated still possesses other slight odors and flavors which may be removed by a subsequent deodorization step carried out by any well-known method such as steam distillation. It is also noted that at this particular phase of the treatment the oil has a negligible peroxide value. This latter condition apparently is due to the fact that the peroxides are absorbed or decomposed by the bleaching earth which has become acidic. The bleaching earth at this point acquires a dark color due to removal of impurities from the oil. Following the bleaching and high temperature phase, the oil is cooled to a temperature below 100° C. and thereafter filtered. Elution of the filter cake with acetone gives a reddish colored solution which apparently is made up of the odor-forming constituents originally present in the untreated oil.

Oil treated in accordance with the teachings of the present invention should be low in free fatty acid content. For maximum results the free fatty acid content should be less than 0.1 percent. If the oil to be treated has a free fatty acid content greater than 0.1 percent, it is advisable to subject the oil to alkali refining, in accordance with methods well-known in the art, prior to subjecting the oil to treatment by the present process.

The following specific examples are set forth as illustrative only of the present invention and are not to be construed as limiting thereto.

Example 1

Two percent (by weight) of a 75 percent solution of phosphoric acid was added to a quantity of well agitated hardened soybean oil (Zeiss butyro Refractive Index 41.0) in an open tank into which air was injected. The temperature of the mixture was raised to within the range of 90° to 95° C. and held within this range for 30 minutes. Following the above treatment, the injection of air was terminated and, while agitation was maintained, 4 percent (by weight) neutral bleaching earth was added to the mixture. The temperature of the agitated mixture

was [illegible] 10° C. [illegible] there for 3 hours. The oil was then [illegible] at a temperature of 60° C., filtered, and steam [illegible] at 235° C. for 3 hours. A control [illegible] was steam deodorized at 235° C. for 3 hours in the conventional manner. Both the control sample and a sample of the oil treated in accordance with the teaching of the present invention were incubated in an oven for 1 week at a temperature of 140° F. The control sample was completely reverted whereas the treated sample was just beginning to develop an off flavor. Actually, the control sample was unacceptable in flavor after 2 days' incubation.

Example II

Hardened cottonseed oil (Zeiss butyro Refractive Index 41.0) was treated in the same manner as set forth in Example I. The flavor stability of a sample of the treated oil was compared with a control that had been steam deodorized only. The control oil had reverted after 3 days, whereas the treated oil was stable for approximately a week.

Example III

When hardened whale oil (Zeiss butyro Refractive Index 42.0) was treated as set forth in Example I, an almost colorless oil was obtained that did not begin to revert until after 3 days in an oven at 140° F. A steam deodorized control sample was highly colored and reverted in less than a day under the incubation conditions.

Fatty oils treated in the manner as shown in the examples exhibit flavor stability during subsequent storage. The typical odors of hydrogenation are removed and odor reversion normally found in oil treated according to known practices is greatly retarded. The amount of phosphoric acid utilized may range from 0.5 to 4 percent by weight where the use of a 75 percent solution is involved. The neutral bleaching earths which may be utilized are those normally known in the art, such as fuller's earth, kieselguhr, etc.

The odor which is present following the bleaching step may be removed by a subsequent deodorization step according to practices well-known in the art. This subsequent deodorization step may not always be necessary. However, normally such a step is necessary and may be carried out by the use of steam, gas, volatile organic liquids, etc. When such a deodorization step is necessary it has been noted that the time normally required under conventional conditions is greatly reduced. Normally, conventional deodorization is carried on for a period of from 3 to 6 hours. When material treated according to the teachings of the present invention is subjected to conventional deodorization the time required is from ½ to 3 hours. Such deodorization is carried out within a temperature range of from 200° C. to 260° C.

It is considered necessary that oxygen be present during the acid treating phase in order to attain the desired results. It has been found that, if the treatment is carried out under nonoxidizing conditions, such as under an atmosphere of nitrogen or in vacuo, the odors of hydrogenation are not removed and the flavor stability of the oil is not as great.

Obviously, many modifications and variations of the invention as hereinbefore set forth may be made without departing from the spirit and scope thereof, and therefore only such limitations should be imposed as are indicated in the appended claims.

I claim:

1. The process of removing hydrogenation odors and flavors from fatty oils which comprises: adding concentrated phosphoric acid to a hydrogenated fatty oil in an amount greater than about 0.5 percent based on the weight of the oil; heating the mixture of acid and oil to a temperature of 60 to 95° C. for at least about one-half hour while subjecting said mixture to aeration and agitation; adding to the aerated mixture at said temperature an amount of bleaching earth; thereafter increasing the temperature of said mixture to about 130°–140° C. for about one-half hour while maintaining agitation and then cooling and filtering the mixture to remove said bleaching earth and impurities absorbed therein.

2. The process of removing hydrogenation odors and flavors from fatty oils which comprises: adding concentrated phosphoric acid to a hydrogenated fatty oil in an amount greater than about 0.5 percent based on the weight of the oil; heating the mixture of acid and oil to an elevated temperature within the range of about 65° C. to about 95° C. for about one-half to one hour while subjecting the mixture to aeration and agitation; adding to the aerated mixture at said elevated temperature an amount of bleaching earth; thereafter increasing the temperature of said mixture to about 130° C.–140° C. for about one-half to one hour while maintaining agitation to insure full bleaching action, and then cooling and filtering the mixture to remove said bleaching earth and impurities absorbed therein.

3. The process of removing hydrogenation odors and flavors from fatty oils which comprises: adding concentrated phosphoric acid to a hydrogenated fatty oil in an amount greater than about 0.5 percent based on the weight of said oil; heating the mixture to an elevated temperature within the range of about 60° C. to about 95° C. for a period of about one-half hour while subjecting the mixture to aeration and agitation; adding to the aerated mixture at said elevated temperature an amount of bleaching earth; thereafter increasing the temperature of said mixture to within the range of about 130°–140° C. for about one-half hour while maintaining agitation to insure full bleaching action, and then cooling and filtering the mixture to remove said bleaching earth and absorbed impurities.

4. The process of removing hydrogenation odors and flavors from fatty oils which comprises: forming a mixture of oil and concentrated phosphoric acid, said acid being present in an amount greater than about 0.5 percent based on the weight of the oil; heating said mixture to a temperature of about 60° to 95° C. for a period of at least about one-half hour while maintaining aeration and agitation thereof; adding bleaching earth to said mixture and reheating said newly formed mixture to a temperature of about 130°–140° C. for at least about one-half hour; cooling said newly formed mixture and adding additional bleaching earth thereto; and thereafter filtering the resultant mixture.

5. The process of deodorizing hydrogenated fatty oils which comprises: forming a mixture of oil and concentrated phosphoric acid, said acid being present in an amount greater than about 0.5 percent based on the weight of the oil; heating said mixture to about 60°–95° C. for at least about one-half hour while maintaining aeration and agitation thereof; adding bleaching earth to said mixture and reheating said newly formed mixture to about 130°–140° C. for at least about one-half hour; cooling said newly formed mixture followed by filtering thereof, and thereafter subjecting the treated oil to gaseous distillation.

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NON-SELECTIVE HYDROGENATION OF FATS AND OILS

Dwight R. Merker, Chicago, Ill., assignor to Swift & Company, Chicago, Ill., a corporation of Illinois

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12 Claims. (Cl. 260—409)

This invention relates to a process for treating oils and more particularly to a process of hydrogenating vegetable and animal oils to produce a partially hardened fat with an improved flavor stability and a wide range of plasticity.

Fatty materials, particularly glyceride oils intended for use in margarine or shortening are ordinarily hydrogenated to convert the liquid oils to semi-solid plastic fats which may be used in margarine or shortening manufacture. The liquid fatty material, a metallic catalyst and gaseous hydrogen are reacted under conditions of pressure, temperature and agitation that result in the addition of hydrogen at the points of unsaturation in the fatty acid portion of the glyceride molecule. Since the degree of hydrogenation can be determined by the amount of unsaturation remaining in the oil, iodine value is considered a good measure of the degree of hydrogenation. It is known that hydrogenation is preferential to the extent that certain unsaturated fatty acid radicals are saturated much more easily than others. In general it can be stated that, irrespective of the conditions of the reaction such as pressure, agitation, and temperature, the more unsaturated radicals will be hydrogenated more readily than those which contain fewer double bonds. The more "selective" the hydrogenation reaction, the greater will be the tendency for the more unsaturated radicals to add hydrogen in preference to the more saturated radicals. More specifically, those radicals containing a greater number of active methylene groups will add hydrogen more readily than more saturated acid chains.

In the hydrogenation of glyceride oils intended for ultimate use in the manufacture of margarine and shortening, it has been the practice heretofore to strive for increased selectivity in the hydrogenation process. The resultant plastic fat in which hydrogenation of monounsaturated acid radicals has been kept to a minimum, is blended with other hard or soft fats to produce an edible product of the desired consistency. It has now been found that by a process of non-selective hydrogenation, it is possible to adjust the hardness and plasticity of the hydrogenated material to any desired range and to produce a harder fat with a higher Wiley melting point, a higher setting point and a higher solid fat index for a given iodine value.

It is therefore an object of this invention to provide a process for treating animal and vegetable oils to produce a partially hardened oil having an improved range of plasticity.

Another object of this invention is to provide an adjustable non-selective method for hydrogenating glyceride oils.

A further object of this invention is to provide a partially hardened, edible plastic fatty material of increased

flavor stability, low susceptibility to color reversion and a high degree of plasticity over a wide temperature range.

Still another object of the present invention is to provide a method for producing a wide plastic range edible fatty material having a higher melting point for a given iodine value.

A further object of this invention is to provide a method for producing a fatty product well adapted to the manufacture of margarine and shortening materials that obviates the necessity of blending to obtain desirable product consistency.

Additional objects, if not specifically set forth herein will be readily apparent to one skilled in the art from the following detailed description of the invention.

In accordance with the present invention a non-selective method of hydrogenating glyceride oils is provided wherein a very small amount of an acidic promoter is added to the oil just prior to hydrogenation. The acidic material which promotes the adjustable non-selectivity of the reaction is represented by mineral acids and organic acids.

More specifically, the present invention comprises adding a mineral acid such as dilute phosphoric or sulfuric acid, to a glyceride oil or mixtures thereof, and catalytically hydrogenating the mixture at conventional hydrogenation pressure and temperature to produce a partially hardened fat having good plasticity over a wide temperature range. Organic acids in aqueous solution such as citric acid, tartaric acid and other acids containing the same functional groups, especially those which are non-volatile, are also well suited to use as the promoter compos[illegible] The concentration and distribution of the acidic material are directly related to the range of plasticity since it has been found that the degree of the plastic range varies with the concentration of the promoter added prior to hydrogenation. Since the breadth of the range of plasticity is dependent upon the amount of promoter added, individual requirements with respect to the consistency desired in the final product will determine the amount of promoter necessary to produce a given product. It has been found, however, that an upper limit of around 0.10% of the promoter material by weight of refined oil is desirable, in order to avoid substantial poisoning of the catalyst. Larger amounts of the non-selectivity promoting agent are desirable when oils containing appreciable amounts of impurities are hydrogenated since the acidic promoter may be bound by impurities in the oil. Among other impurities in the oil with which the acid may react are peroxides, aldehydes, and ketones. The presence of such impurities requires a higher concentration of the promoter acid to produce the same degree of non-selectivity that is obtained when hydrogenating refined and deodorized oils. Although in the preferred form of the present invention deodorized oils have been non-selectively hardened, undeodorized oils may also be treated provided a sufficient excess of the acidic agent is added to react with impurities in the oil.

Although the compounds employed in the practice of this invention as promoters of non-selective hydrogenation have been reported to be catalyst poisons, the very slight poisoning effect is not particularly objectionable in view of the great advantage obtained in permitting adjustment of the plastic range merely by varying the concentration or distribution of the promoter. Moreover, partial poisoning of the catalyst tends to decrease the selectivity of the reaction in the manner desired. It should be

noted, however, that the non-selective nature of the hydrogenation reaction is not necessarily dependent upon or directly related to catalyst poisoning. If the degree of non-selectivity depended only on catalyst poisoning, a reduction in the amount of catalyst employed should produce essentially the same type or degree of non-selective hydrogenation as that induced by the acidic promoters.

In order to show the effect of catalyst poisoning as compared to the effect of acidic promoters, a hydrogenation run was conducted using 0.025% catalyst in the absence of promoter rather than 0.1% catalyst with acid promoter as in the preferred examples set out hereafter. While only 20 to 40 minutes was required to hydrogenate to a given iodine value in the preferred examples, approximately 2 hours were required to hydrogenate the charge containing the reduced amount of catalyst to the same iodine value. Physical constants for the oil hardened in this manner differed substantially from those for oil hardened in the presence of small amounts of acid. Moreover, the degree of non-selectivity obtained under these conditions which simulate catalyst poisoning is only about 5–10% of that realized when the acidic promoters of the present invention are employed.

The following examples will serve to more clearly illustrate the process and products of the present invention.

EXAMPLE 1

An oil charge of 2000 grams of a blend of soybean oil (80%) and cottonseed oil (20%) was placed in a hydrogenator. Approximately 2 milliliters of a 10% aqueous citric acid solution was added to the oil charge and the mixture agitated for several minutes. An adsorbent composition containing 0.1% carbon and 0.1% dicalite was added to the acidified oil and the mixture heated to 135° C. after evacuation of the reaction vessel. About 0.1% nickel catalyst was then added and the charge heated to about 180°–200° C. After thirty minutes reaction time, with gaseous hydrogen at 20 p. s. i. pressure, the iodine number of the hydrogenated oil was 72.8. Analytical data for the products resulting from different concentrations of added citric acid are shown in Table I.

EXAMPLE 2

Two charges, one using the soybean-cottonseed oil blend of Example 1 and one using soybean oil alone were hydrogenated in a manner similar to Example 1 using tartaric acid as the promoter. Physical constants of the partially hardened products are set out in Table II.

EXAMPLE 3

Phosphoric acid (75% aqueous solution) was employed as the promoter in a hydrogenation reaction carried out in accordance with the conditions of Example 1. Table III shows the analytical data for the resultant products.

EXAMPLE 4

Products produced by typical runs carried out in a manner similar to that employed in Example 1 using a 50% aqueous solution of sulfuric acid as the promoter are set out in Table IV.

Because the hydrogenation proceeds in a non-selective course there is considerable formation of saturated glycerides as distinguished from selectively hardened oils. The percentage of polyunsaturated glycerides remaining in the partially hardened product is also higher than is present in hardened fatty materials produced by selective hydrogenation. It is the combination of these saturated and polyunsaturated glyceride molecules which accounts for the unexpected wide plastic range in the products of this invention. The percentage of unsaturated compounds in the non-selectively hardened oils of this invention is shown in the tables which follow.

Table I.—Effect of citric acid (10% aqueous solution) on the hydrogenation of soybean oil (80%), cottonseed oil (20%) blend

	Control	Citric acid promoter		
		0.025%	0.067%	0.1%
Iodine value	76.3	74.6	72.8	66.8
Softening point, ° F.	81	102	113	126
Wiley, ° F.	93	107	115	128
Setting point, ° C.	26.4	29.7	34.4	39.7
Melting point, ° F.	94	111	122	131
Solid fat index:				
50° F.	23.5	32.5	37.9	31.4
70° F.	12.2	21.0	29.1	28.2
80° F.	6.8	17.9	27.6	28.1
92° F.	1.1	9.0	15.9	23.5
100° F.	0.0	3.6	11.9	19.2
Percent linoleic acid	6.0	7.6	9.9	25.6
Percent linolenic acid	0.1	0.32	0.46	1.5
Percent conjugated diene	0.13	0.27	0.24	0.44
Hydrogenation time (minutes)	13	21	30	40

Table II.—Effect of tartaric acid (10% aqueous solution) on the hydrogenation of vegetable oils

	80-20 soybean-cottonseed oil blend		Soybean oil	
	Control	0.067% soln.	Control	0.067% soln.
Iodine value	76.3	75.1	75.8	73.0
Softening point, ° F.	81	110	85	107
Wiley, ° F.	92	116	88	115
Melting point, ° F.	94	122	90	122
Setting point, ° C.	26.4	37.2	26.4	35.9
Solid fat index:				
50° F.	23.5	34.4	33.0	34.4
70° F.	12.2	27.1	18.0	25.6
80° F.	6.8	23.0	10.2	23.1
92° F.	1.1	17.3	1.8	13.1
100° F.	0.0	11.3	0.4	9.1
Percent linoleic acid	6.0	10.7	2.2	8.5
Percent linolenic acid	0.1	0.53	.02	0.37
Percent conjugated diene	0.13	0.39	1.1	0.26
Hydrogenation time (minutes)	13	34	23	28

Table III.—Effect of phosphoric acid (75% aqueous solution) on the hydrogenation of soybean oil (80%), cottonseed oil (20%) blend

	Phosphoric acid added			
	Control	0.006%	0.010%	0.024%
Iodine value	76.3	76.5	79.4	78.4
Softening point, ° F.	81	104	110	122
Wiley, ° F.	92	107	114	130
Melting point, ° F.	94	107	121	137
Setting point, ° C.	26.4	29.6	35.3	43.5
Solid fat index:				
50° F.	23.5	27.5	28.8	23.8
70° F.	12.2	16.4	24.4	32.2
80° F.	6.8	12.4	22.4	30.8
92° F.	1.1	5.1	16.4	28.2
100° F.	0.0	1.3	11.4	26.3
Percent linoleic acid	6.0	9.5	12.6	18.8
Percent linolenic acid	0.1	0.8	1.1	1.7
Percent conjugated diene	0.13	0.20	0.24	0.5
Hydrogenation time (minutes)	13	18	28	50

Table IV.—Effect of sulfuric acid (50% aqueous solution) on the hydrogenation of soybean oil

	Control	0.021% acid added
Iodine value	75.8	76.6
Softening point, ° F.	85	115
Wiley, ° F.	88	118
Melting point, ° F.	90	122
Setting point, ° C.	26.4	33.8
Solid fat index:		
50° F.	33.0	42.2
70° F.	18.0	33.4
80° F.	10.2	30.1
92° F.	1.8	20.6
100° F.	0.0	13.3
Percent linoleic acid	2.2	4.7
Percent linolenic acid	.03	0.49
Percent conjugated diene	1.1	1.9
Hydrogenation time (minutes)	21	23

The sequence of steps for the addition of the reactants to the hydrogenator is not limited to that shown in Example 1. The order may be interchanged or the constituents may be admixed with oil and the mixture employed as a stock solution for subsequent addition to charges in the hydrogenator. The temperature at which the promoter and catalyst are added to the oil may be varied substantially provided sufficient moisture is present.

In order to show the effect of removing impurities by deodorization prior to hydrogenation and also to illustrate a variation in the manner in which the constituents are added to the hydrogenator, the hydrogenation described in Example 5 was run:

EXAMPLE 5

Approximately 2 milliliters of a 10% aqueous citric acid solution was added to 100 grams of deodorized oil (80% soybean, 20% cottonseed). An adsorbent composition of 1.5 grams carbon and 1.5 grams filter aid was added and the entire mixture agitated during heating to about 80° C. After the addition of 3 grams of nickel catalyst (Rufert), the agitation was continued for 15 minutes. The stock mixture was then added to a hydrogenator containing 2900 grams of deodorized oil (80% soybean, 20% cottonseed). The hydrogenator was evacuated, agitated, and heated to 190° C. The charge was then hydrogenated at 20 p. s. i. for 28 minutes. The product had the following physical constants:

Iodine value	73.4
Softening point, °F	126
Wiley, °F	132
Setting point, °C	44.6
Melting point, °F	133
Solid content index:	
50° F	36.2
70° F	32.1
80° F	31.5
92° F	27.1
100° F	22.2
Percent linoleic acid	15.0
Percent linolenic acid	1.1
Percent conjugated diene	0.54

A comparison of the analytical data for the product of the foregoing example in which 0.067% citric acid was used as the promoter, with that set out for a like amount of this promoter in Table I shows the marked advantage obtainable by the removal of traces of impurities prior to hydrogenation.

From the above tables, it can be seen that a method is herein disclosed by which hardness and plastic range of any fatty material can be adjusted within predetermined limits merely by altering the concentration or distribution of the acidic promoter employed in the hydrogenation reaction. For any given iodine value a fat of increased hardness, higher melting point and a broader range of plasticity may be prepared. The degree of polyunsaturation also increases as the amount of promoter employed is increased. The quantitative effect of the promoter on the selectivity of the reaction is best illustrated by the data in Table III. As the amount of promoter employed in the hydrogenation reaction increases, although the iodine value remains relatively constant, the melting point and setting point increase as does the solid content index. It is possible therefore to simulate blended oil such as is used in margarine and shortening manufacture by a one step hydrogenation method. Distribution of the promoter may be altered by use of various inactive adsorbents.

The hydrogenation process may be carried out in the usual manner using any of the well known metallic hydrogenation catalysts such as finely divided nickel or nickel-copper. Although in the examples disclosed herein only soybean and cottonseed oils have been hydrogenated, any of the numerous animal or vegetable glyceride materials known in the art may be hydrogenated by the method of the present invention.

The effect of altering the course of hydrogenation to improve the plastic range has been achieved using only trace amounts of the promoter. At normal hydrogenation temperatures and pressures there is not only an absence of any noticeable deleterious effect of the acidic promoters on the oil, but rather an improvement in flavor stability of oils hardened in accordance with this invention. Oil hydrogenated in the presence of trace amounts of acid, bleached and deodorized, retains good flavor and odor undiminished for a long period of time. Even upon long standing, the odor and flavor that develop are typical of rancidity rather than reversion.

Obviously, many modifications of the invention hereinbefore set forth may be made without departing from the spirit and scope thereof, and therefore only such limitations should be imposed as are indicated in the appended claims.

I claim:

1. A non-selective hydrogenation process for hardening a glyceride oil to produce a partially hardened fat of improved flavor stability and wide plastic range which comprises: incorporating in said glyceride oil in the presence of moisture prior to hydrogenation a small but effective amount of a material selected from the group consisting of mineral acids and aliphatic hydroxy acids.

2. A non-selective hydrogenation process for hardening a glyceride oil to produce a partially hardened fat of improved flavor stability and wide plastic range which comprises: incorporating in said glyceride oil prior to hydrogenation a small but effective amount of an aqueous solution of a mineral acid.

3. The process of claim 2 wherein the mineral acid is sulfuric acid.

4. The process of claim 2 wherein the mineral acid is phosphoric acid.

5. A non-selective hydrogenation process for hardening a glyceride oil to produce a partially hardened fat of improved flavor stability and wide plastic range which comprises: incorporating in said glyceride oil prior to hydrogenation a small but effective amount of an aqueous solution of an aliphatic hydroxy acid.

6. The process of claim 5 wherein the aliphatic hydroxy acid is citric acid.

7. The process of claim 5 wherein the aliphatic hydroxy acid is tartaric acid.

8. A non-selective hydrogenation process for hardening a glyceride oil to produce a partially hardened fat of improved flavor stability and wide plastic range which comprises: incorporating in said glyceride oil in the presence of moisture prior to hydrogenation in an amount not substantially in excess of 0.1% by weight of the glyceride of a material selected from the group consisting of mineral acids and aliphatic acids.

9. A partially hardened fat suitable for shortening prepared by the non-selective hydrogenation of a glyceride oil in the presence of a small amount of an aqueous solution of a material selected from the group consisting of mineral acids and aliphatic hydroxy acids, said fat having an iodine value of from 72 to 81, a solid content index of at least 4% at 100° F. and a melting point of at least 110° F.

10. A partially hardened fat suitable for margarine prepared by the non-selective hydrogenation of a glyceride oil in the presence of a small amount of an aqueous solution of a material selected from the group consisting of mineral acids and aliphatic hydroxy acids, said fat having an iodine value of from 76 to 80, a solid content index of not more than 1.5% at 100° F. and a melting point of not more than 104° F.

11. A method for producing an edible fatty material of improved flavor stability having a wide range of plasticity which comprises: hydrogenating a refined de-

odorized glyceride material in the presence of a small amount of a material selected from the group consisting of mineral acids and aliphatic hydroxy acids in an aqueous carrier.

12. A non-selective hydrogenation process for hardening a glyceride oil to produce a partially hardened fat of improved flavor stability and wide plastic range which comprises: incorporating in said glyceride oil prior to hydrogenation in an amount not substantially in excess of about 0.1 percent by weight of the glyceride of an aliphatic hydroxy acid in an aqueous carrier.

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UDC 542.941.7 : 541.12

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Chem. Pharm. Bull (Tokyo) 13; 843-858 Jul. 1965

110. Ryuichi Miyake, Yoshizo Shimamura, and Hisa Yajima : Kinetics of Fat Hydrogenation.*1

(*Faculty of Pharmaceutical Sciences, University of Chiba**2)

Preliminary remarks: In the previous paper[1] it is concluded that the reaction is of zero order with respect to the concentration of substrate in the hydrogenation of whale oil on copper catalyst, whereas its rate is retarded by self-poisoning. An effort was made to formulate the poisoning effect, however, it was noticed that this condition was not restricted to any special material. This article follows the previous paper wherein examples are presented with the most familiar materials, oleic acid ester and nickel catalyst.

Discussion, however, is centered on the independency of concentration of substrate in greater detail with the advancing of the hypothesis of local mobility of reactants on catalyst surface, and in connection with this the conditions are criticized, under which the most acceptable Langmuir-Hinshelwood theory is applicable.

Under these considerations, it is hoped that liquid hydrogenation may be proved to be providing a potent basis to the development of the theory heterogeneous catalytic reaction keeping pace with ethylene hydrogenation.

Fat hydrogenation, having a tremendous literature, is most pertinent for advancing a general theory of liquid hydrogenation. Nevertheless, it has not played a significant roll in the relevant field of kinetic or the chemical engineering as its studies had been confined chiefly for practical purposes. In other words, it has not established its own theory, from which imaginative endeavors can be evolved.

The principal reason for this retrogression lies without a doubt in the complexities in determining the reaction order with respect to the concentration of substrate, which is at least one of the important starting points to reach a overall self consistent comprehension.

There have appeared many references concerning the reaction order, reporting zero, first, and a combination of both, since the establishment of fat hydrogenation industry. If reviewing the recent literatures appearing in J. Am. Oil Chemists' Soc.,

*1 Presented at the Annual Meeting of Chem. Soc. Japan, April 6, 1961.

*2 Chiba, Japan (千葉大学 [illegible]).

1) R. Miyake, Y. Shimamura : Bull. Chem. Soc. Japan, 29, 611 (1956).

a tendency to follow the consideration of the first order kinetics.[2-4] However, readers must not overlook the fact that even authors of these references do not content themselves with these results. Bailey in his book[5] expresses his firm opinion in relating the complicated fashions of kinetic order in substrate with reaction conditions.

Attention must also be given to the process of determining the reaction order. A straightforward application of an empirical formula $-dc/dt=kc^n$ has been made. In accordance with the conventional mathematical procedure, the cited examples in the present paper give approximately a unity as the value of the order (Fig. 4). Other examples having zero and various numerical orders exceeding unity will be presented in succeeding papers under the thesis of poisoning features of catalysts.

The adoption of mathematical method is a hypothetical extention of the mass law to heterogeneous reaction, due to the lack of the knowledge about the definite specificity of this system. In addition, there is an inevitable characteristic involved in heterogeneous reaction, namely, the deactivation of catalyst. The mathematical treatment, neglecting the deactivation of catalyst, might well be compared with a behavior of organic chemist who attempts to deduce a chemical structure of a substance containing impurities.

One of the simplest method as to examine whether deactivation of catalyst is accompanied, when the reaction is found to be decreasing in rate, is to measure the reaction rate by changing the initial concentration of substrate with its product. This method is, of course, the one which gives a kinetic order with respect to the concentration of substrate based on the original definition.

Unfortunately, this orthodox method in the determination of reaction order has long been ignored, because of too frequent encounters with apparent first order in substrate, which conforms with the researcher's subconscious reasonings.

The same state of affairs is seen in almost all of the liquid hydrogenation of other compounds. The present tendency in this field is to consider zero order manner in substrates as rather an extraordinary case governed by hydrogen diffusion.[6,7]

On the contrary, reviewing precisely all the references available,[8] the reverse of the current tendency is probably valid, and if allowed to run to the extreme, only a discovery of the real unimolecular reaction with respect to the concentration of substrate will deserve a particular notice in the low temperature hydrogenation where no appreciable dehydrogenation will take place. The most doubtfulness is the acceptance of the formulation $-dc/dt=k\,P_1\,V$,[9] as far as the volume of liquid phase (V) is concerned.

Detailed discussion on deactivation by poison will not be given until further examples are presented, but the readers will be able to predict the outline by referring to the previous paper.[3] Also, the hydrogen pressure effect will be likewise laid aside, as confusions will surely arise from it, unless self-poisoning features have been better comprehended.

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[8] As will be stated in the later section, re-examinations will suffice for the substrates involving electronegative groups, others being mostly of zero order, and short critics for crotonic acid and *p*-nitrophenol are given there.

2) L. J. Swicklik, C. A. Hollingsworth, B. F. Daubert: J. Am. Oil Chemists' Soc., 32, 69 (1955).
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6) A. A. Zinovlev: Zhur. Priklad. Khim. (J. Applied Chem.), 23, 99 (1950); C.A., 44, 4320 (1950).
7) H. C. Yao, P. H. Emmett: J. Am. Chem. Soc., 81, 4125 (1959).
8) "Advances in Catalysis" (K), 727 (1957). Academic Press Inc., Publishers, New York.

Many formulations of rate for specific entities, however, should not be merely discarded as worthless, even if, in all the cases self-poisonings are really confirmed, because they are regarded as poisoning features demonstrated in terms of concentration of substrates, which are certainly affording some characteristic informations on poison formation and state of catalyst surface.

There is another facet of problems arising in the experiments presented here. The rate does not increase in proportion to the amount of catalyst; we intentionally avoided dealing with this problem as much as possible, since it is separable in reason from the facet of the kinetic order determination, although it is difficult to make a complete detour of this matter phenomenally. Miyake and Yajima[9] interpreted this phenomenon by introducing the idea of gas liquid interface. According to this theory, reaction occurs exclusively in this hydrogen rich zone, to which catalysts are readily attracted. Other complicated effects on the rate such as temperature, agitation, and hydrogen pressure beside the amount of catalyst, are expected to be understood better on this theory. And also it is true that they were not able to realize the importance of the roll of this specific zone, until having confirmed the kinetic order with respect to the concentration of substrate and accumulated enough informations on the poisoning behavior of catalyst.

Experimental Results

Hydrogenations were carried out on Tsubaki oil and Finback whale oil with nickel barium sulfate catalyst at 180° under normal hydrogen pressure. Measurement of the absorbed hydrogen in the catalyst reduction and the hydrogenation reaction was conducted in the same apparatus and with the same precautions as reported in the previous papers.[1,10]

No effect of the amounts of oil on reaction rate was confirmed up to about ten grams, under 200 r.p.m. horizontal circular agitation, which were applied throughout these experiments.

The Tsubaki oil, which was obtained from Tsubaki seeds, *Thea japonica* Nois, is very similar to olive oil in all respects, having a saponification value of 193, an iodine value of 80 and contained 9.5 % saturated acids (by Twitchell's lead salt alcohol method). The oil with the above characteristics should contain 57% oleic acid and 3 % linoleic acid by weight. The content of linoleic acid in one gram of the oil, an amount used in the experiments, is too small to give any detectable variance in the hydrogenation rates for oleic acid, although its rate is twice as rapid as that of oleic acid.

The saturated oil used as a diluent, having iodine value below 0.1, was prepared from Tsubaki oil by hydrogenation, and was refined carefully by alkali neutralization and bleached by active clay just as in the case of Tsubaki oil, since it involves some injurious substance which was not present before hydrogenation.

The purified Finback whale oil having iodine value of 116 and saponification value of 193, was selectively hydrogenated on nickel catalyst.

The higher unsaturated acids vanish after consuming 37 ml. of hydrogen gas per gram of oil, left most of the oleic acid series acids as unsaturated acids.[11]

The catalyst, developed by Okada in our laboratory, was prepared by mixing a nickel sulfate solution with a barium hydroxide solution, washing the precipitate, and drying and reducing in hydrogen at 350° for 3 hours. An average of 6 ml. of hydrogen was consumed per 0.1 g. of this catalyst, which has substantially similar activity

9) R. Miyake, H. Yajima: Actes du Deuxieme Congrès International de Catalyse, Paris, 2359 (1960).
10) R. Miyake: Yakugaku Zasshi, 68, 8 (1948).
11) *Idem*: *Ibid.*, 68, 1 (1948).

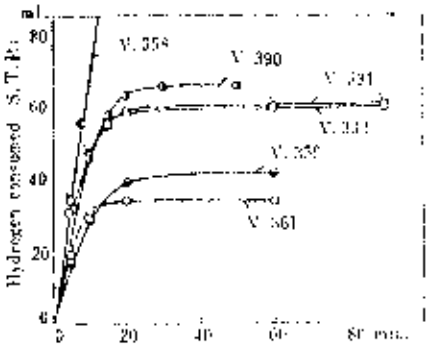

Fig. 1.

Hydrogenation rate of oleic acid in Tsubaki oil is independent of dilution with its hydrogenated product. Initial rates remain constant when amounts of nickel barium sulfate catalyst exceed 0.1 g.

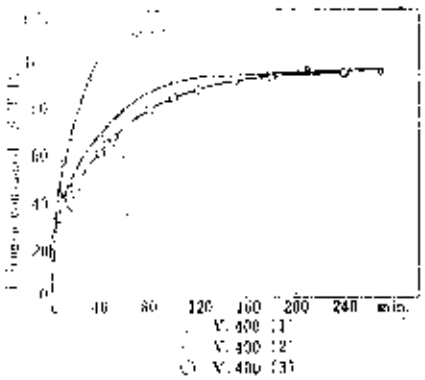

Fig. 2.

With repeated use of the catalyst in the hydrogenations of whale oil, a successive self-poisoning occurred over the whole course in the lower unsaturated acids of each run, but no appreciable degradation in the higher unsaturated acids. Heatings in a vacuum for three hours, marked by arrows, produced little effect on the subsequent rates.

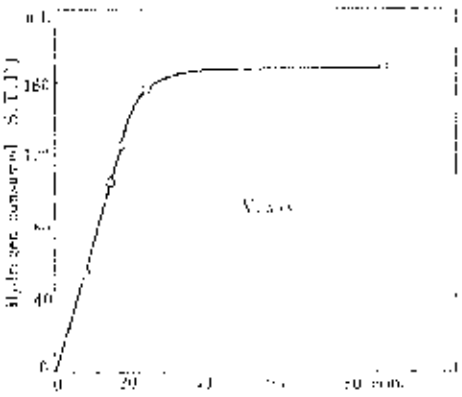

Fig. 3.

Feature of self-poisoning varies frequently with a portion of the catalyst taken even from the same stock. The rate of V. 358 was linear nearly to the end of reaction, an exemplification of prolongation of the (a-b) stage shown in Fig. 4 for V. 332.

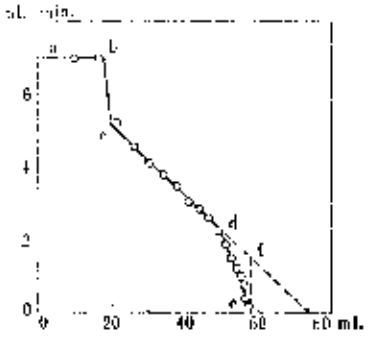

Fig. 4.

dx/dt-x relation for run V. 332. An expression of self-poisoning of catalyst: a-b: a linear portion, c-d: a portion which can be expressed by the typical poisoning equation $dx/dt = k_1(N_0-k_2x)$, where k_1N_0 and k_1k_2 are estimated to be 7.068 ml./min. and 0.0934 min^{-1} respectively. Formally the whole course may be expressed by the first order formulation: $2.3\Omega \log(60/20-x) = 0.1448t+4.1088$.

Table I.

Run No.	Catalyst (g.) (before reduction)	Tsubaki oil (g.)	Completely hydrogenated Tsubaki oil (g.)
V. 331	0.1222	0.8567	1.069
V. 332	0.1125	0.8193	1.010
V. 359	0.0556	0.6026	1.020[a]
V. 361	0.0548	0.1912	
V. 390	0.1052	0.9443	
V. 358	0.2068	2.5400	

a) used without purification after separation of catalyst.

Table II.

Run No.	Catalyst (g.) (before reduction)	Finback whale oil (g.)
V. 400 (1)	0.0701	1.1430
V. 400 (2)		0.9753
V. 400 (3)		0.9870

and function as the usual nickel kieselguhr catalyst in this reaction. The reduced catalyst was used without exposing to air.

Hydrogenation was made with 10 per cent of catalyst at 180° under atmospheric pressure. Tsubaki oil was diluted down to one third with its completely hydrogenated product and hyrogenation rates were compared in two series, using 0.1 g. and 0.05 g. of catalyst respectively (Fig. 1). Similar experiments were done with Finback whale oil, but the catalyst was used in succession for the three times by supplying new oil after completion of each run (Fig. 2, Table I).

Oil and catalyst were subjected to heat treatment for three hours several times, by evacuating the whole assembly during hydrogenation of whale oil, other conditions being unchanged (Fig. 2).

Dilutions evidently did not substantially affect the rate throughout the reaction course with the Tsubaki oil, and also with the portion of higher unsaturated acids of whale oil for the first 36 ml. of hydrogen consumption.

Reaction rates, however, decreased to a considerable degree especially toward the end of each run in Tsubaki oil series, and similarily in the lower unsaturated acids of whale oil which corresponds practically to Tsubaki oil so far as the hydrogenation manner is concerned. The rate, however, became slower than the preceding one as a whole, as would be expected from the repeated use of the catalyst. This is one of the typical form of deactivation of catalyst on repeated use. Comparable experiments with Tsubaki oil and others were omitted though.

V. 358 is introduced as an example following a linearity almost over the entire range (Fig. 1 and 3). A rare case for 10 per cent of this catalyst, in which oil was weighted 2.5 g. greater than other instances, but this linearity could not be attributed to the difference of oil quantity, because the other cases under the identical conditions gave ordinary curved results.

Features of decreasing rate could not be insured to reproduce even with a portion taken from the same stock of catalyst, while the initial rate is rather easily kept uniform for a fixed amount of catalyst.

General attitude of decreasing rate is a slight curvature at the beginning, becoming greater as it approaches the end, that is, in most cases, a straight line reveals itself at the very start of each run (Fig. 1). The range of linearity tends to prolong as the percentage of catalyst is increased.

To summarize, hydrogenation proceeds mostly in a curved, with a straight line appearing in the initial stage and extending longer or shorter according to the nature of catalyst. The catalyst is difficult to be controlled and the rate cannot be connected directly to the concentration of the unsaturated acids.

No deterioration of the oil or the catalyst was incurred by simple agitating and heating to 180°.

Rate of hydrogenation increased with increase in the amount of catalyst between 0.05 to 0.1 g., but no changes occurred between 0.1 to 0.25 g. The upper limit was observed with the oleic acid ester. The total volume of liquid did not affect the rate between 0.5 to 3 g., as had been shown in a test utilizing up to about 10 g. of oil.

Discussion

1. Kinetic Order with Respect to Substrates and Establishment of Adsorption Equilibrium

Discussion will be made principally on oleic acid hydrogenation in order to tackle the problem of independency of rate on the concentration of substrate and only minor comments to the associated problems obtained in the expeiments. This conventional restriction on oleic acid will vanish when all the other factors are fully discussed in the subsequent notes.

The experimental results with Tsubaki oil diluted with its hydrogenated product imply that this is a zero order with respect to the hydrogen acceptors accoording to classical kinetics, in spite of the fact that reaction rates are not linear.

General expressions for diffusion of liquid species are directly induced from zero order dependence of liquid reactants: any steps described as mass transfer are not controlling the reaction, for all these steps evidently depend on bulk concentration, here diffusions in liquid phase, through solid-liquid interface and on the surface of solid being implied.

It is in accord with a kinetic criterion on the occurrence of selective hydrogenation with Finback whale oil, in which the higher unsaturated acids are preferentially hydrogenated in the presence of the lower unsaturated acids irrespective of the quantities of the latter.

As for hydrogen diffusion, the same conclusion should be arrived at, in view of its mass of the lightest. When the hydrogen absorption rate reaches a limiting rate by the increase in the amount of catalyst, as encountered in the experiments with oleic acid, one is apt to consider it as the arrival of the limitation of dissolution velocity of hydrogen on the liquid surface, and no one seem to doubt this concept. But this in itself is hardly acceptable, since the dissolution rate of hydrogen will control the overall process only when the reaction on the catalyst is considerably faster than any diffusional steps. On the contrary, the hydrogenation is the slowest step compared with diffusion processes even in a non agitational condition.[12] Furthermore, there is no theoretical foundation that the rate of hydrogen dissolution on the surface of the oil is slower than the diffusion rates of gas and liquid molecules in the bulk.

An added remark is that the upper limiting rate is specific for a certain combination of oil and catalyst with other factors being constant, otherwise, the comparison of readiness to hydrogenation of many organic substances and the test of the activity of catalysts described in the past references would not be succeeded first of all and become unreliable, as most of the studies were performed with sufficient amounts of catalyst.

For the present, convincing experimental evidences will be given to advance this discussion: A larger consumption of hydrogen than that of the upper limiting rate for oleic acid ester is observable, by merely replacing the nickel catalyst with the more active catalysts such as platinum. The limiting rate is about 1 ml./min. for Cu Cr O catalyst in whale oil,[4] while under the comparable conditions V. 400 gave 10 ml./min. of rate with nickel catalyst.

At any rate under the conditions used in this work, the controlling step is the catalysis, thus, an equilibrium concerning adsorption of reactants and resultants on catalyst was conclusively established.

12) R. Miyake, H. Yajima: Yakugaku Zasshi, 85, 618 (1965). Outline was presented at the 17th Annual Meeting of Chem. Soc. Japan, Apr. 2 (1964).

2. Poisoning as a Main Factor Influencing Change in Reaction Rate

The above conclusion is in accord with the general view that catalyst should be put in the slowest step of reaction. The activity of catalyst is a key on which reaction rate sensibly depends, therefore, the reduction in catalytic activity is only the considerable factor decreasing the reaction rate.

At the same time, as a cause of dropping rate, it is natural to be sought also in the change in the constitution of the substrate, because the isomerized oleic acids (iso-oleic acids) are known to be produced invariably during hydrogenation. Its production is not small, but its proportion to oleic acid is ordinarily maintained almost constant throughout the reaction.

It can be demonstrated that the rate of isomerization is very rapid when compared to hydrogenation rate, and the hydrogenation rates of iso- and normal-oleic acids do not differ to a great degree. Coincidence with this conclusion in many references[13] is supported also by the linear rate of V. 358 (Fig. 3).

A catalyst gradually loses its activity by the unavoidable sintering and self-poisoning in the long run.

Sintering is largely promoted by heating, a part of which being evolved in the exothermic reaction. In the runs of short duration, however, such a permanent deterioration seems not to be so predominately extensive, for which an evidence are being given by the hydrogenation of whale oil (V. 400): rates fall off to a great extent and successively in repeated runs, to be true, in lowered unsaturated acids, but they recovered their activities almost completely on the next addition of higher unsaturated acids.

This is predictable from two standpoints, namely, the nickel catalyst has already been exposed to a temperature higher than 350° in the reduction of nikel oxide, and even the copper catalyst, whose melting point being lower than that of nickel, proved to be eliminated from sintering effect, regardless of its low temperature reduction at 180°.[13]

Before entering upon the problem of self poisoning, some remarks should be made of impurities occurring in the original oils. These probably are phosphatides, oxidation products and other degradation products of oil. Free fatty acid is sometimes mentioned as being a hazard, which was found true in the case of the Cu Cr O catalyst, nevertheless an addition of stearic acid in quite a large amount gave no appreciable problem to our nickel catalyst. Purification with an active bleaching clay after alkali refining is insured to remove any of those common oil bearing impurities.

Of the above impurities, oxidation product generally designated as peroxide, is of a decisive character to halt hydrogenation. Peroxide is not only drastic in its effect but is difficult to be desorbed from the catalyst, that it is proper to designate it as a permanent poison for the procedure used in this study. Peroxide is yielded even in the carefully conducted ordinary refining process, due to the inevitable exposure to air, and its formation is accelerated in highly unsaturated oil like whale oil. However, oils tested were clean enough to satisfying our purpose. If the purification of oil were insufficient, to the contrary of the fact, successive additions of oil would have caused a damaging influence upon the following hydrogenation rate in V. 400.

Gradual lowering of rate, therefore, has to seek its source in production of some material possessing a moderate adsorbing affinity toward catalyst, such as to be removable from catalysing point either by washing with ether[1] or with addition of unsaturated oil as seen in the whale oil hydrogenation (V. 400). It is obviously adsorbed

13) A. E. Bailey: "Industrial Oil and Fat Products", 2nd Ed., 691 (1951), Interscience Publishers, Inc., New York.

in competetion with unsaturated compounds for the catalyst, accordingly it may be termed as temporary and reversible poison. It should be remembered that there has been a thought of recovering the used catalysts by washing with unsaturated oils.[4,14]

The poison in question must be a saturated substance, formed during hydrogenation, present in a minor quantity, as reaction proceeds almost quantitatively, and it is assumed to possess a larger molecular weight than the original fat judging from its relatively strong affinity to catalyst.

As for the removal of this reversible poisonous substances, it is effectively conducted in the preparation of the diluent in our study by alkali refining when in combination of active clay treatment. A mere application of the latter process does not ensure its accomplishment, effective is supposingly an occlusion of poison in soap. In conclusion, this should be some products arising from polymerization reaction.

Unsaturated fats, when subjected to heat treatment, undergo somewhat polymerization, which may incidentally developed by the presence of catalyst. At 180°, however, this unfavorable conversion is too small, it is confirmed by the experiment of heating oil and catalyst in a vacuum causing no drop of the subsequent rate after 3 hours heating, which is long enough to render hydrogenation rate to drop, if hydrogen were admitted. Neither were there any observed improvements of rate, thus an evidence for the absence of volatile poison is furnished at the same time (Fig. 2).

There is, however, formation of some volatile poisonous substance, which is found especially in the early stage of hydrogenation, reaction rate is frequently improved at the very first of evacuation, this is operated several times during reaction in need of supplying hydrogen. The source of volatile poison may be a pre-oxidized state of original fat, which will readily be decomposed at the start of hydrogenation operation.

The above result of simple heat treatments is the correspondence to the negligence of a term of hydrogenation duration in the poisoning equation.[1]

In any event the formation of the poison necessitates the existence of three components, hydrogen, olefinic compound and catalyst, it is concluded eventually one of the side reactions in hydrogenation.

Concerning the self-poisoning in the hydrogenation, it has been mentioned in no rare cases even with gaseous olefines, such as ethylene,[15] propylene,[16] isobutylene,[17] and 1-butene.[18] And at the same time, polymerization products were isolated in the hydrogenation of ethylene.[19,20] These two side reactions are presumably in a close relation.

Turning aside from the catalytic functions of metal, the action of hydrogen atoms produced thermally, photochemically, or from an electric discharge, there occurs a polymerization of ethylene and its homologues[21] and unsaturated compounds including acids to a great extent simultaneously with occurring hydrogenation.[22]

14) K. Omiya: Japan. Pat. No. 39805 (1921).
15) G. Matsuzaki: Shokubai, 1, 32 (1959).
16) O. Toyama: The Review of Phys. Chem. Japan, 14, 83 (1940).
17) R. Kiyama: *Ibid.*, 15, 137 (1941).
18) R. Amemiya: Shokubai, 2, 1 (1960).
19) K. Morikawa: N. R. Trenner, H. S. Taylor: J. Am. Chem. Soc., 59, 1103 (1937).
20) O. Beeck: Discussions Faraday Society, 8, 121 (1950).
21) A. R. Olson, C. H. Meyers: J. Am. Chem. Soc., 49, 3131 (1927); R. Klein, M. D. Schoer, R. Kelley: J. Phys. Chem., 68, 358 (1964).
22) C. Ellis: "Hydrogenation of Organic substances." 3rd Ed., 22, 184, 866 (1930). D. Van Nostrand Co., Inc., New York; H. I. Waterman, C. Boelhouwer, L. J. Revallier: "Hydrogenation of fatty oils," 241 (1951). Elsevier Publishing Co., New York.

To the end of this paragrph, all the liquid hydrogenations are to be reexamined from the view of self poisoning of catalyst as previously reasoned from the standpoint of kinetics.

3. Consideration on the Independency of Rate in Regards to Substrate Concentration

Rate determining in fat hydrogenation is in consequence the pursuit of poisoning of catalytic surface and the adsorption of referring substances in the broad sense.

To exclude the poisoning, zero order behavior with respect to substrate is one of the characteristics of liquid hydrogenation. The final section will be devoted to general consideration on this problem. The authors try to approach the subject from a number of different angles and works reported in the literature, the selection of materials being somewhat arbitrary.

To those concerned with subject pertaining to liquid hydrogenation, this independency of rate on substrate can be considered more or less a complex problem.

3a. Distribution of Reaction Species on Catalyst

Experiments on ethylene hydrogenation give zero order on ethylene under its excess pressure compared with hydrogen pressure and it is ensured especially at low temperature.[33] The situation of liquid hydrogenation with olefines and benzene homologue may be accepted with easiness by analogy, if regarded as that of ethylene in extreme state of high pressures and low temperatures. The reduction of nitro-compounds and aldehydes is to be fixed in the same scope for their identical behavior.

Classical kinetic consideration will immediately indicate an overwhelmingly strong adsorption characteristic of oleic acid, after establishing its zero order kinetics. But it is not unconditionally realizable in general. It is more natural to postulate the adsorption equilibrium on catalyst with every species in liquid bulk. In view of the formation of iso-oleic acids, adsorption of hydrogen is of significance. Adsorption of stearic acid ester cannot be ignored in an admittance of hydrogen adsorption, considering its molecular weight and concentrations is liquid phase.

A concept of likeness must be advanced in the case of selective hydrogenation of whale oil, adsorption of the lower unsaturated acid should be occurring even in the preferential hydrogenation of the higher unsaturated acids. In this case, the concurrent adsorptions of the higher and lower unsaturated acids are evident, as the application of high pressure of hydrogen causes failure of selectivity.[34]

With copper catalyst, the above situation will be more clearly demonstrated. Under atmospheric pressure and temperature below 180°, it hydrogenates exclusively higher unsaturated acids with the lower unsaturated acids remaining unaltered, but it does not guarantee deletion of adsorption of the lower unsaturated acids, because the latter is subjected to saturation above 200°.[35] Furthermore, if applied high temperatures and pressures, saturation of double bonds and reduction of carboxylic radical occur simultaneously to procure corresponding saturated alcohol, where preferential reduction of carboxylic radical to corresponding oleinic alcohol has been achieved with no success. With nickel catalyst, the reaction is more drastic to result in producing hydrocarbons.[36] It seems to provide the information that all the species in liquid bulk should be adsorbed on copper and nickel catalyst.

33) H. Eyring, C.B. Colburn, B.J. Zwolinski: Discussions Faraday Society, 8, 43 (1950).
34) A.E. Bailey: "Industrial Oil and Fat Products," 2nd Ed., 704 (1951), Interscience Publishers, Inc., New York.
35) Y. Shimamura, K. Miyake: Yakugaku Zasshi, 84, 515 (1964).
36) A.E. Bailey: "Industrial Oil and Fat Products," 2nd Ed., 762 (1951), Interscience Publishers, Inc., New York.

3b. Limiting Use of the Langmuir-Hinshelwood Hypothesis

In the present instances, therefore, concurrent adsorption of all the species is naturally to be considered for the whole surface of nickel. The following adsorption, taking the Langmuir isotherm for convenience of illustration, can be formulated, provided its surface is uniform.

$$\theta_E = \frac{k_E(E)}{1 + k_E(E) + k_S(S) + k_H(H)} \qquad (1)$$

where species placed in brackets refer to their concentrations in liquid phase: E, S, and H, are ethylenic compound, saturated compound, and hydrogen molecule respectively.

According to the Langmuir-Hinshelwood hypothesis, accountable for many of the data for catalytic reaction of gas phase, rate (V) is proportional to the product of the fractions (θ) for hydrogen and ethylenic compound.

$$V \propto \theta_H \theta_E \qquad (2)$$

The idea is nothing but a suitable modification of the mass product law in homogeneous reaction. Correspondingly, the traditional explanation of decreasing rate in term of the change in bulk concentration of oleic acid ester is not necessarily itself unreasonable.

An important clue, however, to approach the catalysis is to be sought in this apparent discontinuity between the natural course of thinking and the experimental results.

Equation (1) is derived from the assumption that nickel catalyst consists of uniform surface. On the contrary, surface of nickel catalyst used practically is by not means considered uniform, apart here from barium sulfate, a carrier, which reasonably can be put out of consideration because of its intrinsically inert character.

H. S. Taylor assigned some sensitive area for reaction to an active center, differing from other inactive part with many experimental evidences by using gaseous materials.

Positive evidences for active center theory can also be presented in liquid phase hydrogenation. It is considered that active area is confined in extremely small portion in common catalysts.

Acetic acid has been recommended for a long time as one of the best solvents, in which benzene is well hydrogenated on platinum[8] and rhodium,[9] following zero order, thus benzene may be taken adsorbed strongly in classical consideration. While carrying out the adsorption experiment on metal catalyst, materials to be adsorbed at this time are fatty acids, and benzene as solvent, since the latter is adsorbed in negligibly small amount.[37] To compute the surface of nickel is best done by means of higher fatty acids in benzene.[37] Okada, recently in our laboratory, confirmed acetic acid is adsorbed as much as other fatty acids on Raney nickel, and he also gave comparable hydrogenation rates as zero order with crotonic acid and its ester on Raney nickl in alcoholic solution, while finding the expected results of adsorption in alcohol for acid and ester.

In connection with Okada's data, it is interesting to mention that the rate of consumption of hydrogen was of zero order up to 90 per cent of oleic acid in acetic acid and acetic anhydride with platinum-barium sulfate catalyst.[38]

Active area might as well have a specific adsorption favoring double bond. As a whole, each species, giving their respective distribution pattern throughout the nickel surface, will establish a statistical distribution in term of adsorption potential.

Hence, a modification must be made for adsorption pattern in active portion, where adsorption coefficient k_E for olefinic compound is supposed to be exceedingly greater than that for other species, namely, $k_E \gg k_S + k_H$.

37) H. A. Smith, J. F. Fuzek: J. Am. Chem. Soc., 68, 229 (1946).
38) V. I. Gol'dovskii, S. Yu. Elovich: J. Phys. Chem. (U. S. S. R.), 20, 1085 (1946); C. A., 41, 2978 (1947).

Equation (1), corresponding to the active portion, reduces to

$$\theta_E = \frac{k_E(H)}{1 + k_E(H)} \tag{3}$$

For other patch in the active portion most favoring hydrogen, in the similar way,

$$\theta_H = \frac{k_H(H)}{1 + k_H(H)} \tag{4}$$

And the rate may be expressed by the product of fractions, provided reaction occurs on a overlapping of distribution portions regarding the two species.

$$V \propto \frac{k_E(H)}{1 + k_E(H)} \times \frac{k_H(H)}{1 + k_H(H)} \tag{5}$$

Since the surface is in contact with liquid phase, a further modification is allowed to exercise such that $k_E(H) \gg 1$ in the equation (5), then the velocity becomes independent of the concentration of unsaturated acid. It apparently illustrates the present result, and the kinetic concept of strong adsorption concerning oleic acid ester will be approved, if the account is limited within active portions. Equation (5) is given in ethylene hydrogenation,[3b] but a differential consideration between the active and inactive portions are not taken in.

An additional criterion will be made here about active portion. It is restricted not only to a small area, but its capacity for functioning of hydrogenation seems to be under some restriction. Rates of hydrogenation were identical, whether glyceride or ethylester of oleic acid was used, the bulky glyceride rest does not interfere an approach of double bond to active point. It is a specific place best disposed to hydrogenation of double bond and is not connected with ester structures.

According to the present theory of distribution patterns, adsorption distribution for poison should be located different from the active portions, and an actual poisoning will reveal only after the saturation of this specific area by poison. The linear portion may be proportional to the capacity for accomodating poison, and the large range of linear portion in the catalyst used in V, 338 will be attributed to its large capacity of poison adsorption. There is a trend that a rather low active catalyst possesses the large capacity to be proof against poisoning. We have not suceeded in controlling the proportion of distribution patterns.

3c. Local Mobility of Molecules Adsorbed

Hitherto, discussion, dealing solely with the line of rearrangement of molecules between two phases of liquid and solid, leaves the mobility of molecules untouched after the occurrence of their adsorption, however, suggestions for which have been seemingly provided from liquid and gas hydrogenations.

Selective hydrogenation achieved fairly well under low pressure of hydrogen on nickel catalyst, as shown in whale oil experiment, is injured by an application of high pressure.[3b] The simultaneous hydrogenation of higher and lower unsaturated acids observed under high pressures indicates that all the unsaturated acids are being co-adsorbed on the active portion of nickel catalyst irrespective of hydrogen pressures.

An adsorption pattern in the active portion will be described in a similar way of equation (3) and (4).

3b) D. D. Eley: "Catalysis" Vol. 3 edited by P. H. Emmett, 50 (1955), Reinhold Publishing Corporation, New York.

$$\theta_L = \frac{k_L(L)}{1 + k_L(L) + k_O(O)}$$

$$\theta_O = \frac{k_O(O)}{1 + k_L(L) + k_O(O)}$$

where linoleic acid (L) is assigned as the representative of higher unsaturated acids, and oleic acid as one of lower unsaturated acids. An appropriate mathematical treatment will permit these relations to accomplish a velocity expression after the fashion of deriving equation (5), so as to be compatible with the experimental result for a certain condition.

At any rate, it must be made in this treatment an assumption involving a preferential attraction of hydrogen to linoleic acid, and this assumption appears to illustrate various experimental results under varied conditions.

In the active portion, where a finite number of hydrogen adsorbed, will all hydrogen be attracted to linoleic acid to give a hypothetical couple of them on active nickel atoms, so that the selective hydrogenation will be retained until concentration of linoleic acid becomes less than that of hydrogen, and a comparable relation will be realized in the application of high hydrogen pressure.

After completion of partial hydrogenation of linoleic acid to oleic acid, the hydrogen will be coupled in the same way with oleic acid preferentially than with stearic acid to establish zero order kinetics, even if the concentration of oleic acid decreases on the increase of stearic acid in the active portion.

There is a misunderstanding about selective hydrogenation, this is not a matter of difference in rate of individual species. A rate of linoleic acid was about twice as rapid as that of oleic acid on nickel catalyst at 180° under atmospheric pressure,[7] but the reverse is true in the selective hydrogenation of acetylene, in which its rate is slower than that of ethylene on nickel at some reaction conditions.[10]

An acceptance of the hypothesis of mobility explains in the same manner the kinetics of ethylene at low temperatures. Here it must be recalled of the actual occurrence in the usual experiments undertaken in a constant volume, that is, pressure lowerings of ethylene and hydrogen follow simultaneously.

Rate will be controlled only by a decreasing velocity of the lower concentration species, when assumed it is used up on catalyst after pairing with the excess species, whatever the amount of the latter may be varied. In case the hydrogen is small in amount, for instance, then the rate must remain independent of decreasing ethylene pressure in accordance with experimental results.

3d. Restriction on Mobility

It seems, however, necessary to lay down some restriction on the extent of mobility on catalyst, in excercising this hypothesis of mobility further to the case of hydrogenation with ethylene at high temperatures.

Rate becomes influenced by partial pressures of hydrogen and ethylene on rising reaction temperatures or decreasing each partial pressure to a great degree. Under these circumstances, adsorptions decrease and the distance between adsorbed molecules will increase accordingly.

In this instance, allowed to make an additional postulation of localized mobility, the result is that a coupling of two species may be given by an opportunity to encounter both reactants. At the same time, the extent of mobility is certainly increased at high temperatures, but this effect seems unable to overcome the opposing trend of increasing separation. Thus the chance for hydrogen and ethylene to pair may be in proportion to the product of their concentrations, one of this expression being equation (5).

Therefore, liquid hydrogenation is theoretically predicted to also appear itself as first order with respect to substrate, when its concentration decreases lower than that of hydrogen, however, under atmospheric pressure it will not be realized in a practical sense.

The restricted mobility might as well be considered alternatively as a lateral extension of influence of one species adsorbed to the neighboring species on catalyst. Whatever the real character of localized mobility may be, a solid concept of non-mobility must be modified considering general case.

The weakness involved in the non-mobility will also be pointed out from the following contemplations. In this case, assuming the strong tendency toward pairing, hydrogen and ethylene are brought together only through repeatedly quick hitting from gas phase and bouncing off the surface, which will always cause independent rate on the species of lower partial pressure, irrespective of their concentrations on surface. And if completely ignored the existence of tendency to bring about pairing, a primary controlling factor will be the chance of collision on the surface between both reactants from gas phase, then it is difficult to interprete the occurrence of zero order in either species at low temperatures, because of ensuing steady decrease in partial pressures of the two in the mean time.

Same with the liquid phase hydrogenation, and moreover the selective hydrogenation could not be interpreted.

3e. Intermediate Complex Compound Formation

The final subject is to give any possible suggestion on reasoning for the tendency of hydrogen to approach the high unsaturated acid than to low unsaturated acid on the catalyst surface, to outcome ultimately the selective hydrogenation. Namely, it is on the threshold to realize the occurrence of catalytic reaction.

Nickel atoms constituting the active portion must have a special affinity to double bond, accordingly if any resultant arises from this affinity, it will be a kind of simulated chemical linkage between nickel and double bond. Hydrogen may also have the similar relation with nickel atom.

To give rise to hydrogenation these two quasi compounds must be brought into a close connection, namely it is to construct a pertinent transitional state. As a start of reaction, a concept of complex for the transitional state will be postulated, consisted of three components in a way that hydrogen and double bond being centered on a nickel atom. The central nickel atom is surrounded on the other hand by neighboring nickel atoms, thus, this transitional complex is consequently made up with two ligands of hydrogen and ethylenic compound and several atoms of nickel neighboring the central nickel atom.

The active portion is supposingly such a place where a certain number of nickel atom could participate in the formation of this kind of complex, which, if followed by an intramolecular rearrangement so as to bring hydrogen close to double bond, will finally give rise to addition of hydrogen to double bond.

A complex is generally stabilized by replacing one of the ligands by another ligand which more firmly coordinates the central metal atom,[40] probably due to a resonance, in term of quantum mechanics, working to reduce all the coordination energies to uniformity as much as possible.

In these premises, let us consider two complexes, oleic acid-nickel-hydrogen and linoleic acid-nickel-hydrogen, then the hydrogen linkage of the latter should be stronger

40) R. Tsuchida: "Colours and Structures of the Metallic Compounds." 143 (1944). Zoshindo, Osaka, Japan. H. J. Taufee, M. J. Murray, F. F. Cleveland: J. Am. Chem. Soc., 63, 3500 (1941).

of the two, as the doubly unsaturated linoleic acid will be coordinated more firmly with nickel atom in active areas.

Correspondingly there arises a trend of hydrogen shifting to the more stable complex of linoleic acid-nickel hydrogen in an equilibrium between the two complexes. The equilibrium will govern the selectivity in conformity with the variation of linoleic acid content.

To summarize the above relationships, inasmuch as the ratio of the specific reaction rates for linoleic and oleic acids is valued as approximately two, the degree of selective hydrogenation shown by Bailey as 38/1[9] which though modified here as 38/2 by taking the hydrogenation rate ratio into consideration, will correspond to so-called an equilibrium constant for the above imaginary complexes.

A formation of quasi complex may evidently be the prerequisite for the interaction between hydrogen and unsaturated compound, but if the constitution of it is too stable to pass into the succeeding transformation, the hydrogenation rate will be rather retarded. The acetylene hydrogen that may be considered such a case, although the formation of the quasi complex is preferred, its hydrogenation velocity becomes slower than that of ethylene.

Some additional notices concerning the quasi complex assumption will be given, rather to invoke some interesting connection with catalytic reactions.

It is interesting to refer that transition elements capable of constituting a great deal typical coordinated compounds are also catalyzing a great diversity of reaction, platinum group, iron group, and copper show themselves specific in both fields.

Carriers at the same time may take a part of ligand as well as surrounding metal atoms, the following may be taken as adequate examples, polyvinyl alcohol and related polymers developed by Nord,[10] these are applied as carriers for noble metals.

Even inorganic materials appear to function as ligands for the central metal atom: Kieselguhr seems to enter a molecular arrangement with nickel oxide, because in hydrogen the most difficultly reduced was the mixture of equal weight ratio, although no specificity in hydrogenation rate of fat was found among mixtures of other ratios,[11] the roll of carriers is decisive for copper catalyst in the hydrogenation of higher unsaturated fat; copper alone showed no hydrogenation ability under atmospheric pressure, strikingly differing from nickel catalyst, accordingly such an idea is induced that the active portion for this reaction may be located along the boundary between copper and carriers.

Acknowledgement of the self-poisoning and the independency of rate on the bulk concentration of substrate will surely render liquid hydrogenation progress in a newly systematic and steady way of thinking, with a hope to give stimulations to the studies on various domain of science concerned.

One of these is the analysis of the feature of self poisoning. If one makes an intentional inspection of the countless past reports on hydrogenation of liquid substances, they will be found as one of the way, through which the real matters of catalyst and catalysis will be approached.

To follow this work, it is understandable that a serial study of Maxted and his collaborators on poisoning of catalyst with addition of artificial injurious materials is indispensable for the study of the basic state of affairs. It is felt necessary, however, to modify the expressions made by the original authors in a general fashion into a more practical form, before referring their results to the self poisoning in hydrogenations.

(9) A. E. Bailey: J. Am. Oil Chem. Soc., 26, 611 (1949).
(10) K. H. Hasenhuttl, D. H. Nord: J. Am. Chem. Soc., 65, 2121 (1943).
(11) Yoshida: unpublished.

through which Maxted's fundamental researches will be remembered as well in the practical field of catalysis. A view worked in this manner will be opened elsewhere.

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Summary

Substrates containing electronegative groups have a strong trend to reduce hydrogenation activity of catalysts owing to self poisoning, and fat hydrogenation is not exceptional. Paid due cautions on self poisoning, zero order with respect to substrates is readily confirmed. Discussions, putting aside the problem of poisoning, are concentrated on the behaviors of reactants on catalysts in connection with ethylene hydrogenation.

Fat hydrogenation is considered as a specific instance of ethylene hydrogenation, where partial pressure of ethylene is enormously excess. A theory of local mobilization respecting reactants is advanced to meet the ethylene hydrogenation kinetics of independency on partial pressure of major species at low temperatures and of dependency on partial pressures of both species at high temperatures. Only for the latter case the Langmuir Hinshelwood theory is applicable. Discrimination between active and inactive sites is thereby necessary.

Interpretation for the preferential hydrogenation of linoleic acid to oleic acid on nickel catalyst is made in terms of equilibrium shift between two assumed quasi complex compounds involving all the components concerned, one with linoleic acid and the other with oleic acid as a component, which also accounts for the failure of selectivity under high hydrogen pressures.

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The effect of dose level of essential fatty acids upon fatty acid composition of the rat liver*

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HANS MOHRHAUER† and RALPH T. HOLMAN

University of Minnesota,
The Hormel Institute,
Austin, Minnesota

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SUMMARY

Rats were fed various levels of either ethyl linoleate, ethyl arachidonate, or ethyl linolenate. Weight gain, fat-deficiency status, and fatty acid composition of the liver lipids were determined.

Dietary linoleate, fed in excess of 1% of calories, maintained good growth and cured fat deficiency. Increasing amounts of dietary linoleate were stored in the liver lipids and converted into fatty acids of the linoleate family—20:4 and 22:4. The concentration of 20:3 was decreased. Dietary arachidonate cured fat deficiency three times more effectively than linoleate. Increasing amounts of dietary arachidonate were stored in liver lipids and converted to 22:5ω6. The level of 20:3 was lowered three times more effectively than when linoleate was fed. No fatty acids of the linolenate family were synthesized from linoleate or arachidonate.

Dietary linolenate did not support weight gain as efficiently as did linoleate or arachidonate. Fat deficiency symptoms could not be cured completely. Increasing amounts of dietary linolenate increased the levels of fatty acids of the linolenate family; linolenic acid was stored, and 20:5, 22:5ω3, and 22:6 were synthesized from linolenate. The level of 20:3 was lowered in the same fashion as when linoleate or arachidonate was fed. The level of 20:4 was decreased with increasing amounts of dietary linolenate.

Experiments with radioactive labeled compounds have been of great value in studies concerned with the metabolism of essential fatty acids (EFA). Several basic metabolic conversions in this field, however, have been elucidated in feeding experiments with unlabeled materials. Early investigators have shown, by feeding of linoleate concentrates to rats, that linoleate is converted to arachidonate (1). Proof for this conversion was provided by Mead and coworkers (2) using C^{14}-labeled linoleic acid. Eicosatrienoic acid was found to appear in fat-deficient rats (1); and Fulco and Mead (3) showed that, in the absence of dietary EFA, eicosatrienoic acid was synthesized from C^{14}-labeled oleate.

The conversion of linolenic acid to the highly unsaturated fatty acids of 20 and 22 carbon atoms was established by Widmer and Holman (4) by feeding experiments. This study also showed that arachidonate cannot be synthesized from linolenate. The latter finding was confirmed by Mead and coworkers (5), who used C^{14}-labeled linolenate, and also by Klenk and Oette (6). The intermediate steps in the synthesis of docosahexaenoic acid from linolenic acid via eicosapentaenoic acid and docosapentaenoic acid were elucidated by investigations of Klenk and Mohrhauer (7), who fed synthetic C^{14}-labeled compounds to rats.

Most of the investigators quoted above studied the metabolism of EFA by making comparisons between animals fed fat-free diets and those fed single levels of an EFA. The analytical tools employed in some of the older studies, like alkaline isomerization and ozonolysis of mixtures of polyunsaturated fatty acids, do not allow one to distinguish between certain individual fatty acids of similar structure.

The present study was conducted to show the influence of various levels of intake of linoleic, arachidonic, and linolenic esters upon the fatty acid composi-

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† Present address: Physiologisch-Chemisches Institut, Universität Köln, Germany, Director Prof. E. Klenk.

tion of liver lipids of the rat. Gas-liquid chromatography (GLC), which permits a discriminating analysis of the entire range of tissue fatty acids, was employed in this work. Changes in concentrations of tissue fatty acids under the influence of varying amounts of single dietary EFA are interpreted in terms of fatty acid metabolism. For example, the concentration of a certain fatty acid of the liver lipids might increase with increasing amounts of dietary linolenate, but does not undergo any changes when linoleate or arachidonate are fed. Thus, the appropriate conclusion seems to be that the fatty acid in question is synthesized in the rat liver from linolenate.

EXPERIMENTAL METHODS

One hundred and eighty-four weanling, 27-day-old, male rats of the Sprague-Dawley strain were kept on a basic fat-free diet, the composition of which is given in Table 1. The daily intake of basic diet was recorded for each animal.

Ten groups of rats received daily supplements of highly purified ethyl linoleate (The Hormel Foundation, Austin, Minnesota) varying from 0.01 to 5% of total calories (Table 2). Analysis by GLC of the ethyl linoleate indicated it to be 98.3% pure. The only contaminant was 1.7% oleate.

Ten groups of rats were given diets supplemented with synthetic (9) ethyl arachidonate (20:4), varying from 0.01 to 5% of total calories. This product was obtained from Hoffmann La Roche, Nutley, New Jersey. It contained 81.8% arachidonate, and 8.8% "short-chain" and 9.7% "long-chain" impurities according to GLC analysis. Calculation of dietary arachidonate was based on the actual 20:4 content of the preparation. Santoquine (0.25%) was added as antioxidant.

Thirteen groups of rats were given diets supplemented with ethyl linolenate (The Hormel Foundation, Austin, Minnesota), varying from 0.01 to 10% of total calories. This preparation was found to be 97.3% pure. It contained less than 0.4% linoleate and five other minor unidentified impurities according to GLC analysis.

The ethyl esters of the fatty acids were administered orally by microsyringe. The amount fed was calculated in percentage of calories of the diet consumed by each animal. Groups fed more than 1% of calories as EFA were restricted in size to two to four animals because supplies of EFA were limited. All other groups consisted of six rats. Weight gain, dermal score (10), and fatty acid composition of the liver lipids were determined for each single animal. The results are reported as averages per group of rats. There are more groups in the low-calorie-intake range because previous experiments had shown that the most dramatic changes occurred in this region. Twenty-four animals were kept as controls without any supplements.

TABLE 1. COMPOSITION OF FAT-FREE DIET

Component	Weight
	%
Vitamin test casein	16
α-Cellulose	4
Sucrose	74
Wesson salt mixture	4
Vitamin* mixture	1
Choline chloride*	1

* Vitamins D_2, K_1, B_1, B_2, B_6, B_{12}, Ca-pantothenate, niacin, inositol, p-amino-benzoic acid, folic acid, biotin, and choline chloride were mixed into vitamin test casein in doses recommended by Glaxo Laboratories (8). One kilogram of the diet was mixed with 100 ml of a solution of 4 mg vitamin A acetate (1,000,000 i.u./g) and 280 mg vitamin E (α-tocopherol) in ether. The ether was evaporated before using the diet. All components of the diet but sucrose were obtained from Nutritional Biochemicals Corporation, Cleveland 28, Ohio.

After 100 days the animals were sacrificed by ether anesthesia. The livers were quickly removed and kept in saline solution at −20° until analyzed. The livers were homogenized and extracted with chloroform methanol 2:1 according to Folch et al. (11). The lipids were transesterified by refluxing with 30 volumes of a 5% solution of HCl in methanol. All operations were conducted under nitrogen. The methyl esters were analyzed by GLC, using a Barber-Coleman Model 10 apparatus with argon-ionization detector. A 210-cm glass column of 5 mm i.d., packed with 20% ethylene-glycol-succinate polyester[1] (EGS) coated on Gaschrom P, 80–100 mesh[1] was used. The flow rate was 60 ml argon/min at an inlet pressure of 16 psi.

The inlet heater was kept at 270° and the detector cell at 250°. The esters with retention times shorter than that of 18:3 were chromatographed at 180° column temperature. Approximately 5 μl of a 10% solution of the methyl esters in petroleum ether was injected. The long-chain esters were analyzed on a second run at 200° by injecting approximately 30–50 μl into the chromatographic column.

The individual esters were identified by carbon number (12) and by internal standards wherever feasible. Authentic methyl esters of fatty acids (purity 95–99%), obtained from The Hormel Foundation,

[1] Obtained from Applied Science Laboratories, State College, Pennsylvania.

were used as internal standards. The mixtures of methyl esters were rechromatographed with the added standard, and the peak increased by the standard was identified. Quantification was carried out by triangulation. The fatty acid composition is reported as area per cent, a procedure justified in cases, such as this, where changes in concentration but not the absolute composition are to be measured.

RESULTS AND DISCUSSION

The average weight gain (Fig. 1) and average dermal score of fat deficiency (10) (Fig. 2) for each group of rats were plotted vs dietary EFA. The weight-gain curves and dermal-score curves permitted comparison of the results of the fatty acid analyses in this experiment with older investigations in which weight-gain data and deficiency scores were used as the only parameter to measure fat deficiency. Weight gain increased in almost the same pattern with increasing amounts of all three EFA, although the values were slightly higher for arachidonate and linoleate than for linolenate. An optimum for all three EFA with respect to weight gain seemed to be between 1 and 1.5% of calories. All rats given diets supplemented with EFA in excess of 1.5% of calories showed no further weight-gain increase. In fact, there seemed to be a slight decrease in weight gain, which, however, was inconsistent.

The fat-deficiency signs (i.e., necrotic tail and scaly feet) appeared in increasing severity in all animals fed linoleate at less than 0.6% of calories and arachidonate at less than 0.25% of calories. None of the animals given diets supplemented with linolenate was free of deficiency symptoms. Animals fed levels of EFA at 5 and 10% of calories developed severe dermal symptoms similar to those of fat-deficient rats. This was most striking in rats fed the highest level of arachidonate. It is likely that massive oral doses of polyunsaturated fatty acids exceed the capacity of the antioxidant content of the diet and are subject to oxidation before absorption. The resultant vitamin-E deficiency and the effect of the fatty acid oxidation products (13) could account for the development of dermal symptoms. This reversal of phenomenon is not reflected in the fatty acid composition of tissue.

In general, both weight-gain data and dermal scores obtained in this experiment confirm and extend what is known about the influence of EFA upon fat-deficiency symptoms since the experiments of Burr and Burr (14). Fat-deficiency symptoms are cured by less than 0.5% of calories of dietary linoleate, and by even smaller levels of arachidonate—about 0.25% of calories. Linolenate has some beneficial influence upon dermatitis, but cannot cure fat deficiency completely. However, normal weight gain can be observed when any of the three EFA is fed at a level of approximately 1.5% of calories.

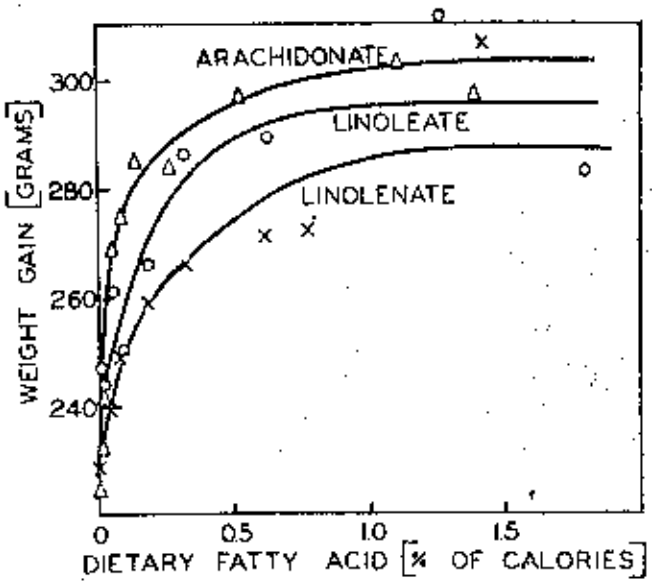

FIG. 1. The effect of varying levels of dietary EFA upon weight gain of rats during the 100-day period of the experiment.

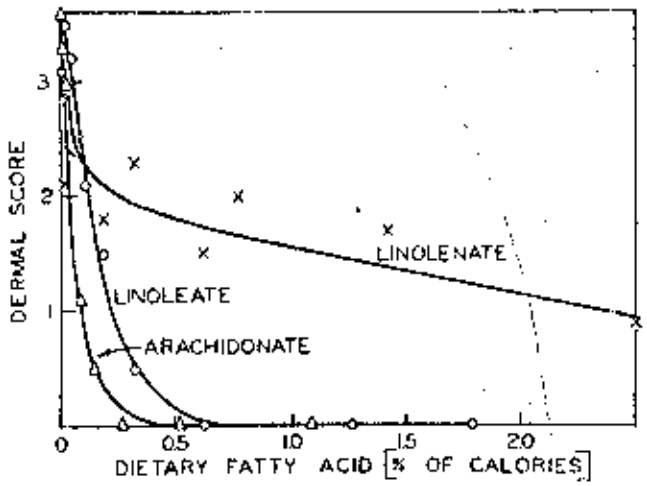

FIG. 2. The effect of varying levels of dietary EFA upon dermal score (the degree of fat-deficiency symptoms on feet and tail of the rats [10]).

The fatty acid composition of the liver lipids as analyzed by GLC is given in Table 2. The saturated fatty acids (myristic (14:0), palmitic (16:0), and stearic (18:0) acids) were identified by carbon number and comparison with authentic standards. Throughout the experiment, no consistent change was observed in the concentration of these saturated acids, indicating that their synthesis is not significantly affected by

TABLE 2. FATTY ACID COMPOSITION OF

Dietary EFA (% of Cal.)	No. of Animals	14:0	16:0	16:1	18:0	18:1	18:2	18:3
None	24	0.94 ± 0.04	27.22 ± 0.43	11.54 ± 0.42	9.56 ± 0.20	40.30 ± 0.58	1.07 ± 0.08	<0.01
Linoleate								
0.008	6	0.91 ± 0.18	26.04 ± 2.16	11.01 ± 1.56	8.75 ± 1.36	40.89 ± 1.79	1.31 ± 0.26	<0.01
0.02	6	0.83 ± 0.20	25.36 ± 0.97	11.98 ± 1.73	9.50 ± 1.92	41.91 ± 1.22	1.22 ± 0.45	<0.01
0.05	6	0.92 ± 0.12	24.12 ± 1.93	12.06 ± 1.93	10.38 ± 1.85	40.32 ± 2.74	1.44 ± 0.36	<0.01
0.10	6	0.94 ± 0.43	23.76 ± 2.89	12.18 ± 2.58	10.34 ± 2.27	41.49 ± 2.09	2.03 ± 0.33	<0.01
0.18	6	0.78 ± 0.23	23.02 ± 2.18	9.42 ± 1.80	14.78 ± 2.22	35.73 ± 3.44	2.20 ± 0.24	<0.01
0.32	6	0.74 ± 0.21	23.76 ± 3.05	9.84 ± 1.37	11.13 ± 1.80	40.24 ± 2.85	2.25 ± 0.42	<0.01
0.61	6	1.28 ± 0.22	25.59 ± 2.24	10.85 ± 1.94	10.99 ± 1.55	38.42 ± 2.00	2.66 ± 0.64	<0.01
1.26	2	1.26	25.64	9.90	12.50	31.50	3.02	<0.01
1.70	2	1.38	27.81	10.81	12.14	27.95	4.28	<0.01
4.87	2	1.00	25.46	7.05	13.60	27.54	5.70	<0.01
Arachidonate								
0.007	6	0.78 ± 0.30	21.14 ± 1.68	8.63 ± 2.58	11.17 ± 1.75	41.01 ± 3.11	1.36 ± 0.19	0.01
0.019	6	0.62 ± 0.16	24.65 ± 2.87	10.56 ± 1.40	10.71 ± 1.69	41.39 ± 2.40	0.88 ± 0.38	<0.01
0.04	6	1.27 ± 0.39	26.24 ± 1.95	10.57 ± 0.81	10.23 ± 0.75	39.07 ± 2.41	1.40 ± 0.24	<0.01
0.08	6	0.86 ± 0.21	24.21 ± 1.52	10.21 ± 1.00	11.19 ± 1.10	38.06 ± 1.70	1.08 ± 0.22	<0.01
0.14	6	0.88 ± 0.58	23.13 ± 1.96	9.41 ± 2.32	11.61 ± 2.99	38.37 ± 3.91	1.20 ± 0.35	<0.01
0.27	6	0.99 ± 0.17	23.50 ± 2.79	9.20 ± 2.14	13.92 ± 2.72	32.91 ± 1.55	1.00 ± 0.51	<0.01
0.52	6	0.99 ± 0.27	25.20 ± 2.28	9.67 ± 1.31	12.53 ± 2.81	33.08 ± 3.75	0.96 ± 0.17	<0.01
1.08	2	0.70	25.26	9.01	12.57	26.64	0.48	<0.01
1.39	2	0.56	25.06	7.53	13.30	21.20	0.42	<0.01
3.75	2	0.50	23.14	5.00	13.31	16.97	0.54	<0.01
Linolenate								
0.009	6	0.62 ± 0.18	24.98 ± 0.86	11.05 ± 1.83	10.88 ± 1.80	41.20 ± 2.91	0.52 ± 0.09	0.01
0.02	6	0.90 ± 0.09	25.16 ± 0.96	11.80 ± 1.34	10.79 ± 1.93	40.53 ± 2.78	0.55 ± 0.17	0.02 ± 0.01
0.04	6	1.00 ± 0.24	24.89 ± 2.11	13.01 ± 2.28	10.45 ± 2.22	40.09 ± 1.96	0.47 ± 0.13	0.02 ± 0.01
0.08	6	1.11 ± 0.26	26.31 ± 3.32	14.18 ± 2.37	9.85 ± 1.65	39.30 ± 4.21	0.44 ± 0.13	0.03 ± 0.01
0.18	6	0.85 ± 0.20	23.62 ± 1.99	13.24 ± 1.35	12.56 ± 2.08	39.08 ± 2.07	0.49 ± 0.10	0.02 ± 0.01
0.32	6	1.10 ± 0.32	23.86 ± 1.30	13.93 ± 1.54	12.23 ± 1.64	38.44 ± 1.81	0.44 ± 0.05	0.21 ± 0.09
0.61	6	0.96 ± 0.26	25.61 ± 1.63	14.82 ± 1.43	11.18 ± 2.47	37.41 ± 3.40	0.42 ± 0.10	0.22 ± 0.06
0.77	4	0.92 ± 0.11	28.85 ± 2.14	12.93 ± 1.96	12.16 ± 2.99	35.03 ± 2.79	0.40 ± 0.16	0.30 ± 0.16
1.42	4	0.78 ± 0.07	30.00 ± 1.90	11.93 ± 0.65	9.74 ± 1.06	36.36 ± 1.40	0.50 ± 0.20	1.03 ± 0.19
2.56	4	0.79 ± 0.18	29.40 ± 3.20	12.26 ± 1.01	12.33 ± 1.07	28.64 ± 2.29	0.39 ± 0.08	1.57 ± 0.56
3.56	4	0.49 ± 0.04	29.55 ± 0.52	10.53 ± 1.23	13.94 ± 0.12	24.60 ± 1.33	0.38 ± 0.12	2.00 ± 0.91
4.14	4	0.68 ± 0.10	27.37 ± 2.04	10.67 ± 0.87	11.64 ± 0.25	28.00 ± 2.45	0.30 ± 0.06	5.40 ± 3.72
9.42	2	0.51	30.30	7.85	13.42	18.23	0.27	5.72

the intake of EFA below 5% of calories. Dhopeshwarkar and Mead (15) reported a significant increase in the concentration of stearic acid in tissues from guinea pigs fed a high percentage of corn oil. The same observation was made for mice by Tove and Smith (16). In both cases the content of linoleic acid in the diet exceeded the amounts fed in the present experiment.

Palmitoleic (16:1) and oleic (18:1) acids were identified by internal standards. The concentration of oleic acid showed a significant increase with decreasing amounts of all three dietary EFA, whereas there was only a slight parallel increase in the level of palmitoleic acid at low EFA intakes (Figs. 3, 4). However, all changes were most pronounced in the experiment with dietary arachidonate and were least significant for dietary linolenate. Increased amounts of monoenoic fatty acids in livers of fat-deficient rats have been reported by Mead (17). It seems that the accelerated synthesis of monoenoic fatty acids under these conditions maintains a certain degree of total unsaturation and physical properties of the tissue lipids that, under normal conditions, are provided by dietary EFA and their metabolites.

Linoleic (18:2) and linolenic (18:3) acids have also been identified by internal standards. All livers analyzed, even from animals on the fat-free diet, contained 18:2, which probably is a mixture of isomers

Liver Lipids* (area percentage on GLC)

Dietary EFA (% of Cal.)	No. of Animals	20:3	20:4	20:5	22:2	22:5ω6	22:5ω3	22:6
None	21	0.54 ± 0.34	1.70 ± 0.13	<0.10	0.24 ± 0.02	0.23 ± 0.03	<0.01	0.30 ± 0.02
Linoleate								
0.009	6	7.61 ± 2.07	2.36 ± 0.66	<0.10	0.26 ± 0.10	0.42 ± 0.14	<0.01	0.42 ± 0.17
0.02	6	6.60 ± 1.81	1.87 ± 0.52	<0.10	0.15 ± 0.07	0.27 ± 0.16	<0.01	0.27 ± 0.12
0.05	6	7.12 ± 1.37	2.54 ± 0.81	<0.10	0.28 ± 0.13	0.12 ± 0.16	<0.01	0.41 ± 0.19
0.10	6	5.56 ± 1.30	2.82 ± 0.47	<0.10	0.16 ± 0.05	0.34 ± 0.10	<0.01	0.15 ± 0.08
0.18	6	7.75 ± 1.33	4.99 ± 1.00	<0.10	0.32 ± 0.12	0.60 ± 0.26	<0.01	0.33 ± 0.26
0.32	6	5.80 ± 1.96	4.80 ± 1.23	<0.10	0.21 ± 0.13	0.72 ± 0.36	<0.01	0.30 ± 0.16
0.61	6	3.44 ± 0.79	5.46 ± 0.73	<0.10	0.22 ± 0.05	0.79 ± 0.31	<0.01	0.31 ± 0.08
1.26	2	3.08	8.46	<0.10	0.16	0.07	<0.01	0.38
1.79	2	1.17	9.59	<0.10	0.34	2.07	<0.01	0.41
4.87	2	0.84	14.70	<0.10	0.05	2.21	<0.01	0.20
Arachidonate								
0.007	6	8.79 ± 2.36	2.81 ± 0.88	<0.10	0.35 ± 0.06	0.48 ± 0.11	<0.01	0.50 ± 0.10
0.019	6	7.64 ± 2.22	2.33 ± 0.73	<0.10	0.27 ± 0.08	0.45 ± 0.12	<0.01	0.31 ± 0.13
0.04	6	7.10 ± 1.08	2.98 ± 0.43	<0.10	0.28 ± 0.01	0.60 ± 0.22	<0.01	0.26 ± 0.04
0.08	6	6.88 ± 0.89	5.50 ± 0.84	<0.10	0.28 ± 0.06	1.28 ± 0.31	<0.01	0.41 ± 0.22
0.14	6	6.12 ± 2.55	6.92 ± 0.90	<0.10	0.39 ± 0.08	1.26 ± 0.46	<0.01	0.36 ± 0.15
0.27	6	4.79 ± 1.21	11.25 ± 1.43	<0.10	0.20 ± 0.07	1.98 ± 0.50	<0.01	0.32 ± 0.15
0.52	6	2.13 ± 0.72	13.71 ± 4.39	<0.10	0.38 ± 0.21	1.90 ± 0.67	<0.01	0.44 ± 0.16
1.08	2	0.61	20.01	<0.10	0.06	2.22	<0.01	0.18
1.30	2	0.42	26.22	<0.10	0.04	2.87	<0.01	0.18
3.75	2	0.25	32.46	<0.10	0.11	2.31	<0.01	0.14
Linolenate								
0.009	6	7.24 ± 1.89	1.97 ± 0.65	0.09 ± 0.02	0.22 ± 0.07	0.27 ± 0.05	<0.01	0.16 ± 0.13
0.02	6	6.09 ± 0.72	2.16 ± 0.65	0.13 ± 0.05	0.17 ± 0.01	0.35 ± 0.10	0.01	0.70 ± 0.30
0.04	6	5.46 ± 1.60	1.74 ± 0.68	0.18 ± 0.09	0.11 ± 0.05	0.19 ± 0.07	0.03 ± 0.32	1.15 ± 0.46
0.08	6	4.86 ± 1.23	1.28 ± 0.64	0.30 ± 0.11	0.11 ± 0.05	0.16 ± 0.10	0.03 ± 0.05	1.50 ± 0.65
0.18	6	4.10 ± 0.61	1.08 ± 0.23	0.74 ± 0.37	0.06 ± 0.02	0.10 ± 0.07	0.22 ± 0.10	3.02 ± 1.09
0.33	6	3.24 ± 0.66	0.73 ± 0.15	1.20 ± 0.30	0.06 ± 0.01	0.11 ± 0.06	0.25 ± 0.29	3.62 ± 0.88
0.61	6	1.88 ± 0.70	0.65 ± 0.20	1.78 ± 0.55	0.05 ± 0.03	0.08 ± 0.05	0.45 ± 0.21	3.96 ± 1.68
0.77	4	1.58 ± 0.23	0.49 ± 0.15	2.28 ± 0.82	0.05 ± 0.03	0.07 ± 0.01	0.62 ± 0.34	3.75 ± 1.37
1.42	4	0.84 ± 0.06	0.46 ± 0.12	3.30 ± 0.35	0.02 ± 0.02	0.05 ± 0.01	0.92 ± 0.24	3.41 ± 0.56
2.56	4	0.48 ± 0.15	0.60 ± 0.21	5.56 ± 1.29	0.07 ± 0.01	0.13 ± 0.07	2.25 ± 1.16	4.96 ± 1.53
3.56	4	0.47 ± 0.09	0.81 ± 0.01	7.55 ± 0.59	0.06 ± 0.03	0.14 ± 0.01	3.10 ± 0.57	5.61 ± 0.37
4.14	4	0.31 ± 0.15	0.74 ± 0.07	7.18 ± 0.32	0.06 ± 0.01	0.11 ± 0.09	2.58 ± 0.32	4.54 ± 0.81
9.42	2	0.19	1.03	12.18	0.13	0.17	3.88	5.31

* Mean values ± standard deviation.

including linoleate and dienes derived from oleic and palmitoleic acids (3, 6). The concentration of linoleate (18:2) in liver lipids was elevated only when linoleate was fed. With increasing amounts of dietary 20:4 and 18:3, a slight decrease in 18:2 levels was noted. The content of linolenate in liver lipids was extremely small, and in most cases not even measurable, in rats fed linoleate and arachidonate. Linolenate increased significantly, however, in the liver lipids of animals given diets supplemented with linolenate.

Recently, Witting et al. (18) reported very similar results for the fatty acid composition of lipids from liver mitochondria and erythrocytes of rats. 18:3 could not be detected in these tissues when diets containing linoleate were fed, whereas the 18:2 content of these tissues could be altered by different amounts of dietary linoleate. These authors found it very difficult to separate linolenic acid (18:3) from eicosaenoic acid (20:1) on an EGS column under the conditions they used. We were able to obtain a very good separation of these two components by using "Hi Eff-2b".

The most striking differences in fatty acid composition of the rat liver under the influence of different amounts of the three dietary EFA occurred in the highly unsaturated acids of C_{20} and C_{22} chain lengths. Eicosatrienoic (20:3) and eicosatetraenoic (20:4) acids were isolated by preparative GLC for further identification. Analysis by GLC of the isolated sample

was carried out to assure the purity of the preparative fraction. Analysis of a hydrogenated aliquot verified the chain length of the individual ester. Reductive ozonolysis was carried out to determine the double bond position. The resulting aldehydes and half-ester aldehydes were identified by analytical GLC.[3]

The 20:3 fraction consisted mainly of 5,8,11-eicosatrienoic acid with small amounts of the 7,10,13-isomer. Both trienoic acids have been shown to appear in liver lipids of fat-deficient rats (2,6). The content of eicosatrienoic acids in liver lipids was decreased by increasing amounts of all three dietary EFA (Fig. 5). Under the influence of dietary linoleate, the amounts of 20:3 decreased rapidly, and the curve leveled off beyond 1.9% of calories. Dietary arachidonate achieved the same level of 20:3 concentration at an intake of only 0.05% of calories, and thus proved to be about three times more efficient than linoleate. This agrees well with results of Turpeinen (19), who found arachidonate to be three times more efficient than linoleate on the basis of fat-deficiency symptoms using the method of "minimum dose for maximum response." Other investigators found arachidonic acid to be less, or more than three times as effective as linoleate (20, 21, 22). The evaluation of *deficiency symptoms* in the present experiment suggests arachidonate to be two-and-a-half to three times more efficient. Our *chemical data* indicate also that arachidonate is three times more effective than linoleate as EFA. Linolenate lowered the 20:3 level in almost the same fashion as did linoleate and arachidonate; in fact, it was even *more* efficient than linoleate in depressing the level of 20:3.

The depression of 20:3 content by all three EFA indicates that the organism prefers polyunsaturated fatty acids of the linoleate or linolenate types; i.e., acids having the first double bond at the 6th or 3rd carbon atoms counting from the methyl group. There may, indeed, be a competition between exogenous linoleate or linolenate and endogenous oleate or palmitoleate for enzyme sites responsible for their conversion to more highly unsaturated fatty acids. When linoleate or linolenate are available, their affinity for the enzymatic systems effectively prevents the synthesis of 20:3. Oleate is converted into 5,8,11-eicosatrienoic acid (20:3) only when dietary EFA are limited. Similarly, 7,10,13-eicosatrienoic acid is synthesized from palmitoleate. Thus the organism is supplied with highly unsaturated fatty acids. But the progress of fat-deficiency indicates that 20:3 cannot meet all the needs of the animal for polyunsaturated fatty acids.

[3] These procedures are described by Privett and Nickell, *J. Am. Oil Chemists' Soc.*, 39: 414, 1962.

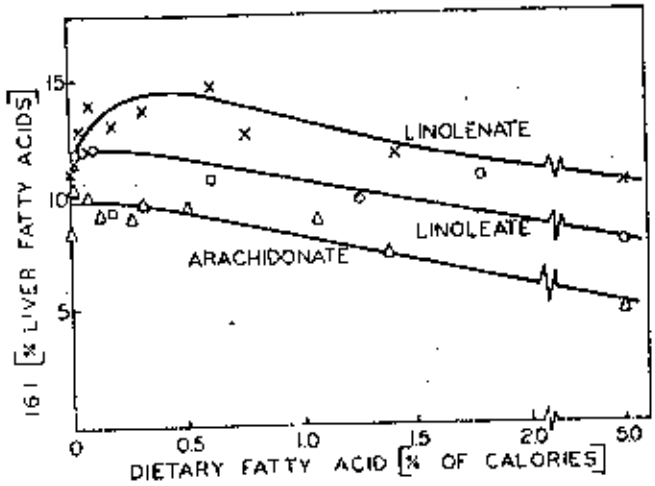

FIG. 3. The effect of varying levels of dietary EFA upon concentration of 16:1 of liver lipids.

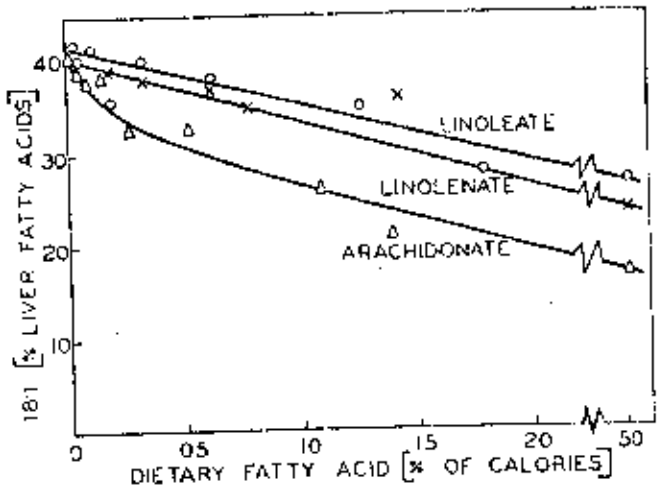

FIG. 4. The effect of varying levels of dietary EFA upon concentration of 18:1 of liver lipids.

Eicosatetraenoic acid (20:4) from liver lipids consists mainly of arachidonic acid (5,8,11,14-eicosatetraenoic acid) and small amounts of 4,7,10,13-eicosatetraenoic acid as determined by reductive ozonolysis. In fat-deficient animals, the latter increased to almost one-quarter of the 20:4. The concentration of total 20:4 increased in proportion to dietary linoleate (Fig. 6). The level of 20:4, at approximately 5% of caloric intake, exceeded the level in fat-deficient rats by a factor of eight. Arachidonate, fed at high-caloric-intake levels, was stored in the liver lipids to make up almost one-third of all liver fatty acids. With increasing amounts of dietary 18:3, the concentration of arachidonate decreased. Recently, it has been reported that linseed oil in the diet depresses the concentration of arachidonate in the lipids of chicken liver (23). The influence of dietary linolenate upon the synthesis of

arachidonate in the rat liver is currently under investigation in our laboratory.

Eicosapentaenoic acid (20:5), docosapentaenoic acid (22:5ω3), and docosahexaenoic acid (22:6) have been identified by comparison with internal standards, which were isolated from fish oils.[3] They were 5,8,11,-14,17-eicosapentaenoic acid and 7,10,13,16,19-docosapentaenoic acid from menhaden oil, and 4,7,10,13,16,19-docosahexaenoic acid from tuna oil. In all cases where linoleate and arachidonate were fed, the 20:5 and 22:5ω3 contents of the liver lipids were extremely low, and were estimated to be less than 0.1% and 0.01%, respectively. The values for docosahexaenoic acid were significantly higher but did not show any consistent changes with increasing amounts of dietary linoleate and arachidonate. Feeding linolenate, however, dramatically increased all three of the above fatty acids (Fig. 7). Approximately 10% of calories of linolenate in the diet increased 20:5, 22:5ω3, and 22:6 in liver lipids more than 100, 100, and 10 times, respectively, in comparison with the amounts present in rats not supplemented with linolenate. Thus, it has been shown that, in this experiment, these pentaenoic and hexaenoic fatty acids were synthesized only from linolenate. Their structures, therefore, can most likely be deduced by the simple rules of chain lengthening by acetyl groups and by dehydrogenation in the divinylmethane rhythm (skipped double bonds) between the existing unsaturation and the carboxyl groups. Therefore, the pentaenes and hexaene synthesized from linolenate should be 5,8,11,14,17-eicosapentaenoic, 7,10,13,16,19-docosapentaenoic, and 4,7,10,13,16,19-docosahexaenoic acids. The polyunsaturated fatty acids of the linolenate type, exceeding in some cases the amounts of arachidonate synthesized from similar amounts of dietary linoleate, do not provide the functions of the linoleate-type polyunsaturated fatty acids; not all deficiency symptoms can be cured by dietary linolenate (Figs. 1, 2).

Docosadienoic acid (22:2) has been designated by carbon number only, and the identification is tentative. No significant and consistent changes in concentration of 22:2 could be observed throughout the experiment.

An ester having a calculated carbon number of 25.2 on an EGS column was tentatively identified as 22:4 by extrapolation of retention times of methyl 7,10,13,-16,19-docosapentaenoate (C = 25.7) and methyl 4,7,10,13,16,19-docosahexaenoate (C = 26.3) standards. However, when this substance was isolated and its structure determined,[4] it was found to be methyl

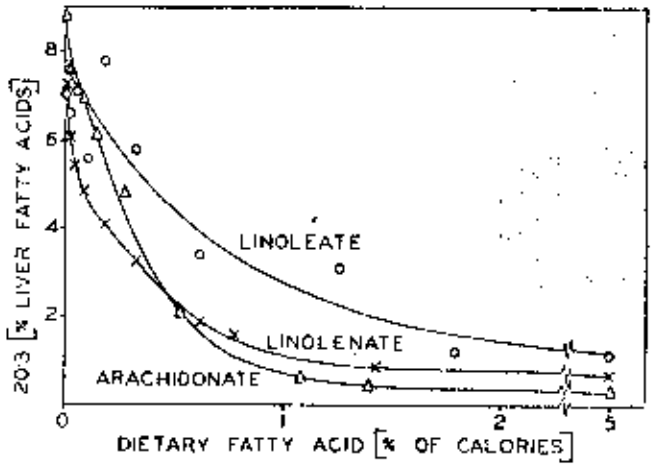

FIG. 5. The effect of varying levels of dietary EFA upon concentration of 20:3 of liver lipids.

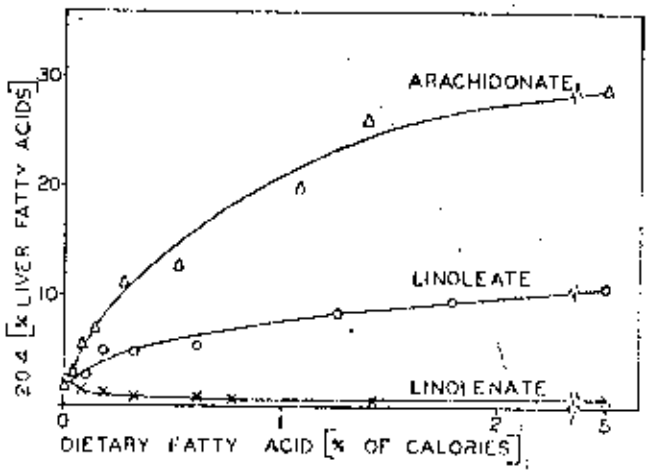

FIG. 6. The effect of varying levels of dietary EFA upon concentration of 20:4 of liver lipids.

4,7,10,13,16-docosapentaenoate, the pentaene of the linoleate family. In order to distinguish the two isomers of 22:5 we have used the suffixes ω3 and ω6 in the shorthand formulae to indicate at which carbon atom the first double bond from the ω carbon lies. Thus 22:5ω3 is the linolenate type pentaene and 22:5ω6 is the linoleate type docosapentaene. The concentrations of 22:5ω6 increased with dietary linoleate and arachidonate (Fig. 8) in almost the same pattern in liver lipid as did arachidonate (Fig. 6). Dietary linolenate did not give rise to 22:5ω6. Thus we conclude that the fatty acid is synthesized from linoleic acid via arachidonic acid by chain lengthening and dehydrogenation. This conclusion is substantiated by findings of Klenk and Mohrhauer (7), who showed that 22:5ω6 and 22:6 are synthesized from linolenate via 8,11,14,17-20:4 in analogy to the conversion demonstrated here. With increasing amounts of dietary

[3] The isolation procedures and analyses of these fatty acids will be published by Privett and Nickell of this institute.

[4] Unpublished results of J. Rabin and R. T. Holman.

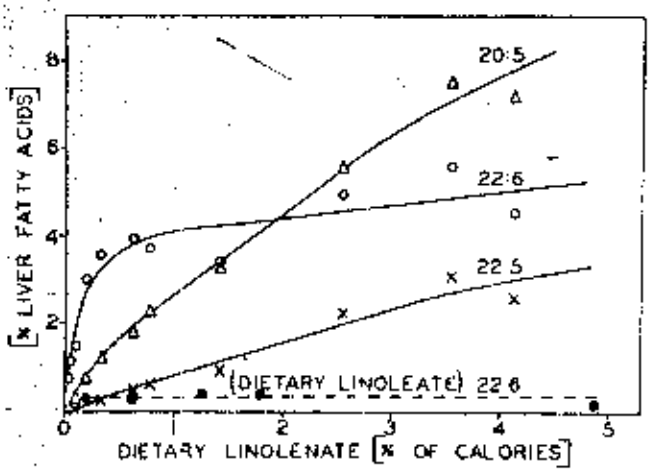

FIG. 7. The effect of varying levels of dietary linolenate upon concentration of 20:5, 22:5ω3, and 22:6 of liver lipids. The dotted line shows the effect of dietary *linoleate* upon the concentration of 22:6.

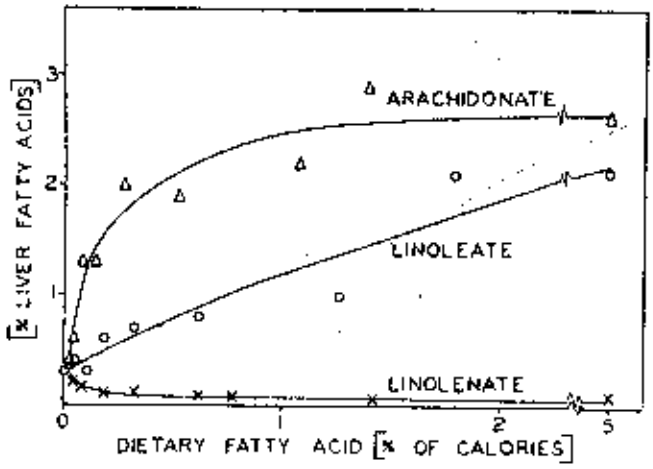

FIG. 8. The effect of varying levels of dietary EFA upon concentration of 22:5ω6 in liver lipids.

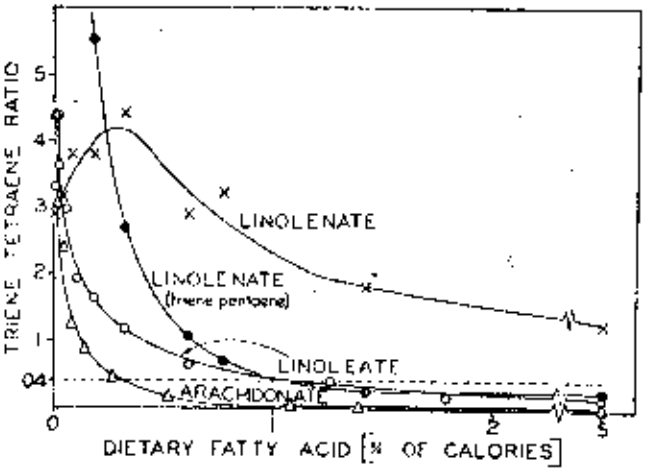

FIG. 9. The effect of varying levels of dietary EFA upon the ratio of 20:3 and 20:4 of liver lipids. Solid circles show the effect of dietary linolenate upon the ratio of 20:3 and 20:5. The dotted line represents a level of the triene/tetraene ratio below which the metabolism of EFA is considered to be normal.

linolenate, a slight decrease of 22:5ω6, in analogy to the performance of 20:4, can be noted.

It has recently been shown by Holman (24), that the ratio of trienoic to tetraenoic acids can be used to indicate the degree of EFA deficiency in rats and other species (25). In those earlier experiments, mixtures of natural fats were fed, and the analyses of tissue lipids were carried out by alkaline isomerization. That technique does not distinguish between isomeric trienes and tetraenes, nor between polyenes of different chain length. In the present experiments, highly purified single essential fatty acids were fed. The analyses of tissue fatty acids by GLC made it possible to measure specifically the pronounced changes in concentrations of 20:3 and 20:4. The curve relating the triene/tetraene ratio to dietary linoleate was also constructed from data obtained by alkaline isomerization analyses of the same liver lipids. This curve was almost identical to those found in the original experiment (24) and the one reported here (Fig. 9). Therefore, the triene/tetraene ratio, or the ratio of 20:3 to 20:4, proved to be a useful parameter for describing linoleate metabolism.

Below a triene/tetraene ratio of 0.4, no major changes in the curves occur, which corresponds to a normal EFA status in rats. This level of 0.4 is reached at a linoleate intake of 1% of calories, which is equivalent to a daily supplement of approximately 40 mg. linoleate per rat. One per cent of calories represents a level of linoleate below which the normal metabolism of polyunsaturated fatty acids no longer persists. Below this level, normal conversion of linoleate to arachidonate does not appear to take place at a sufficient rate, and the 20:3 synthesis from oleate and palmitoleate becomes predominant.

The curve relating the triene/tetraene ratio to dietary arachidonate reaches the 0.4 level at 0.3% of calories, which indicates again that arachidonate is approximately three times more effective as EFA than is linoleate. The triene/tetraene ratio reaches lower levels than in the case of dietary linoleate, but the two curves follow the same pattern.

The plot of triene/tetraene ratio vs dietary linolenate, however, is of a different type. The concentration of 20:4 does not change with increasing amounts of dietary linolenate, whereas the 20:3 decreases in the same fashion as when linoleate or arachidonate is fed. By using the ratio of eicosatrienoic acid (20:3) to eicosapentaenoic acid (20:5) instead, a curve was obtained, which is very similar to the curves relating the

triene/tetraene ratio vs dietary linoleate or arachidonate. This triene/pentaene ratio might be used to describe the metabolic status of the rat in regard to linolenate metabolism. A triene/pentaene ratio smaller than 0.4 indicates that the synthesis of the highly unsaturated fatty acids of the linolenate type is sufficient to replace the synthesis of 20:3 from oleate. The level of 0.4 is reached at a dietary intake that also supports maximum growth (Fig. 1). However, both the triene/tetraene and triene/pentaene ratios are derived for the metabolism of pure dietary linoleate or arachidonate and linolenate, respectively. Since all three EFA have similar activities in lowering the levels of 20:3 of deficient rats, the interactions of these three EFA must be studied before the proposed ratios can be used as strict indicators of the overall EFA status of an animal. Experiments to evaluate these interrelationships are currently under way in this laboratory.

The basic accomplishment of the present experiment is the description of the influence of carefully measured doses of highly purified EFA upon the composition of the liver lipid fatty acids as analyzed by employing the discriminating method of G.L.C. The previously known relationships between dietary EFA and weight and dermal symptoms in fat deficiency have now been related in a more precise manner to specific biochemical changes in tissue fatty acids. These relationships can be used also to estimate more precisely the EFA requirement of the rat and to develop prediction equations, which allow calculation of the dietary EFA intake from tissue analysis. The estimation of EFA requirement and the prediction equations for dietary EFA will be treated in a forthcoming publication.

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Effect of Linolenic Acid Upon the Metabolism of Linoleic Acid[1]

HANS MOHRHAUER[2] AND RALPH T. HOLMAN
University of Minnesota, The Hormel Institute, Austin, Minnesota

ABSTRACT Weanling rats were fed a fat-free diet supplemented with highly purified linoleate and linolenate combined in several ratios. The fatty acid composition of lipids of liver, heart and adipose tissue was analyzed by gas chromatography. Increasing amounts of dietary linolenate suppressed the levels of arachidonate in tissue lipids, thus showing that the conversion of linoleate to arachidonate is inhibited by dietary linolenate.

Recent investigations in this laboratory have shown that dietary linolenate, as well as linoleate, decreases the concentration of 5,8,11-eicosatrienoic acid in liver lipids of the rat. Moreover, a slight decrease in the concentrations of arachidonate and docosapentaenoate was observed when increasing amounts of linolenate were fed to rats supplied with a fat-free diet (1). These experiments led to our investigation of the interrelationship of linoleate and linolenate when fed together at different levels.

Greenberg et al. (2) have described a "sparking" effect in which the growth-promoting activity of linolenate was enhanced by minimal doses of linoleate. Privett et al. (3) noted a similar effect by grading dermatitis of fat-deficient rats. They also observed that dietary linolenate depressed the content of tetraenoic acid in rats. Klenk and Oette (4) reported that the content of arachidonate in liver phospholipids of rats supplemented with linolenate was less than in those fed a fat-free diet. Marco et al. have reported (5) that the linoleate-arachidonate ratio in mitochondria from chicken liver and cerebellum was increased by feeding linolenate-containing oils. During the course of our present investigation Machlin reported (6) that linseed oil fed to chickens doubles the amount of linoleic acid deposited in the liver. The arachidonate content, however, is decreased significantly. Thus, he proposed a theory that linolenic acid inhibits the conversion of linoleic acid to arachidonic acid.

All of these observations indicate that linolenate has an influence upon the metabolism of linoleate. It is our purpose in the present investigation to study this interaction systematically by feeding highly purified linoleate and linolenate in several ratios. Intake levels chosen are in the range which enables one to study the changes in fatty acid composition of tissue lipids at the onset of essential fatty acid (EFA) deficiency. Thus, the results of this experiment can be compared with those of the previous one, when linoleate and linolenate were fed singly (1).

EXPERIMENTAL

One hundred eight weanling, 24-day-old, male rats of the Sprague-Dawley strain were maintained with a basic fat-free diet as described previously (1). The major constituents of the diet were vitamin-free casein, sucrose, cellulose and the required salts and vitamins. The daily intake of basic diet was recorded for each animal. The rats were divided into 18 groups of 6 animals each. Linoleate and linolenate were fed in daily oral doses by microsyringe as indicated in table 1. Three levels of linoleate were combined with 6 levels of linolenate.

The ethyl linoleate was prepared according to the methods used by the The Hormel Foundation, Austin, Minnesota. It contained 2.3% oleate, but no linolenate could be detected by gas-liquid chromatography (GLC). The ethyl linolenate (The Hormel Foundation) was free from oleate

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[2] Present address: Bundesministerium für wissenschaftliche Forschung, Bad Godesberg, Germany.

TABLE 1

Fatty acid composition of liver lipids (% of total methyl esters)

Group no.	Dietary fatty acid 18:2	Dietary fatty acid 18:3	14:0	16:0	16:1	18:0	18:1	18:2
	% of calories							
1	0.06	0.05	0.79 ± 0.22[1]	21.76 ± 3.09	9.10 ± 1.30	13.75 ± 1.49	37.98 ± 2.68	1.33 ± 0.3[illegible]
2	0.07	0.09	0.77 ± 0.32	23.43 ± 2.72	10.44 ± 3.18	12.57 ± 3.08	38.05 ± 3.54	1.53 ± 0.33
3	0.09	0.21	0.99 ± 0.27	25.22 ± 2.05	11.00 ± 2.47	11.15 ± 3.12	37.75 ± 4.52	1.34 ± 0.29
4	0.09	0.37	0.70 ± 0.16	21.08 ± 0.66	9.81 ± 0.99	14.42 ± 0.96	33.53 ± 2.35	1.53 ± 0.20
5	0.07	0.72	0.70 ± 0.39	22.78 ± 1.75	10.51 ± 2.46	12.47 ± 2.17	35.63 ± 4.22	1.38 ± 0.31
6	0.08	1.83	0.85 ± 0.35	26.06 ± 2.07	11.36 ± 2.16	11.20 ± 1.59	31.08 ± 2.05	0.88 ± 0.10
7	0.34	0.05	0.68 ± 0.09	26.63 ± 2.77	8.12 ± 0.98	14.00 ± 2.04	32.68 ± 2.80	2.64 ± 0.8[illegible]
8	0.34	0.11	0.83 ± 0.21	22.30 ± 1.39	9.22 ± 1.75	15.18 ± 2.46	31.86 ± 2.06	2.61 ± 0.69
9	0.27	0.16	0.76 ± 0.12	24.04 ± 2.14	10.01 ± 1.26	13.60 ± 1.22	32.82 ± 1.62	2.04 ± 0.41
10	0.28	0.33	0.71 ± 0.09	25.01 ± 2.02	8.07 ± 0.79	13.73 ± 2.01	33.10 ± 1.70	2.04 ± 1.07
11	0.30	0.68	0.68 ± 0.19	25.58 ± 2.01	10.68 ± 1.35	13.41 ± 0.69	31.97 ± 0.79	1.95 ± 0.27
12	0.30	1.60	0.84 ± 0.15	21.61 ± 2.40	10.92 ± 0.66	13.21 ± 1.83	30.02 ± 1.34	2.39 ± 0.43
13	0.73	0.06	0.78 ± 0.20	24.07 ± 1.33	8.22 ± 2.62	13.85 ± 1.76	31.08 ± 3.39	3.21 ± 0.3[illegible]
14	0.58	0.09	0.68 ± 0.09	26.83 ± 2.77	8.12 ± 0.98	14.00 ± 2.04	32.68 ± 2.80	2.61 ± 0.8[illegible]
15	0.59	0.16	0.82 ± 0.10	22.58 ± 2.01	8.42 ± 1.02	14.50 ± 1.13	31.59 ± 1.46	3.40 ± 1.0[illegible]
16	0.62	0.37	0.74 ± 0.08	24.79 ± 3.30	9.38 ± 0.66	13.56 ± 1.57	30.47 ± 2.37	3.05 ± 0.67
17	0.64	0.75	0.72 ± 0.18	23.81 ± 1.46	9.38 ± 1.71	13.22 ± 1.57	30.52 ± 2.03	3.09 ± 0.8[illegible]
18	0.67	1.92	0.87 ± 0.21	21.91 ± 1.34	9.56 ± 1.29	13.56 ± 1.67	29.01 ± 2.73	5.40 ± 1.92

[1] See Experimental section of text for explanation.
[2] SD.

and contained traces of linoleate. At the end of the experiment, the intake of fatty acids for each animal was calculated in percentage of calories. One per cent of calories of linoleate and linolenate is equivalent to approximately 40 mg/day/animal.

After 87 days the animals were killed by ether anesthesia. The livers, hearts and epididymal fat were quickly removed and kept in saline solution at −20°C until analyzed.

The tissues were homogenized and the lipids extracted with chloroform:methanol (2:1) according to Folch (7). The lipids were transesterified by refluxing with 30 volumes of a 5% solution of HCl in methanol. The methyl esters were analyzed by GLC using a Barber-Coleman Model 10 apparatus with argon ionization detector.

The column was 210 cm by 5 mm i.d., packed with 20% ethylene glycol succinate (EGS) coated on Gaschrom P, 80–100 mesh.[3] The flowrate was 60 ml argon/min, and the temperatures were at the inlet heater 270°C, detector cell 250°C. The column was held at 180°C for methyl esters with retention times shorter than that of 18:3 and at 200°C for long-chain esters.

The individual esters were identified by carbon number and by internal standards. Isolation of the composite 20:3 fraction by preparative GLC followed by ozonolysis showed that 5,8,11-eicosatrienoate was the major component, and that the 8,11,14- and 7,10,13-eicosatrienoate isomers were present. The 8,11,14-isomer varied depending on the amounts of dietary linoleate. Hereafter, 5,8,11-eicosatrienoate will be referred to as 20:3ω9 and 8,11,14-eicosatrienoate will be designated 20:3ω6. This notation is used to denote relationship between polyunsaturated fatty acids (PUFA) which exist in families, the terminal structures of which are the same. Thus, acids designated ω3 are related to linolenate, ω6 to linoleate and ω9 to oleate. The calculated carbon numbers on EGS columns for 20:3ω9 and 20:3ω6 were found to be 21.85 and 22.14, respectively. These isomers probably correspond to the 2 reported by Morin et al. (8). 4,7,10,13,16-Docosapentaenoate (22:5ω6) has been identified by isolation via GLC and ozonolysis[4] and has a calculated carbon number of 25.22 on EGS columns. Quantitation was carried out by triangulation. The fatty acid composition of tissue lipids is

[3] Applied Science Laboratories, State College, Pennsylvania.
[4] Rahn, J. J. 1963 The effects of dietary fatty acids upon lipids of subcellular particles. Ph.D. Thesis, University of Minnesota (June).

TABLE 1 (Continued)

Fatty acid composition of liver lipids (% of total methyl esters)

18:3	20:3ω9[1]	20:3ω6[1]	20:4	20:5	22:5ω6[1]	22:5	22:6
0.50 ± 0.26	6.39 ± 1.76	0.96 ± 0.23	3.36 ± 0.71	0.46 ± 0.09	0.63 ± 0.23	0.13 ± 0.07	2.14 ± 0.76
1.37 ± 0.06	5.51 ± 2.17	0.48 ± 0.03	3.38 ± 1.26	0.54 ± 0.18	0.46 ± 0.17	0.24 ± 0.15	3.26 ± 1.30
1.35 ± 0.07	3.42 ± 1.42	0.72 ± 0.18	2.28 ± 0.39	0.71 ± 0.12	0.23 ± 0.02	0.36 ± 0.11	4.32 ± 1.00
1.88 ± 0.09	4.26 ± 0.90	0.74 ± 0.15	2.49 ± 0.15	1.88 ± 0.44	0.20 ± 0.03	0.58 ± 0.26	7.85 ± 1.16
1.83 ± 0.40	1.83 ± 0.71	0.28 ± 0.15	1.74 ± 0.46	2.13 ± 0.62	< 0.1	1.07 ± 0.47	8.25 ± 1.27
1.25 ± 0.34	0.93 ± 0.30	0.35 ± 0.31	1.14 ± 0.32	4.76 ± 0.94	< 0.1	2.28 ± 0.42	7.87 ± 1.52
0.31 ± 0.07	3.31 ± 0.93	0.92 ± 0.19	6.94 ± 1.62	0.37 ± 0.08	1.16 ± 0.31	0.18 ± 0.07	1.87 ± 0.41
1.35 ± 0.03	6.13 ± 1.14	1.30 ± 0.36	6.33 ± 0.78	0.51 ± 0.07	0.88 ± 0.13	0.27 ± 0.13	3.25 ± 0.75
0.40 ± 0.03	4.23 ± 0.70	0.59 ± 0.17	5.12 ± 1.04	0.76 ± 0.15	0.64 ± 0.19	0.29 ± 0.13	3.91 ± 0.85
1.14 ± 0.17	3.29 ± 0.64	0.79 ± 0.25	4.30 ± 1.10	1.15 ± 0.34	0.28 ± 0.11	0.53 ± 0.18	6.31 ± 1.60
0.45 ± 0.09	2.21 ± 0.57	0.56 ± 0.11	2.79 ± 0.33	1.90 ± 0.37	0.11 ± 0.01	0.78 ± 0.18	6.01 ± 0.63
1.51 ± 0.51	1.03 ± 0.19	0.42 ± 0.08	2.22 ± 0.37	5.00 ± 0.68	< 0.1	2.47 ± 0.61	8.16 ± 0.84
0.41 ± 0.09	3.00 ± 1.03	1.13 ± 0.29	8.48 ± 1.83	0.75 ± 0.17	1.83 ± 0.23	0.20 ± 0.09	2.40 ± 0.51
0.31 ± 0.07	3.31 ± 0.93	0.92 ± 0.19	6.94 ± 1.62	0.37 ± 0.08	1.16 ± 0.31	0.18 ± 0.07	1.87 ± 0.41
0.41 ± 0.12	3.33 ± 0.79	1.09 ± 0.18	7.70 ± 0.99	0.56 ± 0.05	0.95 ± 0.21	0.37 ± 0.16	4.26 ± 0.95
0.51 ± 0.09	2.59 ± 0.59	0.62 ± 0.10	6.00 ± 0.97	1.03 ± 0.21	0.41 ± 0.10	0.63 ± 0.17	6.08 ± 1.25
1.06 ± 0.20	1.51 ± 0.44	0.50 ± 0.17	4.45 ± 0.67	1.84 ± 0.17	0.18 ± 0.05	1.19 ± 0.36	7.95 ± 1.06
1.83 ± 0.98	0.58 ± 0.29	0.51 ± 0.15	3.20 ± 0.37	3.64 ± 0.54	0.10 ± 0.03	2.13 ± 0.40	7.41 ± 1.25

reported as area per cent, a procedure justified in cases where changes in concentration, but not the absolute composition, are to be measured.

Liver lipids were analyzed for each animal. The results reported are averages for 6 animals with their standard deviations. Hearts, as well as epididymal fat, were pooled and one GLC analysis was performed per group.

RESULTS

The animals showed approximately equal growth with all linoleate-linolenate supplementations. The average weight gain was 240 g during the 3-month period of the experiment. No severe fat deficiency symptoms could be observed, although mild dermatitis was detectable, especially in the groups (1–6) with the lowest linoleate supplement (0.08% of calories). The fatty acid composition of the liver lipids is shown in table 1.

No consistent changes can be observed in the concentrations of the saturated fatty acids — myristic (14:0), palmitic (16:0) and stearic (18:0) — throughout the experiment.

Increasing levels of dietary linoleate lead to the following changes in the fatty acid composition of the liver lipids:

1) The levels of palmitoleic (16:1) and oleic (18:1) acids are decreased slightly.

2) The concentrations of the fatty acids of the linoleate family — 18:2, 20:4 and 22:5ω6 — are increased significantly.

3) The level of 5,8,11-eicosatrienoic acid (20:3ω9) is decreased.

4) No significant changes can be observed in the concentrations of the fatty acids of the linolenate family — 18:3, 20:5, 22:5 and 22:6.

Increasing amounts of dietary linolenate influence the fatty acid composition of the liver lipids at all 3 levels of linoleate intake as follows:

1) The concentrations of the fatty acids of the linolenate family — 18:3, 20:5, 22:5 and 22:6 — are increased significantly.

2) The levels of 20:3 and the fatty acids of the linoleate family — 20:4 and 22:5ω6 — are lowered significantly.

3) No pronounced influence upon the concentration of the monoenoic and dienoic acids of the liver lipids can be observed.

In table 2 the fatty acid composition of heart lipids is shown. The data represent analyses of the pooled hearts of 6 animals for each group. In general, the same changes are observed as described for liver lipids. At all 3 levels of linoleate intake,

TABLE 2

Fatty acid composition of heart lipids (% of total methyl esters)

Group	Dietary fatty acid		14:0	16:a1[1]	16:0	16:1	18:0	18:1	18:2	18:3	20:3ω9[2]	20:3ω6[2]	20:4	20:5	22:5ω6[2]	22:5	22:6
	18:2	18:3															
	% of calories																
1	0.08	0.05	0.58	1.15	17.32	6.66	18.43	33.74	3.55	< 0.1	10.60	0.58	5.74	< 0.1	0.39	0.14	1.11
2	0.07	0.09	0.77	1.10	16.08	6.80	16.95	32.63	3.91	< 0.1	6.75	0.49	7.34	0.1	0.42	0.36	2.32
3	0.09	0.21	0.89	1.12	20.17	7.75	17.17	33.20	4.40	< 0.1	6.01	0.52	5.28	0.21	0.20	0.43	2.64
4	0.09	0.37	0.83	1.20	20.09	7.22	17.17	34.57	4.08	0.48	4.05	0.37	4.67	0.66	< 0.1	0.65	3.96
5	0.07	0.72	0.82	1.28	18.83	6.59	18.16	33.28	4.23	1.24	2.21	0.45	4.52	1.66	< 0.1	1.22	5.54
6	0.08	1.83	1.04	1.23	21.80	9.93	15.37	34.79	3.33	2.57	0.95	0.21	3.00	—	—	—	—
7	0.34	0.05	0.74	1.50	17.24	5.25	18.74	29.47	8.27	< 0.1	4.13	1.01	11.21	< 0.1	0.92	0.17	1.36
8	0.34	0.11	0.75	1.45	18.34	5.56	18.72	29.05	8.05	< 0.1	3.88	0.68	10.45	< 0.1	0.77	0.25	2.05
9	0.27	0.16	1.05	1.02	21.50	7.73	15.69	33.37	6.15	< 0.1	3.47	0.56	7.02	< 0.1	0.41	0.26	1.82
10	0.28	0.33	0.67	1.54	16.85	5.46	19.70	29.50	8.25	< 0.1	3.60	0.74	8.09	0.30	0.25	0.82	4.23
11	0.30	0.68	0.93	1.06	19.96	7.89	15.32	34.13	7.44	< 0.1	1.84	0.48	5.18	0.94	< 0.1	0.85	4.00
12	0.30	1.80	0.81	1.18	18.08	5.19	18.01	26.96	9.85	1.51	1.39	0.74	6.09	1.53	< 0.1	2.64	6.02
13	0.73	0.06	1.39	0.94	21.31	8.46	13.57	32.45	8.17	< 0.1	1.74	0.50	9.35	< 0.1	0.93	0.23	0.96
14	0.58	0.09	0.52	1.67	17.56	4.30	19.39	27.35	10.64	< 0.1	2.48	0.73	12.68	< 0.1	1.09	0.08	1.49
15	0.59	0.18	0.71	1.27	18.06	4.52	18.01	29.96	9.53	< 0.1	1.92	0.53	12.01	< 0.1	0.59	0.26	2.54
16	0.52	0.37	0.63	1.40	17.71	5.14	17.35	30.66	10.70	< 0.1	1.49	0.53	10.14	0.09	0.31	0.48	3.36
17	0.64	0.75	1.00	1.45	16.93	6.20	15.82	31.11	11.15	< 0.1	0.95	0.56	7.37	0.48	0.09	0.91	3.97
18	0.67	1.92	0.70	1.44	16.94	4.71	17.36	25.27	16.99	0.78	0.56	0.49	6.15	1.49	< 0.1	2.05	5.10

[1] 16:a1 identified as the C-16 saturated aldehyde.
[2] See Experimental section of text for explanation.

TABLE 3

Fatty acid composition of the lipids of depot fat (% of total methyl esters)

Group	Dietary fatty acid		14:0	16:0	16:1	18:0	18:1	18:2	18:3
	18:2	18:3							
	% of calories								
1	0.08	0.05	1.55	30.54	17.82	1.71	48.05	0.32	< 0.1
2	0.07	0.09	1.70	34.27	18.36	1.51	43.75	0.42	< 0.1
3	0.09	0.21	1.85	31.04	21.00	1.33	44.41	0.37	< 0.1
4	0.09	0.37	1.91	27.56	19.86	1.54	48.46	0.35	0.32
5	0.07	0.72	2.33	37.42	26.86	1.79	30.29	0.56	0.75
6	0.08	1.83	1.38	38.91	19.69	1.31	37.05	0.39	1.28
7	0.34	0.05	2.06	26.17	18.48	2.07	50.60	0.66	< 0.1
8	0.34	0.11	1.87	27.83	18.34	2.03	49.33	0.61	< 0.1
9	0.27	0.16	1.99	32.50	19.96	1.28	43.65	0.52	< 0.1
10	0.28	0.33	1.83	31.92	18.80	1.39	45.40	0.67	< 0.1
11	0.30	0.68	1.82	29.10	20.92	1.75	45.29	0.82	0.49
12	0.30	1.80	1.61	35.01	21.53	1.34	37.25	0.74	1.50
13	0.73	0.06	1.80	31.26	19.56	1.72	44.66	0.98	< 0.1
14	0.58	0.09	1.67	31.44	20.57	1.48	44.10	0.75	< 0.1
15	0.59	0.18	1.79	28.82	16.05	1.95	46.05	0.86	0.48
16	0.62	0.37	1.79	30.60	19.70	1.62	44.98	1.01	0.31
17	0.64	0.75	1.87	31.89	20.70	1.50	42.15	1.28	0.63
18	0.67	1.92	2.70	31.24	20.01	1.77	40.46	1.70	2.06

the decrease in concentration of 20:4 and 22:5ω6 with increasing amounts of dietary linolenate is several fold. At constant linolenate intake the concentrations of 18:3 and 20:5 appear to decrease with increased linoleate intake.

The analysis of depot fat (table 3) shows that different amounts of dietary linoleate and linolenate are reflected in the 18:2 and 18:3 concentrations of the lipids. The saturated and monoenoic fatty acids are not significantly influenced. The concentration of arachidonic acid was too low to be measured.

DISCUSSION

The effect of dietary linolenate upon the concentration of arachidonate and 22:5ω6 in lipids of rat liver as described recently (1) could be confirmed and extended by the results of the present experiment. The percentage of arachidonate in liver lipids of rats fed 1.8% of calories as linolenate is less than one-half the level observed in livers of rats fed 0.05% of calories. This is true for all 3 levels of linoleate fed (fig. 1). The same effects were observed in heart tissue, although the changes were not quite as consistent as in liver lipids.

The increase in linoleate content at higher levels of dietary linolenate as observed by Machlin in chickens (6) is not so pronounced in lipids of rat tissue at the low levels of linoleate we fed. At the highest intake level (0.6% of calories linoleate) an increase of linoleate content can be noted, however, especially in heart lipids.

The ratio of linoleate to arachidonate (precursor to metabolic product) increases sharply at all linoleate intake levels with increasing amounts of dietary linolenate (table 4). These changes are most pronounced at the highest linoleate level fed.

Concurrent with the decrease of 20:4, the synthesis of 22:5ω6 is suppressed by increasing amounts of dietary linolenate. This is to be expected because this docosapentaenoate is synthesized from linoleate via arachidonate (1).

From our observations it might be concluded that the conversion of linoleate to arachidonate in the rat is partially inhibited by dietary linolenate. If no external dienoic or trienoic fatty acids are available, oleic acid is converted into 20:3ω9 (9). If linoleate or linolenate are present in sufficient amounts, they will occupy the enzymes responsible for dehydrogenation and chain lengthening and are converted into the polyunsaturated fatty acids (PUFA) of their corresponding

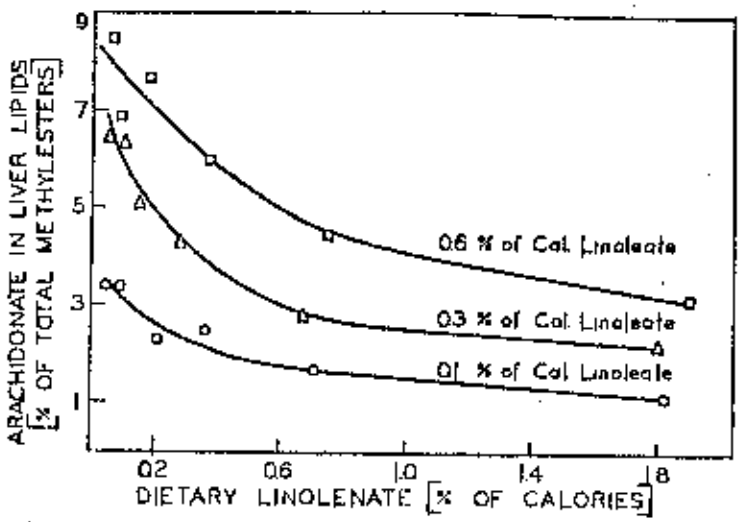

Fig. 1 The effect of varying levels of dietary linolenate upon the biosynthesis of arachidonate in liver lipids.

TABLE 4

Effects of dose level of dietary linolenate upon the linoleate-arachidonate ratio in liver and heart lipids

Dietary fatty acid		Linoleate-arachidonate ratio	
18:2	18:3	In liver lipids	In heart lipids
% of calories			
0.08	0.05	0.39	0.62
0.07	0.09	0.45	0.53
0.09	0.21	0.59	0.83
0.09	0.37	0.61	0.87
0.07	0.72	0.79	0.94
0.06	1.83	0.77	1.11
0.34	0.05	0.37	0.74
0.34	0.11	0.41	0.77
0.29	0.16	0.39	0.88
0.28	0.33	0.47	1.02
0.30	0.69	0.69	1.44
0.30	1.60	1.08	1.62
0.73	0.06	0.38	0.87
0.58	0.09	0.38	0.84
0.59	0.18	0.44	0.79
0.62	0.37	0.51	1.06
0.64	0.75	0.83	1.51
0.67	1.02	1.46	2.70

families (1). In case linoleate and linolenate are fed together, especially if the linolenate concentration exceeds that of linoleate in the diet, linolenate is converted preferably, and the linoleate-arachidonate conversion is inhibited.

This concept of substrate competition leads to the assumption that the same or similar metabolic pathways are responsible for all conversions of oleic, linoleic and linolenic acids to the PUFA of their respective families. The degree of unsaturation between the 9-position and the terminal methyl group of the substrate fatty acid determines the affinity to the enzymes involved. The triene, linolenate, blocks the conversion of the diene, linoleate. Either linoleate or linolenate blocks the conversion of the monoene, oleate. Thus, the affinities for the enzyme sites are linolenate > linoleate > oleate. A similar mechanism may be applicable for the conversion of 20-carbon to 22-carbon PUFA. The biosynthesis of the PUFA of the linolenate type, 22:5 and 22:6, may inhibit the conversion of arachidonate to 22:5ω6, or the inhibition could be due to simple lack of 20:4 substrate.

Recently, we have reported that the synthesis of 20:3ω9 from oleic acid is inhibited by feeding linolenate to fat-deficient rats. This effect is confirmed in the present experiment. At all 3 levels of linoleate fed, additional amounts of linolenate in the diet decrease the 20:3ω9 content of liver and heart lipids even farther. Thus, the abilities of linolenate and linoleate to lower the content of eicosatrienoate in tissue lipids are additive. This may be the

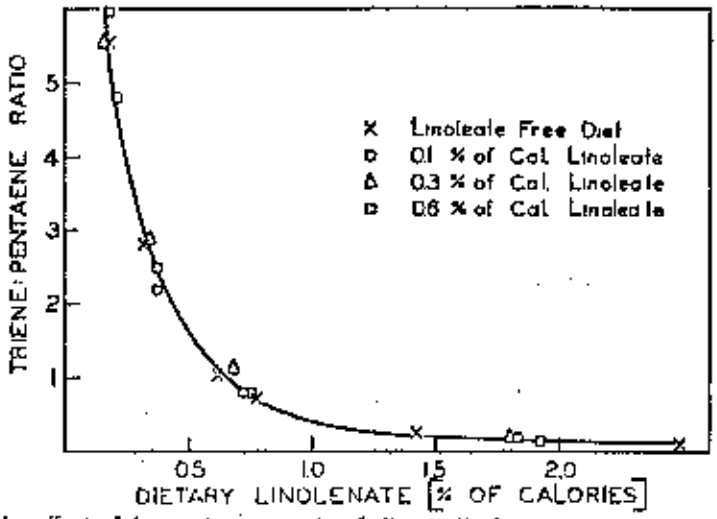

Fig. 2 The effect of increasing amounts of dietary linoleate upon the triene-pentaene ratio at different levels of linoleate intake.

biochemical basis for the "sparking" effect reported by Greenberg et al. (2).

The ratio of triene-to-tetraene was proposed to determine the fat deficiency status of rats (10), swine (10, 11) and other species. Experiments with purified fatty acids which were fed singly to rats supplied with fat-free diets supported the usefulness of the triene-tetraene ratio as a parameter for describing linoleate metabolism (1). It was shown, however, that the proposed ratio could not be used in cases where linolenate is the only dietary fatty acid. The present experiments show that the triene-tetraene ratio is not meaningful either when the linolenate concentration exceeds the linoleate content of the diet. Since most normal diets, especially human diets, contain much more linoleate than linolenate, the triene-tetraene ratio still has practical value.

The ratio of eicosatrienoic (20:3ω9) to eicosapentaenoic acid (22:5ω3) proposed to describe the fat status of the rat with respect to linolenate metabolism (1) has been found to be useful in the whole range of linoleate intake studied in the present experiment. Figure 2 shows the effect of dietary linolenate upon the triene-pentaene ratio at 4 different levels of linoleate intake. The triene-pentaene ratio for linoleate-free diets is taken from an earlier publication (1). All points relating the triene-pentaene ratio to dietary linolenate lie on the curve derived for linoleate-free diets. Thus, the triene-pentaene ratio is independent of dietary linoleate within the range studied here.

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UNITED STATES PATENT OFFICE

2,602,807

RECOVERY OF CATALYST FROM HYDROGENATED OIL

Charles E. Morris and Frank P. Khym, Chicago, Ill., assignors to Armour and Company, Chicago, Ill., a corporation of Illinois

No Drawing. Application December 26, 1945, Serial No. 637,292

8 Claims. (Cl. 260—409)

1

This invention relates to recovery of catalyst of the removable type from hydrogenated oil, as through filtering and the like.

In the hydrogenation of edible oils it is common to mix the oils with a catalyst and subsequently, after hydrogenation, to remove the catalyst from the oil. In continuous hydrogenation processes the catalyst is mixed with the oil and hydrogenation is accomplished under elevated temperatures and pressures in the presence of the catalyst and thereafter the hot oil is passed through a deodorizing and degassing tower and finally through a cooling tank to storage. In the course of such a process, the catalyst is normally removed shortly after the hydrogenation step and sometimes it is removed after the deodorizing step. In some processes, the filtering operation is employed at both stages.

In the filtering operations, considerable difficulty has been experienced in removing the catalyst, which is usually a nickel catalyst. In the ordinary filtering of hydrogenated oil, complete removal of the catalyst is not effected. The resulting oil is quite dark in color, assuming an appearance which is similar to orange pekoe tea. Even in subsequent bleaching operations, the catalyst remained in the oil and the above color was not modified substantially. The ordinary bleaching clays were not effective for removing the catalyst.

An object of the present invention is to provide a method for the complete removal of the hydrogenation catalyst so that a clear filtrate is obtained. A further object is to provide a method whereby the removal of the hydrogenation catalyst can be accomplished with a slight modification of the present filtering operation to produce a clear product. Other specific objects and advantages will appear as the specification proceeds.

We have discovered that if a slight amount of acid is incorporated in the clay slurry employed in the filtering of the oil, the clay which was theretofore ineffective in the removal of the catalyst becomes completely effective in bringing about the removal of the catalyst. The acid may be incorporated in the clay at any desired stage prior to the filtering operation. If desired, an acidified or acid leached clay may be used for this purpose. For example, a clay which has been leached out with a solution of sulphuric acid and then later pulverized is found satisfactory.

As an example of the process, we have brought about clarification of oils which otherwise were unfilterable by the addition of a few one hundredths of 1% of 85% syrupy phosphoric acid.

2

The acid was added directly to the bleaching clay in the bleaching kettle. More specifically, good results were obtained by adding from .01% to .04% of such phosphoric acid to the clay at the time the bleaching or clarification operation was performed.

Instead of phosphoric acid, sulphuric acid and other acids which do not interfere with the edible properties of the oil may be used, and so long as the amount of the acid is sufficient to bring the clay over to the acid side. Any such acid which is non-toxic and does not interfere with the edible properties of the oil, either because it is non-toxic per se or because it is used in such relatively small amounts that it has no injurious effect, will be found satisfactory. We have found, however, that phosphoric acid and sulphuric acid are particularly advantageous in the carrying out of the process.

As an example of the use of acid activated clay, we find that from 0.50 to 1.0%, when incorporated in the oil prior to its being pumped to the hydrogenation tank, was effective in the obtaining of a clear filtrate after the hydrogenation step.

Another effective method in the use of the acidified clay is to pump a 10% clay and oil slurry into the filter so as to coat and cover the surfaces in the filter just prior to the passing of the oil and catalyst mixture into the filter. The acidified clay slurry is found to be very effective when mixed with the oil at or just prior to the filtering operation.

The process is inexpensive in that very minute amounts of acid are required and relatively small amounts of the acid treated clay are required. The oil is immediately freed of the catalyst and a clear filtrate, which could not heretofore be obtained by the use of large amounts of untreated clay, is obtained.

While in the foregoing process, we have set forth specific details as illustrative of the process, it will be understood that such details may be modified widely by those skilled in the art without departing from the spirit of our invention.

We claim:

1. In a process for the recovery of a removable nickel catalyst from hydrogenated oil, the steps of incorporating clay and an acid taken from the group consisting of sulphuric and phosphoric acid, with the oil, and filtering the mixture.

2. In a process for recovering removable nickel catalyst from hydrogenated oil, the steps of incorporating clay and an acid selected from the

group consisting of phosphoric acid and sulfuric acid with the oil and filtering the mixture.

3. In a process for the recovery of a removable nickel catalyst from hydrogenated oil, the steps of incorporating a slurry containing clay and an acid selected from the group consisting of phosphoric acid and sulfuric acid with the oil and filtering the mixture.

4. In a process for the recovery of a removable nickel catalyst from hydrogenated oil, the steps of incorporating clay acidified with an acid selected from the group consisting of phosphoric acid and sulfuric acid with the oil and filtering the mixture.

5. In a process for the recovery of a colloidal suspension of nickel catalyst from hydrogenated oil, the steps of filtering the oil to remove substantially all of the nickel catalyst except a colloidal suspension thereof, incorporating clay acidified with an acid selected from the group consisting of phosphoric acid and sulfuric acid, and filtering the mixture to remove said colloidal suspension of catalyst and clay.

6. In a process for the recovery of a removable nickel catalyst from hydrogenated oil, the steps of incorporating phosphoric acid and bleaching clay with the oil and filtering the mixture.

7. In a process for the recovery of removable

nickel catalyst from hydrogenated oil, the steps of mixing a clay, leached with a solution of sulphuric acid, with the hydrogenated oil and filtering the mixture.

8. In a process for the recovery of removable nickel catalyst from hydrogenated oil, the steps of mixing clay, leached with a solution of sulphuric acid, with a small amount of the oil to form a slurry, mixing the slurry with the mass of the hydrogenated oil just before filtering the mixture and then filtering the mixture.

CHARLES E. MORRIS.
FRANK F. KHYM.

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Soy

SUPPLEMENTATION OF HUMAN DIETS WITH VITAMIN E

A Statement of the

FOOD AND NUTRITION BOARD

DIVISION OF BIOLOGY AND AGRICULTURE

NATIONAL RESEARCH COUNCIL

(Prepared by the Committee on Nutritional Misinformation)

NATIONAL ACADEMY OF SCIENCES

June, 1973

Supplementation of Human Diets with Vitamin E

Among all the vitamins probably none has commanded more public attention or been the subject of more clinical controversy than vitamin E. The list of ailments claimed to be relieved by this vitamin includes most noninfectious diseases, e.g., heart disease, sterility, muscular weakness, cancer, ulcers, skin disorders, burns, and shortness of breath. As for apparently healthy subjects, vitamin E has been claimed to promote physical endurance, enhance sexual potency, prevent heart attacks, protect against the health-related effects of air pollution, and slow the aging process and alleviate its accompanying ailments.

How did these claims come about? To some extent they arose from a misinterpretation of the results of research on experimental animals. Vitamin E was shown to be among the factors required to prevent sterility in male rats and to permit normal pregnancy in female rats. This discovery naturally led to examination of the possible beneficial effect of the vitamin in connection with various problems of reproduction in men and women. The relevant tests on human subjects are difficult to evaluate because they cannot be conducted in a laboratory setting in which the effects of other factors can be eliminated. Some instances of beneficial effects were reported but, on the basis of results obtained over the past 35 years, leading scientific and medical opinion does not support the view that supplemental vitamin E has any value in preventing male impotency or sterility or in altering the outcome of pregnancy.

Advocates of vitamin E supplementation in human beings overlook the fact that an effect on reproduction in animals can be demonstrated only when the animals have been fed for long periods on diets free of vitamin E. The widespread presence of the vitamin in human diets has prevented a deficiency, such as seen in animals under experimental condition, from developing in man.

In most animal species that have been studied, severe vitamin E deficiency causes muscle wasting or dystrophy. Understandably, vitamin E has therefore been tested as a treatment for various muscle diseases of man, including hereditary muscular dystrophy. Again, results have been negative. The muscles of persons with dystrophy contain normal amounts of vitamin E, and there is no evidence that the condition in man is associated with a dietary deficiency of this vitamin.

Abnormalities in the heart muscle of vitamin E-deficient animals are less common and less severe than those seen in the skeletal muscle. In cattle and sheep, however, heart-muscle abnormalities can be severe, whereas severely deficient monkeys with skeletal dystrophy and bone-marrow failure have shown no cardiac involvement. Similarly, there is no evidence that cardiac disease is a consequence of vitamin E deficiency in man and, to date, extensive tests have failed to demonstrate therapeutic benefit from supplemental vitamin E.

Why has supplemental vitamin E been so ineffective in treatment of disease? Clearly, it is because the reproductive failure, heart disease, and muscular dystrophy observed in man are not attributable to dietary deficiency of this vitamin. Likewise, there is no satisfactory

scientific or clinical evidence that supplemental dietary vitamin E is beneficial in the treatment of such other conditions as burns, skin disorders, poor physical performance, and cancer.

Surveys of the United States population indicate that adequate amounts of vitamin E are supplied by the usual diet. The recommended daily dietary allowances of this vitamin range from 5 to 30 international units, depending upon age, sex, and physiologic state, and are deemed to exceed the actual needs of most individuals. Dietary vitamin E is supplied in substantial amounts by most vegetable oils as well as by margarine and shortening made from these oils, and significant inputs are made by many vegetables and by whole-grain cereals. Meats, fish, poultry, milk, eggs, legumes, fruits, and nuts also contribute to the dietary supply. Thus, supplementation of the diet with vitamin E is unlikely to be useful in alleviating any of the ailments mentioned above.

Are there any special cases in which supplementary vitamin E is beneficial? Some physicians prescribe vitamin E for premature infants, who frequently have low blood levels of the vitamin because of limited transfer from the mother's blood before birth. Patients afflicted with conditions that interfere with normal digestion or absorption of fats and fat-soluble vitamins require supplements of these vitamins, one of which is vitamin E. Such individuals should be under the care of a physician; for others vitamin E supplements are unnecessary. Self medication with vitamin E in the hope that a more or less serious condition will be alleviated may indeed be hazardous, especially when appropriate diagnosis and treatment may thereby be delayed or avoided.

Summary

Misleading claims that vitamin E supplementation of the ordinary diet will cure or prevent such human ailments as sterility, lack of virility, abnormal termination of pregnancy, heart disease, muscular weakness, cancer, ulcers, skin disorders, and burns, are not backed by sound experimentation or clinical observations. Some of these claims are based upon deficiency symptoms observed in other species. Careful studies over a period of many years attempting to relate these symptoms to vitamin E deficiency in human beings have been unproductive. The wide distribution of vitamin E in vegetable oils, cereal grains, and animal fats makes a deficiency in humans very unlikely. Premature infants or individuals with impaired absorption of fats may require supplemental vitamin E, but they should, in any event, be under the care of a physician.

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Effects of dietary fish oil on the composition and stability of turkey depot fat

By T. S. NEUDOERFFER and C. H. LEA

Food Research Institute, Low Temperature Research Station, Cambridge

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1. Beef fat (2·5%) and anchovy oil (2·5%; 2·5% plus 0·02% ethoxyquin; 5%) were incorporated into isocaloric cereal-based diets A, B, C and D and given to turkeys from 2 to 19 weeks of age. Diets A, B, and C contained 13 mg and diet D 26 i.u. α-tocopheryl acetate/kg. The lipids of diets B and D, which contained fish oil without ethoxyquin, autoxidized in the feeding troughs, but not seriously in the brief period of exposure permitted. 2. The birds all remained healthy and grew well, the only nutritional effects of the fish oil being a depressed storage of vitamin A in the liver at both levels of feeding (prevented by ethoxyquin) and a slightly adverse effect, at the higher level only, on food conversion ratio. 3. The skin fats of the birds given fish oil contained seven major and two minor fatty acids, derived from the fish oil, which were not present in the skin of the control group given beef fat, as well as one minor fish oil acid present in much greater concentration in the birds given fish oil than in the controls. All these acids were present in the skin fats at about a half or two-thirds of their concentrations in the dietary lipid, except for acid 22:5, which reached two or three times this level. 4. The stabilities towards autoxidation of the skin fats decreased in the order A > C > B > D, as their content of polyunsaturated fatty acids increased, but the greater stability of group C as compared with B was probably due, in the main, to the higher tocopherol content of the skin fat of the birds on the ethoxyquin-containing diet. Fishy flavours which developed on cooking showed a similar relationship to diet.

Though adequate supplies of polyunsaturated fatty acids (PUFA) are necessary for the animal body to produce lipids of proper biological function, adverse effects can ensue if such acids are present in the diet in concentrations that are high in relation to those of vitamin E and other substances of related protective activity. In chicks, when the diet is deficient in vitamin E, polyunsaturated acids of the linoleic-arachidonic series cause encephalomalacia, and Dam & Sondergaard (1964) have shown that approximately 0·8 mg D-α-tocopherol is required to balance each g of linoleic acid present in the diet. Neither selenium nor sulphur-containing amino acids protect against this sign of vitamin E deficiency, though certain synthetic antioxidants do so, e.g. ethoxyquin (EMQ; 1,2-dihydro-6-ethoxy-2,2,4-trimethylquinoline). On diets low in selenium and vitamin E (or other biologically active antioxidant) the giving of PUFA causes or greatly increases the severity of exudative diathesis. On diets low in sulphur amino acids and vitamin E, muscular dystrophy develops, though this sign is less dependent on the fat content of the diet and some evidence has recently been produced disputing any direct relation between muscular dystrophy in the chick and increased susceptibility of the tissue lipids to oxidation (Desai, Calvert & Scott, 1964). Nevertheless, Calvert, Desai & Scott (1964) found that the amount of vitamin E required to prevent muscular dystrophy in chicks increased with the amount of linoleic acid ingested up to 0·5% (though thereafter no further increase in vitamin E was required up to 2·5% linoleate), and Miller, Leong, Knobl &

Gruger (1965) have demonstrated that sufficiently high levels of highly unsaturated fish oil esters or glycerides will cause both these vitamin E deficiency syndromes in the presence of normally effective levels of protectants. Miller *et al.* (1965) assess the relative protective potencies of selenium, DL-α-tocopheryl acetate and EMQ for this purpose as approximately 1:500:3750, on a weight basis.

It may be that PUFA increases the requirement for vitamin E and related protective substances partly because of increased destruction of the vitamin by free radicals produced during autoxidation of the unsaturated fatty acids, either before or during absorption. Probably more important, however, is the resulting increased deposition of these readily peroxidizable fatty acids in important lipid structures, such as the membranes of mitochondria and lysosomes, where their biological function could be damaged by peroxidation under the influence of catalysts now believed to consist of both haem proteins and inorganic iron (Wills, 1966).

Another important consequence of giving fats containing high levels of PUFA to non-ruminant farm animals is an increased tendency for the development of 'fishy' odours and flavours on cooking: at lower dietary levels of PUFA 'off' flavours may result which are not recognizable as 'fishy'. Taints of this kind probably arise from peroxidation of the increased quantities of oxidatively unstable fatty acids present in the tissues, with subsequent fission to volatile, malodorous fragments.

In a previous experiment (Lea, Parr & Carpenter, 1960) we failed to detect any fishy taint in the eggs or flesh of hens on diets containing 8% of fresh or stored herring meal, equivalent to a dietary content of nearly 1·4% of chloroform–methanol extractable fish lipid or approximately 1·0% of ether or light petroleum extract. More recently (Lea, Parr, L'Estrange & Carpenter, 1966) we have found that the presence of fish oil (in fresh fish meal), at a level of 2·2% of the diet, caused serious tainting of the flesh of roast turkeys; the same amount of oil oxidatively polymerized by storage of the meal before feeding caused only negligible tainting. Stabilization of the oil in fish meal by the use of antioxidants is therefore likely to have an adverse effect on the flavour of the meat produced, though the protein of stabilized meal may be better nutritionally because the oxidized oil can cause damage to the protein (Lea *et al.* 1960; Carpenter, Morgan, Lea & Parr, 1962). At levels likely to be met in practice the oxidized oil of stored fish meal is not itself directly toxic to chicks (Carpenter, Lea & Parr, 1963).

In a recent review of the rather contradictory literature on the subject of tainting by fish oil Fry, van Walleghem, Waldroup & Harms (1965) have concluded that 'off' flavours most commonly appear when the fish oil content of the diet reaches 1·5–2·0%, indicating a necessity to keep it below 1·0–1·5%. There have been some reports that the addition of extra tocopherol or of synthetic antioxidants to diets containing fish oil or meal reduces tainting of the flesh of pigs or poultry to a limited degree, but in other instances no apparent improvement has resulted.

Since the physiological and organoleptic consequences of feeding fish oil to poultry presumably stem alike from the transference of some of the characteristic PUFA (with or without modification) from the fish oil to the body tissues, it was of interest to apply the improved techniques now available for lipid analysis to trace this trans-

ference into the storage depot fat and into the lipids of the muscular tissue. The present paper, therefore, compares diets containing anchovy oil at the 2·5% (with and without antioxidant) and 5% levels with a control containing 2·5% beef fat, for their effects on the growth of turkeys, and on the chemical composition, oxidative stability and palatability of the skin fat (mainly triglyceride). In a subsequent paper the detailed composition of the fractionated lipids of the breast and leg muscle will be presented, to establish the extent to which individual PUFA of the fish oil enter individual lipids of the muscle.

EXPERIMENTAL

Thirty-six 1-day-old female turkey poults, of a strain (10·20 Broad Breasted White) of fast-maturing birds specially suited for slaughter at 5–6 lb weight, were obtained from Norfolk Manor Farms Ltd, Great Witchingham Hall, Norwich. For the first 2 weeks the birds were fed on a 'young bird diet' which, with the exception of a slightly higher protein content (26·3% v. 22·1%) was the same as the diet given subsequently to control group A. The birds were then weighed and eight randomized groups of four birds each selected to minimize differences between group weights. Four sets of two groups each were then given diets A, B, C and D respectively for a further 8 weeks. After the 2nd week on the experimental diets, when the birds were seen to be tolerating the diets well, one from each cage was discarded, leaving six birds on each diet. Feeding was *ad lib.* but residual food was removed from the troughs every 24 h and discarded, to minimize autoxidative changes in the lipids.

Diets

Each of the four diets contained 92 parts of a common basal mixture supplying the necessary growth factors in an economically feasible form. To this was added beef fat or fish oil and maize starch or cellulose powder, as indicated below, to adjust the four diets to the same calorific value.

Diet A: (beef fat control) 92 parts basal diet, 2·5 parts beef fat and 5·5 parts maize starch.

Diet B: 92 parts basal diet, 2·5 parts anchovy oil and 5·5 parts maize starch.

Diet C: as diet B, but with the addition of 0·02% EMQ.

Diet D: 92 parts basal diet, 5·0 parts anchovy oil and 3·0 parts cellulose powder (Whatman Standard Grade). The vitamin E content of this diet was raised by doubling the amount of Rovimix E (Roche Products Ltd) added.

Immediately after mixing, all four diets were packed under nitrogen in A 2½ cans, the end seams of which were coated with a low-melting alloy (Wood's metal) to ensure complete gas-tightness. Storage was at −10° until a few hours before opening for use.

Basal mixture. The 92 parts basal diet contributed: ground wheat 28, finely ground barley 28, extracted soya-bean meal 17, dried unextracted yeast 5, casein 8, sterilized steamed bone flour 2 and mineral, vitamin, antibiotic premix (in bone flour) 4. The premix, which was similar to that used in a previous experiment (Lea *et al.* 1968) with the addition of vitamin B_{12} (0·02 ppm of the final diet), contributed to the diet

7700 i.u. vitamin A/kg and 13 i.u. vitamin E/kg as α-tocopheryl acetate (26 i.u./kg for diet D).

Fats used. Beef fat was rendered from fresh beef adipose tissue. The anchovy oil, of Peruvian origin, in solution in light petroleum, was passed through a column of activated silicic acid and alumina to remove autoxidation products and other impurities, followed by distillation and deodorization with steam under reduced pressure to remove the solvent. After removal of a sample for fatty acid analysis by gas chromatography both fats were packed in sealed cans under nitrogen and stored at −80° until required.

Oxidation in the feeding troughs. Spare troughs half filled with diet were held under conditions similar to those in which the birds were feeding, and representative samples were removed daily to test for oxidation. The lipid from each sample was extracted by shaking with chloroform for 0·5 h, after which the peroxide value (pv) was determined on the extracted fat by the method of Lea (1952).

Performance of the birds

Weight increase and food consumption were measured throughout the experiment, and after the birds were killed their livers were weighed and analysed for vitamin A content (Lea, Parr, L'Estrange & Carpenter, 1964).

Analysis of the lipids

Extraction of the fat. Since practically no abdominal fatty tissue had been laid down, the skins were examined as the main fat storage depot. For this purpose the skins of each group were combined, cut up with scissors and mixed, and a representative sample was freeze-dried. The fat was then extracted from the dry tissue with peroxide-free ether, in darkness under nitrogen in an apparatus of the hot extraction type, and the solvent removed at low temperature under reduced pressure.

Conversion to methyl esters and analysis by gas chromatography. The fat (1 g) was refluxed in 400 ml dry 5% (w/v) sulphuric acid–methanol, with the addition of 25 ml benzene to ensure solution, for 4 h under nitrogen. The methyl esters were washed and dried by the method of Bowyer, Leat, Howard & Gresham (1963). After removal of a sample for determination of the iodine value a 10% (w/v) solution of the methyl esters in pure isooctane containing 5 mg % BHT (2,6-di-tert.butyl-4-methyl-phenol) was prepared for gas chromatography.

Hydrogenation. To assist identification of the very complex mixture of fatty acids present in the anchovy oil a sample (0·2 g) of the methyl esters from this oil was hydrogenated in methanol solution for 16 h at 37° with Adam's platinum oxide catalyst (50 mg), and the recovered saturated esters were subjected again to gas chromatographic analysis.

Thin layer chromatography. As a further aid to the identification of individual fatty acids the anchovy oil methyl esters were fractionated according to degree of unsaturation on a 20 × 20 cm 20% silver nitrate–silica gel H plate, using a modification of the method of Kenney, Komanowsky & Wrigley (1965). The sample of methyl esters (80–100 μl of the 10% solution in isooctane) was applied as a narrow band 2 cm from the bottom of the plate, and developed in a constant temperature room at 1

with hexane–benzene (20:80, v/v) containing, per 100 ml, 10 mg BHQ (butylhydroquinone, Neudoerffer & Lea, 1966), which was found to be the most suitable solvent. We preferred to make the separated ester bands visible by spraying with rhodamine–fluorescein (rhodamine 100 mg and dichlorofluorescein 32 mg dissolved in ether 150 ml, ethanol 70 ml and water 17 ml), rather than with water as used by Kenney *et al.* (1965). After elution of the four separated fractions from the support with 3 × 5 ml light petroleum the solvent was distilled off and the residue redissolved in 0·2 ml isooctane containing 5 mg BHQ/100 ml for gas chromatographic analysis.

Gas chromatographic analysis. Analysis of the fatty acid methyl esters was carried out on a Pye Series 104, model 24 (dual column) flame ionization gas chromatograph, but only one of the columns was used. The 5 ft glass column (purchased from W. G. Pye and Co. Ltd, Cambridge) was packed with 100–120 mesh Celite, carrying 10% of polyethylene glycol adipate (PEGA) as liquid phase, and the temperature was programmed at 4°/min from injection at 150° to 200°, after which the analysis was completed isothermally at 200°. The detector temperature was held at 220° throughout, in a specially constructed compartment, to maintain uniformity of response. Argon at a flow rate of 40 ml/min was used as carrier gas and a 1:1 mixture of hydrogen and nitrogen supplied the flame. The voltage applied to the jet of the flame detector was increased from 45 to 90 V, to extend its linear dynamic range. With a sample injection of 1·5 μl of a 10% solution of the esters in isooctane the attenuation of the amplifier sensitivity was varied between 2000 and 50000 times (i.e. full-scale deflection varied from 2×10^{-9} to 5×10^{-8} A) and the chart speed was usually 30 in/h.

For calibration, mixtures of methyl myristate, palmitate, stearate, oleate, linoleate, linolenate, arachidonate, eicosapentaenoate and docosahexaenoate (obtained from the Hormel Foundation, Austin, Minn. and the National Institutes of Health, Bethesda 14, Md) were prepared and assayed repeatedly. The last three of these esters were less pure (about 95%) than the others (> 99%) and were themselves assayed by gas–liquid chromatography (GLC), so that appropriate corrections could be made to the compositions of the mixtures containing them.

Tocopherol determination. Tocopherol (presumed to be mainly α- because of less efficient absorption and deposition of the other homologues) was determined on portions of the ether extracts of the skin by a slightly modified version of the method of Erickson & Dunkley (1964) using 2,7-diphenyl-1,10-phenanthroline. Replacement of the 0·1 M-orthophosphate specified by a 0·2 M solution was found to give more complete protection against photoreduction of residual ferric iron, and elution with 20 ml solvent, in place of the 10 ml suggested, was found to be necessary to achieve complete recovery of added tocopherol. A standard reference curve was constructed for hexane:benzene (2:3, v/v) solutions of pure DL-α-tocopherol.

Stability of the depot fat. For comparison of the stabilities of the skin fats towards autoxidation 0·2 ml portions were pipetted into a series of small glass cups and held at 60°, with determination of the peroxide value (pv) at suitable intervals, as previously described (Lea, 1960), but omitting the peroxide 'starter'.

RESULTS

Oxidation in the feeding troughs. The rates of peroxidation of the lipids of the four diets when stored in the feeding troughs under the conditions of the 1st week of the experiment (27° and bright fluorescent lighting) are shown in Fig. 1. Thereafter the temperature at the feeding troughs (which were remote from the infrared heating lamps) was reduced to 20°, from which it fell gradually to 15° at the conclusion of the experiment. The illumination was also much less bright than during the 1st week.

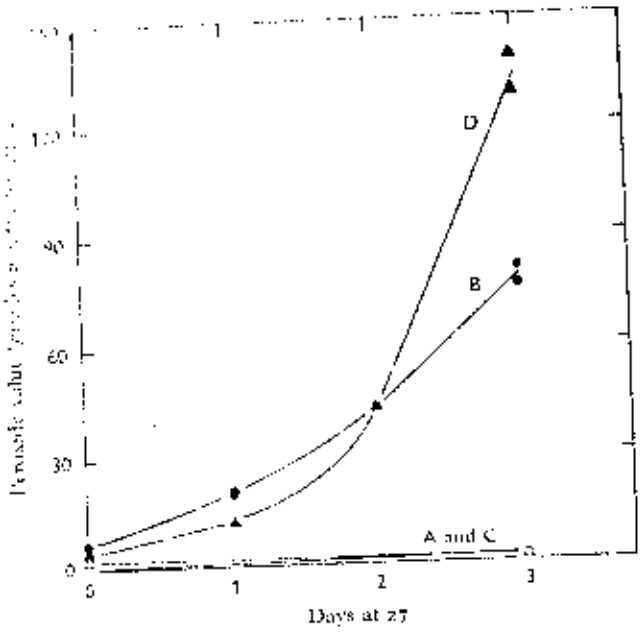

Fig. 1. Peroxidation of dietary lipids in the feeding troughs of turkeys. Diet A, 2·5% beef fat; diet B, 2·5% anchovy oil; diet C, 2·5% anchovy oil + EMQ; diet D, 5·0% anchovy oil.

Table 1. *Response of the turkeys to the experimental diets containing different fats*

Diet	Live-wt gain 2–10 weeks (g/poult)	Food conversion ratio (food consumed/wt gained)	Liver wt (as % body-wt)	Vitamin A reserves (i.u./liver)
A (2·5% beef fat, control)	2516	2·38	1·49	7060
B (2·5% anchovy oil)	2574	2·43	1·50	5680
C (as B, + 0·02% EMQ)	2564	2·41	1·50	7280
D (5·0% anchovy oil)	2552	2·58	1·59	5530
Standard error of treatment means*	±38	±0·041	±0·055	±248

* In the analysis of variance the food conversion and the vitamin A content of the livers were significantly affected by treatment. In the former, diet D was just significantly higher than the other three ($P < 0·05$); in the latter, diets A and C were higher than diets B and D ($P < 0·05$).

On both these counts, therefore, peroxidation of the lipids of diets B and D during the exposure of less than 24 h in the trough would have been considerably slower during most of the experimental period than is shown in Fig. 1.

Table 2. *Composition of the skin lipids of the turkeys on diets containing different fats*

Diet	Lipid content of the skin (%)	Tocopherol content of the lipid (μg/g)
A (2·5% beef fat, control)	18·4	22
B (2·5% anchovy oil)	19·2	21
C (as B, +0·02% EMQ)	19·8	38
D (5·0% anchovy oil)	16·8	31

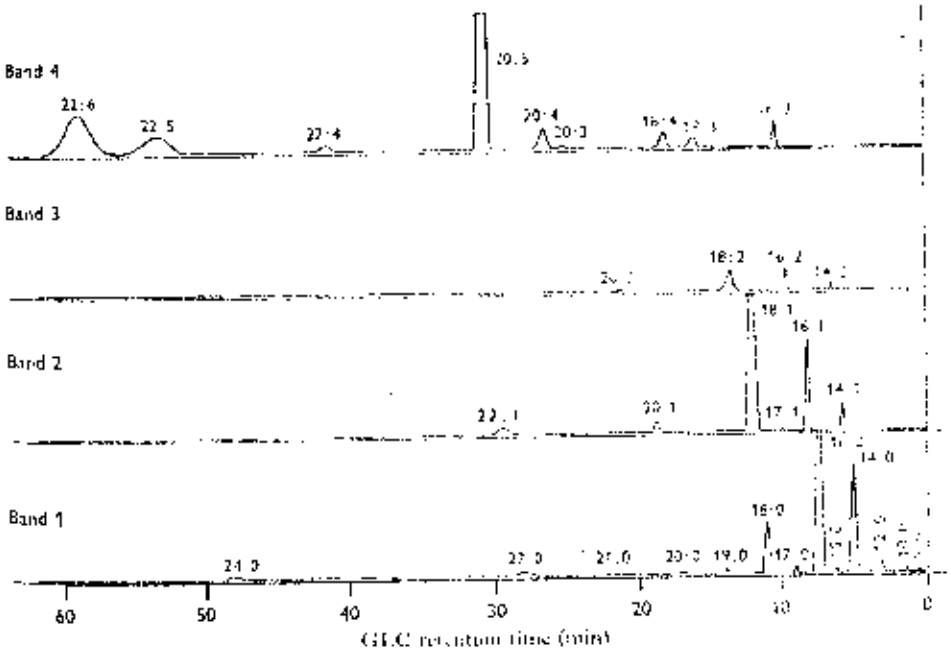

Fig. 2. Fractionation of anchovy oil methyl esters according to unsaturation on a silver nitrate–silica gel thin-layer plate at 1°.

Performance of the birds. Growth and apparent health were normal on all four diets (Table 1). There did, however, appear to be a slightly inferior food conversion ratio ($P < 0·05$) and perhaps a slightly higher liver weight ($P = 0·05$) when 5% anchovy oil was given, as compared with values on the other three diets. Replacement of beef fat by anchovy oil slightly depressed storage of vitamin A in the liver, as previously observed (Lea *et al.* 1966), but diet D (5% anchovy oil), which also contained extra vitamin E, was not appreciably worse in this respect than diet B (2·5% anchovy oil). Vitamin E is known to conserve or protect vitamin A. Addition of EMQ with 2·5%

anchovy oil (diet C) restored the liver vitamin A to the level on the control (2·5% fat) diet A.

Analysis of the lipids. The fat contents of the skins of all the birds were rather low (Table 2), but negligible values for lipid phosphorus showed that the extracted lipid was virtually all neutral fat. Whereas fish oil may have marginally reduced the tocopherol content of the depot fat (diets B and D), EMQ (diet C) apparently raised it considerably. With the method of tocopherol estimation used, a recovery of 96% of added α-tocopherol was obtained and no interference by carotenoid with the determination was experienced.

Table 3. *Results of analysis of the anchovy oil methyl esters after hydrogenation*

	Hydrogenated esters	
Fatty acid	Found	Calculated from determined composition of the unhydrogenated esters (Table 4)
14:0	0·1	Trace
16:0	8·0	8·0
17:0	0·5	0·4
18:0	28·0	27·6
19:0	1·1	1·2
18:0	21·2	21·2
19:0	0·5	Trace
20:0	27·8	27·8
21:0	0·5	Trace
22:0	12·1	12·2
24:0	0·2	Trace
	Total 100·0	Total 98·4

Thin layer chromatography of the methyl esters of anchovy oil separated them cleanly into four fractions (Fig. 2), the fastest moving band (no. 1) containing the saturated components, followed by bands 2 and 3 of monoenes and dienes respectively, leaving polyenes in a fourth band near to the origin. To obtain sharp separation of dienes from polyenes it was essential to avoid overloading the plate.

Hydrogenation of the methyl esters of anchovy oil reduced their iodine value from 196·2 to 1·2 and greatly simplified the gas chromatographic pattern. Comparison of the analysis of the hydrogenated esters with that expected from the direct analysis of the unhydrogenated esters (using in the latter, identification facilitated by the separation achieved on the thin layer silver nitrate plate) gave satisfactory agreement (Table 3). Further confirmation of the validity of the identifications used was afforded by good agreement between the iodine value calculated for the fatty acid composition found (194·8) and that of the original ester mixture analysed (196·2).

The fatty acid compositions of the basal diet, of the fats added to it, and of the skin fats of the four groups of turkeys are summarized in Table 4. For ease of reference, the acids have been arranged in three groups according to whether they were major (> 1%), minor (1·0–0·1%) or very minor (< 0·1%) constituents.

Stability and flavour of the skin fats. The fats extracted from the skins of the birds

on diets B and D, despite precautions against autoxidation during handling and extraction, already showed small peroxide values (3·2 and 2·2 μmoles/g respectively), whereas those from the birds on diets A and C were zero. The colour of the extracted fats also increased progressively from A (white to cream) through C (definitely yellow) and B (deeper yellow) to D (very yellow). On incubation at 60° the fats developed peroxide in the same order (Fig. 3), the beef fat control diet (A) giving the most and the 5% anchovy oil diet (D) the least stable fat.

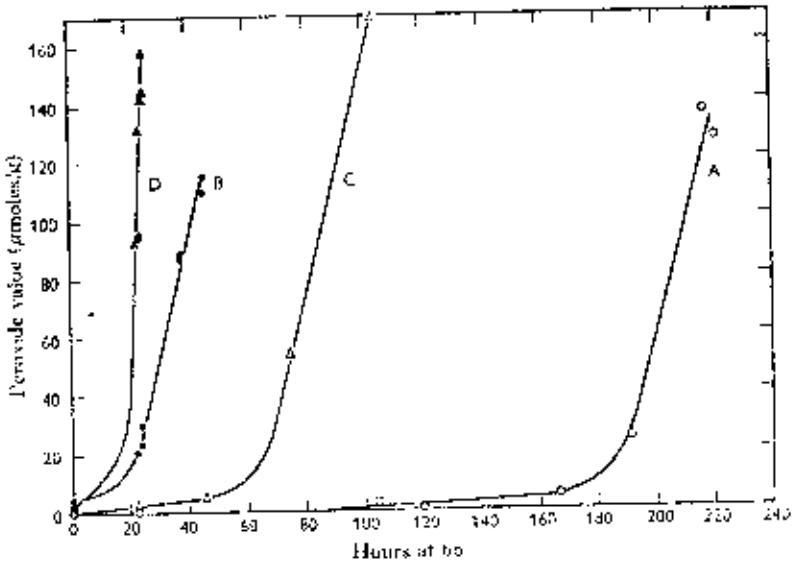

Fig. 3. Relative rates of autoxidation at 60° of the skin fats of turkeys on diets with different fats. Diet A, 2·5% beef fat; diet B, 2·5% anchovy oil; diet C, 2·5% anchovy oil + EMQ; diet D 5·0% anchovy oil.

Taste panel results on the roasted breast and leg meats from this experiment will be given in Part 2, together with the composition of the muscle lipids to which they directly relate. Full taste panel tests could not be done on the skin tissue alone, through lack of material, but it was obvious that fishy taints were present in all the groups given fish oil, with D certainly the most tainted and C probably less tainted than B.

DISCUSSION

Oxidation of the dietary lipids. Peroxidation of unstable lipids such as fish oils during the storage and feeding of experimental diets containing them can invalidate the results obtained unless a stabilizing antioxidant is also present, and this is not always practicable in view of possible nutritional effects. In the experiments of Rasheed, Oldfield, Kaufmes & Sinnhuber (1963), in which 15% fresh clay-bleached menhaden oil was given to rats in a semi-synthetic basal diet, toxic signs including anorexia, steatitis and death were attributed to rapid and extensive oxidation of the oil during 24 h exposure of the mixed diet before consumption. The same authors also

quote an experiment in which menhaden oil mixed in unspecified proportion with a synthetic diet increased in thiobarbituric acid (TBA) value from 32 to 1220 in 24 h at room temperature. On the other hand, we have previously found (Lea *et al.* 1966) that 1·5% anchovy oil incorporated into a normal type of cereal-based diet for feeding to turkeys did not oxidize appreciably in 24 h at 32°. The experiment now reported, in which 2·5% or 5·0% anchovy oil was included in a similar diet, represents an intermediate example, in that peroxidation of the oil during 24 h exposure at 27° was readily detectable (Fig. 1), but was too small to be nutritionally serious. Probable reasons for the diversity of these results are (*a*) the smaller proportions of oil used in our experiments, and (*b*) the protective action of natural antioxidants present in the cereal, soya and yeast constituents of the normal diet. Some of the nutritional effects of fish oils reported in the earlier literature may have been due rather to peroxidized fish oils.

Performance of the turkeys. The turkeys tolerated 5% of anchovy oil surprisingly well; Table 1; a result, however, in general agreement with the recent observation of Lewis, Knobl, Snyder & Grager (1964) that at 5% of the diet menhaden oil was equal to maize oil in feeding value for broiler chickens. Our result was, no doubt, influenced by the presence in the diet of 26 i.u./kg of added stabilized α-tocopheryl acetate and Leong *et al.*'s by the presence of 0·0166% EMQ.

Gas chromatographic analysis of the lipids. GLC provides a rapid means of separating and tentatively identifying the components of mixtures of fatty acid esters, but with so complex a mixture as a fish oil some supplementary aid is advisable to assist differentiation between close-running pairs such as the esters of 17:0 and 16:2, 18:4 and 22:1, and 20:5 and 22:1, where the opposing effects of increasing chain length and unsaturation can make identification difficult. For this purpose a preliminary fractionation of the ester mixture on a thin layer silica silver nitrate plate, and an additional GLC analysis of the fully hydrogenated ester mixture were both found useful.

Quantitative operation can present more difficulty. Though a majority of users of the GLC technique have observed (or, more usually, assumed) proportionality between peak area and weight percentage (Horning, Ahrens, Lipsky, Mattson, Mead, Turner & Goldwater, 1964), some authors have found better agreement with mole percentage (Grager, Nelson & Stansby, 1964), and the difference between these two methods of calculation can be large for a fish oil containing major components so far apart in molecular weight as C14 and C24. Still others (Kilgore & Luker, 1964; Pons & Brampton, 1965) have found it necessary to use a different conversion factor for each individual fatty ester. Because of these conflicting reports it has become fairly common practice to test the performance of the instrument and the conditions used on test mixtures of known composition, but these have often consisted of saturated esters only, or of saturated plus unsaturated esters up to linolenate (18:3). Only very rarely have esters of the 20:4, 20:5 and 22:6 acids been included, though these late-running components are the most likely to suffer loss under the conditions of high temperature or long time required to elute them. Gerson, McIntosh & Shorland (1964) reported losses of 51% and 86% during separation of the methyl esters of the C_{20} and C_{22} acids of shark-liver oil. In repeated runs with known mixtures con-

taining these highly unsaturated esters we found no evidence of any systematic loss or of deviation from proportionality between chart area and weight percentage, and no correction factors have been applied to any of the results reported in this paper.

Effect of dietary lipids on the composition of the skin fats. Replacement of 2·5% beef fat in the diet by the same amount of anchovy oil raised the iodine value of the skin

Table 4. *Fatty acid composition of the dietary fats, and of the skin fats of the four groups of turkeys receiving diets (A–D) with different fats*

Carbon number	Probable identity	Basal diet* IV 105	Beef fat† IV 47	Anchovy oil‡ IV 196	Skin fats A IV 68	B IV 90	C IV 94	D IV 133
			Major constituents (> 1%)					
14·00	14:0	1·1	3·5	7·8	1·7	4·5	4·3	5·9
16·00	16:0	22·4	24·0	14·0	25·0	26·3	27·0	22·4
16·49	16:1	1·8	4·7	9·2	7·0	6·7	6·6	7·9
17·20	16:2		—	2·1	—	0·7	0·7	1·1
17·70	16:3		—	2·0	—	0·7	0·6	1·1
16·95	17:0	Tr	1·4	1·2	0·7	1·0	1·0	1·0
18·00	18:0	6·7	18·9	2·2	10·3	8·7	8·9	6·1
18·40	18:1	19·3	41·0	17·3	37·4	20·8	21·4	19·7
18·95	18:2	43·2	8·9	6·2	13·9	15·8	15·5	15·9
19·80	18:3	4·7	0·4	0·4	1·3	1·5	1·7	1·0
20·30	18:4	—	—	3·0		0·8	0·7	1·5
20·35	20:1	Tr	Tr	2·1	0·5	1·2	1·2	1·5
21·75	20:4	—	—	2·2	—	0·5	0·5	0·7
24·48	20:5	—	—	23·4		5·9	4·6	12·0
24·55	22:5	—	—	3·0		2·2	2·2	2·6
24·85	22:6	—		7·5		1·9	1·7	3·9
			Minor constituents (1·0–0·1%)					
14·55	14:1	—	1·0	0·2	0·5	0·4	0·3	0·4
15·09	15:0	—	0·7	0·4	0·3	0·5	0·4	0·5
17·35	17:1, 18 br		0·8	—	0·4	—		—
20·00	20:0	Tr	Tr	Tr	0·5	0·4	0·6	0·6
22·20	22:1	—	—	0·1	—	0·1	0·1	0·1
23·35	22:4	—	—	1·0		Tr	Tr	Tr
			Very minor constituents (< 0·1%)					
10·00	10:0	—	Tr	Tr	Tr	Tr	Tr	Tr
12·00	12:0	—	Tr	Tr	Tr	Tr	Tr	Tr
14·75	15 br	—	Tr	NS	NS	NS	NS	NS
15·60	14:2, 16 br	—	0·4	Tr	0·1	Tr	Tr	Tr
16·80	17 br	—	0·8	NS	Tr	NS	NS	NS
19·02	19:0	—	Tr	Tr	Tr	Tr	Tr	Tr
19·50	20 br	—	Tr	Tr	Tr	Tr	Tr	Tr
19·65	19:1	—		Tr	Tr	Tr	Tr	Tr
20·90	20:2		—	Tr		Tr	Tr	Tr
20·95	21:0		—	Tr	Tr	Tr	Tr	Tr
21·30	20:3			Tr	—	Tr	Tr	Tr
22·00	22:0	—		Tr	Tr	Tr	Tr	Tr
24·00	24:0	—		Tr		Tr	Tr	Tr

Iodine values (IV) are the means of observed and calculated, rounded to the nearest unit. Tr = < 0·1%; NS (no significant amount) = < 0·1%; — = absent.

* Contributes 2·3% lipid to all diets.

† Contributes 2·5% to diet A. Also contains traces of 14 br and 19 br acids.

‡ Contributes 2·5% to diets B and C, 5·0% to diet D.

fat by about 30 units, and doubling the amount of anchovy oil raised it by another 20 units (Table 4).

Linoleic acid at the basal diet, 2·3%, in all four diets, was very much richer in linoleate than either the beef fat or the anchovy oil, and was presumably the main source of this acid in the skin fats; its concentration, therefore, did not vary very much. The beef fat contained more branched chain saturated acids, all in small amount, than did the anchovy oil; some of these branched chain acids were detectable also in the skin fat of the birds from group A. The anchovy oil contained seven major and two minor acids which were not present in either of the other dietary fats, and all of these were also present in the skin fats of groups B, C and D, which had received fish oil, but were absent from the skin fats of group A, which had not. One major acid (20:1), present at 2·1% in the anchovy oil and at the trace level only in the other dietary fats, was also present at much higher levels in the skin fats of the groups given fish oil.

Table 5. *Transference of individual fatty acids from dietary fish oil to the skin lipid of turkeys receiving diets B, C and D*

Acid	Concentration in anchovy oil (%)	Concentration in skin fat: No. of times level in diet B	C	D	As % of level in dietary lipid* B	C	D
16:1	2·1	13·3	13·3	10·3	64	64	76
18:1	2·0	14·0	12·0	11·0	67	58	80
18:4	3·0	10·7	9·1	8·7	51	45	63
20:1†	2·1	13·3	13·3	9·5	64	64	69
20:4	2·2	9·2	9·2	6·4	44	44	46
20:5	23·4	10·1	8·4	8·5	48	40	62
22:5	3·0	19·4	20·4	17·3	141	143	126
22:6	7·3	10·4	9·3	8·2	50	45	60
Mean (excluding 20:1)		11·6	10·7	9·0	55	51	65

* Total dietary lipid = 4·8% for diets B and C, 7·3% for diet D.
† Present also in skin fat of group A and calculations therefore based on increases.

The efficiencies with which the eight major fatty acids of fish oil were transferred from the dietary to the skin lipid followed a generally similar pattern, except for acid 22:5. When the fish oil level was 2·5% (diets B and C) the other seven acids were deposited in the skin fats at a level about eleven times greater than their concentrations in the whole diet, or approximately half their concentrations in the dietary lipid. When the fish oil content of the diet was raised to 5·0% (diet D) the corresponding figures were approximately nine times and two-thirds (Table 5). Acid 22:5, however, accumulated between two and three times as efficiently with diets B and C and nearly twice as efficiently with diet D. This could be the result of a more efficient deposition or less efficient mobilization of this particular acid, but it could, alternatively, be due to chain lengthening of a small proportion of the 20:5 acid, of which the anchovy oil contained as much as 23·4%, before deposition.

Marion & Woodroof (1962) gave 1% of menhaden oil to chickens and found approximately 1% of the 20:5 acid in the skin fat, but no acids of longer chain length were

detected, though they were present in the lipids of the muscle. Reed (1964) found that deposition of PUFA in the depot fat of rats did not follow the proportions in which they were present in the cod-liver oil given; though the 20:5 and 22:6 acids were present in the cod-liver oil in a ratio of nearly 2:1 the depot fat of rats receiving the oil showed elevated contents of hexaene but not of pentaene acids. The method (alkali isomerization) used for analysis of the depot fats gave fatty acid composition according to degree of unsaturation, but not according to chain length. In our experiment with turkeys there was no difference between the behaviour of the 20:5 and 22:6 acids.

Effect of diet on fat stability and flavour. The reason for the greatly reduced stability of the skin fats of the birds given fish oil as compared with the control group given beef fat (Fig. 3) can be seen from their compositions. The fats of groups A, B and D all contained approximately the same amount of tocopherol (Table 2) and the large differences in their stability therefore derived, presumably, from the higher levels of unsaturation in the groups given fish oil. The total concentrations of fatty acids with three or more double bonds in groups B, C and D were respectively 13·5, 12·3, and 20·3%, mainly pentaene and hexaene, whereas in group A the polyene level was only 1·5, all linolenate (18:3). The considerably greater stability of skin fat C as compared with B is not likely to have been due to its only slightly lower PUFA content, but was more probably due to a sparing of tocopherol by the antioxidant (EMQ) in this diet, which resulted in a tocopherol level nearly twice as high as in the other skin fats. Whether traces of EMQ itself might have been deposited in the skin fat of group C could not be determined with the methods of estimation and limited amounts of material available. The extra tocopherol in diet D did not result in increased deposition in the depot fat, presumably owing to a destructive effect of the high level of fish oil present in this diet.

Even dietary linolenic acid in sufficient quantity can cause 'off' flavours described as 'fishy' in roast turkey, as shown by Klose, Mecchi, Hanson & Lineweaver (1951) who gave 5% of linseed oil, but fishy flavours resulting from dietary additions of marine animal oils probably arise mainly from oxidative degradation of fatty acids still more highly unsaturated. The skin, because of its high fat content and maximum exposure to high temperature and oxygen during cooking, is particularly susceptible to the development of 'off' flavours of this kind.

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Hydrogenated Marine Fat, Effect on Growth and Liver Weight in Young Rats given a High Fat, Low Protein Ration

L. R. NJAA, O. R. BRÆKKAN, G. LAMBERTSEN and F. UTNE

Government Vitamin Laboratory, Norwegian Fisheries Research Institute, Bergen

Abstract. Hydrogenated marine fat and some other commercial fats were fed to young rats in a low protein diet at a level of about 50% of the calories. The diet was marginal in choline, α-tocopherol and linoleic acid. The experimental procedure involved littermate control and equalized feeding within litters.

Rats given hydrogenated marine fat, as well as those given hydrogenated soya bean oil and hydrogenated rapeseed oil showed less weight gain than rats given lard and herring oil. When the conditions were not complicated by the occurrence of fatty livers, the rats given the hydrogenated fats showed a higher liver weight: final body weight ratio than the rats given the natural fats.

Supplementing the hydrogenated marine fat with 2.5% sunflower seed oil improved its caloric efficiency.

Additions of α-tocopherol were without any effect.

There was a high degree of correlation between the growth efficiency of calories above basal needs and the liver weight: final body weight ratio.

Key Words
Young rat
Hydrogenated marine fat
High fat diet
Growth
Liver weight

Hydrogenated marine fat (HMF) is used in the production of margarine. Few studies report on the nutritive value of HMF. BRÆKKAN *et al.* [1968] fed a moderate level of HMF (m.p. 32°C) to young rats on a semisynthetic diet. The rats given HMF grew slightly slower than the control rats given a mixture of lard and olive oil (9:1) of the same melting point (m.p.). UTNE *et al.* [1966] gave graded amounts of 2 types of HMF to growing mink. The melting points were 44 and 33°C, and lard (m.p. 38°C) was used as a control. Growth was best on the lower melting HMF, followed by lard and the higher melting HMF. The content of fat and fatty acids in the faeces was highest in the higher melting HMF, followed by the lower melting HMF and lard. LAKSESVELA [1966] found in experiments with chicks that hydrogenation of herring oil to give HMF's with melting points ranging from 31 to 50°C

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reduced the digestibility of the fat progressively. The degree of hydrogenation had, however, little effect on the growth of the chicks. HERSTAD [1970] used 2 types of HMF (melting points 43 and 33°C) as supplements to broiler feeds. He found that the feed utilization was best with the lower melting type and that soya bean oil was slightly better utilized than HMF. AAKS-JØRGENSEN and HOLMER [1969] gave partly-hydrogenated herring oil and similarly hydrogenated arachis oil and soya bean oil as the sole dietary fat to young rats and studied the development of essential fatty acid deficiency and the changes in the fatty acid composition of lipids from the testes. Essential fatty acid deficiency and lipid changes in the testes developed on all the hydrogenated fats, but the effects were most profound with partly-hydrogenated herring oil.

We now report growth studies with young rats given HMF and some other fats in a high fat, low protein diet which was also low in choline. This type of diet, which was also used by PLATT [1959], was chosen because it is reported to be suitable for detection of differences between different types of fat. LUCAS and RIDOUT [1967] make the following statement on this point: 'BENTON *et al.* point out that the difference in the effect of different fats can be shown only when the diet is deficient in either protein or choline and that under normal conditions (adequate diet) the type of fat has no effect on the deposition of liver lipids. We agree with this and also with DAMODARAN and SIVA RAMAN that the response to fats fed with an unbalanced diet gives no indication of the nutritive value of the fat when fed in a normal ration.'

Seven experiments were performed over 2 years using different batches of commercial available HMF. Each experiment lasted 4 weeks and the 4-week weight gain was used as a criterion for fat utilization, further, littermate control and equalized feeding within litters were applied. The ratio between liver weight and final body weight was also recorded because results with oxidized fats [JOHNSON *et al.*, 1957], laboratory heated fats [POLING et al., 1960, POTTEAU *et al.*, 1970] and polymerized marine oils (own unpublished results) have shown that this ratio may be an indication of some unknown changes in fat metabolism.

Materials and Methods

Fats. The fats used were commercially available, HMF, hydrogenated soya bean oil (HSO), and 85:15 mixture of hydrogenated rape seed oil and tallow (R+T), a 1:1 mixture of coconut oil and tallow (C+T), a herring oil (HO) and lard. In the tables the fats are

Table I. Some analytical data on the fats used

	Lard	HO	Hydrogenated fats		
			HMF	HSO	R+T
Melting point	34–40	–	36–40	40.5	36
Percent trans-bonds (in sum of fatty acids)	0	0	45	50	38
Percent 18:2-acids (incl. isomers in hydrogenated fats)	7.5–8.5	1.1	1.5–2.0	0.8	–
Percent C_{20}-acids (saturated and monoenes)	1.5	15.8	13–18	0	8.0
Percent C_{22}-acids (saturated and monoenes)	0.1	22.4	15–20	0	38.4

identified by the codes given in parentheses. Some information on these fats are given in table I.

Lard was used as a standard of reference and the melting points of the other fats, except that of herring oil, were adjusted near to that of lard. All fats except lard contained little linoleic acid, and the hydrogenated fats contained 38–50% of trans double bonds, calculated on the basis of the total fatty acids. As the experiments were done over 2 years, several samples of HMF and lard were used, and these were not analyzed individually for their fatty acid composition. The type of HMF used has been analyzed for its fatty acid composition [Lambertsen *et al.*, 1966] and for its isomer content [Lambertsen, 1971]. HMF had fatty acids of chain lengths from 14 to 24 carbon atoms and about 35% of saturated and monoene C_{20} and C_{22} acids. 75% of the monoenoic acids had trans configuration at their double bonds, and the positions of the double bonds showed a statistically normal distribution around a maximum at the middle of the chain. This isomer composition was interpreted to indicate that the fatty acids had been isomerized to an equilibrium state in the hydrogenation process.

The mixture of coconut oil and tallow, which was not analyzed for its fatty acid composition, was included as a fat having a wide range of fatty acid chain lengths (C_{10}–C_{18}) compared to lard with 65% C_{16}-acids and to hydrogenated soya bean oil with 90% C_{18}-acids. Herring oil, a raw material for HMF, does not contain 'unnatural' isomers and has less saturated acids and more highly-unsaturated acids than HMF. The fatty acids of herring oil have been analyzed by Lambertsen and Brækkan [1965]. The batches of lard used were of Danish origin supplied in 250 g packages.

Samples of HMF from different oil refineries are identified by a, b, and c. The numbers 1, 2.5, 4, 5 and 10 given together with the fat codes indicate percentage additions of sunflower seed oil (containing 60% linoleic acid) to the experimental fats. 5 r designates addition of 5% sunflower seed oil stripped of α-tocopherol by molecular destillation. The additions of α-tocopherol are indicated by α-toc_1, α-toc_2 and α-toc_3 which refer respectively to 0.07 and 0.032% α-tocopherylacetate, and to 0.031% saponified α-tocopherylhydrogen succinate in the diet.

Diets. The fats were tested at the 28-percent level (corresponding to about 50% of the calories) in a diet which further consisted of 44% partly dextrinized potato starch, 9% sucrose, 10% spray dried egg albumine, 4% salt mixture [Sure, 1941] and 5% oat hulls. The vitamin supplements given in 1% starch, were per 10 g diet: Thiamine, riboflavine and pyridoxine 50 μg, vitamin B_{12} 0.1 μg, d-biotin 1 μg, folic acid 10 μg, d-calcium pantothenate 150 μg, niacin 250 μg, *p*-aminobenzoic acid 500 μg, i-inositol 2.5 mg, cholin chloride 5 mg, menadion 25 μg. Vitamin A and D were given by dropper 2 times weekly or mixed into the diet in amounts corresponding to 200 IU vitamin A and 20 IU vitamin D per rat per week.

Experimental procedure. Each experiment comprised 4 treatment groups of 6 rats except experiment G which comprised 5 groups of 5 rats. The rats were 4–5 weeks old at the start of the experiment. 6 (or 5) litters of 4 (or 5) rats of the same sex and of similar body weight within litters were allotted to the treatment groups so that each litter was represented in all treatment groups. The rats were kept in separate cages with wire bottoms, suspended over trays covered with filter paper. The urine was absorbed by the paper, thus the separation of spilled food and faeces was facilitated. The diets were mixed weekly and stored at 2–5°C. In all experiments except in experiment F and G the rats were put directly on to the experimental diets, in experiments F and G all rats were given the lard diet for 1 week before the experiment started. The appetite factor was eliminated by keeping the food intake constant within each litter throughout the experiment, including the preliminary periods in experiments F and G. The rat with the lowest food intake determined the amount offered to the other rats within the litter. Spillings were returned to the feeding cup several times daily and by careful supervision the littermates were induced to eat the same amount of food during the 4-week feeding period. The rats were weighed weekly. In 2 experiments (A and B) faeces were collected from each rat during the last 2 weeks, air dried and weighed. After 4 weeks on the experimental diets the rats were weighed and killed, the livers were removed and weighed.

Analytical methods. Fat in the livers was determined by ethyl ether extraction in a Soxhlet apparatus of individual livers (experiment A) or of samples of pooled livers (experiment B) after grinding with anhydrous sodium sulphate. Fat in faeces was determined by ethyl ether extraction of samples of dry faeces without and with previous treatment of the samples with 18 N sulphuric acid. The extraction procedure was described by Soutar *et al.* [1958]. The amount of fat extracted without previous acid treatment was termed free fat, the amount extracted after acid treatment was termed free plus bound fat. The fat fractions used to determine the fatty acid chain length composition of faeces fat were obtained by first extracting extensively with ethyl ether, then the faeces residue was acidified with hydrochloric acid and the bound fat was extracted by continued use of ethyl ether. From these fractions methyl esters were prepared, these were completely hydrogenated and analyzed by GLC.

The fatty acid composition of some of the fats used have been analyzed at intervals, partly in connection with other experiments. Details of the analytical procedures including GLC were described by Lambertsen *et al.* [1966]. The percentage of trans double bonds was determined by IR-spectrophotometry of the total fatty acid methyl esters [Lambertsen, 1971].

The trans-values given, therefore, refer to the sum of the saturated, monoenoic and polyenoic fatty acids in the fat (table I).

Protein (N × 6.25) was determined by a micro Kjeldahl technique.

Dry matter was determined by drying in an oven at 105°C for 2 h.

Ash was determined by heating in an oven at 800°C for 4 h.

Calcium was determined in the ash by oxalate precipitation and titration with potassium permanganate.

Details of the experiments

Experiment A. HMF was compared with 2 natural fats, lard and a mixture of coconut oil and tallow (1:1) and further with hydrogenated soya bean oil.

Vitamins A and D were given by dropper 2 times weekly in 50 mg sunflower seed oil which supplied about 5 μg α-tocopherol and 9 mg linoleic acid per rat per day. Individual livers were analysed for fat. Faeces collected from each rat during the last 2 weeks of the experiment were weighed air dry and analysed for free and free plus bound fat, and for nitrogen.

Experiment B. 3 samples of HMF from different oil refineries were compared with lard. One package of lard was used each week, the melting point varied from package to package between 34 and 40°C. Vitamin A and D were supplied as in experiment A. Fat, protein and ash were determined in the pooled livers from each group. Faeces collected from each rat as in experiment A were analysed for free and free plus bound fat, ash and calcium.

Experiment C. HMF was compared with the hydrogenated soya bean oil as in experiment A and with hydrogenated rapeseed oil to which had been added 15% tallow to raise the melting point to 36°C. Sunflower seed oil containing fat soluble vitamins was either given by dropper as in experiments A and B or for comparison mixed with HMF.

Experiment D. HMF containing 1% sunflower seed oil was compared with HMF containing 4% sunflower seed oil and extra α-tocopherol, with lard and with herring oil.

Experiment E. HMF without added sunflower seed oil was compared with HMF with added α-tocopherol and with HMF with 5% added sunflower seed oil which had been stripped of α-tocopherol by molecular distillation. Lard was included as a standard of reference. Vitamins A and D were given by dropper as in experiments A–C, except that arachis oil was used as vehicle instead of sunflower seed oil to reduce the intake of α-tocopherol and linoleic acid.

Experiment F. HMF was compared with HMF containing 5% added sunflower seed oil, with HMF containing 5% sunflower seed oil stripped of α-tocopherol and with HMF to which was added α-tocopherol corresponding to the concentration in the original sunflower seed oil. Vitamins A and D were mixed into the experimental fats.

Experiment G. HMF containing graded amounts of sunflower seed oil (0, 2.5, 5 and 10%) was compared with lard. Vitamins A and D were given as in experiment F.

Statistical treatment of the results. 4-week weight gains, liver weight: final body weight ratio and values of liver and faeces components determined on individual rats were treated in analyses of variance and the significance of the treatment effect was ascertained. When significant treatment effects were found, the treatment sum of squares was subdivided into comparisons with one degree of freedom as described by COCHRAN and COX [1950]. Of

these comparisons only the significant ones are given in the tables, the significance levels being indicated by 3–1 asterisks corresponding to the levels 0.001, 0.01 and 0.05, respectively. For instance: In experiment A (table II) the following comparisons were made between weight gains: HMF (2) against the mean of the 3 other groups $(\frac{1+3+4}{3})$, hydrogenated soya bean oil (3) against the mean of the 2 remaining groups $(\frac{1+4}{2})$, and finally lard (1) against the mixture of coconut oil and tallow (4). Only the 2 first comparisons were significant and are indicated in the table, 2 vs. $\frac{1+3+4}{3}$ is given on the same line as HMF with 3 asterisks, indicating that this comparison is very highly significant.

An attempt was made to correlate the liver weight: final body weight ratio with the efficiency of caloric utilization for growth. As the starting weight and the food intakes were not the same in all experiments this was taken into account by calculating the efficiencies of the calories consumed above the basal requirement. In the variable thus calculated: $(C-C_0)/\triangle W$, C is the calorie intake obtained by multiplying the food intake by the calorie content of the diet (4.8 kcal/g), C_0 is the amount of calories required for basal metabolism obtained from the formula $C_0 = 70$ kcal $W^{0.73}$, 28, where W is the mean body weight in kg and 28 is the length of the experimental period in days [MITCHELL and PAYNE, 1964]. $\triangle W$ is the 4-week weight gain in g.

Results

The 4-week weight gains and the liver weight: final body weight ratios are summarized in table II. The results in experiment A showed less weight gain in the group given HMF than in the other groups, further the group on hydrogenated soya bean oil grew less than the rats given the 2 natural fats. The liver weight: final body weight ratio increased with decreasing weight gain. These findings were substantiated when 3 different samples of HMF were compared with lard in experiment B and when HMF was compared with hydrogenated soya bean oil and hydrogenated rapeseed oil in experiment C. There was no significant difference between the 3 samples of HMF (experiment B). Hydrogenated soya bean oil and hydrogenated rapeseed oil gave similar weight gains, but the liver weight: final body weight ratio was significantly lower for the group given hydrogenated soya bean oil than for the 3 other groups (experiment C). There was no significant effect of giving the sunflower seed oil containing vitamins A and D in the diet instead of by dropper.

In experiment D was tested whether the effects found for HMF were inherent in unhydrogenated herring oil, and further whether these effects were due to low intakes of linoleic acid and α-tocopherol. There was a tendency for the group given 4% sunflower seed oil and α-tocopherol in HMF to grow better than the group given 1% sunflower seed oil. The 2 HMF groups grew

Table II. Weight gain and liver weight: final body weight ratios in rats given HMF and some other commercial fats

Experiment	Group	Fat	Starting weight g	Food eaten g	Weight gain g	Significance of comparison	Liver weight/ final body weight ratio g/g	
A	1	Lard	65.1	175	38.4		0.031	
	2	HMF	65.4	176	22.2	vs. $\frac{1+3+4}{3}$***	0.040	vs. $\frac{1+3+4}{3}$**
	3	HSO	65.5	176	31.7	vs. $\frac{1+4}{2}$**	0.034	vs. $\frac{1+4}{2}$**
	4	C+T	65.5	175	35.8		0.031	
B	1	Lard	53.7	175	40.2	vs. $\frac{2+3+4}{3}$***	0.030	vs. $\frac{2+3+4}{3}$***
	2	HMF_a	53.7	175	26.6		0.047	
	3	HMF_b	54.0	176	23.3		0.049	
	4	HMF_c	52.7	176	24.2		0.047	
C	1	HMF	51.1	164	24.0	vs. 3+4***	0.042	
	2	HMF 1	51.1	164	25.9		0.041	
	3	HSO	50.9	164	30.7		0.035	vs. $\frac{1+2+4}{3}$***
	4	R+T	50.1	164	29.7		0.040	
D	1	Lard 1	59.3	184	46.5		0.038	
	2	HMF 1	59.5	184	38.7	vs. 1+4**	0.036	
	3	HMF 4 α-toc_1	59.0	184	41.1		0.038	
	4	HO 1	58.6	184	44.6		0.046	vs. $\frac{1+2+3}{3}$**
E	1	Lard	68.5	208	46.4	vs. $\frac{2+3+4}{3}$**	0.034	
	2	HMF	69.3	208	34.3		0.042	vs. 1+4***
	3	HMF α-toc_2	68.1	208	32.9		0.043	
	4	HMF 5 r	69.5	208	36.5		0.036	
F	1	HMF	60.2	198	31.8		0.045	
	2	HMF 5	60.3	198	41.0	vs. 1+4***	0.036	vs. 1+4***
	3	HMF 5 r	59.6	198	42.4		0.036	
	4	HMF α-toc_3	60.9	198	29.5		0.048	
G	1	Lard	95.0	288	68.9	vs. $\frac{2+3+4+5}{4}$***	0.046	vs. $\frac{2+3+4+5}{4}$**
	2	HMF	95.8	288	56.4	vs. $\frac{3+4+5}{3}$*	0.041	vs. $\frac{3+4+5}{3}$*
	3	HMF 2.5	95.5	288	61.9		0.037	
	4	HMF 5	95.5	288	60.1		0.035	
	5	HMF 10	96.4	288	60.9		0.036	

less than the 2 groups given the natural fats, lard and herring oil. Fatty livers were observed in some of the rats in the herring oil group and to a smaller extent in the lard group, accounting for the higher liver weight: final body weight ratio observed in these groups. In experiment E the tendency observed in experiment D for the supplemented group was investigated further by separate additions of α-tocopherol and sunflower seed oil stripped of α-tocopherol. The HMF groups showed no significant growth differences but all grew slower than the lard group. The liver weight: final body weight ratio was of the same magnitude in the HMF group with added stripped oil and in the lard group, the mean of these 2 groups were significantly lower than in the 2 other HMF groups. In experiment F the rats given HMF containing 5% sunflower seed oil and 5% of the stripped oil grew significantly better and had lower liver weight: final body weight ratios than the rats given HMF without supplement or supplemented with α-tocopherol alone. Experiments E and F showed that when HMF was supplemented with α-tocopherol in amounts corresponding to the content in 5% sunflower seed oil neither weight gain nor liver weight: final body weight ratio changed significantly as compared with the values found for the unsupplemented groups. In fact, weight gains were slightly less and the liver weight ratios slightly higher in the groups supplemented with α-tocopherol than in the unsupplemented groups. The effect of graded amounts of sunflower seed oil was studied in experiment G. The growth was significantly improved when HMF was supplemented with sunflower seed oil, the 2.5% level showing maximum effect as no further improvement was found with the 5- and 10-percent levels. The weight gains in the supplemented groups did not reach the level attained in the lard group. The supplemented groups showed lower liver weight: final body weight ratios than the unsupplemented group. The high liver weight: final body weight ratio in the lard group was due to the occurrence of fatty livers.

The analyses of the livers in experiments A and B (table III) showed lower fat contents in the groups given HMF or hydrogenated soya bean oil than in the groups given lard or the mixture of coconut oil and tallow. In experiment A, where individual livers were analyzed, the difference was very highly significant. The fat levels found were, however, below the range usually found in fatty livers [LUCAS and RHOUR, 1967]. The dry matter, protein and ash contents of the pooled livers in experiment B showed only small differences. The faeces collected during the last 2 weeks of experiments A and B weighed more from rats given HMF than from the other rats. This was due to more fat being excreted in the HMF groups. This difference in fat excretion was

Table III. Chemical analyses of livers and faeces of the rats in experiments A and B

	Experiment A				Experiment B			
	1 Lard	2 HMF	3 HSO	4 C+T	1 Lard	2 HMF_a	3 HMF_b	4 HMF_c
Liver								
Dry matter, %					29.9	29.2	30.5	33.6
Fat, %	5.2	3.0	3.2	4.3	7.9	4.2	3.6	3.8
Protein, %					23.6	24.2	24.6	24.7
Ash, %					1.47	1.48	1.58	1.54
Fat comparisons	1+4 vs. 2+3***							
Faeces								
Weight, g	10.5	12.4	10.6	10.4	10.1	11.8	12.0	11.4
Weight of fat free, g	8.1	8.0	7.7	7.8	7.6	8.0	7.7	7.5
Free fat, % of eaten	0.64	1.80	1.49	0.80	0.69	1.63	1.71	1.37
Free plus bound fat, % of eaten	8.9	16.7	11.1	9.9	9.6	14.5	16.7	15.1
Nitrogen, % of eaten	16.6	19.4	14.8	14.2				
Calcium, % of fat free					2.98	2.80	3.00	2.91
Ash, % of fat free					13.0	11.6	11.9	11.7
Free fat comparisons	2+3 vs. 1+4***, 2 vs. 3*				1 vs. $\frac{2+3+4}{3}$***			
Free plus bound fat comparisons	2+3 vs. 1+4***, 2 vs. 3***				1 vs. $\frac{2+3+4}{3}$***			
Nitrogen comparisons	2 vs. $\frac{1+3+4}{3}$*** 1 vs. $\frac{3+4}{2}$*							

For code, see text.

seen both for free and for free plus bound fat. The data given for fat free faeces are the differences between faeces weight and the weight of free plus bound faeces fat. The HMF rats lost up to about 8.5% of the calories as fat in the faeces whereas the other rats lost about 4–5%. In experiment A the HMF group also lost slightly more nitrogen in the faeces than the other groups. In experiment B the fat free faeces contained more ash in the lard group than in the other groups, but there was no corresponding differences in the calcium contents.

The chain length analyses of the experimental fats used in experiment A and of the corresponding fat fractions extracted from the faeces (table IV) showed a general agreement between food fat and faeces fat. There was,

Table IV. Experiment A: Fatty acid proportions by chain length of diet fats, and free (f) and bound (b) faeces fat fractions

Fatty acid chain length	Group 1 Lard	f	b	Group 2 HMF	f	b	Group 3 HSO	f	b	Group 4 T+C	f	b
12	–	–	–	–	–	–	–	–	–	31.1	11.9	5.7
14	1.2	1.6	0.6	10.4	4.7	1.8	–	0.5	0.1	11.9	12.5	9.5
16	29.4	22.7	23.3	24.9	14.7	12.4	9.4	12.6	19.1	19.4	26.4	34.2
18	67.9	64.2	73.6	20.2	20.8	18.0	90.6	82.5	79.2	37.6	45.9	49.5
20	1.5	7.6	2.0	20.6	24.0	27.8	–	2.9	1.1	–	2.6	0.8
22	–	3.9	0.5	23.8	35.8	40.0	–	1.5	0.5	–	0.7	0.3

however, a tendency for the longer chain lengths to be more concentrated in the faeces fraction than in the food fats and a correspondingly lower concentration of the short chain fatty acids.

Discussion

At the start of these experiments we assumed that the long chain saturated and trans monoene fatty acids present in HMF would be more difficult to metabolize than those in lard, particularly at the high fat, low protein and low choline levels used. However, the first 3 experiments showed that this was not the case. All fats tested were well digested. The different digestibility of HMF and lard (table III) could not explain the growth effect: The calorie efficiencies for lard and HMF (A1 and A2 in fig. 1) were 13.5 and 24.5, respectively. When the calorie intakes were corrected by the calorie content in the faeces fat, these values changed only to 12.4 and 21.3. The slower weight gain of the groups given HMF compared to the lard groups point in fact to a slightly increased catabolism of the HMF fatty acids. The low choline level used did not result in fat accumulation in the livers of the HMF groups. Table III shows that the fat content in the livers were higher in the lard groups than in the HMF groups, and the few cases of fatty livers observed were confined to 2 lard groups and the herring oil group. Experiments D–F showed that α-tocopherol did not influence the growth differences. The α-tocopherol content of HMF varies between 25 and 100 μg/g

[LAMBERTSEN *et al.*, 1964] and the amounts present seem to be adequate under our experimental conditions. Experiments E–G showed that growth improved when sunflower seed oil was added to HMF, 2.5% addition giving optimal effect.

The results obtained from 29 experimental groups in 7 experiments were compared in the graph given in figure 1. This graph shows that calories above the basal level required for 1 g of weight gain and the liver weight: final body weight ratio were highly correlated ($r = +0.92$). The observations on HMF with up to 1% added sunflower seed oil show a high degree of spreading along the regression line, whereas those with 2.5% or more added sunflower seed oil cluster together near the lower part of the regression line. The 1-percent level thus seems to be marginal. In the lower set of values we also find one observation on HMF with 1% added sunflower seed oil (D2), the observations on hydrogenated soya bean oil (A3 and C3) and one obser-

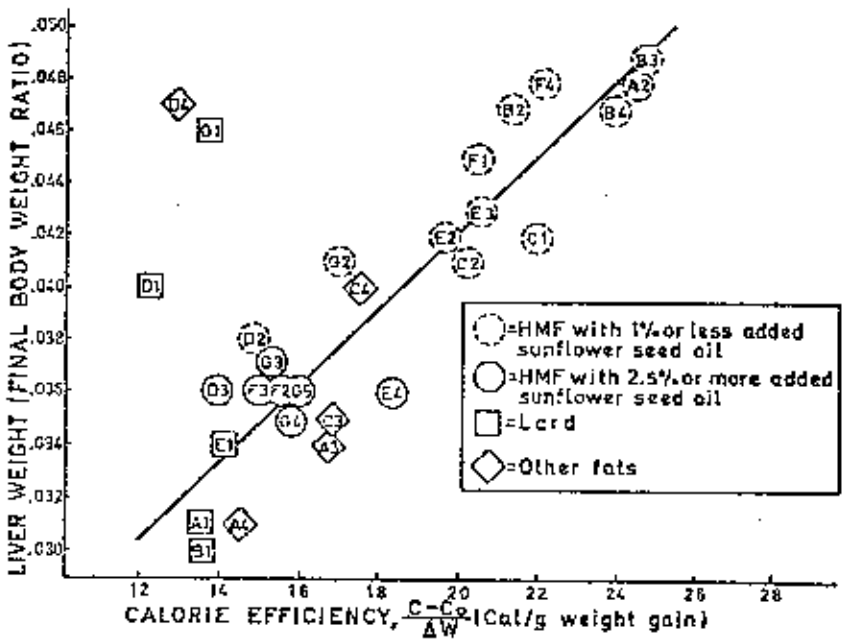

Fig. 1. Linear regression of liver weight: final body weight ratio (Y) on efficiency for growth of calories consumed above basal needs (×). Regression equation $Y = 0.0015^{*} \times +0.012$, correlation coefficient $r = +0.92$. Calculation based on 26 points excluding the groups with fatty livers (D1, D4 and G1). C = calories consumed, C_0 = basal calories, ΔW = 4-week weight gain. Letters refer to experiments, numbers to groups.

vation on lard (E1). Two observations on lard (A1 and B1) and the observation on the mixture of coconut oil and tallow (A4) are found at the lower end of the line. The observation on hydrogenated rapeseed oil (C4) is found near the middle of the line. The graph illustrates the effects of adding sunflower seed oil to HMF on its calorie efficiency as well as on the liver weight: final body weight ratio. We have pointed out [BRÆKKAN *et al.*, 1968] that the content of about 2% 18:2 fatty acids in HMF is mainly in the form of other isomers than true linoleic acid, and the effects of adding sunflower seed oil to HMF are probably due to its content of linoleic acid.

The requirement of linoleic acid for the rat is not well established. HOLMAN [1960] estimated the requirement for the maintenance of a normal trienoic: tetraenoic ratio in tissue lipids to be 1% linolate calories in the diet. The 2.5-percent level of sunflower seed oil in HMF corresponds to about 0.5% linolate calories in the diet. This may be regarded as an estimate of linoleic acid requirement for the proper utilization of HMF for growth on a diet which in contrast to that used by HOLMAN [1960] is low in protein and choline and which is high in a fat of a type very different from those used by him. No symptoms of fatty acid deficiency were seen in any groups in this study. The hydrogenated soya bean oil showed calorie efficiencies and liver weight: final body weight ratios near those found for HMF with 2.5% or more added sunflower seed oil, in spite of its low residual linoleic acid content. Probably the lack of long chain fatty acids accounts for this. In comparison the one group given hydrogenated rapeseed oil gave a decidedly higher liver weight: final body weight ratio.

CHENITT *et al.* [1967] found that sunflower seed oil improved the utilization for growth of partly-hydrogenated rapeseed oil. The rapeseed oil was fed at about 40% of total calories and the linoleate levels corresponding to the 3 levels of sunflower seed oil tested were about 1, 2 and 3% of total calories. Although the experimental conditions were very different from ours, the requirement for added linolate for maximum improvement of hydrogenated rapeseed oil was not very far from that found by us to be required for improved utilization of HMF. In both cases the levels found to give maximum effect did not differ much from HOLMAN's [1960] estimate of total linoleate requirement.

We mentioned in the introduction that high liver weight: final body weight ratios have been reported in rats fed laboratory heated fats or polymerized fats. Generally the high ratios were found in rats showing growth depression or impaired food utilization. KAUNITZ *et al.* [1955] reported that the growth depression and liver enlargement observed in rats given heated and aerated

cottonseed oil could be counteracted by mixing untreated oil into the diet, or to a lesser extent by giving untreated oil by dropper. POLING *et al.* [1960] showed that unsaturated fats which had been damaged in the laboratory by long heating at high temperatures under oxidative conditions reduced food utilization and caused increases in liver weight. ALEXANDER [1966] pointed out that POLING *et al.* [1960] make no mention of how often the diets were mixed and of storage conditions. This also apply to KAUNITZ *et al.* [1955]. ALEXANDER [1966] found that the untoward effects of heated fats could be counteracted by daily mixing of the diet and by keeping it stored under refrigeration. The conditions in these experiments may well have been such that the linoleic acid supplied was oxidized in the stored diets. POTTEAU *et al.* [1970] found growth depression and enlarged livers in rats given soya bean oil heated in an inert atmosphere. Analyses showed that about 75% of the linoleic acid present in the original oil were found in the heat-treated oil. Therefore, their results can not be ascribed to the destruction of linoleic acid.

Altogether this indicates that liver enlargement is observed when the liver is subjected to a metabolic stress. Such stress conditions may due to a deficiency of an essential substance (fatty acids, vitamins) or to the presence in the diet of substances which are inadequately metabolized. The liver enlargements mentioned above all pertain to the fat metabolism, but similar enlargements have been observed in vitamin deficiencies [GUGGENHEIM and DIAMANT, 1959; CHATTERJEE *et al.*, 1970].

In conclusion, HMF in the type of diet used gave a slower growth and an increased liver weight: final body weight ratio compared to 'natural' fats (lard and a mixture of coconut oil and tallow). The 2 criteria measured were positively correlated as shown in figure 1. Three dietary factors were marginal in the diet. Of these the low choline content resulted in fatty livers in 2 of the lard groups, and in the herring oil group, i.e., in 3 groups given 'natural' fats. The increased liver weight: final body weight ratios seen in the HMF-fed groups were not caused by an increased fat content in the livers, and were, therefore, probably not related to a choline deficiency.

A supplementation with α-tocopherol did not improve the HMF diets as judged by our criteria.

HMF as well as the 2 hydrogenated vegetable fats were all very low in linoleic acid. Supplementing HMF with sunflower seed oil improved calorie efficiency and lowered the liver weight final: body weight ratio, 2.5% giving maximum effect. However, supplementation with sunflower seed oil did not result in calorie efficiencies equal to those found for 'natural' fats. This residual difference may be caused by a combined effect of long chain fatty

acids (20 and 22 carbon atoms) of low unsaturation and a substantial content of 'unnatural' isomers (trans and positional) in HMF. Hydrogenated soya bean oil with a similar isomer content but without long chain fatty acids showed better growth efficiency than unsupplemented HMF and hydrogenated rapeseed oil, the latter containing 'unnatural' isomers as well as long chain fatty acids. Herring oil containing natural long chain fatty acids gave good growth whereas the liver weight: final body weight ratio was obscured by the occurrence of fatty livers.

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Author's address: Dr. L. R. NJAA, Dr. O. R. BRÆKKAN, Dr. G. LAMBERTSEN and Dr. F. UTNE, Government Vitamin Laboratory, Norwegian Fisheries Research Institute, P. O. Box 187, *5000-Bergen* (Norway)

Fish Oils "c. Advisory Ec. 1960"

CHAPTER 11

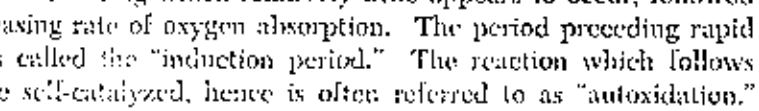
H. S. Olcott

Antioxidants

An antioxidant may be defined as a substance which, when present in an oxidizable substrate in relatively low concentration, markedly inhibits the rate of reaction with oxygen. Information about this phenomenon is particularly important with fish oils, the fatty acids of which are generally highly unsaturated and hence unusually susceptible to attack by the oxygen of air. The results of such oxidation of fish oils may be favorable if a resistant, insoluble paint film is the desired product, but if food values are to be preserved oxidation must be avoided. Otherwise highly offensive tastes and odors develop (rancidity).

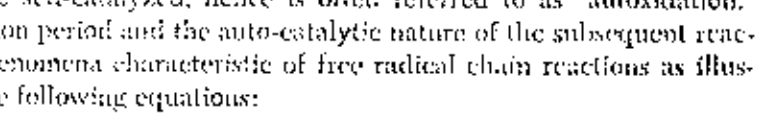
When unsaturated oils are stored in contact with air or oxygen, there is a period of time during which relatively little appears to occur, followed by an increasing rate of oxygen absorption. The period preceding rapid oxidation is called the "induction period." The reaction which follows seems to be self-catalyzed, hence is often referred to as "autoxidation." The induction period and the auto-catalytic nature of the subsequent reaction are phenomena characteristic of free radical chain reactions as illustrated in the following equations:

Initiation Step



(1)

Propagation Steps



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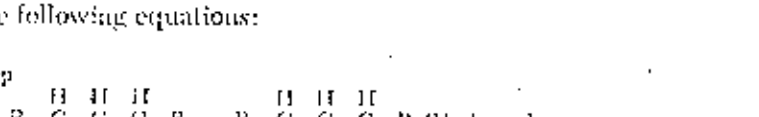

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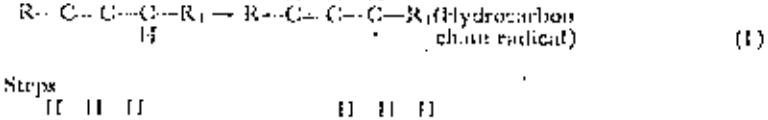
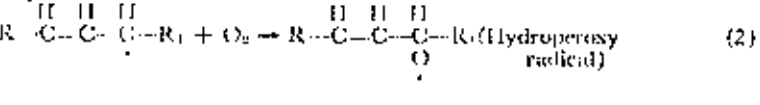


Propagation of the chain involves both hydrocarbon and hydroperoxy free radicals, and a non-radical hydroperoxide is an end-product of the

reaction. Decomposition of the hydroperoxide forms additional radicals (Eq. 4)



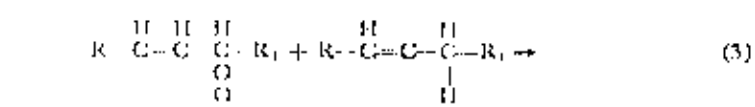
(4)

which in turn initiate new chains. This is assumed to account for the accelerating rate of oxidation once the reaction rate has become appreciable.

The length of the induction period is a function of temperature, the availability of oxygen, and the presence or absence of pro- or antioxidants. Oxidation is accelerated, that is, the length of the induction period is decreased, by increase of temperature, in pure oxygen as compared to air, in light as compared to dark, with certain added heavy metals, peroxides, or other sources of free radicals, etc.

Antioxidants are those substances that interfere either with the initiation step or with the early stages of the propagation steps. The initiation step in a purified substrate is of obscure origin. An occasional cosmic ray or some other adventitious event may achieve the first hydrogen abstraction. Uri has suggested that it is catalyzed by minute traces of heavy metal salts (Uri 1961). In the following, we refer specifically to antioxidant systems whose effect is recognized by an increase in the length of the induction period. They may also change the rate of the subsequent oxidation but this is a more complicated problem and of less immediate utility. That is, once a fat has become inedible, the rate with which it continues to oxidize is not of practical consequence.

An antioxidant reacts with either the original free radical or with one formed in the early stages to give an intermediate which is not capable of continuing the chain. The following are simplified versions of these reactions: AH represents the antioxidant:



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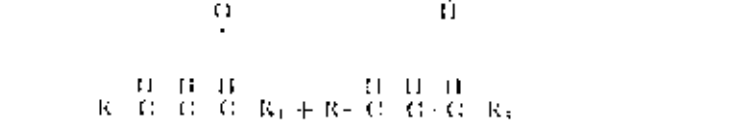

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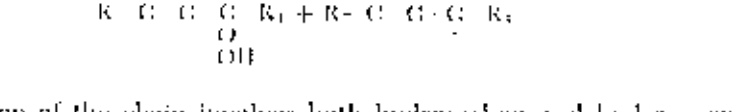


(7)

$$A^{\cdot} + A^{\cdot} \rightarrow AA \text{ (Terminates)} \qquad (8)$$

Substances capable of acting as antioxidants should have readily removable protons and the remaining free radicals should be incapable of

abstracting a proton from another molecule of an unsaturated substrate. Hence the reaction chain would be stopped.

The best-known free-radical acceptors of this nature are aromatic phenols and amines. However, the simplest of these, phenol and aniline, are almost devoid of antioxidant activity. The active antioxidants have side-groups which activate the hydroxyl or amino groups. Thus, hydroquinone and catechol (1,4- and 1,2-dihydroxyphenol) are good antioxidants but resorcinol (1,3-dihydroxyphenol) is not. Similarly pyrogallol 1,2,3-trihydroxyphenol and hydroxyhydroquinone (1,2,4-trihydroxyphenol) are effective whereas phloroglucinol (1,3,5-trihydroxyphenol) is not (Olcott 1934). Substitutions in the ortho- and para-position are thus more important than substitution in the meta position.

Many organic phenols have been investigated for antioxidant effectiveness. Their relative activity has been shown to vary markedly depending upon the methods used in the assays. The general inconsistencies in such comparison have so far prevented the development of a comprehensive theory of antioxidant action (*cf.* Uri 1961).

Several phenolic compounds have been accepted for use in food products in the United States (Table 12). They are known to be safe at the

TABLE 12

PARTIAL LIST OF FOOD ADDITIVES PERMITTED IN THE UNITED STATES[1]

Antioxidants[2]	Synergists[3]
Butylated hydroxyanisole (BHA)	Ascorbic acid
Butylated hydroxy toluene (BHT)	Ascorbyl palmitate
Propyl gallate (PG)	Citric acid
Nordihydroguaretic acid (NDGA)	Isopropyl citrate
Guaiaguaiac	Lecithin[4]
	Phosphoric acid

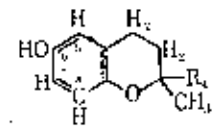
[1] Usually at levels not over 0.02%.
[2] BHA, BHT, and PG are all by the major uses of edible phenolic antioxidants in the United States.
[3] Commercial antioxidant mixtures often contain a phenolic or mixture of phenolic antioxidants, such as BHT plus BHA, together with synergist such as citric acid.
[4] See Table 6.

levels indicated. Because of the difficulties mentioned above, it is not possible to rate these compounds in order of activity. For practical purposes, the potential user would be best advised to assay their antioxidant activities in the specific product he wishes to protect and for the specific environment he wishes to protect it from.

Aromatic amines corresponding in structure to the phenols are equal to or even more effective as antioxidants. However, since their toxicity is probably greater, they have not been studied for food use. One exception is ethoxyquin (6-ethoxy-2,2,4-trimethyl-1,2-dihydroquinoline) (EQ) which is a useful antioxidant for preserving the carotene (precursor of vi-

tamin A) of dehydrated alfalfa and is permitted for this purpose and for animal feed mixtures at levels not to exceed 0.015%.

The antioxidants mentioned above are synthetic chemicals which have been discovered to have this property. However, certain naturally occurring substances are strong antioxidants and their presence accounts, at least partially, for the stability of the products in which they occur. The most important and widely distributed of such substances are tocopherols of which eight have been identified, all methyl derivatives of the mother substance tocol, in which side chain R_1 is either a hydrocarbon chain (C_{17}) or a triply unsaturated chain group (*cf.* Table 13).



Tocol

The names, structures, and estimation of antioxidant activity of seven of the tocopherols are shown in Table 13. They occur in small amounts in almost all plants but are particularly rich in seed oils. α-Tocopherol is the normal constituent of animal tissue. For the nutritionist these substances are of particular interest since α-tocopherol is identified with vitamin E; the other tocopherols occur less widely and are less active as vitamin E. The antioxidant activities of the tocopherols may explain their biological activities but this is by no means completely proven or accepted. For example, certain physiological manifestations of vitamin E deficiency, such as sterility in the female rat, can be cured by the administration of di-

TABLE 13

STRUCTURE AND RELATIVE ANTIOXIDANT ACTIVITY OF TOCOPHEROLS IN SQUALENE (50°)[1]

	Methyl Groups	Relative Antioxidant Activity
Tocols		
Alpha	5,7,8	1
Beta	5,8	5
Gamma	7,8	18
Delta	8	11
Tocotrienols		
Zeta	5,7,8	1
Epsilon	5,8	4
Eta	7,8	10

[1] Relative antioxidant activity. R_1 side chain of tocol is $-CH_2-CH_2-CH(CH_3)-CH_2-CH_2-CH(CH_3)-CH_3$; R_1 side chain of tocotrienol is $-CH_2-CH_2-CH(CH_3)-CH=CH_2$.

[2] $-CH=CH_2$.

phenylphenylenediamine (DPPD), an active fat antioxidant without any structural relationship to the tocopherols. On the other hand, the ability of the different tocopherols to overcome vitamin E deficiency symptoms in no way parallels their antioxidant activity.

The tocopherol contents of several fish oils are recorded in Table 14. Such data will differ with season and the diet of the fish and, therefore, can be considered to be only rough estimates. Sablefish oil has been found to contain a singularly high amount of tocopherol which may account in part for its relatively great stability (Dolev and Olcott 1965).

Other naturally occurring antioxidants are sesamol in sesame oil, gossypol of cottonseed, quercetin of various vegetable products, various unidentified phenols in smoked products, and others (Olcott and Mattill 1941).

TABLE 14

TOCOPHEROL CONTENT OF SEVERAL FISH OILS

Fish	Tocopherol Content μgm/Gm. of Oil
Muscle[1]	
Sardine	40
Menhaden	70
Tuna	160
Herring	140
Salmon (pink)	220
Whale	200
Sable	630
Liver[2]	
Haddock	90
Ling Cod	90
Cod	115
Hake (Atlantic)	200
Skate	220
Sole (lemon)	540
Catfish	1100

[1] Kinsset, E., Olcott, H. S., and Scarsby, M. E. (1957).
[2] Ward, R. J., private communication.

SYNERGISTS

The term "synergist" is used to define a substance which has little or no antioxidant activity of its own but when present with an antioxidant markedly enhances its protective properties. Table 12 includes a list of commonly used synergists.

The mode of action of synergists is incompletely understood. In some cases the ability of chelating substances such as citric or phosphoric acids to bind heavy metals could account for the activity. However, remarkable synergistic effects are often observable in the absence of added heavy metals. Hence other explanations are required.

The biochemist and biologist are particularly interested in the synergistic activity of the phospholipids, since tocopherols and phospholipids are

closely associated in animal and vegetable tissue. Both purified phosphatidyl choline (lecithin) and phosphatidyl ethanolamine are effective synergists, but phosphatidyl inositol is not (Table 15). These observations lead to the conclusion that the nitrogen moiety is involved. Further studies of the antioxidant activity of aliphatic amines showed that tertiary and secondary amines were more effective synergists than primary amines.

TABLE 15

EFFECT OF PHOSPHOLIPIDS AS SYNERGISTS FOR ETHOXYQUIN (EQ) IN PURIFIED MENHADEN OIL AT 50°[1]

Antioxidant	Synergist[2]	Induction Period Days
None	None	1
0.05% EQ	None	7
0.05% EQ	2%PC	45
0.05% EQ	2%PE	25
0.05% EQ	2%PI	7
None	2%PC	1
None	2%PE	1
None	2%PI	1

[1] Olcott and Van der Veen (1963).
[2] PC, phosphatidyl choline (lecithin); PE, phosphatidyl ethanolamine; PI, phosphatidylinositol.

The tertiary amine was very protective at temperatures of 50° or 60°C. but less so at lower temperatures. Examination of this phenomenon led to the discovery that aliphatic tertiary amines (such as trioctylamine) are oxidized by peroxides to aliphatic secondary hydroxylamines (Harris and Olcott 1966) (Eq. 8).

$$R_3N + ROOH \rightarrow R_2NOH + RC{=}O \qquad (8)$$

The substituted hydroxylamines have antioxidant properties and thus represent a new type of antioxidant of potential utility although their toxicities have not been investigated in detail.

It is of interest and some importance that the effectiveness of antioxidants is adversely affected by the presence of free fatty acids (Olcott 1959). Thus it may be expected that refining processes would, on the one hand, reduce the stability of oils by removing traces of tocopherols and phospholipids and, on the other, increase the effectiveness of residual tocopherols or of added antioxidants by removing free fatty acids.

The interrelationships between antioxidants and synergists and the conditions under which maximum lipid protection can be achieved are not yet understood. A considerable amount of research will be required to throw light on these obscure problems, the solutions to which will yield enormous dividends in the optimal utilization of the world's food supply.

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JANUSZ PALUSZAK

EFFECT OF EXTREMELY HYDROGENATED FAT ON THE LIPID METABOLISM

Department of General and Experimental Pathology, Medical Academy, Poznań
Head: Professor A. Horst, M.D.

[illegible] *Effect of Extremely Hydrogenated Fat on the Lipid Metabolism*, Acta Physiol. Polon. 18 (4): 6[illegible]-6[illegible], 1967. — The aim [illegible] effect of fatty hydrogenated fats on fat [illegible] of depot fat atrophy in rats [illegible] fatty acids is [illegible] fatty acids is [illegible] in the [illegible] of [illegible] in the [illegible] of [illegible] CoA. The nonmetabolized acetyl-CoA [illegible] the development of ketonemia.

Recent years brought about a renewed interest in the effect of dietary fats on the organism and especially on the fat metabolism. Numerous studies have revealed an importance of dietary fatty acid saturation and composition in the development of circulatory diseases [2, 27, 29, 37]. Studies of *Horst et al.* [22, 23] on biological effects of hydrogenated vegetable fats have revealed an interesting phenomenon; rats fed on the diet of hydrogenated soybean and rape oil have lost their tissue fat depots [illegible].

Partial or complete atrophy of the adipose tissue in experimental animals has been achieved by diets which contained pure palmitic acid [3], triclantin [30], fats of a high peroxide and polymer content [5] and conjugated dien fatty acid of a *trans* type [10] respectively. The deleterious effects of fatty acids peroxides could be explained by their toxicity to-

The investigation was supported by a Grant From the VI-th Department of Medical Sciences, Polish Academy of Sciences.

[illegible] of the living cell [39]. These [illegible] of fat culture [illegible] [illegible]. They [illegible] are left out in blank the [illegible] of [illegible] is also known that diets of a high [illegible] of [illegible] [illegible] [illegible] the rate of growth of animals [illegible] due to the resulting deficiency of [illegible] [illegible] to [illegible].

[illegible] of the [illegible] can be brought about by diets which contain large amounts of [illegible] [illegible] such a diet results in biotin deficiency. Biotin is [illegible] by the action of egg white.

The general causes which might produce the disappearance of the depot fat are considered, following mechanisms of fat impaired synthesis, [illegible] [illegible] [illegible]:

1. Lack of substrate for fat synthesis, as for example in starvation, lack of [illegible] in hypothyroidism, diabetes as well as in other disturbances of the [illegible] [illegible].
2. A deficiency of vitamins and [illegible] as cofactors of the fat metabolism,
3. The presence of inhibitors as for example of substances which bind [illegible] [illegible] [illegible] of [illegible].
4. [illegible] due to the impaired [illegible] of [illegible].

[illegible] behavior and excludes the first three mechanisms. An impairment of biosynthesis of the enzymes which catalyze the production of [illegible] from a deficiency of dietary antigenic factors or from the errors or lacking of genetical information.

The detailed pathway of the biosynthesis of fatty acids in both animals and plant cells are present [16, 31, 41]: mitochondrial and extramitochondrial systems. Mitochondrial system is responsible for the bulk of fatty acid synthesis in the living body and is biotin dependent.

The aim of the present study was to investigate the effect of extremely hypocaloric diets on the fat metabolism and to explain the phenomenon of depot fat atrophy in rats fed with extremely hypocaloric vegetable oils.

METHODS

Studies were performed in 80 adult male and female rats which weighed between 210–250 g. The animals were separated into groups according to the type of diet they have obtained. In order to characterize the role of biotin in the fatty hypocaloric diet of [illegible] animals were given the diet which was deficient in [illegible]. A phosphorated preparation of vitamin B (vit. B fatty product) was the main source of B [illegible] in the diet. This preparation does not comprise biotin. The types of diet employed in the study were as follows:

Group I — [illegible] diet according to *Hirsch et al.* [22, 23].

Group II — [illegible] diet enriched with biotin 0.2 mg/100 g of the diet.

Group III — [illegible] which consisted: 100 g of the basic diet, 100 g of [illegible] (in the amount [illegible] 5) and 1 g of phosphorated [illegible] of [illegible] per 100 g of diet.

Group IV — the diet as of Group III but without cholesterol and enriched with [illegible] portion of the diet.

Group V — the diet as of Group III but without cholesterol.

Group VI — the diet as of Group III but enriched with biotin 0.2 mg/100 g of [illegible].

[illegible] animals. The animals of Group IV were fed with the [illegible] diet for 11 weeks and then for additional three weeks with the same diet [illegible].

[illegible] of rats all were [illegible] at 110–120°C in the presence of [illegible]. The [illegible] for displaced iodine number 5 and melting point [illegible] were [illegible].

The animals were weighed and their skin changes noted [illegible] weekly schedule. The diets were weighed and [illegible] consumption was recorded weekly.

The [illegible] was determined by the method of Van de Kamer [43]. [illegible] were performed in 3 rats of each group according to *Hanson* [15] using [illegible] and *Bold* [35]. The determinations were carried out after 3 weeks since placing the rats on experimental diets. After 11 weeks, total fat was determined in these rats on experimental diets. After 11 weeks, total fat was determined in these [illegible] of each group [33, 37]. [illegible] of [illegible] was measured in two [illegible] of Group I and 6 rats of Group IV by the microdiffusional technique of *Harkins-Wright* [9].

The results were analyzed statistically by Student's "t" test. Differences were considered significant at $p < 5\%$ probability level.

RESULTS

1. *Intake of food, weight and appearance of the rats*

The intake of food in different groups was as follows:

a) in rats of Group I and II it amounted to 17 g /day/ rat,

b) in rats of Group III, IV, V, and VI it amounted to about 35 g /day/ rat.

The rats of Group I weighed much the same after 11 weeks of experiment while the weight of rats of Group II was slightly increased. In Group III and IV the rats displayed a considerable loss of weight in the first two weeks thereafter the weight of the rats of Group IV became slightly higher. The weight of rats of Group III was further decreased. At the end of the experiment these rats displayed 23 percent loss of weight. In the rats of Group V and VI (whole cereal-free diet) loss of weight was slower and less marked (Fig. 1).

The appearance of the rats of Group I and II was not changed during the experiment. Their fur was smooth, fluffy and shiny. Rats of Group

III displayed hair loss and bristly fur already after two weeks of the diet. Change of pigmentation was evident in the skin of the tail (brown patches and scales) which resembled lion's tail in appearance. Brown patches were also visible in the skin of ears as well as comb-like shedding of the ear margin. The animals became drowsy and weakly responsive towards external stimulation. The movements were spastic and the spine humped (kangaroo posture).

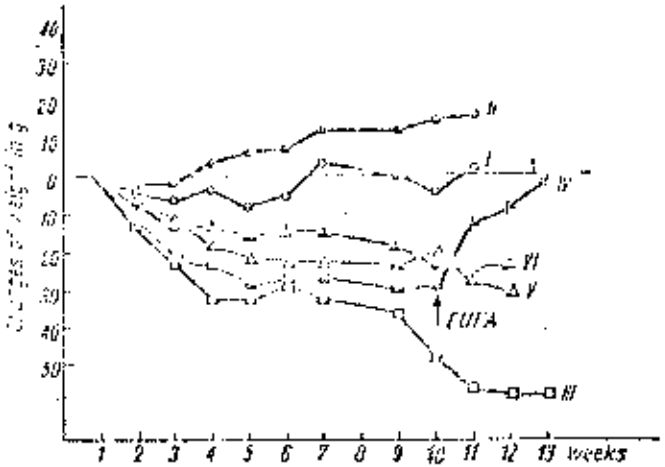

Fig. 1. Weight gain curves in rats. Groups of diet: I. Basal stock diet. II. Basal stock diet + biotin. III. Basal stock diet + 50 percent of maximally hydrogenated oil + 1 percent cholesterol. IV. Basal stock diet + 50 percent maximally hydrogenated oil + 1 percent cholesterol + 1 percent biotin. V. Basal stock diet + 50 percent maximally hydrogenated oil. VI. Basal stock diet + 50 percent maximally hydrogenated oil + biotin. The arrow shows administration of essential unsaturated fatty acids (EUFA).

Dissection of the rats was performed after 11 weeks of feeding with fully hydrogenated fat. The adipose tissue was completely absent in its normal localization. Internal organs were diminished in size.

Rat of Group IV which obtained a similar diet but fortified with biotin displayed similar symptoms after two weeks. However the pathological changes developed at a faster rate and patches of baldness appeared at various places already at the end of the seventh week. These patches contained heavily pigmented spots. Exudative eczema was evident. In spite of this the dissection of rats revealed the presence of adipose tissue especially in the neighborhood of kidneys and genital organs and in the mesentery. The fat was darkly colored and the individual lobules of the tissue were only loosely connected.

After eleven weeks, a part of the animals of Group IV received EFA (essential unsaturated fatty acids). Fatty infiltration of skin of the tail

disappeared within three weeks. Fur regained its normal appearance. The animals gained weight.

The changes of appearance of the animals of Group V and VI were not so dramatic as in those of group III and IV. The former displayed bristle fur, partial hair loss and desquamation of skin of the tail but only after four weeks.

2. *Results of chemical analyses*

The results are presented in Table 1.

a) Digestibility of fat. The coefficient of the digestibility of fat was low in the animals of Group III and V. It amounted to 42·1 ± 3·1 and 41·4 ± 3·6 percent respectively.

Table 1. Blood ketone levels and total fat concentration in rats fed experimental diets

Groups of diet	N	Ketone bodies (expressed as acetone mg percent)	N	(Total fat percent)
I	6	4·42 ± 0·33*	3	6·81 ± 1·63*
II	6	3·72 ± 1·81	3	8·95 ± 3·02
III	6	8·65 ± 2·68	3	2·58 ± 0·54
IV	6	5·14 ± 0·14	3	4·93 ± 0·61
V	6	6·34 ± 1·78	3	2·78 ± 2·15
VI	6	5·41 ± 1·64	3	4·23 ± 1·89

* Mean ± standard deviation, N — Number of animals

Statistical analysis

Ketone bodies	I	II	III	IV	V	VI	Carcass fat
I		NS	$P<0·001$	$p<0·05$	$p<0·05$	$p<0·05$	I
II	NS		$P<0·001$	$p<0·05$	$p<0·05$	$p<0·05$	II
III	$p<0·01$			$p<0·01$	$p<0·05$	$p<0·05$	III
IV	$p<0·05$	$p<0·01$			NS	NS	IV
V	$p<0·05$	$p<0·05$	$p<0·05$			NS	V
VI	NS	NS	NS	NS			VI
Carcass fat		NS	NS	NS	NS		

NS — Nonsignificant

b) **Ketone bodies.** The concentration of ketone bodies in blood was especially high in the animals of Group III and V. It was somewhat lower in the animals of Group IV and VI which received biotin. Statistical analysis of the results confirmed ketogenic properties of fully hydrogenated fats. The effect was especially evident in the animals of Group III which received cholesterol, and it was counteracted by biotin.

c) **Total fat content.** Total fat content percentage of rats was the reverse of the concentration of ketone bodies. Fat content was highest those of IIIrd Group which were fed with extremely hydrogenated fat content was displayed by rats of Group III and V. Animals which received biotin enriched diet displayed a higher fat content than those on a biotin free diet. This was evident both in the animals on the high-fat and fat-free diets.

d) **Biotin concentration in caecum content.** The results of the microbiological assay revealed a five-fold higher concentration of biotin in caecum content in the animals of the control group (I) than in those of IIIrd Group which were fed with extremely hydrogenated fat ($p < 0.001$), (17·6 ± 2·2 and 3·6 ± 2·4 γ/g respectively).

DISCUSSION

Feeding with a diet of extremely hydrogenated fats produced skin changes and adipose tissue atrophy in rats. It is known that catalytic hydrogenation of fats might yield numerous toxic products which induce the aforementioned pathological changes. These products comprise peroxides, of biotin and EFA in the diet.

However it seems that these toxic products, i.e. polymers, peroxides and fatty acids isomers were not present in the hydrogenated rape oil employed in the present study. The animals which had been fed with this fat fully regained a normal weight and a healthy skin after inclusion of biotin and EFA in the diet.

Therefore it should be assumed that the biological effect of extremely hydrogenated fat results from its high content of saturated fatty acids. Excessive intake of saturated fatty acids leads to two deficiency syndromes:

a) biotin deficiency,

b) EFA deficiency.

Such a statement is allowed because of a prophylactic and therapeutic effect of EFA and biotin.

Our observations concerning the changes of external appearance of rats are fully consistent with the experimental EFA and biotin deficiency symptoms in rats [1, 3, 11, 14, 19].

A probable deficiency of EFA in our rats is suggested by the lack of linoleic, linolenic and arachidonic acid respectively in the employed diet. The rats received at the same time other foodstuffs which are known to increase the utilization of endogenous EFA, i.e. cholesterol and saturated fatty acids [1, 3, 21].

Symptoms of biotin deficiency are more difficult to explain. Biotin deficiency could arise from:

a) inadequate supply of biotin in the diet,

b) impaired absorption of biotin from the digestive tract,

c) inhibition of biotin synthesis in bacterial flora of the digestive tract,

d) increased utilization of biotin in tissues.

ad a) The first possibility can be excluded since rats on a basic diet without fully hydrogenated fat were completely normal in respect to weight and fat content.

ad b) In rats which received "defattening diet" and biotin, fat depots were maintained. Thus impaired absorption of biotin from the digestive tract can be excluded.

ad 3) Biotin like other B vitamins is synthetized by bacterial flora of caecum [36]. Under normal conditions this amount of biotin is more than adequate and a part of the vitamin is excreted in feces. Some fatty acids, as for example oleic acid, are essential for a normal growth of rat caecum bacteria [42]. Likewise, linoleic and linolenic acids are also essential for bacterial growth [12]). Polyunsaturated fatty acids were absent in the diet employed in the present study and the content of oleic acid was negligible. Biotin equivalent of hydrogenated rape oil as calculated by an empirical formula [9] amounted to only 0·3 milimicrograms per 1 mg of fatty acids (0·3 mμg/mg). This equivalent is much higher for other fats as for example for butter, margarine and soybean oil it amounts to 9·4, 11·7 and 7·2 respectively [9]. Low equivalent of the fully hydrogenated fat was due to its high content of saturated fatty acids. Saturated fatty acids can even inhibit the growth of bacteria [34]. The present study indicates the magnitude of biotin synthesis by intestinal bacteria since the concentration of biotin in caecum content of the control animals was five times greater than in the caecum of the rats on hydrogenated fat diet. Thus extremely hydrogenated fat inhibited the growth of biotin synthetising bacterial flora.

ad d) The possibility that biotin utilization in tissues was higher is indicated by the following data. Fats of a high melting point are only weekly absorbed from the intestine; this in turn might impair the absorption of water-insoluble vitamins (A, D, E, K). Deficiency of vitamin A results in a higher production of branched fatty acids which need biotin for their metabolism [16]. Deficiency of EFA and the resulting uncou-

piling of oxydative phosphorylation as well as a possible deficiency of vitamin E all increase biotin utilization via the mechanism of activation of cellular oxidative processes. This increased utilization of biotin is due to its participation in carboxylation reactions [17, 25]. Thus the two hypotheses, one on the inhibition of biotin synthesis in the intestine and the second on the increased utilization of biotin in tissues assume a common primary causative factor which is the deficiency of EFA.

The effect of these deficiency syndromes on fat metabolism should be finally discussed. Fat biosynthesis occurs mainly in the adipose tissue.

The role of biotin in this respect, as pointed out in the introduction, is obvious. Biotin deficiency produces the inhibition of fatty acid synthesis and the resulting loss of weight. Lipogenesis seems to be inhibited at the step of transformation acetyl-CoA into malonyl CoA. This reaction is catalysed by acetyl-CoA carboxylase which comprises biotin in the molecule. The accumulated active acetate is metabolised via an alternate pathway to *beta*-hydroxybutyric acid, acetoacetic acid and acetone. This produces ketonemia.

It seems that essential unsaturated fatty acids (EFA) take active part in fat metabolism and probably are a component of the reductase system and electron transfer. Therefore the activation of fat synthesis as produced by biotin application might increase the utilization of EFA depots. Further studies will probably elucidate the role of EFA in the synthesis of fats.

The deficiency of EFA brings about the activation of cellular metabolism. The effect is due to a disturbance of oxidation and phosphorylation. The electron transfer chain is directed by lipids of mitochondrial membrane which contains mainly EFA [13, 38]. The uncoupling of phosphorylation results in an imbalance of energy equilibrium and impairment of protein metabolism [32]. This explains the loss of weight of these animals which partly maintained their fat depot. It is also known that biotin deficiency might result in a diminished biosynthesis of proteins since the substance is a cofactor of deamination reactions [28, 34].

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Author's address: Department of General and Experimental Pathology, Medical Academy, Swiecicki Str. No. 6, Poznań (Poland).

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High Oleic Oils by Selective Hydrogenation of Soybean Oil[1]

OVIDIU POPESCU,[2] SAMBASIVARAO KORITALA and H. J. DUTTON,
Northern Regional Research Laboratory,[3] Peoria, Illinois 61604

Abstract

High oleic (monoene) oils were obtained from soybean oil by selective hydrogenation with copper catalysts. A mixture of nickel and copper chromite catalyst had activity suitable for producing the high monoene oils. A new catalyst (copper-on-Cab-O-Sil) prepared in the Laboratory was more active than commercial copper catalysts. Hydrogenated oils contained 61–72% monoenoic and 14–24% dienoic acids, and there was essentially no increase in stearic acid. The *trans*-isomer content of these oils varied between 17% to 32%. Double bonds in the monoene were distributed along the molecule from C_8 to C_{15}, but were located preferentially in the C_9 position for the *cis*-monoene and in the C_{10} and C_{11} positions for the *trans*-monoene. When the iodine value of these high monoene oils was about 90–95, they remained liquid above 28 C. Citric acid treatment reduced the copper content of hydrogenated oils to a level that was comparable to that of the original soybean oil.

Introduction

Copper-chromite catalysts possess high selectivity for linolenate over linoleate and an infinite selectivity towards linoleate over oleate (7). Hydrogenation of polyunsaturated oils with these catalysts stops at the monoene stage. Therefore, oils with a high monoene (oleic) content should be readily prepared from soybean oil (SBO) by selective hydrogenation with copper catalysts. Some preliminary findings are reported here on the hydrogenation of SBO at low and high temperatures and at low and high pressures. Hydrogenation experiments with a catalyst mixture of nickel and copper chromite are included, as well as a two-step hydrogenation, nickel followed by copper.

Experimental Procedures

Apparatus

Two different hydrogenators were used in the production of high monoene oils: a Parr hydrogenator with a modified stirrer as described by Beal and Lancaster (2), and a 3 gal stainless steel convertor, provided with electric thermostated heating and with cooling coils.

Analytical Methods

Methyl esters were prepared from triglycerides by transesterification with 0.5% sodium methoxide catalyst and their compositions were determined by GLC. A Pye chromatograph, equipped with a 4 ft × ¼ in. glass column packed with 11% EGSS-X on Gas-Chrom P, 100–120 mesh (organosilicon polyester packing from Applied Science Laboratories, Inc., State College, Pa.) and with a radium D ionization detector, was operated at 170 C with an argon gas flow of 45 ml/min. The area under each peak was determined from an electronically integrated curve.

Contents of conjugated dienoates, linolenate and linoleate were estimated by the AOCS Official Method (1) with a Cary Model 14 recording spectrophotometer. *Trans*-isomers were estimated with a Perkin-Elmer 621 infrared spectrophotometer by comparing the absorption at 10.36 μ to that of methyl elaidate.

Methyl esters of selected hydrogenated products were separated into saturates, monoenes and dienes on a rubber column (5). Monoenes were further separated into *cis*- and *trans*-fractions by chromatography on a silver-saturated cation exchange resin (4). The position of the double bonds in *cis*- and *trans*-isomers was determined with microreactor gas chromatography (3).

Hydrogenation

Soybean oil (1 liter for a Parr apparatus; 3 to 5.7 liters for a 3 gal convertor) and the catalyst were charged into the hydrogenator, purged with hydrogen gas, and then heated under vacuum with stirring. At 20 degrees below the hydrogenation temperature, stirring was stopped and hydrogen was introduced. When the proper temperature was attained, stirring was restarted and the progress of hydrogenation was measured by the drop in hydrogen pressure in an external reservoir. The drop in iodine value was calculated from the amount of hydrogen uptake. When the required amount of hydrogen was reacted, hydrogenation was stopped by evacuating the gas and cooling the oil under vacuum. The conditions of hydrogenation, the catalysts and the amounts used, as well as the type of apparatus, are shown in Table I.

Discussion of Results

Activity of Catalysts

Activities of the catalysts were evaluated by the drop in iodine value versus time (Fig. 1). Initially, commercial catalysts showed high activity (Exp. 1

[1] Presented at the AOCS Meeting, Chicago, October 1967.
[2] Food and Agricultural Organization representative from Rumania. Present address: Bd. Schitul Magheranu 28, Bucharest, Rumania.
[3] No. Utilz. Res. Dev. Div., ARS, USDA.

TABLE I
Conditions of Hydrogenation of Soybean Oil

Experiment no.	Catalyst: Source	Catalyst: %, w/v	Temp., C	Pressure, psi	Time, min	Type of apparatus
1	Cu-1106P[a]	1.0	170–175	80	300	Parr
2	G-22[b]	0.5	200–220	80	270	Parr
3	Cu-1106P	1.0	180	400–100	218	3 Gal
4	G-49A[c]	0.14	170–205	15	12	3 Gal
5	Cu-1106P	1.0	220	55–80	350	3 Gal
6	G-49A+	0.03+	180–238	55–25	85	3 Gal
	Cu-1106P	1.0				
7	10% Copper on Cab-O-Sil[d]	1.0	170–195	55–35	37	3 Gal

[a] Commercial copper-chromite catalyst (CuO, 39%; Cr_2O_3, 43.5%; BaO, 10%).
[b] Commercial copper-chromite catalyst (CuO, 42%; Cr_2O_3, 40%; BaO, 12%).
[c] Commercial nickel catalyst reduced and stabilized (55% Ni).
[d] Reference 6.

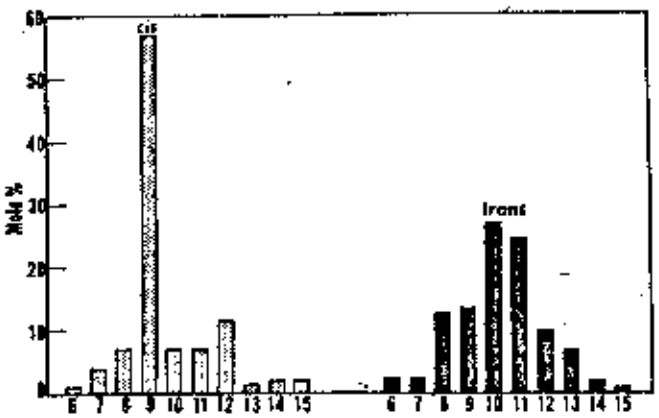

FIG. 3. Double bond distribution in cis- and trans-monoene fractions (Exp. 5).

of a 33% citric acid solution, stirring for ½ hr, applying vacuum at about 110–115 C to remove the moisture and, finally, filtering the cooled oil under vacuum over Celite; (b) the same procedure as under (a) except 0.2% concentrated phosphoric acid was used instead of citric acid; and (c) refining the oil with sufficient 10% NaOH solution that corresponded to 20% in excess of the theoretical amount required to neutralize the free fatty acids. The results given in Table III show that copper contaminant can be readily removed from the hydrogenated products either by a citric or phosphoric acid treatment or by a refining process.

TABLE III

Copper Content of Oils

Sample	Copper, ppm[a]
Original soybean oil	0.04
Original soybean oil	0.03
Experiment 5	1.53
Experiment 3	0.44
Experiment 3 (citric acid treatment)	0.02
Experiment 3 (phosphoric acid treatment)	0.02
Experiment 3 (alkali refining)	0.04

[a] Copper analyses made by an industrial laboratory with atomic absorption spectrophotometry.

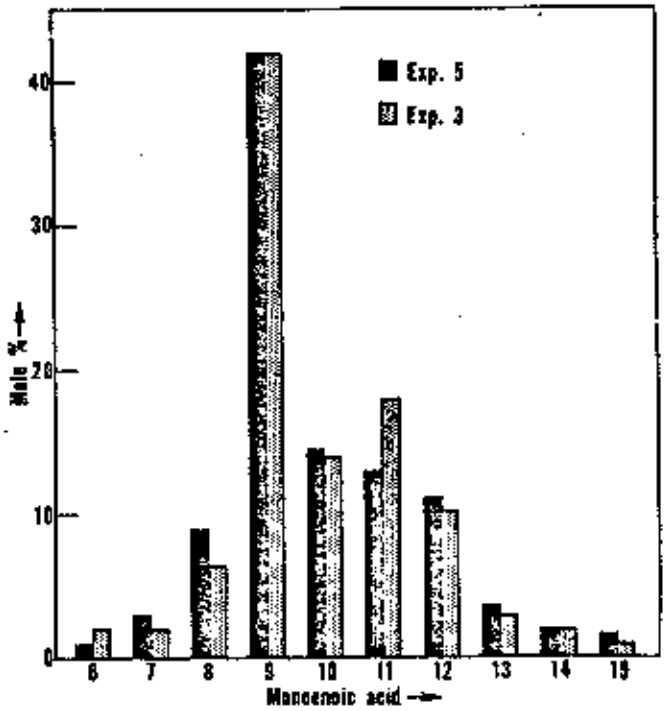

FIG. 4. Double bond distribution in monoenes.

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CONTROLLED TRIAL OF SOYA-BEAN OIL IN MYOCARDIAL INFARCTION

REPORT OF A RESEARCH COMMITTEE TO THE MEDICAL RESEARCH COUNCIL*

Summary A trial is reported of the value of a serum-cholesterol-lowering diet in the prevention of relapse in men aged under sixty recently recovered from a first myocardial infarction and discharged from four district hospitals in the north and west of London. 199 men randomly allocated to the experimental group were given a diet low in saturated fats and containing 85 g. of soya-bean oil daily; the 194 control patients took their ordinary diet. Both groups weighed their food regularly and a high degree of cooperation was achieved. The shortest individual period in the trial was two years and the longest six and three-quarter years. About half of the men were in the trial for four years or more. The test diet lowered the serum-cholesterol from a mean initial figure of 272 to 213 mg. per 100 ml. at six months (22% fall); the level in the controls fell from 273 to 259 mg. per 100 ml. (6% fall). Suspected relapses were assessed at regular intervals by a review committee unaware of the patient's diet group. 62 men on the test diet suffered at least one relapse during the period of observation compared with 74 of the controls. 40 of the first relapses in the test group were major (i.e., definite reinfarctions or deaths from coronary heart-disease) compared with 39 major first relapses in the controls. The total number of men who had a major relapse at any time in the trial was 45 in the test group and 51 in the controls; of these major relapses 25 in each group were fatal. None of the differences found is significant. Relapses were not related to initial cholesterol level, to change in cholesterol level during the trial, nor, in any consistent way, to observance of the dietary regimen. The results are compared with those from a similar trial in Oslo. There is no evidence from the London trial that the relapse-rate in myocardial infarction is materially affected by the unsaturated fat content of the diet used.

* Members of the committee were: Prof. J. N. Morris (chairman), M.R.C. Social Medicine Research Unit; Dr. K. P. Ball (secretary), Central Middlesex Hospital; the late Dr. A. Antonis, St. George's Hospital, London W.1; Dr. W. W. Burbus (consultant to the trial), The London Hospital, E.1; Dr. C. J. Burns-Cox; Dr. Mary Fulton; Dr. Margaret Haigh, M.R.C. Social Medicine Research Unit; Dr. G. J. L. Hall; Dr. Irena Hanington; Dr. J. A. Heady (statistician to the trial), M.R.C. Social Medicine Research Unit; Dr. P. M. McAllen, West Middlesex Hospital; Dr. T. R. E. Pilkington, St. George's Hospital, London S.W.1; Dr. G. R. Royston, Barnet General Hospital; Dr. D. E. Sharland; Dr. G. S. C. Sowry, Edgware General Hospital; and Prof. Clifford Wilson (consultant to the trial), The London Hospital, E.1. Mrs. J. A. C. Clarke, Miss E. Holley, and Mrs. J. Wood were the dietitians. Miss J. W. Marr, M.R.C. Social Medicine Research Unit, advised on dietetic matters and acted as independent observer. Dr. Burns-Cox, Dr. Fulton, Dr. Hall, Dr. Hanington, and Dr. Sharland were the registrars responsible for the day-to-day administration of the trial. Dr. Haigh and Dr. Heady analysed the results and drafted the report.

Introduction

We report here a controlled dietary study carried out for two to seven years on 393 male patients who had recovered from their first myocardial infarction, and who were discharged from four large district general hospitals in the north and west of London (Central Middlesex, West Middlesex, Edgware General, and Barnet General). The trial lasted from 1960 to 1967, and patients were entered up to March, 1965.

The objective of the trial was to determine whether a diet in which saturated fats were replaced by polyunsaturated fats would reduce the incidence of myocardial reinfarction in men who had survived a first attack.

Selection, Management, and Methods

SELECTION

Criteria for Inclusion

All men, discharged from hospital after admission for a first infarction, and aged under sixty at the time of the infarct were considered for entry to the trial. First infarction was diagnosed on a first history of pain with Q-wave evidence of infarction on the electrocardiogram (E.C.G.), or a history of at least one hour's rest pain plus S-T changes on E.C.G. plus previously agreed ancillary evidence (fever, leucocytosis, raised erythrocyte-sedimentation rate, or raised enzyme levels).

Criteria for Exclusion

73 patients who had fulfilled the above criteria were excluded for the following previously agreed reasons: gross obesity (2); diabetes diagnosed on routine urine testing (8); syphilis diagnosed by positive Wassermann reaction (11); hypertension where the diastolic pressure was consistently over 110 mm. Hg or if potent hypotensive drugs were required (6); patients with cardiac lesions which significantly prejudice the prognosis (e.g., complete heart block, gross cardiomegaly cardiac aneurysm, or ruptured ventricular septum) (7); previous significant modification of dietary fat intake (2); inability of the patient to understand or comply with the requirements of the diet regimen (14); unsuitability for prolonged anticoagulant therapy (e.g., patients on steroid therapy or with peptic ulceration or haemorrhoids) (28); and previous anticoagulant therapy (5). (Myxoedema was a reason for exclusion, but no patient had this condition.)

Allocation to Test or Control Group

After selection for the trial on the basis of these criteria, 199 patients were allocated at random, within hospitals, to the diet regimen under test and 194 to the control group.

Description of Patients and Comparison of Groups at Entry to the Trial

Table I compares the two groups in terms of information available at entry to the trial (taken as the first outpatient visit). There was no significant difference in any of the factors examined with the exception of height, the controls being significantly shorter than the men on the test diet ($0.01 < P < 0.02$). With so many factors it is not surprising that one should show a significant difference at this level. Apart from height, which

attendances each year until the end of the trial or death. In addition 28 men attended fairly well, although some had moved away from London. Only 8 men (3 on the test diet and 5 control) were uncooperative from the start, attending sporadically and then failing to resume contact with the trial despite repeated attempts to see them again.

Dietary Control

Patients in the test and control groups were issued with scales and diet sheets, and asked to record the weight of all food consumed on a different day each week for the first seven weeks after admission to the trial, and thereafter on the first day of each month. These records were used as the basis of the dietary supervision and analysis.

An independent dietitian (J. W. M.) interviewed a sample of patients' wives in each group on one occasion (99 in all, 65 on the test diet and 34 controls). She found little discrepancy between statements made to her and those made to the trial dietitians. There was some evidence that the control patients were eating less fried food than they did before their infarcts.

At the end of the trial the dietitians classified the 199 patients on the test diet into three groups comprising 169 men who cooperated in taking the required amount of oil and who cut down their intake of saturated fat, 5 men who lapsed partially from the diet although they still took some soya-bean oil (the proportion of oil in their diet falling below 50% of the total fat), and 25 men who lapsed completely from the diet. Lapses in the last two categories affected 11% of the total possible time in the trial for the men in the test group.

The average amounts of fat and soya bean oil are given for both groups in fig. 1 in terms of oil and of total fat from all sources, and show remarkable constancy throughout the trial time. The average amount of oil recorded (80 g. per day) and the average amount of other fat recorded daily by the men on the test diet (40 g. per day) were very similar to those in the planned diet (85 g. and 35 g. respectively). The ratio of saturated to polyunsaturated fat in the test diet was approximately 1:2 on average and in the control diet was 6:1. In only two instances do oil and fat consumption seem to have changed after a clinical relapse. 40% of calories were derived from fat on average in the test diet.

Dietary Constituents Other than Oil and Fat

The average consumption of carbohydrates and sugar was remarkably steady, and in each case was slightly higher in the diet group than in the controls. Men in the control group averaged 228 g. per day carbohydrate of which 60 g. per day was sucrose, and the men in the test group ate 243 g. per day carbohydrate which included 66 g. per day sucrose. In this context it must be remembered that more controls than men in the test group were on a reducing diet which involved primarily

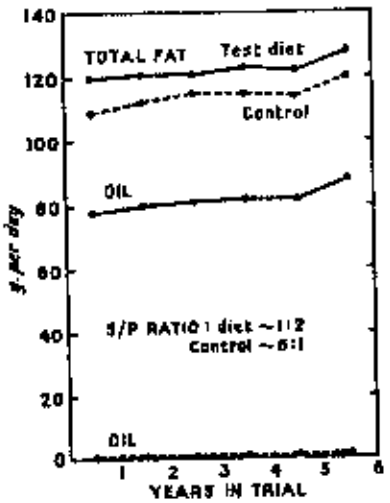

Fig. 1—Dietary fat.

TABLE II—MEAN WEIGHT AND BLOOD-PRESSURE (B.P.)

Time	Weight (lb.)		Systolic B.P. (mm. Hg)		Diastolic B.P. (mm. Hg)		No. of men*	
	Test diet	Control	Test diet	Control	Test diet	Control	Test diet	Control
Entry to trial	164	163	133	135	85	84	193	184
6 mo.	161	162	133	133	83	84	175	173
1 yr.	160	161	132	132	83	84	175	175
2 yr.	161	162	134	133	83	84	168	165
3 yr.	160	162	135	133	85	85	126	126
4 yr.	164	160	135	133	84	87	88	89
5 yr.	164	158	137	137	84	85	45	41

* The figures are the numbers of men for whom weight was determined at each stage. The time given (apart from entry to the trial) is to the nearest three months since patients could not always conform to a rigid programme. The numbers for blood-pressure are very similar. The total numbers in the trial at each stage (using exact time) are shown in the legend to fig. 3.

sucrose and carbohydrate reduction. The average calorie intake per day by the patients on the test diet was 2380 C. and by controls 2274 C.

The consumption of dietary cholesterol was more than twice as great in the controls (588 mg. per day) as in the test group (258 mg. per day). Calcium intake (excluding that from the water supply) was consistently higher in the control diet (930 mg. per day compared with 803 mg. per day for the men on the test diet); this was mainly because most cheeses were forbidden in the test diet. The average protein intake of the men on the test diet was 80 g. per day and of the controls 88 g. per day. A detailed report of the dietary analysis and method of control will appear elsewhere.

Anticoagulant Control

It had originally been intended to compare the effects of long-term and short-term anticoagulation, but unfortunately there was great difficulty in maintaining adequate anticoagulant dosage and uniform prothrombin control in the four hospitals. The anticoagulant part of the trial was therefore abandoned in the 4th year since it was considered that it would not provide a fair comparison of the anticoagulant treatments. The patients (both test and control, except for an early "pilot" group of 27 patients who were all on "short-term" anticoagulation) had been allocated at random, within each hospital, to two anticoagulant regimens—"long term" (or indefinitely prolonged) and "short term" (full anticoagulation for four months from the date of first infarction followed by six weeks tailing-off). This meant that anticoagulation within the diet and control groups was equally distributed, so that a fair assessment could be made of the diet independently of any effect that the anticoagulants might have. It also meant that the anticoagulant treatment in each hospital could be changed without affecting the dietary result provided that, within any hospital, the change was made in everyone concerned. In fact, all patients on long-term anticoagulants were tailed off over six weeks when they had been on anticoagulants for two years or longer, except at Barnet General Hospital where patients on long-term anticoagulants continued on this regimen. This change has no effect on the dietary part of the trial.

Clinical and Laboratory Findings

Blood-pressure

Mean systolic and diastolic blood-pressures are given in table II. There is little difference between the two groups at any stage, and there was no tendency for blood-pressure to rise during the trial.

Weight

The average weight of the patients in each group remained remarkably similar and constant around 160 lb. (73 kg.) in both groups (table II).

Serum-cholesterol

Mean levels during the trial are shown in fig. 2 and

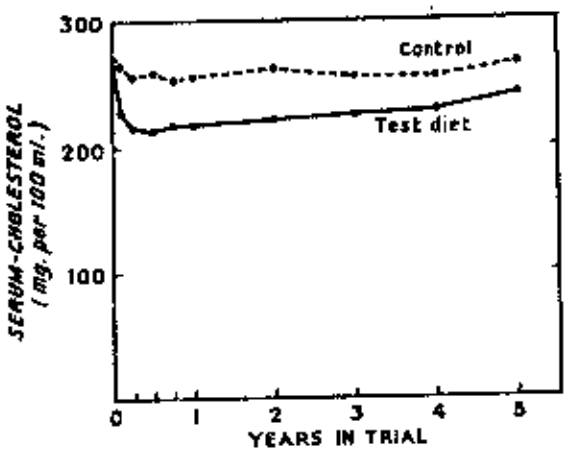

Fig. 2—Serum-cholesterol.

table III. There was an immediate and highly significant fall of 22% (59 mg. per 100 ml.) in the test group to a minimum at six months. There was a steady rise from this low point to the end of the 5th year in the trial when the fall from the starting value was 33 mg. per 100 ml. or 12%. From an almost identical start the level in the control group fell by 6% (14 mg. per 100 ml.) in the first six months and also showed a rise later so that the difference between the groups was maintained.

The cholesterol response of the group on the test diet was affected by the degree of observance of the dietary instructions. Compared with the 169 men who maintained their diet, there was distinctly less fall in the 30 who lapsed from it though their cholesterol level was at all times below that of the control patients. Those 27 men who consistently took less than 60 g. per day of oil had a generally higher serum cholesterol level than those who consistently took more than 60 g. per day.

Serum-cholesterol was estimated by the method of Abell et al. (1952) and later by the automated procedure of Block et al. (1965) as modified by Antonis (1967), giving excellent correlation with the Abell method, the mean difference being −1·4 mg. per 100 ml. These and all chemical estimations were carried out at St. George's Hospital.

Serum-triglycerides (Fasting)

A single estimation was made by the automated fluorimetric method of Kessler and Lederer (1965) as modified by Antonis (1967) during 1965 or 1966 on every man then in the trial in two of the hospitals. 110 were on the test diet and 107 in the control group. More of the patients on the test diet (81 men) had serum-triglyceride values below 150 mg. per 100 ml. than the controls (70 men), but the difference was not significant. There was a wide range of individual values (from 37 to 472 mg. per 100 ml.). In a 10% sample, estimations were made on a second occasion. The mean difference between pairs of observations made on the same individual was 33 mg. per 100 ml.

TABLE III—SERUM-CHOLESTEROL LEVELS AT SUCCESSIVE POINTS IN THE TRIAL

Time	Mean serum-cholesterol (mg. per 100 ml.)		No. of men*	
	Test diet	Control	Test diet	Control
Entry to trial	272	273	173	171
6 mo.	213	259	175	174
1 yr.	218	255	177	170
2 yr.	221	262	166	158
3 yr.	226	257	118	127
4 yr.	229	255	89	86
5 yr.	241	265	46	41
6 yr.	239	269	10	12

* Because of lack of information, the number of men shown at any stage is less than the number in the trial. In particular, there are 49 missing values for cholesterol at entry to the trial. For 44 of these men (26 on the test diet, 18 controls) values obtained while the patient was in the ward, and determined by the hospital laboratory, are available. If these are included, the mean values at entry are 268 mg. per 100 ml. for the patients in the test group and 273 mg. per 100 ml. for the controls.

Tissue-fat Biopsy

Fat-biopsy specimens were also taken from 50 patients (28 on the test diet and 22 controls). Results, which will be reported elsewhere, confirm that the tissue fat of the men on the soya-bean oil diet was less saturated than that of the controls.

Relapses and Deaths

Suspected events were classified by the review committee using the criteria listed above under "Assessment of Relapse".

We have analysed these relapses and deaths in three ways: (1) all first relapses; (2) "major" relapses, and (3) all deaths from cardiovascular causes.

TABLE IV—FIRST RELAPSES

Relapse*	Diet group	Control group
A. Definite†		
(a)	10	10
(b)	16	16
(c)	5	5
B. Probable:		
(a)	4	8
(b)	0	0
(c)	0	1
(d)†	4	0
C. Possible:		
(a)	1	4
(b)	1	0
(c)	13	18
(d)	3	4
(e)†	0	2
D. Other ischaemic cardiac death†:		
(a)	4	5
(b)	1	1
All first relapses	62	74

* See text under "Assessment of Relapse".
† Major relapse.

We emphasise first relapses because, at the start of the trial, we had agreed that the main criterion of success or failure of the diet under test would be occurrence of a first relapse, and that after this the clinician responsible for the patient was free to change the treatment. Details are given in table IV. In the event, however, little or no change was in fact made in the patient's diet after a first or later relapse, and we have therefore also considered events in later relapses.

The concept of a "major" relapse was introduced partly for comparison with the Oslo trial (Leren 1966) and also as a useful category of serious events. A major relapse is defined as a "definite relapse" (categories A[a] to A[c]) or a death in any other relapse (categories B[d], C[e], D[a] or D[b]) (table IV).

Results are shown in table V. The number of first relapses is greater, though not significantly so, in the men in the control group (74 compared with 62). The number of major first relapses (fatal or not), however, is virtually the same (39 and 40), and the excess of first relapses in the control group is entirely due to those not classified as major, which include "acquired angina" (35 compared with 22).

TABLE V—RELAPSES AND DEATHS DURING TRIAL

Category*	Test diet		Control	
No. of men	199		194	
First relapses	62		74	
Major		40		39
(a) Fatal		15		14
(b) Non-fatal definite reinfarctions		25		25
Other non-fatal relapses		22		35
Men who had a major relapse at any time during trial:		45		51
Fatal		25		25
Non-fatal definite reinfarctions		20		26
All deaths from cardiovascular disease		27†		25

* 1 death was from subarachnoid haemorrhage and 1 from cerebral embolism.
† In addition there were 7 deaths from carcinoma (6 controls and 1 in the test group). The patients concerned were excluded from the trial from the time malignant disease was diagnosed. There were no other deaths during the trial.

More men in the control group had a major relapse at some stage in the trial (51 compared with 45) but again the difference is not significant—i.e., the occurrence of a major relapse as a second or later relapse was commoner in the controls (12 compared with 5).

The total number of deaths ascribed to coronary heart-disease (i.e., fatal major relapses) was the same in each group, and the two deaths from other cardiovascular disease were in the test group, giving a total of 27 cardiovascular deaths in the test group and 25 in the controls.

The number of relapses and deaths in each group takes no account of the stage of the trial at which the relapse occurred nor the numbers at risk at the various stages. "Life-table" analysis, calculating the percentage of men "surviving" (without a relapse or alive) at each stage, based on successive numbers at risk at each stage up to that point, gives results which are in fact very similar to the crude results presented in table v. There is no difference in cardiovascular mortality, little difference in terms of all major relapses, and slightly more difference in terms of all first relapses (fig. 3). In no case is the curve for the test group significantly different from that for the control group.

Relapses and Deaths in Relation to Serum-cholesterol at Entry to the Trial

All men in the trial for whom an initial cholesterol was recorded (347) are considered in table VI. Apart from the fact that men with cholesterol levels under 225 mg. per 100 ml. seem to have suffered fewer relapses and deaths there is no general tendency for rates to be higher in the men with higher initial cholesterol levels.

Change in Serum-cholesterol in Those Who Relapsed and Those Who Did Not

The cholesterol level for controls who relapsed was consistently slightly higher than for controls who did not, but no such distinction was found in the test group. Similar results are available for deaths and major relapses. Thus, in the test group at least, the amount of cholesterol change was not related to the occurrence of death or a relapse.

Relapses and Deaths in Relation to Dietary Observance in the Test Group

The evidence on this point is conflicting. There is no difference in terms of first relapses, major relapses, or cardiovascular deaths between the 30 patients who were classified as having lapsed (either partially or completely) from full maintenance of the test diet and the remainder of the test group. Nor is there any relationship between recorded oil consumption and mortality. The first relapse rate, however, showed a consistent trend from a low rate in those who recorded 80 g. of oil or more a day to a high rate amongst the 27 men who recorded less than 60 g. a

TABLE VI—RELAPSES AND DEATHS IN RELATION TO SERUM-CHOLESTEROL LEVEL AT ENTRY (MEN IN THE TEST DIET AND CONTROLS COMBINED)

Initial serum-cholesterol (mg. per 100 ml.)	No. of men	First relapses No.	Major relapses No.	Cardiovascular deaths No.
< 225	61	16 (26%)	7 (12%)	11 (18%)
225–274	128	47 (37%)	22 (17%)	34 (27%)
275–324	98	38 (39%)	17 (18%)	27 (28%)
≥ 325	60	20 (33%)	5 (9%)	13 (22%)
Total	347	121 (35%)	51 (15%)	85 (25%)

Cholesterol was not recorded for 49 patients at their first visit. Ward values were available for 44 of these men (see footnote to table III). Inclusion of these makes no material difference to the results.

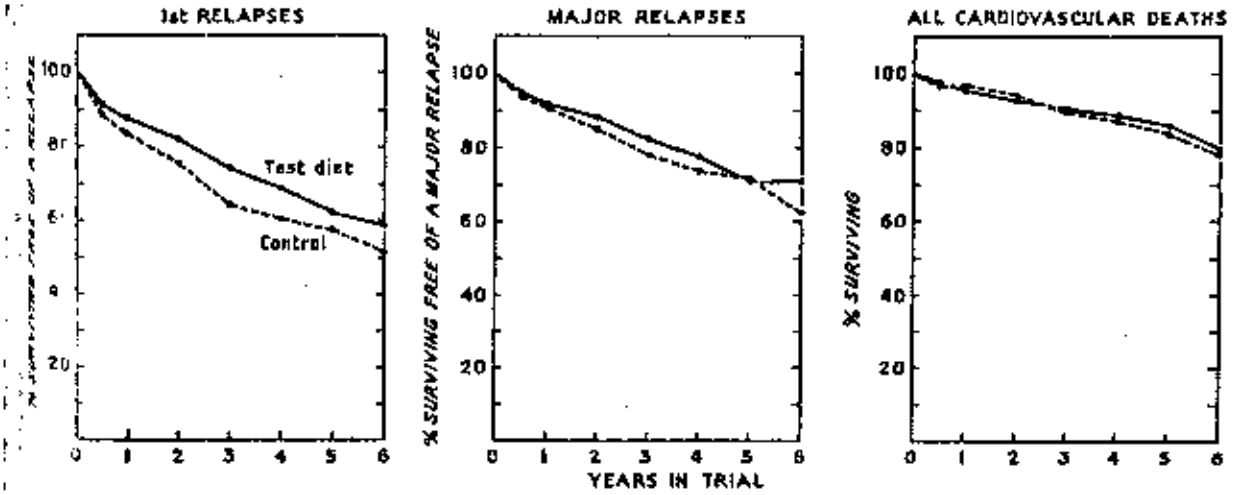

Fig. 3—Percentages free of relapse and surviving, calculated by "life-table" methods.

Numbers surviving in the trial at different stages were as follows:

Stage	Test diet	Control	Stage	Test diet	Control
6 mo.	192	186	4 yr.	91	85
1 yr.	187	185	5 yr.	48	42
2 yr.	181	172	6 yr.	8	9
3 yr.	138	128			

day. This last rate was, paradoxically, higher than in the control group.

Discussion

The design and supervision of clinical trials in men who have survived a myocardial infarct has proved difficult, and the majority of such trials are open to criticism. Oliver (1967) and the review by the standing subcommittee of the National Heart Foundation of Australia (1967) give full details both of drug trials and of dietary trials designed to lower the serum-cholesterol in order to prevent relapse in ischæmic heart-disease, and we will therefore comment on only three.

A research committee of similar composition to ours found no difference after four years in the relapse-rate or death-rate between two groups of patients who entered a trial of a low-fat diet soon after their first infarct (Research Committee 1965). Controls took their normal diet (129 men) and the test group of 123 men a low-fat diet (40 g. daily). In spite of random allocation to treatment, there was a small but significant difference in initial weight between the groups. Both control and treated groups experienced a fall in serum-cholesterol levels but this was greater in the treated group, particularly in the early stages of the trial. The mean difference in serum-cholesterol levels between the two groups was 28 mg. per 100 ml. at six months and 21 mg. per 100 ml. after two years.

Bierenbaum et al. (1967) report a five-year study in two groups of 50 men aged twenty to fifty with "coronary artery disease", who were given one of two different low-fat diets. A matched control group of 100 men with coronary-artery disease was recruited from industry. The controls had a reinfarction-rate 100% higher and a mortality-rate 233% higher than the men on the two diets. This striking result is statistically significant but difficult to assess since there was no randomisation between the diet and control groups at the start of the trial.

Leren (1966) published the results of a five-year trial of men aged under 65 in Oslo with ischæmic heart-disease using a 2300 C. diet containing 75 g. of soya-bean oil. There were 206 men on this diet and 206 randomly chosen controls who ate a normal Norwegian diet. Comparisons between the present trial and the Oslo trial are summarised in table VII. The main differences are those affecting the conditions at the beginning of the trials. For instance, the mean cholesterol levels in London were considerably lower than in Oslo (and tests have shown that this was not due to laboratory technique); the time from primary infarct to the start of the trial averaged twenty months in Oslo and five weeks in London; 14 Oslo men had had a second infarct before they entered the trial whereas none of the London men had had a reinfarction before entry: and of the Oslo men only 61% were aged under sixty at the start of the trial. There was little dietary supervision of the controls in the Oslo trial and in the test group diets taken by only 17 "especially conscientious" patients were fully analysed. The Oslo groups were well randomised apart from the starting blood-pressures which were slightly higher in the diet group. In London height was the only factor to show a significant difference.

The comparison of the results of the two trials in the men aged under sixty is also shown in the lower half of table VII. Major relapses in our trial have been compared with the sum of "sudden deaths" and "myocardial reinfarctions" found in Oslo. We have also attempted to classify the major relapses in the London trial using the Oslo criteria which (for reinfarctions) are somewhat more conservative. Thus, as table VII shows, some London reinfarctions would not be accepted as such by Leren. (The reverse is also true, but less often.)

The 6 men on the test diet and 12 controls in the Oslo trial who relapsed but did not have a major relapse were classified as "acquired angina". In contrast to the assessment of "myocardial reinfarction" in that trial, which was made in ignorance of the treatment which the patient was receiving, the assessment of acquired angina was not "blind".

The results as a whole show little difference between the trials at the ages at which they can be compared. Indeed the patterns are remarkably similar. In both, there is little difference in total cardiovascular mortality or in sudden deaths from ischæmic heart-disease, more difference in major relapses (as defined), more still in myocardial reinfarctions alone using the Oslo criteria and most in the remaining, non-major relapses. None of the differences between the men in the test diet and the controls in table VII, however, is significant with the exception of all relapses in the Oslo trial (33/47) and here, already noted, the methods of assessment in the two trials are not comparable. The overall relapse and death rates in the two trials are remarkably similar considering the different exposure-times and the fact that the Oslo criteria cannot be exactly applied to the London data.

The possibility must therefore be considered that the results of both trials are consistent in showing no effect on mortality but some difference in the rate of reinfarction and less serious relapses such as acquired angina. Neither trial is large enough to make even an approximate estimate

TABLE VII—COMPARISON OF LONDON AND OSLO TRIALS

Factor	London		Oslo	
No. of men in trial	393		412	
Aged under 60 at entry	390* (99%)		257 (61%)	
Time from first infarct to entry	12 to 90 days (average 36 days)		13 to 24 mo. (average 20 mo.)	
Second infarction before entry	0		14 (3%)	
Initial cholesterol level (mg. per 100 ml.)				
(a) test diet	272		296†	
(b) control	273		290†	
Soya-bean oil in test diet (g./day)	85		75‡	
Saturated/polyunsaturated ratio in test diet	1:1·8		1:2·4‡	
Time from entry to trial to maximum cholesterol reduction	6 mo. (22% reduction)		3 yr. 3 mo. (21% reduction)	
Period of observation	2-6½ yr.		5 yr.	
Frequency of outpatient visits	3 monthly		6 monthly	
	Test diet	Control	Test diet	Control
Results for men aged under 60:				
No. of men	199	194	134	123
Relapsed at any time during trial	62	74	33§	47§
Major relapse at any time‖	45	51	27	35
Myocardial reinfarction (Oslo criteria)	30	38	17	27
Myocardial reinfarction (London but not Oslo criteria)	7	4	N.A.	N.A.
Sudden death (Oslo criteria)	8	9	10	8
Relapsed, but no major relapse at any time¶	17	23	6	12
All deaths from cardiovascular disease	27	25	18	23

* Three men had their 60th birthday between their first infarct and entry to the trial.

† At all ages; at ages under 60 corresponding figures were 299 and 302 (Leren, personal communication).

‡ Based on diets from 17 "especially conscientious" patients.

§ The only significant difference (at the 5% level) between diet and control groups in this part of the table is in those who relapsed at any time in the Oslo trial ($p = 0.027$).

‖ In London, as defined in text; in Oslo, myocardial reinfarctions and sudden death.

¶ In London, probable relapse, *B.a)-B.c)*, or possible relapse, *C(a) C(b)*; see table IV; in Oslo "acquired angina".

of the size of these benefits. The London trial was designed to detect or exclude with sufficient assurance a 50% reduction in all relapses. This it has done: there was no such reduction. But its results are nevertheless consistent with the possibility of a smaller reduction. A trial to detect or exclude, say, a 25% reduction in all relapses would have had to be about four times as large, and this would have been beyond our resources. Even combining the results of the two trials (which is of doubtful validity) can only produce estimates with large margins of error. For myocardial reinfarction alone, which, combining the two trials at ages under sixty, does show a significant difference ($p < 0.05$), a best estimate of 31% reduction is obtained. Using conventional 95% confidence limits for a difference in proportions, limits as wide apart as 53% and 2% can be roughly estimated for the reduction (i.e., it is possible to be reasonably sure only that the reduction in myocardial reinfarction does not lie outside these limits). For all major events together the best estimate is a 20% reduction, but the corresponding limits are 40% reduction and 5% increase in the risk. To examine myocardial reinfarction alone is reasonable since there are theoretical grounds for supposing that this, rather than death due to electrical instability, is the type of relapse which the diet under test might be expected to prevent. Moreover, in the Oslo trial, the same benefit occurs in men aged over sixty and, in that trial, the difference between the test group and the controls in myocardial reinfarction at all ages is statistically significant.

Not shown in table VII are the relationships between initial cholesterol and relapses in the two trials. Leren (1966) found that initial cholesterol was highly related to subsequent relapses, the higher the cholesterol at the start of the trial the higher the relapse rate. In our trial no such picture emerged (table VI), possibly due to the lower initial cholesterol levels in the men, and the amount of cholesterol change was not related to death or to relapse.

The place of our trial in the various published attempts to prevent relapse by lowering serum-cholesterol using dietary means can now be seen. There could be several factors that influenced the result.

In the first place there could have been deficiencies in the experimental design. The two groups were successfully randomised at the start of the trial (table I) except for height, the controls being significantly shorter. It is unlikely that this masked any effect the diet might have had; rather the reverse, since there is evidence (Morris et al. 1966) that short people are more likely to develop coronary heart-disease. Indeed, in this trial the shorter men had a slightly higher total relapse-rate.

The trial itself was not and could not be "blind". The staff and the patients knew the treatment and there may have been room for bias in the registrars' selection of incidents for consideration by the review committee. To test this, an independent assessment of all the serial E.C.G.'s of the 257 patients who did not relapse (table V) was carried out "blind" by a cardiologist, Dr. John Robinson of the London Hospital, not connected with the trial, who used his own criteria of assessment. He was not told initially of the reasons for his analysis, and at no time were details of the patient's history, diet or clinical investigations made available to him. This check revealed (in Dr. Robinson's opinion) 7 fresh Q waves not previously identified in 137 patients in the test group and 4 fresh Q waves in 120 in the control group. On review of the records there was no note of symptoms in any of these incidents. These results do not suggest any material bias in bringing cases before the review committee.

The excess of control patients in our trial with nothing but non-fatal, non-major relapses (23 against 17) though not statistically significant is also interesting in this connection. One possibility is that the men in the control group themselves may have felt that they were having less active treatment, and might have been psychologically motivated both to complain more and more readily of cardiac symptoms. The other possibility, of course, is that the diet may have had a real effect in these cases of minor ischæmia.

The diet under test proved effective in reducing serum-cholesterol. Using the formula of Keys et al. (1965) it was calculated that the fall in serum-cholesterol should theoretically have been 55 mg. per 100 ml. In fact the maximum observed fall in the diet group (at six months) was 59 mg. per 100 ml.

Dietary cholesterol reduction such as occurred in the men in the diet group, may have contributed towards reducing the serum cholesterol level. What effect other changes had, for instance a lower intake of vitamin A (1000 I.U. lower in the men on the test diet) and of calcium (122 mg. lower in the men on the test diet), is unknown.

In practical terms, although the men in this trial had suffered a recent infarct and were highly motivated to cooperate fully, some found the diet rigorous and not easy to adapt to life in the office or factory. The idea that a diet with an even greater ratio of poly-unsaturated fats to saturated fats would lower the serum-cholesterol further and therefore possibly act as a better secondary preventive of reinfarction is not easy to justify on practical quite apart from theoretical grounds, at any rate as a routine measure.

Indeed, the results of this trial alone lend little support to this suggestion or to the suggestion that a diet of the kind used should be recommended in the treatment of patients who have suffered a myocardial infarction. Taken together with the results of the Oslo trial there is no indication that this type of diet affects mortality. The combined evidence, however, suggests that a proportion of non-fatal reinfarctions might be prevented, though more evidence is required to confirm this effect, and to make a useful estimate of its size.

It must be emphasised, finally, that these results apply to secondary prevention, that is to the treatment of men who have had an attack of coronary heart-disease. The importance of such diets in primary prevention remains to be assessed.

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Requests for reprints should be addressed to J. A. H., Social Medicine Research Unit, London School Hygiene and Tropical Medicine, Keppel Street, London W.C.1.

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Damage to Proteins, Enzymes, and Amino Acids by Peroxidizing Lipids

W. T. ROUBAL[1] AND A. L. TAPPEL

Department of Food Science and Technology, University of California, and Bureau of Commercial Fisheries, Davis, California

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Transient free-radicals are produced in peroxidizing lipid protein reaction systems. The pattern of damage to proteins, induced by these radicals, is similar to that observed in the case of radiation damage; proteins and enzymes lose solubility and constituent amino acids are destroyed. Lipid peroxidation damage appears to be about one-tenth as effective as radiation damage. Amino acid destruction was measured in lipid-peroxidation damaged γ-globulin, catalase, serum albumin, hemoglobin, and and ovalbumin. Among the most labile amino acids are methionine, histidine, cystine, and lysine. Major products of lipid peroxidation-cystine interaction are hydrogen sulfide and cystine.

Transient free-radicals, generated in peroxidizing unsaturated lipid-protein mixtures, participate in the chain of reactions leading to considerable damage. Chemical evidence for damage to cytochrome c has been reported (1). A recent paper describes the mechanism for lipid peroxidation induced free-radical polymerization of proteins and enzymes (2); it was also shown that soluble polymeric materials are produced in an irradiated cytochrome c solution.

This paper reports evidence for the radiomimetic effects of lipid peroxidation intermediates to proteins and amino acids.

MATERIALS AND METHODS

Experiments conditions of the reaction systems have been given (2); ethyl arachidonate was used unless otherwise indicated. Proteins and enzymes used in experiments on yield of insoluble protein were: trypsin (2X-crystallized, Mann), pepsin (2X-crystallized, Mann), α-chymotrypsin (3X-crystallized, Mann), ovalbumin (crystalline, Nutritional Biochemicals Corp.), and hemoglobin (2X-crystallized, Sigma). In order to initiate and maintain peroxidation in reactions containing nonmetalloproteins, the buffer employed was 0.05 *M*, pH 7.0 phosphate containing 10^{-3} *M* ascorbate and 10^{-5} *M* copper sulfate. Control reactions were run in lipid-free systems at 37°.

In studies of lipid peroxidation damage to constituent amino acids, the proteins and enzymes were: γ-globulin (Bovine, Calbiochem), catalase (Crude, Sigma), bovine serum albumin (Crystalline, Pentex, Inc.), hemoglobin and ovalbumin of the purity and source given above. Lipid peroxidation was allowed to proceed until sufficient protein was insolubilized; insoluble proteins were extracted and stored as previously described (2). Amino acid analyses of the acid hydrolyzed insoluble protein were made either with a Beckman or Technicon automatic amino acid analyzer.

In studies of peroxidation damage to cysteine, a reaction system composed of 1 gm of L-cysteine, 1 gm of ethyl arachidonate, and 5 ml of buffer was employed. Hydrogen sulfide was measured by the method of Marbach and Doty (3). Nonvolatile cysteine degradation products were characterized by thin-layer chromatography using silica gel G plates and a migrating solvent composed of phenol-acetic acid-water (70 wt.:10 vol.:20 vol.). Spots were detected with the polychromatic ninhydrin spray devised by Moffat and Lyle (4).

RESULTS

Expression of protein insolubilized per mole of peroxy radical in Table I is appro-

[1] Present address: U. S. Bureau of Commercial Fisheries, Technological Laboratory, 2725 Montlake Blvd., Seattle, Washington 98102.

priate because it allows a comparison with protein damage by ionizing radiation where product formation per free radical is given as ionic yield. Peroxy radicals are approximately equal to oxygen reacted in this well known lipid peroxidation mechanism involving free-radical intermediates.

Table II gives the percentage loss for each amino acid in reacted protein or enzyme. Extent of oxidation was 2.5, 1.6, 1.9, 1.9, and 1.6 moles of oxygen per mole of lipid for γ-globulin, catalase, bovine serum albumin, hemoglobin, and ovalbumin reaction mixtures, respectively. Lysine, histidine, tyrosine, methionine, and cystine, in descending order, are the most labile amino acids in γ-globulin; in catalase, lysine, serine, valine, methionine, and histidine are the most labile amino acids. Glycine, cystine, histidine, alanine, and valine are labile in bovine serum albumin while tyrosine, methionine, lysine, and histidine are labile in hemoglobin. Finally, methionine, histidine, threonine, proline, and glycine are the most labile amino acids in ovalbumin.

TABLE I

PROTEIN INSOLUBILIZED BY PEROXIDIZING LIPID

Protein	Moles O/mole lipid	Yield (moles protein insolubilized/mole peroxy radical)
Trypsin	0.28	0.012
Pepsin	0.15	0.017
Ovalbumin	0.29	0.0067
α-Chymotrypsin	0.22	0.0025
Cytochrome c	0.25	0.018
Hemoglobin	1.03	0.0021

[a] Lipid consisted of a mixed ethyl ester containing 73% docosahexaenoate and 17% cis-sapentaenoate.

Peroxidizing arachidonate-cysteine interaction leads to sulfhydryl cleavage with the production of hydrogen sulfide. The data of Fig. 1 show that H_2S production increased in a linear fashion with extent of lipid peroxidation. Of the nonvolatile products of lipid-cysteine interaction, only cystine and a trace of alanine could be detected on thin-layer chromatograms (Fig. 2). Both cystine and alanine would be expected to react further to yield both ninhydrin-positive and ninhydrin-negative products and could account for the low yield of alanine at the termination of the experiment. However, no intermediate oxidation products of cysteine or cystine, based on spot color, could be identified. No in-

TABLE II

LIPID PEROXIDATION DAMAGE TO AMINO ACIDS OF PROTEINS

Amino acid	% Amino acid loss				
	γ-Globulin	Catalase	Bovine serum albumin	Ovalbumin	Hemoglobin
Lysine	58.8	42.4	30.8	21.6	58.6
Histidine	51.8	18.2	51.1	38.3	57.6
Arginine	26.5	8.2	11.5	Slight damage	24.1
Aspartic acid	11.3	0	39.8	0	7.2
Threonine	11.8	10.9	46.2	28.0	37.7
Serine	24.4	22.4	43.2	25.0	43.0
Glutamic acid	24.1	[a]	11.0	25.0	39.0
Proline	40.0		Slight damage	27.9	38.8
Glycine	21.2	12.0	82.5	27.6	53.8
Alanine	18.8	10.5	50.0	24.5	33.7
½ Cystine	52.8	[a]	64.0	[a]	[a]
Valine	21.0	21.1	47.7	25.8	31.3
Methionine	38.3	21.3	47.5	80.3	58.5
Isoleucine	20.0	12.3	12.8	21.4	[a]
Leucine	22.2	2.6	34.1	5.4	32.8
Tyrosine	50.7	10.5	15.2	8.5	91.0
Phenylalanine	32.0	6.8	11.1	20.9	36.4

[a] Amino acid analyses were not suitable for an accurate measurement of loss.

[b] These amino acids are only present in small amounts in the protein.

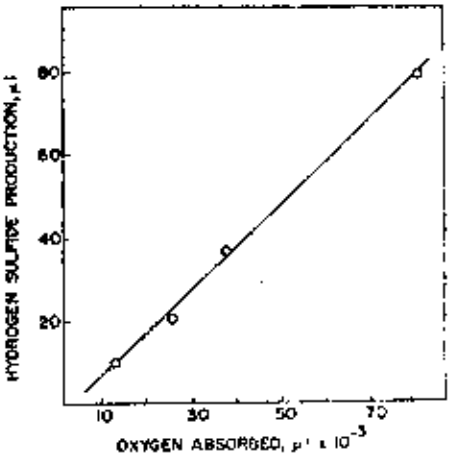

FIG. 1. Formation of hydrogen sulfide as a function of oxygen absorption in a cysteine-arachidonate system.

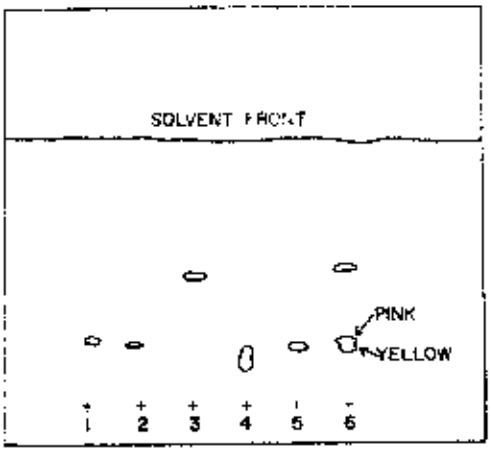

FIG. 2. Thin-layer chromatography of cysteine-arachidonate reaction products.

		R_f	Color
1	Cysteine standard	0.28	Pink
2	Cystine standard	0.27	Yellow
3	Alanine standard	0.50	Pink
4	L-Cysteic acid standard	0.22	Blue
5	L-Cysteine sulfinic acid standard	0.27	Blue
6	Aqueous cysteine-arachidonate reaction products	0.20 (pink and yellow), 0.57 (pink)	

solubilized material was produced even under prolonged exposure of reaction mixture to high oxygen tension; tests for sulfate and free sulfur were negative.

DISCUSSION

Comparison of lipid peroxidation damage with that of ionizing radiation is appropriate because both reactions involve free-radical intermediates and because analogous information is available from studies of radiation damage to proteins and cysteine. Results of lipid peroxidation damage in Table I can be compared to radiation damage to proteins. Yield values of protein damaged per ion pair are 0.05, 0.03, 0.10, and 0.48 for invertase, catalase, cytochrome c, and ribonuclease, respectively (5). Another yield value is 0.20 for trypsin (6). Lipid peroxidation damage is less than radiation damage, an inequality caused by the biphasic lipid reaction system; many of the peroxy free-radicals are prevented from reacting with protein. Since both radiation and lipid peroxidation give rise to free-radical intermediates, it does not seem surprising that both types of damage should show similarities; Haissinsky has indicated that both ionizing radiation and lipid peroxidation may have a similar mechanism of damage at the molecular level (7).

The relationship between radiation and peroxidation damage is also able to explain the observation that the over-all pattern of amino acid damage in proteins is similar in both processes. In irradiated ovalbumin, histidine, cystine, methionine, phenylalanine, and threonine suffer greatest damage (8). Irradiation studies with hemoglobin show the most radiolabile amino acids to be methionine, histidine, threonine, tyrosine, and phenylalanine (8). Likewise, cystine, methionine, phenylalanine, histidine, and tyrosine are the most radiolabile amino acids of catalase (9).

Irradiated proteins often show an increased alanine content with increase in dose; however, the content of this amino acid drops at still higher dosage and remaining amino acids suffer increased destruction. Studies of lipid peroxidation damage to proteins have shown that there are no increases in alanine. Furthermore, α-amino-α-butyric acid, produced by cleavage of the terminal CH_3S group from methionine, has not been detected. Evidently, the magnitude of peroxidation damage was sufficient to destroy these primary damage products.

Hydrogen abstraction reactions, initiated by a variety of free radical intermediates would account for disulfide production:

$$\left.\begin{matrix}ROO\cdot\\ R\cdot\\ RO\cdot\\ HO\cdot\end{matrix}\right\} + R'SH \rightarrow R'S\cdot + \left\{\begin{matrix}ROOH\\ RH\\ ROH\\ H_2O\end{matrix}\right.$$

followed by $R'S\cdot + R'S\cdot \rightarrow R'SSR'$. Free-radical cleavage of the R—SH bond would lead ultimately to H_2S production. The radiolability of sulfur amino acids is well documented (10, 11).

The lability of histidine is of interest. Data of Table II show this amino acid to be labile in most of the examples given. Histidine acts prooxidatively to initiate a rapid uptake of oxygen in arachidonate emulsions with the formation of soluble polymeric products which are Folin reagent-positive (2). Recently, Saunders and Hampson have shown that the prooxidative effect of histidine in methyl linolenate emulsions appears to be associated with both the imidazole and amino groups (12). In the presence of activators, the histidine complement of β-casein is photolabile (13).

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Time factors in obesity[1]

Humphrey F. Sassoon,[2] *Ph.D.*

Many fat people are convinced they do not overeat and in fact may consume 500 kcal/day less than comparable persons who stay lean (1). To reckon by calories alone is too simplistic because the corollary, that calories stored must be surplus to requirements, is demonstrably false under certain conditions. It is suggested that these conditions can be rationalized in terms of time factors.

Convertibility of calories to body fat differs according to dietary source, intake pattern, metabolic adaptations, and other conditions that are variable, unstable, and hard to summate. "Threshold intakes," defined as supplying the largest number of calories that can be handled per day without storage of fat, obviously vary by over 500 kcal/day. Thus, a low threshold intake may supply fewer calories than are in fact required for expenditure, so that to remain comfortable at his desired level of activity, a person must either accumulate fat or raise his threshold intake by changing his diet, eating habits, and resultant metabolic adaptations. This proposition will be amplified in terms of time factors, which operate mechanistically in most of the above variables and so qualify time to be a practical common measure of comparison. Priorities will be discussed rather than actual time measurements because the priorities tend to be more stable. For simplicity, the present argument is directed only to intakes at or below threshold amounts.

Time factors interact but for convenience they are discussed under three heads: *a*) those governing availability of dietary calories, i.e., how often, how soon, and at what rate calorifers[3] are delivered to the plasma; *b*) those governing metabolism, including uptake from the plasma, storage and retrieval from stores, and diet-induced adaptations; and *c*) longer range time factors.

Availability

Meal frequency affects the propensity to store calories. Fábry and co-workers (2) reported more obesity-related disorders with three or fewer meals per day than with five or more, and Pawan (1) noted more obesity with two meals per day than with four or more. Many people eat six unequal meals per day: breakfast, midmorning, lunch, midafternoon, supper, and a night snack. The fewer the meals per day, the longer the intervals between them. The longest interval counts most, for meal-frequency experiments merge into fasting–refeeding experiments, and even a 16-hr overnight fast enhanced the lipogenic adaptation of rats (3). When protein was not a calorifer, such adaptations were found to be wholly carbohydrate dependent (4). Such adaptations tend to create a one-way flow, by channeling calories from the diet into the fat store within a diurnal cycle and preventing equivalent mobilization within that same cycle (see the section on longer range factors below).

After a mixed meal, the bulk of the lipids may be released from the stomach up to approximately 4 hr after most of the original sugars; if the meal was balanced, the order of release (of median amounts) may be: sugars, digesta of animal proteins, vegetable proteins that were encapsulated in cellulose, starches, and fats. The later the release, the more gradual it may be.

Transit through the small gut may further separate carbohydrates and amino acids from

[1] From the Life Sciences Research Office, Federation of American Societies for Experimental Biology, 9650 Rockville Pike, Bethesda, Maryland 20014.

[2] Formerly, Research Associate.

[3] Literally "energy-carriers," a convenient word to describe a property that is transferred from the original nutrients to their successive metabolites until it is spent.

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lipids by 20 to 60 min. Problems with fructose start here; large amounts given experimentally on an empty stomach, or any amounts hydrolyzed from sucrose at the duodenal brush border, are absorbed as well and as quickly as glucose (5, 6). This, however, does not entirely dismiss the old view that small amounts of fructose, e.g., released gradually from vegetables, that are mixed with other digesta may be absorbed poorly or slowly. On the other hand, in North America today, large intakes of sucrose are virtually unavoidable owing to its use as a universal preservative (7).

Lipids over 12-carbon chain length (milk is virtually the only source of shorter chain lipids) are still further separated in time from other absorpta because they tend to enter the lymph, and the bulk of them take many hours to filter gradually into the circulation. In Table 1, some foods and nutrients usually present in a balanced meal are listed roughly according to how soon and at what flow rate they deliver calorifers to the portal plasma and so become available for use or storage. Recurrent diurnal alternations of storage and hunger obviously are less severe when these calorifers become available gradually or in sequence than when they enter the plasma in surges and together. A physiological role of digestion and absorption is to smooth out irregularities of intake and so protect against storage of more calories than can be retrieved diurnally from the rapidly mobilizable body pools. Rapidly available calorifers evade this mechanism. Large amounts of rapidly available carbohydrates also evade glucose-operated satiety mechanisms. When rats were fed single energy diets ad libitum, they consumed 33% more calories of fat diet than of sucrose diet (8, 9); stomachs were continually distended with the fat diet but not with the sucrose one, indicating satiety effects of sucrose (9) which, as further discussion will indicate, gave little physiological protection.

TABLE 1

Availability of nutrients ranked according to time factors[a]

Food source	Digestion[b]		Absorption[c]		Availability[d]		Total ranking[e]
	Rapidity	Rate	Rapidity	Rate	Rapidity	Rate	
Protein							
Animal	3	2	1	1	4	3	7
Vegetable	3	3	1	1	4	4	8
Carbohydrates							
Starches	3	4	1	1	4	5	9
Glucose	1	1	1	1	2	2	4
Sucrose	1	1	1	1	2	2	4
Fructose	1	1	1	1	2	2	4
Vegetable Fructose	2	3	3	2	5	5	10
Fat							
Under 12-C chains	3	5	2	2	5	7	12
Over 12-C chains	4	5	4	3	8	8	16

[a] The numbers in columns 2 to 8 are rankings, not time units, so items in a column scored 1 are judged equal first, and so on. [b] Digestion is from ingestion to release from stomach. Items scored 1 for rapidity are dissolved on ingestion; scored 2 are dissolved on release from cells; scored 3 or more are chemically altered. Rate scores reflect time spread; the greater the spread, the smaller the volume of flow. [c] Absorption is from stomach release to appearance in the plasma. Items scored 1 for rapidity are absorbed mostly at the duodenum and go directly into the plasma. [d] Availability columns summate for rapidity and rate, respectively. The relative starts and durations of flow of nutrients from a mixed diet into the plasma should give a perspective for assembling real time measurements. [e] Total ranking summates the availability columns. The smaller the score, the more sudden and meal related is the flow of calories into the plasma.

Uptake and metabolism

The three relevant "energy" pools are plasma glucose, cell (mainly liver and muscle) glycogen, and body fat. The plasma supplies glucose for use at the rate of approximately 180 g/day (720 kcal), 80% to the brain, but contains only approximately 3 to 5 g (60 to 100 mg/100 ml) at any one time. Thus, the plasma must receive an average 7.5 g glucose/hr. This can be supplied either endogenously by continuous gluconeogenesis (GNG) regulated by the lower limit of plasma glucose (e.g., 60 mg/100 ml) in which case inflow never significantly exceeds outflow, or from the diet in which case inflow periodically exceeds outflow and the plasma glucose is then controlled at its upper limit (e.g., 100 mg/100 ml) by storage of the excess.

This use of the upper limit seems to be the troublemaker.

Cells accumulate monosaccharides, either by uptake from the plasma or by GNG following uptake of excess amino acids, and store them as glycogen; this pool is rapidly entered, quickly mobilized, and finite. A usual body total with modern diet is approximately 150 g or 600 kcal (5), with a probable upper limit of nearly 250 g, or less than can accumulate from one normal meal. In rats fed diets supplying no available glucose, liver glycogen levels become stable at 10 to 20% of their upper limit; then if carbohydrate is fed, the glycogen level rises, and evidence suggests that this initiates rapid glycolysis (GL) and adaptive induction of GL pathway enzymes (8, 9). In these rats, the first stage of the induction occurs between 4 and 8 hr after the carbohydrate meal but in rats prefed carbohydrate diets, there is an important difference; if they are fasted and then given a meal containing sucrose, initiation of induction resulting in additional GL capacity may take only 1 to 2 hr (8). This mechanism clearly deserves further study in relation to meal intervals.

If a man uses his plasma glucose at a rate of 8 g/hr for 1 hr, at most 10 g of dietary glucose can enter the plasma during that hour without raising his liver glycogen level. If much more becomes available, fat will be synthesized, and if that fat leaves the liver some of it may be used by the muscles but most of it will rapidly enter the fat store. Furthermore, if the plasma regularly receives each day more glucose than is expended (ca. 180 g, 720 kcal), the excess must be converted into fat.

The capacity of the body fat store is virtually infinite, but mobilization is relatively slow. Dietary lipids become available gradually and, as a sole source of calories, they avoid the need for short-term storage; also, fat mobilization is no immediate problem when the liver is switched to continuous GNG (see Cahill's pathways in (10)). However, with mixed diets, the bulk of blood lipids come from dietary carbohydrates by GL in the liver (11). At times when these lipids are entering the fat store rapidly, a switch to mobilization of fat (e.g., after carbohydrate deprivation) is delayed well beyond one diurnal cycle by the need to reverse GL enzyme adaptations in the liver; in man, the delay may be several days (5). This contrast of adaptation times is seen also in muscle in which adaptation to surplus nutrients, mainly FFA, is measured in minutes, but to deprivation in hours (12). Plasma FFA flow is approximately 160 g/day and FFA half-life in plasma is 2 to 4 min (10).

The carbohydrate pathways of liver cells (kidney contributes at most 10% (10)) usually are switched either to GNG or to GL. GNG seems to be the physiologically normal state, and GL a tachyphylactic response because *1*) body cells accept glucose from the outside only (or in liver more readily) when stimulated from the outside and not always then; *2*) enzymes rate limiting for GNG are structural but for GL are cytoplasmic and far less stable; *3*) binding of insulin to the cell wall organizes ribosomes as for protein synthesis rapidly (13); and *4*) subsequent GL enzyme increases appear to be substrate induced and start to revert promptly on disappearance of substrate (8, 9). Thus, GL, in terms of cellular physiology, may possibly be a detoxification mechanism. The liver also can be switched to uptake or release of glucose by its "substrate cycle" independently of external factors (14). The pivotal metabolite seems to be glucose-6-phosphate (G-6-P), which in GNG is converted enzymatically to glucose, or in GL to glycogen, gluconolactone-6-P and fructose-6-P, so that in GL there are at least three simultaneous pathways for G-6-P turnover, separately rate limited by inducible enzymes. Despite these complications, 2-deoxyglucose studies suggest that the currently maximal rate of cellular G-6-P turnover limits the rate of glucose uptake by the cells absolutely and regardless of extra insulin (8).

Diet-derived fructose is taken up preferentially by many tissues, and insulin is neither influenced nor required, even by adipocytes (5). Most of the fructose not taken up by the gut wall and portal red cells is scavenged by the liver (11), though less effectively after a sucrose meal (15). In liver cells fructose is first converted, again pref-

erentially over glucose, to glycogen resulting in GL, evidenced by more rapid enzyme inductions (16), but as fructose accumulates in the cell it is also converted directly to triose and so to lipids (5), and this presumably retards G-6-P turnover by feedback. These lipids return to the plasma more so in men than in young women (15). Such findings confirm and amplify some old reports (15, 17, 18), and seem even more like detoxification. In addition, the body beneficially uses no exogenous fructose for the small amounts in tissues such as lens, seminal vesicles, and red cells have been found to be endogenous by way of polyols (15, 19).

Meanwhile, glucose probably must accumulate in the plasma, its uptake being further inhibited by metabolic lipids (20); if so, it will continue to stimulate insulin release, probably via islet-cell G-6-P (21–23). As the fructose block clears, the extra insulin should accelerate both lipid storage (24) and glucose uptake, so that a glucose tolerance test might reveal nothing unusual. Alternatively, there might be a syndrome of glucose intolerance and insulin resistance. Such an explanation would be consistent with a number of reports (25–27), but it requires experimental verification.

During food deprivation, the plasma is supplied with glucose by GNG from amino acids initially and the hormonal picture is quasi-diabetic (28). The fact that GL enzyme levels are higher when meals are fewer has been related to the hormone picture (29), as well as to accelerated induction (9); and a permissive effect of the direction of amino acid flow also seems inferrable. Evidently, the same mechanisms respond when intracellular amino acid pools are saturated by too much dietary protein. That this metabolic glucose induces GL enzyme increases is clear from the data of Szepesi and Freedland (30) in rats fed 90% protein diets. This may be the only situation in which a cell is switched to both GNG and GL at the same time, and also the only situation in which it accumulates great excesses of glucose independently of insulin. Thus, if the primary stimulus to enhancement of fat stores is the recurrent flooding of the liver with high concentrations of monosaccharides (11), nothing should do this more efficiently and rapidly than a typical athlete's diet of animal proteins and sucrose eaten twice a day.

Another effect of "rapidly available" diets may be "functional hypoglycemia" between meals (31) or at least hypothalamic discomfort. If the top of the 60- to 100-mg/100 ml plasma glucose range is taken as normal and used in the calculation of daily caloric requirements, such "requirements" will be exaggerated. Any glucose that now becomes available from the diet will be especially likely to be taken up and converted to fat because of the axiom that reactions occur when the conditions for them are present.

The summation columns of Table 1 together with the metabolic time factors suggest that, calories being equal, some diets conduce less than others to flooding of the plasma with combinations of calorifers. For example, glucose from dietary sugars seems unlikely to connect significantly with lipids from the same meal. Glucose from starch may connect with such lipids, but both flow relatively slowly. On the other hand, glucose from starch may connect with fast-flowing metabolic lipids derived either from accompanying sugars or from excessive proteins that also permit GL capacity to maximize; there has been a suggestion that such glucose may potentiate lipid deposition (32). By contrast, low protein, permitting less expansion of GL capacity (16), should produce a slower and smaller flow of metabolic lipids, though perhaps more prolonged. If time factors act so, there is a case for their further study with regard to "balance" in diet composition.

So far as social competition demands maximal utilization of diets, food intakes of those who compete always will approach thresholds for obesity. When work emphasis shifts from muscle effort to brain effort, there is a corresponding shift of importance from fatty acid availability to glucose availability. Such trends have been reflected in diet changes with the result that caloric intakes have diminished and obesity problems have increased. Our reliance on animal production models has been partly blamed for this; objectives such as rapid growth, body products, and slaughter at puberty, may be

inconsistent with health and longevity of adults. Thus it is clear that longer range time factors are also involved.

Longer range time factors

Dietary fats and carbohydrates are well-known to affect utilization of dietary protein in different ways that are hard to study separately with mixed energy diets, so that long-term studies using single energy diets have been advocated (27). This additional precision is useful only when there is equal precision in the design of experiments.

For example, when weanling rats were fed virtually single energy diets, carbohydrate, or fat for 22 days, the fat was judged wasteful of protein on the basis of carcass composition (17% less protein/rat), elevated GNG enzyme levels, and presumed diversion of proteins to glucose (33). On the other hand, nitrogen balances and GL enzyme determinations in adult rats fed single energy diets for 13 days showed that fat gave 35% better protein economy and 44% better growth per calorie of intake than sucrose, which also greatly elevated the GL enzyme activities (9). When single energy diets were fed for 12 weeks to unprotected weanling rats, mortality from respiratory infection in the fat-fed groups was repeatedly 10 to 14% of that in the sucrose-fed groups (34).

These data are all in agreement because the GNG enzymes in question are structural, whereas the GL enzymes are cytoplasmic. Thus, the GL enzymes should turn over faster when activities are high; evidence for this was revealed by the nitrogen balances but not by the intakes and carcass compositions alone. The long-term study suggested that GNG from protein, rather than from fat, probably was important only during the relatively slow adaptation to a carbohydrate-free diet. It can be inferred that the GNG state was not merely normal but also advantageous, compared with the GL state. The relatively slow availability of calories from dietary fats probably was also advantageous. However, such advantages may disappear when even small amounts of rapidly available calorifers enter the diet, unless all the time factors have been taken into consideration. These are hypotheses that can be tested experimentally if controls are precise enough.

Adipose tissue has approximately 2% of the GL activity of the liver by weight, but equal activity by protein content (35). By calculation, a burden of 150 lb (67 kg) of adipose tissue may contain as much GL capacity as a liver. The capacity to sequester plasma monosaccharides and amino acids must increase with cellularity, which in fat people may be multiplied threefold (27). Because multiplication of such cellularity usually stops at approximately age 6, early childhood obesity should and does conduce greatly to intractable adult obesity (1, 36). Vascularity may be an additional factor; weight reduction removes stored fat, but the cells and vessels remain, thus conserving the full capacity of the tissue for uptake and metabolism. Therefore, in the foregoing example up to one-half of the glucose and amino acids entering the plasma from the diet might continue to be sequestered. Surprisingly, this factor seems to have escaped quantitative study.

It would be wrong to shirk the final question, whether dietary carbohydrate is a physiological requirement or merely a cultural, economic, and ecological necessity. This discussion has suggested the latter (and why do we use the word "tolerance"?); also, ruminants have evolved to what amounts to substantial (90%) protection against entry of diet-derived glucose into their plasma (37, 38). Rats thrive on a carbohydrate-free diet (4, 9), and this raises the possibility for future study that ketosis may be merely a transient problem of carbohydrate withdrawal. Relative time factors, important in evaluating mixed energy diets, may permit man to consume slowly available starches in some time and quantity patterns without unphysiological storage, or at least without notable pathological results. This is borne out by the increasing frequency of simple obesity along with descending status in the cultural and socioeconomic scales (39), contrasted with the well-known increase of obesity-related complications (such as atherosclerosis) in the upward direction. One is tempted to try to relate this to animal protein intakes, but of course, there are other important influences including heredity. For example, a

genotype that would maintain relatively high carbohydrate stores on a sparse diet of gradually available calorifers would enhance the stamina of people in a "primitive" state, but on their transfer to modern diets and meal patterns, would logically conduce to low glucose tolerance. This "thrifty genotype" hypothesis was first proposed by Neel (40) and seems now to be accepted (25, 26). North American instances appear to exist among Indian tribes such as the Pima (41) and the Tarahumara (Sassoon, Watson, Diecker and Sassoon, unpublished data). Laboratory studies (8) indicate that transcription of fresh RNA from the gene is a required first step for adaptive increases of a key glycolytic enzyme induced by dietary carbohydrate.

Atherosclerosis in man is a gradual disorder now recognized (15) as closely related to diets of rapidly available calorifers. Its somewhat accelerated appearance in athletes (42) may be partly due to high protein diets, as discussed above. But experiments that produce it rapidly in short-lived and normally resistant laboratory animals may be largely irrelevant to the human disorder.

Summary and conclusions

Evaluation of major nutrients in terms of time factors has suggested that some modern diets and daily meal patterns may require consumption of calories that are stored, in order to avoid periodical deficits. This suggestion is centered on availability of calorifers in the plasma, whether from diet or from metabolic sources.

Gradually available calorifers from dietary fats (and some starches) seldom lead to irregular storage. Rapidly available calorifers (e.g., sugars) tend to surge into the plasma, evading protective barriers of digestion, absorption, and satiety, and then to surge in again as metabolic fats. When plasma glucose is supplied entirely from cellular gluconeogenesis, its level is regulated at a lower limit; when it is supplied from diet, the level is regulated at an upper limit by uptake and storage mechanisms. These involve enzyme adaptations that promote rapid storage of fat and delay its mobilization; the adaptations are inferred to resemble tachyphylactic responses by cells that are stimulated, by insulin, to take up glucose. Fructose and glucose that is elaborated intracellularly from amino acids evade the insulin barrier. Intervals between carbohydrate containing meals that elicit a fasting state may accelerate the enzyme adaptations so that they are reinforced after every meal. Thus, a synergism seems to arise between a rapid availability of calorifers, an increased rate of metabolism, and an adaptation for quick storage of fat. It is inferred that under such conditions, the longer the intervals between meals, the smaller will be the threshold daily caloric intakes for obesity, and this effect would be separate from genetic and endocrine influences. Validation of this inference will require further study of these time factors and their interactions and of longer range time factors involved in cumulative obesity and related disorders. Such long-range time factors include consequences of the weaning diet and of prepubertal adaptations that persist into adulthood. Thus, the limitations on experiments, e.g., those involving meal frequency changes in adults, man or rat, should always be considered.

If all this is so, an optimal diet for man would supply restricted amounts of protein and energy mainly from fats and slowly available starches in order to maximize both the daily caloric intakes and the intervals between meals that will not lead to obesity. Furthermore, such a diet would be desirable at least from weaning to puberty, and for children who are not breast fed, there are precautions regarding the formula that warrant attention (36). Such patterns of nutrition used to be traditional and still prevail, e.g., in Eskimos (5). However, modern food technology, distribution problems, and popular preferences have generated social and economic demands resulting in a recognized need to try to establish relatively safe use patterns for some rapidly available calorifers including carbohydrates. One approach to this difficult problem may be through study of its time factors.

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Dietary Regulation of Liver Glucose-6-Phosphate Dehydrogenase in the Rat: Starvation and Dietary Carbohydrate Induction[1]

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HUMPHREY F. SASSOON,[2] YOSEF DROR,[3]
JULIA J. WATSON AND B. CONNOR JOHNSON
Biochemistry Section, Oklahoma Medical Research Foundation, Oklahoma City, Oklahoma, and Department of Biochemistry and Molecular Biology, College of Medicine, University of Oklahoma Health Sciences Center, Oklahoma City, Oklahoma 73104

ABSTRACT The previously reported dietary carbohydrate dependence of glucose-6-phosphate dehydrogenase, 6-phosphogluconate dehydrogenase and pyruvate kinase of rat liver has been further explored in rat liver cytoplasm under various conditions of starvation and refeeding. Similar but smaller influences of dietary carbohydrate were found on adrenal and on liver mitochondrial enzyme levels. In liver cytoplasm, glycogen accumulation appeared to be a precursor of the carbohydrate-dependent enzyme increases and, if so, could explain the increase in these enzymes following high dietary protein as well as carbohydrate. Orotate incorporation into liver nuclear RNA was seen after challenge of fasted rats with the high sucrose diet but not after challenge with the high fat diet. Actinomycin D and cycloheximide blocking of enzyme synthesis at zero time and cycloheximide but not actinomycin D blocking of enzyme increase several hours after sucrose indicates transcription level induction of synthesis following high sucrose diet feeding. The data seem consistent with regulation of these enzymes by the tissue levels of their specific substrates. The enzyme increases appear to involve fresh messenger RNA synthesis. J. Nutr. 103: 321–335, 1973.

INDEXING KEY WORDS G6PD · 6PGD · PK · sucrose · starvation–refeeding · actinomycin D · cycloheximide · induction · rat

The metabolism of dietary carbohydrates is regulated in liver, partly by limitations on the potential rates of flow of metabolites through the nonoxidative (Embden-Meyerhof) and oxidative (pentose shunt) pathways of glycolysis. The pentose shunt is so called because its metabolites that are not used as pentoses are reconverted to the initial substrate, glucose-6-phosphate (G6P) or to fructose-6-phosphate: in addition, by reducing two molecules of NADP per molecule of G6P metabolized, the pentose shunt furnishes a permissive feed-forward stimulus to lipogenesis. Flow rate of metabolites through the pentose shunt depends on the availability of two enzymes, G6P dehydrogenase (EC 1.1.1.49, G6PD) and 6-phosphogluconate dehydrogenase (EC 1.1.1.44, 6PGD). Flow rate through the Embden-Meyerhof pathway is limited primarily by the availability of pyruvate kinase (EC 2.7.1.40, PK) to metabolize accumulations of phosphoenolpyruvate (PEP).

A continuation of studies on the effects of different diets on these enzyme systems (1–5) is reported. Previously, use of two contrasting diets, one essentially carbohydrate-free and the other almost fat-free, had shown that levels of G6PD, 6PGD and PK above very low "resting" amounts were quantitatively dependent on carbohydrate intakes (1). Responses were rapid, especially to sucrose (3), and were preceded by

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[2] Present address: Federation of American Societies for Experimental Biology, Life Sciences Research Office, 9650 Rockville Pike, Bethesda, Md. 20014.
[3] Present address: Department of Animal Nutrition and Agricultural Biochemistry, Faculty of Agriculture, Hebrew University of Jerusalem, Rehovot, Israel.

precipitous rises in liver glycogen levels (3). The first enzyme to respond was G6PD, and it responded more than did 6PGD, so that with increasing flow through the pentose shunt 6PGD, which responded less, tended to take over the rate-limiting function (4). Increases under maximally stimulating conditions (starvation–refeeding experiments) tended to reach peak levels after 3 days, and this interval was used to determine the greatest potential increases (1–4). Although no enzyme increases were seen on refeeding carbohydrate-free, high fat diet, all other parameters indicated good recovery from the effects of starvation (1).

Further experiments have been aimed at identifying the direct inducers of the enzyme increases, and also the step in initiation of protein synthesis at which induction takes place. A major concern has been to evolve control conditions, for work in intact animals, that will isolate the events to be studied without inhibiting them.

MATERIALS AND METHODS

Preparation of rats. Male rats were caged singly in suspended wire-bottom cages. The room was kept nominally at 24° and lit by windows and fluorescent lights dimmed at night to 33% of daytime intensity. To compensate for possible position effects, cages were allotted in turn to one rat from each treatment group. Water was always available.

High fat, essentially carbohydrate-free diet (F diet) and high sucrose diet (S diet) were prepared as previously described (1). The F diet contained (% of weight) casein 33, lard 51, corn oil 8, vitamins and minerals 8. The S diet contained casein 20, sucrose 69, corn oil 5 (for essential fatty acids), vitamins and minerals 6. A third diet (M) provided a mixed energy source as follows: (g/100 g) 25.3 lard, 6.7 corn oil, 34.9 sucrose, 26.7 casein, 4.9 mineral mixture, 0.64 vitamin mixture, 0.15 choline, 0.15 DL-methionine. The M diet provided (kcal/100 g diet) 107 from protein, 288 from fat and 140 from carbohydrate. The percentage of calories as protein and the protein/vitamin–mineral ratio were essentially the same in the three diets.

A commercial cereal diet (Teklad, Inc., Monmouth, Ill.) was used in some cases to carry rats prior to starvation. Its analysis was protein 24.27%, carbohydrate 56.23%, fat 4.15%, fiber 4.86% and ash 7.78%.

Preparation of rat livers. Livers were quickly washed in 0.9% NaCl (saline), weighed to 0.1 g and homogenized using a Teflon pestle in a 1:1 w/v of ice-cold saline with 1 μM EDTA (EDTA–saline). Otherwise the 105,000 × g (average) supernatant cytoplasmic fraction was obtained as previously described (1).

Preparation of rat liver mitochondrial fraction. All operations were performed in the cold. Five milliliters of whole liver homogenate were mixed with 5 ml of cold 10% Ficoll[4] (6) solution and centrifuged in a horizontal rotor at 550 × g (average) for 10 minutes. Two milliliters of the supernatant mixed with 30 ml of cold EDTA–saline were centrifuged in an angular rotor at 11,000 × g (average) for 10 minutes. The mitochondrial pellet was twice washed in fresh 30-ml aliquots of cold EDTA–saline by mixing with a Vortex mixer and recentrifuging. The final pellet was brought to 4 ml with cold EDTA–saline, 0.9 ml was placed in a glass tube, 10.5 mm internal diameter, 0.8 mm wall thickness, 0.1 ml of 10% w/v Triton X-100 was added, and the mixture was thrice sonicated for 5 seconds using a Biosonic[5] probe, width 4 mm, 20,000 cycles, set at 80% intensity. Probe and tube were cooled in ice-water for 10 seconds after each 5-second sonication. The suspension was sedimented in a clinical centrifuge for 10 minutes, and 50-μl aliquots of the supernatant were used for enzyme determinations.

Preparation of rat adrenals. Each gland with its capsule was washed in cold saline, cleaned, blotted and weighed. The glands from each group of rats were pooled and homogenized in 10 parts of 1% w/v bovine serum albumin[6] solution in a 2 ml Potter-Elvehjem glass mortar with Teflon pestle. The 60-minute 105,000 × g (average) supernatant was assayed without further dilution.

Fresh tissue content of analyzed liver samples. All additions to the weighed tis-

[4] Pharmacia, Uppsala, Sweden. Ficoll is a synthetic high molecular weight polymer made from sucrose and epichlorohydrin. Average molecular weight 400,000. It is used to prepare an inert sedimentation medium.
[5] Branwill Scientific, Rochester, N. Y.
[6] Bovine Serum Albumin, Sigma Chem. Co., St. Louis, Mo.

sue were recorded. Calculations were as described previously (1).

The ratio: (water content of whole liver homogenate)/(water content of its 105,000 × g supernatant) was found to vary according to nutritional state. Samples were dried at 105° and the following ratios were calculated: after 7 days starvation, 0.95; after continuous feeding of F diet or 7 days starvation and 1 day refeeding of S diet, 0.91; after 7 days starvation and 3 days refeeding of S diet, 0.84. Calculations of fresh tissue content of supernatant samples were corrected to take account of these ratios.

Analytical methods. G6PD and 6PGD activities were determined by methods developed during the course of these studies (5). PK activity was determined as previously (1) or by the following method developed during the course of these studies. Two microliters of liver supernatant or 50 μl of mitochondrial preparation were added to the Bucher and Pfleiderer (7) reaction mixture also containing 0.5% bovine serum albumin and, in later experiments, 1 mM D-fructose-1,6-diphosphate (FDP) (8). This was done in order to maximize any "contaminant" activity of glycerophosphate dehydrogenase (GPD) and then, after a separate assay for GPD activity (7), the PK activity of the presence of FDP was calculated by difference. All enzyme activities are expressed as units each representing net redox alteration of 1 μmole of the appropriate nucleotide cofactor per minute at 30° under the described conditions.

Cytoplasmic protein was determined as before (1). Glycogen was estimated by a modification of the method of Carroll et al. (9) using 0.1 to 0.3 g samples from the distal tip of the left liver lobe. These were homogenized in 5% trichloroacetic acid (TCA) for 1 minute in a 5-ml capacity blender,[5] centrifuged at low speed and the pellet was rehomogenized similarly, the two supernatants were pooled, brought to 20 ml with 5% TCA and further diluted to a concentration of 10 to 200 μg of glycogen per milliliter. In a further modification, aliquots of whole liver homogenate were added to 5% TCA, blended for 1 minute as above, and the supernatant was diluted as above.

Blood glucose was determined by an automated procedure[6] based on the method of Hoffman (10).

The data are expressed per 100 g body weight in order to be able to correctly compare amounts per liver, for animals of different size. Hence these data reflect the relative enzyme activities per liver cell irrespective of content of liver tissue in water, glycogen, protein, etc.

Orotate incorporation. At various times (1 to 32 hours) after intraperitoneal (ip) injection of orotate-6-^{14}C[7] in conjunction with various dietary treatments rats were killed and their livers pooled and homogenized. The fraction containing nuclei was prepared by the method of Widnell and Tata (11), and the Chandler and Neuhaus (12) method was used to prepare 50° and 65° phenol RNA extracts. The ^{14}C content of these RNA fractions was determined by liquid scintillation counting. The total RNA content of each fraction was estimated by the orcinol method of Ceriotti (13) using yeast RNA as standard. The data were subjected to analysis of variance according to Cochran and Cox (14).

RESULTS

Starvation. The progressive effects of starvation on a number of parameters (table 1) were determined in rats previously accustomed to S diet ad libitum. Liver glycogen fell by 93% overnight and virtually to nil for 2 days; it then recovered to 8% at 4 days and 12% at 8 days. Changes in blood glucose levels were parallel. Overnight mean losses of enzyme activities were: G6PD 44%, 6PGD 19% and PK 14%. Liver weights, cytoplasmic protein contents, and activities of all three enzymes continued to decrease throughout the 8 days. Thus glycogen and G6PD levels responded most rapidly to starvation, as earlier they had responded most rapidly to refeeding (fig. 3) (18).

Diurnal variations, i.e., hourly effect of time after eating. For control purposes 16 groups of six rats, 350 to 400 g, were fed F or S diet ad libitum for 2 weeks and were then killed at 3-hourly intervals over a 24-hour period. Since these rats ate mainly at

[5] Omni Mixer, Ivan Sorvall Inc., Midland, Mich.
[6] Technicon Instrument Corp., Tarrytown, N. Y.
[7] Nuclear Chicago, Des Plaines, Ill.

TABLE 1

Effects of starvation on rats previously fed S diet[1]

Period of starvation[2]	No. of rats[3]	Body weight at death	Body weight loss	Liver weight	Liver protein	Blood glucose	Liver glycogen	Liver 6PGD	Liver G6PD	Liver PK
		g	%	g	mg	mg/100 ml	mg	units	units	units
Nonstarved controls	5	306±7	—	4.1±0.2	2.1±0.3	115±6	312 ±64	24.9±4.8	16.0±1.8	49.6±3.5
12 hours (overnight)	6	338±22	—	3.2±0.2	2.1±0.2	102±8	22 ±11	20.1±4.5	8.9±2.1	42.6±2.6
1 day	6	343±26	—	2.8±0.1	2.2±0.2	84 ±4	0.8± 1.8	14.0±1.7	8.8±2.8	32.9±2.2
2 days	5	321±20	9±1	2.5±0.1	1.9±0.2	84±9	1.6± 0.8	10.0±1.0	6.5±2.2	23.5±2.1
4 days	6	291±9	—	2.5±0.1	1.8±0.2	96±6	24 ±7	8.6±1.1	2.0±1.2	18.0±1.4
8 days	6	265±14	28±2	2.2±0.2	1.1±0.1	106±6	38 ±14	5.5±1.1	2.1±0.6	3.9±1.3

[1] All liver content data are expressed per 100 g body weight. Standard deviations are shown. Abbreviations: S diet, was a balanced diet with energy derived from sucrose; G6PD, glucose 6-phosphate dehydrogenase; 6PGD, 6-phosphogluconate dehydrogenase; PK, pyruvate kinase. [2] Sucrose diet intake of control and of starvation groups before starvation was 20.8±0.9 g daily. [3] Three-month-old male Charles River rats were used.

night, eight groups fed S diet only by day (9 AM to 5 PM) were added. By necessity this study was spread over seven occasions in July and one in September; all data for any one time (fig. 1) were obtained on the same occasion. The glycogen curves show influences of diet and feeding period, consistent with reports by others (15–17), and the general 9:00 AM "low" indicates that rats killed in our laboratory under "normal" feeding patterns at about this time would show least circadian influences. Under conditions of continuous starvation no such rise and fall, but only a continuous gradual fall, in these enzyme levels occurs indicating that this "circadian" curve is in fact merely an "after feeding" response curve (18, 19).

Liver mitochondrial enzymes. Since others (20, 21) had reported differences between cytoplasmic and mitochondrial activities, these were measured for G6PD in 6-month-old Sprague-Dawley rats, and for 6PGD and PK in 3-month-old Charles River rats, after various dietary treatments (table 2). Although, throughout, mitochondrial activities were less than cytoplasmic activities by one to two orders of magnitude (per gram of liver), most responses to the treatments were parallel. In both systems G6PD activity exceeded 6PGD activity only when fully "induced" by S diet, and was not decreased to starvation level after transfer to F diet and feeding it for 10 days.

These experiments permitted comparison of the cytoplasmic G6PD levels of starved rats with those of rats fed the carbohydrate-free F diet for 6 months. The levels after starvation were essentially similar in the two strains of rats (cf. table 1); appreciably lower cytoplasmic G6PD levels were found in the Sprague-Dawley rats that had never been fed dietary carbohydrate than in *any* of the starved groups. However, this difference was absent in the mitochondrial fraction (table 2).

Adrenal G6PD. As an example of an organ functionally very different from liver, we examined the diet dependence of G6PD in adrenals. Forty-eight 3-month-old Sprague-Dawley rats fed the cereal diet from weaning were starved for 6 days, or starved for 6 days and refed S diet for 3 days, or fed S diet ad libitum for 10 days. All rats were killed at 8:00 AM and each

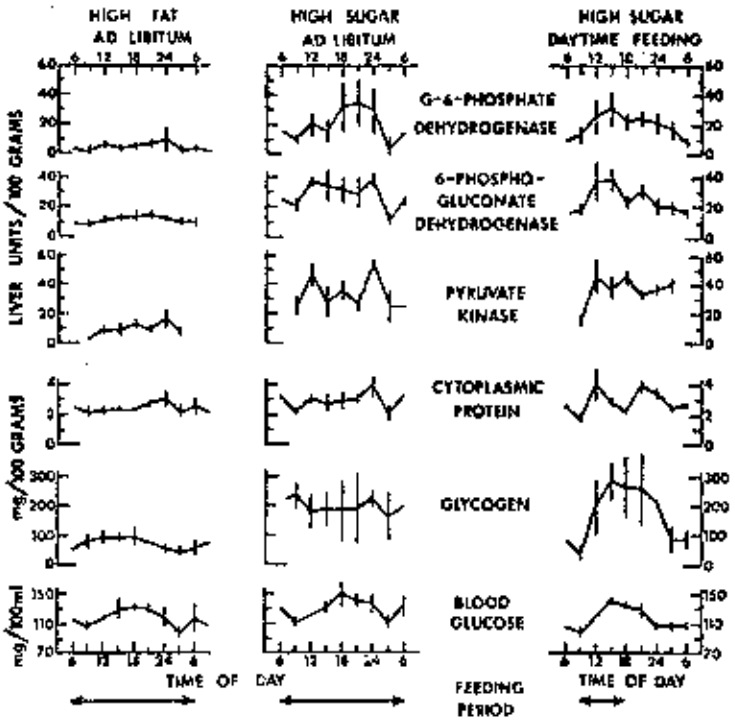

Fig. 1 Diurnal variations in rats kept at the Oklahoma Medical Research Foundation and fed F diet ad libitum, S diet ad libitum or S diet only between 9 AM and 5 PM. Six rats per point.

group was randomly divided into subgroups of eight, of which livers and adrenals were pooled after weighing. Agreement between subgroups was close (table 3); adrenal G6PD activity in the group fed S diet continuously for 10 days was

TABLE 2

Liver mitochondrial and cytoplasmic enzyme activities compared[1]

	G6PD			6PGD			PK		
	Cytoplasmic	Mitochondrial	Ratio[2]	Cytoplasmic	Mitochondrial	Ratio	Cytoplasmic	Mitochondrial	Ratio
	units	units		units	units		units	units	
				Six-month-old rats					
F diet from weaning	1.2	0.13	9						
Stock diet, then F diet for 10 days	7.9	0.19	41						
				Three-month-old rats					
Stock diet from weaning	15.8	0.24	66						
Starved 7 days (exp. 1)	2.3	0.12	19	5.3	0.18	29	4.3	0.05	85
(exp. 2)	3.4	0.08	43	5.5	0.10	55			
S diet refed 1 day	7.6	0.06	127	8.1	0.15	54			
2 days	48.9	0.44	111						
3 days (exp. 1)	31.4	0.47	67	22.8	0.18	127	26.7	0.27	99
(exp. 2)	44.3	0.33	134	28.3	0.50	57			

[1] Livers of six to eight rats were pooled for each determination. Enzyme units are expressed per gram of liver. Abbreviations: S diet was a balanced diet with energy derived from sucrose; composition of S (sucrose) and F (fat) diets is given under Methods.
[2] Units in cytoplasm per unit in mitochondria.

TABLE 3

Adrenal and liver G6PD activities with S diet[1]

	G6PD activities[2]				
	Adrenals		Liver		Adrenal/liver Ratio
	Average	(Pool values)	Average	(Pool values)	
	units[3]	units	units	units	
Fed S diet 10 days	18.13	(18.53, 17.72)	15.07	(14.83, 15.30)	1.20
Starved 6 days	15.26	(14.93, 15.59)	2.14	(1.91, 2.37)	7.13
Starved 6 days, refed S diet 3 days	20.02	(20.36, 19.67)	27.34	(27.63, 27.05)	0.73

[1] Abbreviations as in table 1. [2] In each treatment group two subgroups of eight rats were randomly made up after killing, and livers and adrenals of each subgroup were (respectively) pooled for assay. [3] Micromoles NADP+ reduced/minute/gram of fresh tissue.

slightly higher than the liver activity but the responses, especially to starvation, were much smaller (see "ratio" column). Szepesi and Freedland (22) have reported that liver G6PD responds to dietary fructose and to glucose, whereas adrenal G6PD does not and it is apparent from table 3 that the effect of major dietary change is much less in the adrenals than in the liver.

Nutritional effects of inhibitors. Since single doses of actinomycin D or cycloheximide essentially block subsequent food intake, making the effect of the antibiotic per se difficult to assess, the effects of single doses of actinomycin D or cycloheximide were compared with the effects of overnight starvation. Three-month-old Sprague-Dawley rats were fed S diet for 2 weeks; at 5:00 PM on day 15 food was removed. Actinomycin D, cycloheximide or water was injected intraperitoneally (ip) into different groups. All rats were killed 16 hours later. It can be seen from table 4 that the inhibitors produced no significant extra response in body weight, liver weight/body weight, liver cytoplasmic protein, G6PD or 6PGD; however, the inhibitor-treated groups did have depressed PK activities and elevated blood glucose levels (table 4). These data indicated that one could use such protein synthesis-blocking agents, if done in such a way that they did not damage food intake and absorption, i.e., if given after the inducing food intake had been achieved or if food was given immediately after the blocking and before damage to the intestinal wall. Because of the finding of little difference between starvation and starvation plus blocking agent, controls could be treated identically with regard to time of feeding and

TABLE 4

Effects of actinomycin D or cycloheximide compared with effects of overnight starvation[1, 2]

	Daily intake before test	Weight gain before test (15 days)	Weight loss during test (16 hr)	Liver weight	Liver cytoplasmic protein[3]	Blood glucose[4]	6PGD	G6PD	PK
	kcal/100 g/day	%	%	g	mg	mg/100 ml	units	units	units
Control (water) (5 rats)	19±0.9	12±3.3	3±0.3	3±0.1	2±0.4	111±6	21±3.6	9±3.9	30±7.5
Actinomycin D (4 rats)	20±0.4	10±1.5	3±1.5	3±0.2	2±0.4	246±44	30±1.5	9±3.0	10±2.2
Cycloheximide (6 rats)	18±1.8	10±2.0	4±0.7	3±0.3	2±0.4	179±63	23±5.3	10±7.0	20±1.7

[1] Injections were given ip, at 0.25 ml/100 g body weight and contained antagonist at 1 mg/ml (except controls). All liver and intake data are per 100 g of body weight. Abbreviations as in table 1. [2] Standard deviations. [3] Determined in 105,000 × g supernatants of homogenates as previously described (1). [4] P for control and actinomycin D less than 0.01. P for control and cycloheximide or cycloheximide and actinomycin D, not significant.

food withdrawal but without the blocking agent.

Thus we next examined the effect of blocking agents given to animals starved and already partially refed and in which food was withdrawn at time of treatment from both control and treated groups. The period of G6PD response to diet had been ascertained as 6 to 98 hours after refeeding a starved rat (18, 23). Enzyme peaks representing maximum increase had indicated 48 to 72 hours after refeeding, and we estimated that rates of increase were such that observable increases should be found 12 to 36 hours after refeeding. We decided to administer inhibitors after refeeding for 24 hours, to remove food and to kill 6 hours later. Thirty 3.5-month-old Charles River rats fed the cereal diet from weaning were divided randomly into five groups of six rats and starved 7 days. One group was then killed and four were refed S diet for 24 hours. One more group was killed, and three were injected ip with water, actinomycin D or cycloheximide, deprived of food and killed 6 hours later. Each group was killed at 9:00 AM (table 5).

After refeeding for 24 hours, marked increases of G6PD, 6PGD and PK levels were apparent. During the next 6 hours actinomycin D did not affect levels of blood glucose, liver cytoplasmic protein, or further increases of G6PD and 6PGD activities as shown by the water-injected control rats; PK activity was actually enhanced in the rats given actinomycin D, an unexplained effect also reported by others (24). These results indicate that at this time after refeeding these enzyme increases were independent of DNA to RNA transcription.

Cycloheximide on the other hand, totally blocked the 6-hour synthesis of these enzymes. A second experiment in which rats were initially starved for 10 days instead of 7 produced essentially similar results.

These experiments indicated that by 24 hours after refeeding adequate mRNA was available for continued translation to yield enzyme in the presence of actinomycin D, or alternatively that the site of induction might be at the translational rather than the transcriptional level. Further progress seemed to demand more precise control over the initial dietary stimulus, and the following experimental design was explored.

Two-hour refeeding model. Enzyme activities were minimized by feeding rats F diet for 25 days, and training them to eat each day's ration in one 2-hour mealtime (15, 25). They were then fasted for 1 day, refed one 2-hour meal of S diet and killed 3, 6, 12 or 24 hours afterwards. Liver G6PD nearly doubled (fig. 2) between 12 and 24 hours, while 6PGD rose by 20%. On the other hand, the adrenal increases of G6PD and 6PGD were complete before 12 hours. In general terms the data of

TABLE 5

Effect of inhibitors on rats refed S diet for 24 hours after 7 days' starvation[1]

	Units	Starved 7 days	Starved 7 days, refed 24 hr	Starved 7 days, refed 24 hr restarved 6 hr[2]			SEM	$P<$
				Water	Actinomycin D	Cycloheximide		
Group no. (each 6 rats)		1	2	3	4	5		
Liver weight	% of BW	2.5	4.6	4.3	4.2	3.9	0.3	0.01
Blood glucose	mg/100 ml	93	111	95	105	196	17	0.01
Liver cytoplasmic protein[3]	mg/100 g BW	141	178	205	196	157	14	0.05
G6PD	units/100 g BW[4]	10	46	66	73	37	9.3	0.01
6PGD	units/100 g BW	12	30	41	39	23	4.4	0.05
PK	units/100 g BW	5	31	48	72	18	14.0	0.01

[1] Abbreviations: BW, body weight; otherwise as in table 1. Male rats 14 weeks old were used. [2] Injections were given ip at the start of restarvation, 0.5 ml/100 g body weight with inhibitor concentration 4 mg/ml. [3] For description see table 4. [4] Units each represent oxidation or reduction of 1 nmole of cofactor/minute under the described assay conditions.

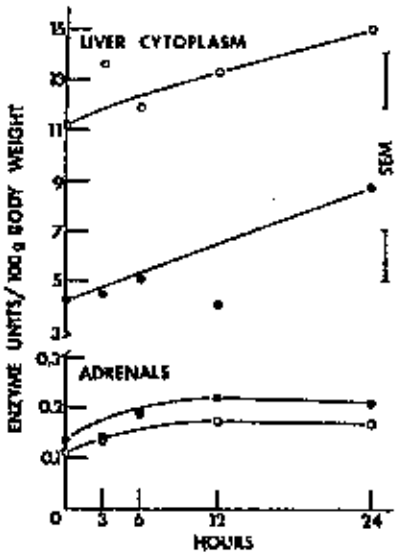

Fig. 2 G6PD and 6PDG activities in liver cytoplasm (mean of six assays on six rats) and adrenals (one assay on pooled adrenals of six rats) at times after the start of a 2-hour meal of S diet given to rats with minimized enzyme levels. ●——● G6PD, ○——○ 6PGD; bars at right show SEM.

table 6 indicate clearly that even using this controlled once a day 2-hour feeding, 24 hours were required after the start of feeding to be able clearly to detect increases in liver levels of G6PD and 6PGD. This time scale was important to establish along with this controlled feeding system before more definitive actinomycin D experiments could be conducted.

Clearly, controls of this type held promise for such studies. It was also found that sensitivity could be improved by selection of responsive animals. For example, towards the end of the phase of accustoming the rats to 2-hour-per-day consumption of their requirement of F diet, rats were rejected for failure to eat enough to gain weight. In addition, after starvation, the group to be killed was chosen so that the average weight of the rats in the group equalled that of the rats remaining for the refeeding groups. The justification for selection of this sort is that one is studying the response rather than the rat. In the following experiments various degrees of selection were used.

Inhibitors and initiation of enzyme increases. The 2-hour refeeding model was used. F diet was offered from 8:00 to 10:00 AM daily for 14 days to 56, 3-month-old Charles River male rats divided into seven groups of equal average weight and then starved for 4 days. Group 1 received water ip and was killed at 8:00 AM. Groups 2 to 7 were refed S diet for 2 hours and were killed 24 hours later, having received ip water, actinomycin D or cycloheximide, as shown in table 7, which summarizes the results.

TABLE 6

Early effects of time-limited dietary "induction" of G6PD and 6PGD increases[1]

	Fasted	Hours after start of 2-hour refeed with S diet				SEM
		3	6	12	24	
No. of rats in group	6	5	6	6	6	
Group average BW, g	497	519	511	404	510	38
Liver wt, g/100 g BW	2.3	2.6	2.7	2.8	2.4	0.09
Adrenals wt, mg/100 g BW	10.0	9.8	11.4	12.5	10.1	
G6PD, units/g fresh tissue[2]						
Liver cytoplasm	1.0	1.8	1.9	1.5	3.2	0.37
Adrenals[3]	13.4	14.0	16.4	17.3	20.4	
Adrenal/liver concentration ratio	7.2	8.0	8.7	11.6	6.5	
6PGD, units/g fresh tissue[2]						
Liver cytoplasm	5.1	5.4	4.8	4.8	6.1	0.36
Adrenals[3]	13.1	13.5	16.4	13.9	16.9	
Adrenal/liver concentration ratio	2.4	2.5	3.4	2.9	2.8	

[1] Enzyme levels were minimized by 25 days' feeding F diet for 2 hours/day, followed by a 1 day starvation. "Induction" was started by 2 hours refeeding S diet. No further feed was given. Abbreviations as in tables 1 and 5. [2] The data based on body weight are shown in figure 2. [3] Adrenals from each group were pooled for these determinations, while livers were assayed individually.

TABLE 7

Effects of inhibitors on rats refed S diet for 2 hours after 4 days starvation[1]

Parameter	Unit	Starved 4 days	Fed F diet; starved 4 days; refed S diet for 2 hours (8 to 10 AM) and injected as below at times starting from 8 AM. Assayed 12 hours after start of refeeding					
			Water[2] 0 hr	Actinomycin D[3]			Cycloheximide[3]	
				0 hr	3 hr	4 hr	0 hr	2 hr
Group no.		1	2	3	4	5	6	7
Number of rats		8	7	7	7	8	7	7
Intake of S diet	kcal		39	33	28	31	9	35
Liver wt	% of BW	2.4	3.1	3.2	3.0	3.3	2.5	2.9
Blood glucose	mg/100 ml	70	108	109	110	114	81	168
Liver glycogen	mg/100 g BW	20	138	146	129	123	22	53
LCP[4]	mg/100 g BW	110	162	155	145	142	118	130
G6PD	units/100 g BW	8.1±1.30[5]	19.7±3.1	12.8±1.6	12.4±2.4	18.3±3.8	6.0±0.7	7.3±0.8
6PGD	units/100 g BW	14.3±0.7	27.3±2.7	23.0±0.8	22.7±1.9	24.8±1.2	14.5±0.9	18.5±1.5

Significance of differences between the various groups presented in table 7 (t test)

		$P <$ Groups			
		1	2	5	2 & 5
Groups 2	G6PD	0.005		NS	
	6PGD	0.005		NS	
3 & 4	G6PD	0.02	0.012	0.05	
	6PGD	0.005	0.02	NS	
6 & 7	G6PD	NS	0.005		0.005
	6PGD	NS	0.005		0.005

[1] Male rats 3 months old. Groups were selected for similar average BW of all groups at time of selection. Abbreviations as in table 5. NS, not significant. [2] Amount of all injections 0.5 ml/100 g BW; concentration of each inhibitor in injection 0.2 mg/ml. [3] Liver cytoplasmic protein as in table 4. [4] Standard errors.

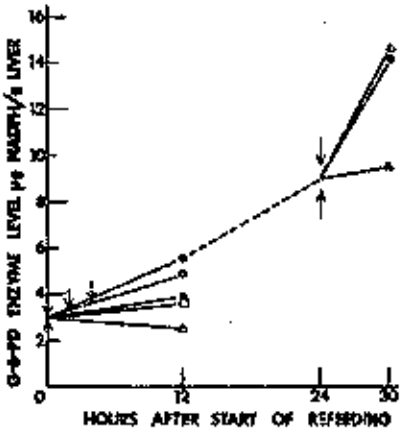

Fig. 3. Summary of effects of actinomycin D and cycloheximide on rats with enzyme levels minimized by starvation and then refed with S diet. Time of administration of actinomycin D, denoted by (↓) and of cycloheximide by (↑). Actinomycin D given (ip) at 100 μg/100 g body weight at 0 hours (■), 2 hours (□), 4 hours (○) and at 2 mg/100 g body weight at 24 hours (◇). Cycloheximide given (ip) at 100 μg/100 g body weight at 0 hours (△) and 24 hours (▲). No blocking agent given (◆). Each point represents six rats.

Under these conditions of a 2-hour meal at the start of the experiment actinomycin at 0 time did not interfere significantly with this 2-hour intake and absorption of diet or with the uptake and utilization of glucose and amino acids by the body cells as evidenced by the blood glucose, liver glycogen and cytoplasmic protein data. Nevertheless, G6PD increases were blocked significantly when actinomycin D was given either at 0 time or 2 hours, but not 4 hours, after the start of refeeding. Leader and Barry (26) have shown G6PD increases in organ cultures of mammary tissue to be actinomycin D and cycloheximide sensitive until 3.5 hours after the induction stimulus but not after. 6PGD synthesis following S diet did not appear to be blocked by actinomycin D even when given at 0 time. These observations indicated that the primary site of action of S diet in promoting greatly increased G6PD synthesis was prior to the site of action of actinomycin D, i.e., at the level of transcription. They also indicated that once transcription had taken place, message was stable for some hours to continue to act as template for the increased synthesis of the G6PD enzyme.

The cycloheximide given blocked protein synthesis and thus enzyme induction. In the organ culture systems used by Leader and Barry (26) cycloheximide did not block G6PD synthesis if added after 3.5 hours indicating that in their system protein synthesis was finished by 3.5 hours. Figure 3 summarizes the effects of actinomycin D and cycloheximide in this series of experiments and shows the effects of cycloheximide in blocking G6PD synthesis at all times given while, for the 24-hour period studied, actinomycin D only blocked the enzyme increase when given during the early stages of induction.

Starvation-refeeding and orotate incorporation into RNA. The effects of carbohydrate challenge on nuclear RNA synthesis were more directly observed in two different types of experiments. In the first, 3-month-old, Charles River rats fed a cereal diet from weaning were fed diet M for 10 days, and 12 groups of three rats each, of equal average weight, were starved for 3 days ending at 7:00 AM when each rat received 5 μCi of orotate-6-^{14}C. Four groups were then refed S diet, four F diet and four were not refed: one group from each set was killed after 1, 2, 4 and 6 hours. Although the F diet groups had all eaten more protein and energy than the respective S diet groups (table 8), the S diet groups showed a major uptake of ^{14}C in the 50° phenol extracts of liver nuclei at 2 hours, while the F diet groups showed a slightly lower uptake even than the starved groups (fig. 4, left). No significant peak of ^{14}C was detected in the 65° phenol extracts (fig. 4, right).

In the second experiment, 3-month-old rats were prepared on F diet, starved overnight, given orotate, refed S or F diet ad libitum and killed after 4, 8, 16 and 32 hours. The S diet rats showed a peak of ^{14}C in the 50° phenol extracts at 8 hours, and the F diet rats did not (fig. 5, left) although again they had eaten more protein and energy (table 8).

Inhibition of G6P metabolism. The capacity of the cell to metabolize G6P is vital; if it be blocked in the rat by ip injection of > 0.5 g/100 g (body weight) of 2-deoxyglucose (2DG), most rats die in 20

TABLE 8

Orotate-6-^{14}C experiments dietary intakes of protein and calories

	Protein	Energy	Protein	Energy	Protein	Energy	Protein	Energy
	All values to the nearest 1 kcal							
First experiment[1]								
Hours after refeeding	1		2		4		6	
S diet	6	28	6	28	8	40	6	30
F diet	8	38	7	33	9	44	11	53
Second experiment[2]								
Hours after refeeding	4		8		16		32	
S diet	4	19	7	39	6	28	7	36
F diet	9	44	9	44	15	75	12	62

[1] Averages of three rats. Refeeding ad libitum. The "energy" in S diet was sucrose, in F diet lard in the same calorie ratio to the protein. Intakes were measured when rats were killed. [2] Averages of two rats. Refeeding ad libitum.

to 30 minutes.[10] 2DG is rapidly taken up and converted to 2DG-6-P, which in normal mammalian liver is not metabolized further (27), and this blocks utilization of G6P by the cell (28).

Barely nonlethal single doses of 2DG (0.5 g/100 g body weight, ip in water 1/1) were given to Sprague-Dawley rats fed F diet; blood glucose and liver glycogen measurements taken during the next 12 hours[11] indicated that a) uptake and utilization of blood glucose was blocked within 15 minutes and was virtually at a standstill for at least 6 hours; b) during this time liver glycogen stores were depleted (table 9). Similar conclusions concerning inhibition of glucose uptake by 2DG were reached by Horecker (29).

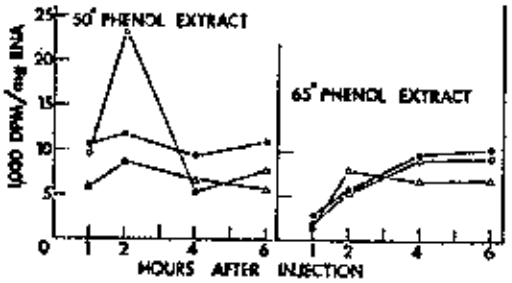

Fig. 4 Specific radioactivity of RNA in 50° and 65° phenol extracts (see Methods) of liver nuclei from rats injected intraperitoneally with 5 μCi each of orotate-6-^{14}C after 10 days' exposure to a mixed-energy diet and 3 days' starvation; after injection four groups of three rats each were refed S diet (○——○), or F diet (●——●), or were not refed (△——△). One group of three from each treatment was killed at the times shown.

The liver G6PD, 6PGD and PK activities of these rats were not increased following administration of 2DG.

DISCUSSION

The continuing aim of this research (1–5, 18, 23) is the understanding of the relative roles of carbohydrate and fat, the principal dietary sources of energy, in nutrition. Gross differences between the effects of carbohydrate versus fat on the cardiovascular system in starvation–refeeding experiments (30) may be related to the biochemical effects of the more "stressful" carbohydrate in the induction of certain enzymes following starvation–refeeding. In considering enzyme induction in the intact organism by dietary carbohydrate the aims of the research continue to be to determine the site of induction, the actual compound which is the inducer at this site, the mechanism of its induction and the mechanism whereby dietary carbohydrate produces the inducer.

To measure early increases in G6PD, 6PGD and PK rats were prepared with F diet to ensure minimal levels (1), and then starved to enhance the response to challenge with carbohydrate (2). To measure decreases, rats were prepared with S diet to establish maximal enzyme levels. When rats were fed ad libitum, a chart of the diurnal fluctuations (i.e., changes in enzyme levels related to time after eating) in rats in our laboratory (fig. 1) was used

[10] Sasson, H. F., J. J. Watson and D. R. Roller 1967 Unpublished data.
[11] Sasson, H. F. 1968 Federation Proc. 27: 419 (abstr. 1152).

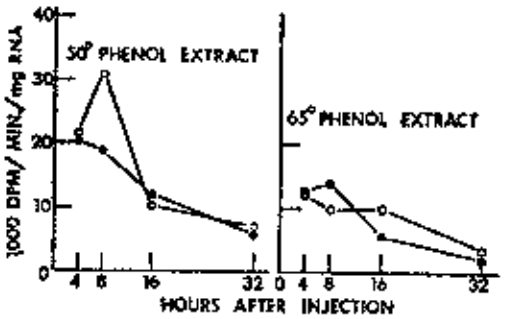

Fig. 5 Specific radioactivity of RNA in 50° and 65° phenol extracts (see Methods) of liver nuclei from 3-month-old rats injected intraperitoneally with 5 μCi each of orotate-6-^{14}C after 2 months' exposure to F diet and 18 hours' (overnight) starvation; after injection four pairs of rats were refed S diet (○——○), or lard-energy diet (●——●). All pairs were of the same average body weight. One pair from each treatment was killed at the times shown. Three rats per point.

in the timing of observations to reduce variability.

To further reduce variability, experiments were performed in which rats trained to eat a day's intake within 2 hours were utilized (15). Levels of blood glucose and liver glycogen served as checks on absorption and uptake of carbohydrate by rats that were treated with actinomycin D or cycloheximide. In addition to the liver cytoplasmic fraction, the liver mitochondrial fraction and the adrenal gland were examined briefly, and many similar responses to diet of the enzymes studied were found (tables 2 and 3, fig. 2). The "fasted" rat adrenal/liver concentration ratios (table 6) of 7.2 for G6PD and 2.4 for 6PGD are similar to those reported by Zaheer et al. (20).

A close time relationship is evident between the liver glycogen level and the levels of G6PD, 6PGD and PK. This has been observed under conditions of greatest increase (18), greatest decrease (table 1), smaller changes (table 7), and "steady state" of rats fed F or S diets (tables 7 and 9). Changes of glycogen level precede those of the enzymes ((18) and table 1). Glycogen is the major body carbohydrate store. Thus it is suggested that either the level of glycogen or the products produced from its turnover mediate the influence of dietary carbohydrate intake on the levels of G6PD, 6PGD and PK. When 2DG, a carbohydrate, failed to increase the glycogen level (table 9), it also failed to increase the levels of these enzymes. Mediation by glycogen would also explain the reported increases of these enzymes "induced" by 90% protein diets (31), because most surplus amino acids are converted to carbohydrate and stored as glycogen.

The apparent responses of G6PD, 6PGD and PK to glycogen level or metabolic product differed in rapidity and amount ((1, 3) and table 1). Since G6PD responded the most rapidly, the relationships of glycogen to 6PGD and PK, and probably to all three enzymes, were inferred to be indirect. During glycolysis glycogen breaks down to G6P, the specific substrate of G6PD. G6P also is metabolized through fructose-6-P and further steps to PEP, the specific substrate of PK. In the latter pathway there are no known rate-limiting steps

TABLE 9

Blood glucose and liver glycogen levels after 2-deoxyglucose (2DG) treatment[1]

	Times after treatment[2]					
	15 min	30 min	1 hr	3 hr	6 hr	12 hr
Blood glucose, mg/100 ml						
2DG rats	217±16[3]	202±14	183±10	267±10	266±12	102±16
Controls	147±18	—	115±22	—	106±6	113±4
Liver glycogen, mg/100 g						
2DG rats	85±14	57±25	64±7	45±17	18±10	86±20
Controls	85±21	—	64±38	—	76±25	111±44

[1] Male Sprague-Dawley rats were fed F diet for 10 days and were given single ip injections of either 2DG, 0.5 g/100 g body weight suspended 1:1 w/v in water, or water alone (controls). [2] All rats were killed at 9:00 AM to minimize diurnal variability (fig. 1).
[3] Each measurement is the mean ±SD of a group of five or six rats.

previous to the PK reaction. However, the rate at which G6P is metabolized to 6-phosphogluconic acid by way of gluconolactone-6-P is limited by the G6PD reaction (5), and so the 6GPD reaction should not come under pressure until the level of G6PD approaches that of 6PGD. These considerations seem to be reflected in the observed responses to diet, of 6PGD and PK, relative to G6PD. Thus the most economical inference from the observed dynamics is that under these conditions each of the three enzymes is regulated by its specific substrate.

This idea has received little attention since Tepperman and Tepperman (31) found no rise in hepatic G6P level before diet-induced increases of G6PD. G6P is, however, rapidly metabolized to glycogen and to PEP and thus its net level may be no guide to its availability for the induction of G6PD, which would have to depend on the site and dynamics of G6P turnover. At one time insulin was proposed as a direct inducer of G6PD increases (32). However, after we showed that neither insulin nor certain other hormones promoted such increases in rats fed F diet (1), other workers confirmed that insulin is not a direct inducer (33, 34), and it is now known that binding of insulin to the cell wall facilitates entry of carbohydrate into the cell and enhances the organization of polysomes (35).

Since, of the three enzymes G6PD had responded most rapidly to dietary stimuli, G6PD was the logical choice for studies on the site of enzyme induction. The question that was asked at this stage was whether transcription synthesis of fresh RNA was required for any increase to occur, or whether the primary event was translational, perhaps along the lines of the Cline and Bock model (36).

It quickly became evident that the starvation-producing effect of inhibitors such as actinomycin D and cycloheximide, previously shown in 3-day studies (3), operated even overnight (table 4, in which the inverse association of blood glucose and PK levels may be noted). Therefore it was necessary to plan such inhibitor experiments over short periods so that extended food intake after administration of the blocking agent was not required. Meal-trained rats were able to eat for the usual 2 hours after actinomycin D or cycloheximide before damage to the digestive system precluded further food intake and absorption. In this way the lower part of the graphs of figure 3 was obtained. By permitting feeding for 24 hours and hence with enzyme increases proceeding steeply, the blocking agents could be used without requiring further intake and this was done in the upper part of the graph of figure 3. The cycloheximide data indicated that all of the enzyme increases involved enzyme protein synthesis rather than activation of existing enzyme or assembly of existing subunits.

The enzyme increases appeared sensitive to inhibition by actinomycin D until about 4 hours after the first dietary stimulus but not thereafter (table 7, also fig. 3). This is analogous to the observations of a 2-hour initial sensitivity to actinomycin D of serine dehydratase induction by casein hydrolysate, which Pitot and co-workers (37) had interpreted as an initial requirement for transcription of fresh RNA. In their case the fresh RNA was apparently stable for about 6 hours, and in ours for at least that amount of time (fig. 3). The incubation of labeled orotic acid into RNA under two sets of experimental conditions was a further indication of fresh RNA synthesis (figs. 4 and 5). The increased incorporation of orotic acid into RNA occurred between 1 and 2 hours after stimulus when the rats had been fasted (fig. 4) but took approximately four times longer when rats were merely switched from F to S diet (fig. 5). This observation may help explain the higher levels of glycolytic pathway enzymes reported when meal frequency is decreased (38). There are two manifestations of specific nutrient intake on enzyme induction. One is the response seen following starvation and refeeding. The maximum enzyme level is reached several days after start of refeeding and is an "overshoot" which may be 10 to 20 times the starvation level. The other is the response seen to a single meal per day, this so-called diurnal variation response is of the order of two times the premeal level and is reached in a matter of a few hours after eating (fig. 1). Presumably the inducer site and mechanism of induction of any

specific enzyme is the same in both cases. There is probably a continuum ranging from maintenance of a certain enzyme level by "nibbling" to the "overshoot" observed following starvation–refeeding. It is evident that the time required for clearly demonstrable induction following prolonged starvation (24 hours, table 6) is greater than that required with daily food intake. This may be related to the time required to regenerate normal polysomes after starvation and refeeding.

Of immediate practical importance may be the observation that during starvation or high fat, carbohydrate-free diet consumption, the resultant low levels of certain carbohydrate metabolizing enzymes may result in the reduced glucose tolerance which has been reported (39, 40).

In summary it appears from our previous work and the present report that the direct inducer of G6PD increases in vivo, following ingestion of carbohydrate, is probably derived from glycogen, may be G6P, and that it acts initially to stimulate transcription of fresh RNA.

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ticipates in the activity of the glutathione peroxidase of rat erythrocytes. These findings have been extended in the author's laboratory[2] where it has been found that the glutathione peroxidase of chick plasma is high in chicks receiving selenium, but drops to a low level in selenium-deficient chicks just prior to onset of exudative diathesis.

Several interesting interrelationships have been discovered concerning toxicities of cadmium, mercury, thallium, arsenic and selenium. These effects appear to be concerned with the influence of one or more of these elements upon the rate or mode of excretion of the others (19, 20). Work by Mason and Young (21) has shown that selenium is approximately one hundred times as effective as zinc in preventing the injury to the testes of rats produced by injection of cadmium chloride.

Problems of selenium deficiency in commercial poultry and livestock. Numerous cases of selenium deficiency have occurred in poultry and livestock on farms in those areas of the world where the selenium content of the soil is low, and therefore the local feeds and forages contain insufficient selenium to meet the nutritional requirements of the animals. The first recognized field case of selenium deficiency occurred in a large flock of turkeys in Ohio. Myopathies of the gizzard and heart resulted in severe mortality in the turkeys at approximately 5 to 6 weeks of age. The problem was found to be due to the very low level of selenium in both the corn and the soybean meal produced in Ohio (22). Subsequently, numerous flocks of turkeys and broilers throughout the eastern half of the United States have suffered poor growth, poor efficiency of feed utilization, and high mortality shown to be due to deficiency of selenium in the feeds fed to these flocks.

In lambs and calves, nutritional muscular dystrophy, recognized as an endemic disease in New York State and other areas of the eastern United States for half a century, can now be controlled by supplementing the diet of the pregnant cow or ewe with approximately 0.1 mg selenium per kilogram of total diet. This level of selenium also will prevent exudative diathesis in chicks; however, the studies with turkeys indicated that 0.2 mg selenium as sodium selenite is needed per kilogram of practical diets to completely prevent heart and gizzard myopathy in young poults.

The very high incidence of selenium deficiency in the eastern part of the United States appears to be due not only to the low selenium content of the feedstuffs but also to a very low biological availability of selenium in many feedstuffs, averaging approximately 85% for grains, 65% for soybean meal, but only 33% for fish meal, meat scraps and other products of animal origin.[3]

The studies referred to above, and numerous other investigations, demonstrate that selenium is an essential nutrient. The studies have shown further that many commercial diets for poultry and livestock are severely deficient in selenium. A review of the voluminous literature demonstrating the need for selenium supplementation of many commercial poultry and livestock feeds has been published by the National Academy of Sciences. The review was made by a seven-member subcommittee on selenium acting for the Committee on Animal Nutrition of the National Research Council. This publication, entitled, "Selenium in Nutrition," describes the overwhelming evidence for the need to supplement many commercial poultry and livestock feeds with selenium.

On March 9, 1970, the American Feed Manufacturers Association submitted a formal request[4] to the Food and Drug Administration for approval to add sodium selenite or sodium selenate to feeds for chickens, turkeys and swine in amounts not to exceed 0.25 ppm total Se in feeds for chickens and swine, and 0.35 ppm in feeds for turkeys. It was requested that the additive be incorporated into feeds as a premix containing a suitable marker for qualitative control identification and that these premixes be labeled to identify the form of selenium additive used and the quantity of selenium present. Request was made also that the finished feeds be labeled to bear the name of the form of selenium which had been added to the feed.

The Food and Drug Administration officials requested information on tissue levels

[2] Scott, M. L., and T. Noguchi 1973 Metabolic function of selenium in prevention of exudative diathesis in chicks. Federation Proc. 32: 3747 (abstr.).

[3] Cantor, A. H., and M. L. Scott 1972 Biological availability of selenium in several feedstuffs of plant and animal origin. Poultry Sci. 51: 1700 (abstr.).

[4] Number MF-3433-V, amended May 12, 1971.

of selenium in broilers, turkeys and swine fed graded levels of selenium below and somewhat above the nutritional requirements. Studies by Cantor and Scott [5] demonstrated that the selenium content of the blood and muscle of broilers and turkeys did not increase when dietary selenium levels were increased from 0.2 to 0.8 ppm. Ullrey [6] obtained similar results with swine. The maximum blood levels observed with 0.8 ppm of selenium in the diets of broilers or turkeys was no higher than 0.25 ppm. This is the average selenium value found by Allaway et al. (23) in the blood of human blood donors in Rapid City, South Dakota, where the selenium content of the soil and therefore of local vegetables, meats and milk is about as high as in any other locale in the United States.

As a result of these findings, the Food and Drug Administration published in the Federal Register for June 17, 1971, the Notice of Filing of the AFMA petition. However, to this date, because of the legislation under which the Food and Drug Administration must operate, final approval has not been given for the addition of nutritional levels of selenium to animal feeds.

In the meantime, almost all feed manufacturers in the northeastern United States have reported case after case of severe selenium deficiency in broilers and turkeys receiving their feeds. Veterinarians in the New York State Diagnostic Laboratories have encountered numerous flocks of broilers and turkeys showing high mortality from exudative diathesis or gizzard and heart myopathies. Growth and efficiency of feed utilization were reduced. These cases responded promptly to "experimental" supplementation of the diet or drinking water with sodium selenite at levels to provide 0.1 to 0.2 mg selenium per kilogram of diet. Losses to poultrymen due to the illegality of adding this nutrient to feeds have been calculated to be millions of dollars per year. Insidious losses due to poor growth and poor efficiency of feed utilization in chronically selenium-deficient animals may be far greater than that due to acute outbreaks of severe selenium deficiency.

The case for the nutritional essentiality of selenium is unquestioned. The need for supplementation of feeds has been proven beyond any doubt. It has been shown that nutritional amounts of selenium can be added to the diets of poultry and swine without increasing the blood or muscle tissue levels of selenium above those found in people and animals living all of their lives in South Dakota, Wyoming and other areas of the United States. Why then are we not adding selenium to feeds? The answer is simple—selenium has been claimed to be a carcinogen.

Cancer studies using toxic levels of selenium. In 1943, Nelson et al. (24) of the Food and Drug Administration reported that 11 out of 53 rats which had been made cirrhotic by feeding a low protein diet developed adenoma or low grade carcinoma in the cirrhotic livers and four others showed advanced adenomatoid hyperplasia after surviving 18 to 24 months on diets containing 5, 7 or 10 mg of selenium per kilogram. No tumors occurred in any of the rats during the first 18 months of the experiment. No tumors occurred in livers that were not cirrhotic. No encapsulation was noted, and no metastases were seen. The authors stated that the tumors observed . . .

> "were very similar to many of the low grade carcinomas of the rat liver seen after the ingestion of *o*-aminoazotoluene, and anyone who has studied a series of the latter tumors will appreciate the difficulty of deciding just when the borderline between nonmalignant and malignant tumor has been passed, and also just when hyperplasia has passed into tumor."

Because the above selenium toxicity study a) was complicated by being produced only in animals suffering for long times from liver cirrhosis; b) did not unequivocally identify the liver disorder as being cancer; and c) was conducted largely with selenium contained in seleniferous feeds and corn and thus the effects observed could not be ascribed definitely to selenium, an elaborate investigation was undertaken at Oregon State University to determine the possible carcinogenicity of selenium. The results of the study were reported by Tinsley and associates (25) and Harr and associates (26). Eight hundred twenty-nine rats were fed various levels of either sodium selenite or sodium selenate ranging from 0.5 to 16 ppm

[5] Cantor, A. H., and M. L. Scott. 1972. Effect of dietary supplements of sodium selenite on tissue selenium levels in market age turkeys. Poultry Sci. 51: 1790 (abstr.).

[6] Ullrey, D. E. 1972. Personal communication.

of selenium. Two hundred seventy-four rats received control diets containing no selenium; 88 rats were given N-2-fluorenylacetamide (FFA), a known hepatocarcinogen. Of the total number of rats started, 175 lived 2 years or more.

One thousand, one hundred twenty-six rats were autopsied in the course of the experiment. Sixty-three neoplasms were found. Forty-three of these occurred in 88 rats fed the carcinogen, FFA. Eleven neoplasms occurred in the 274 rats receiving the control diets; 9 neoplasms were found in the 829 rats that had received selenium at levels ranging from 0.5 to 16 ppm. None of these rats showed hepatic neoplasms. The Oregon workers did find hyperplastic lesions in the livers of about 50% of the selenium-fed rats which lived 282 days or longer. The liver cells were sometimes three to five times normal size and contained multiple nuclei and chromatin granules. The surfaces of the livers were mottled, with pale yellow or white areas. Hydrothorax, ascites, pericardial edema and icterus were common. Chronic toxic hepatitis occurred in many of the rats receiving selenium at 2 ppm or more. These livers showed 1) small hobnailed surfaces; 2) irregular mottled surface; and 3) diffusely enlarged liver. Fibroblastic proliferation occurred around the central vein. Bile duct cell proliferation formed cystic ducts, multiple ducts with fine chains of cells which extended between and into the hepatic lobules. Reticuloendothelial proliferation formed small granulomas around Glisson's Islands.

Thus, although toxic levels of selenium were found by these workers to produce drastic changes in liver and other organs of rats, this element even at high levels did not produce cancer.

At this point the controversy appeared to center on the ability of the researcher to distinguish between hyperplasia and true tumor cell development. The Oregon workers used a recognized hepatocarcinogen to produce cancer in 43 rats. Their investigation, sponsored by the National Cancer Institute, was done with the express purpose of determining whether or not the toxic effects of selenium levels on liver do indeed lead to cancer. Under the conditions of their experiments they found that dietary selenium, as either sodium selenite or sodium selenate, did not produce cancer of the liver in rats.

Several possibilities may explain the differences between the Oregon studies and those of Nelson and associates: 1) Nelson and associates used the Osborne-Mendel strain of rats while the Oregon investigators used the Wistar strain; it is possible that conflicting results were due to genetic differences. 2) While the Oregon workers fed sodium selenite or sodium selenate to provide the levels of dietary selenium, the Nelson group used various levels of seleniferous corn and wheat to provide the selenium levels in most of their work. They did, however, report tumors in two rats which had received 10 ppm of selenium in a mixture of ammonium potassium sulfide and ammonium potassium selenide. They did not feed a control lot ammonium potassium sulfide alone, and they did not have a spectrophotometric analysis of the seleniferous grains to determine whether or not these grains may have been contaminated with a known carcinogen. 3) Nelson et al. reported hepatomas only in rats that also showed cirrhosis. 4) The hyperplasia and "hobnail" appearance of the livers which the Oregon workers observed may have been much more severe in rats already suffering from cirrhosis, thereby forming foci which resembled tumors.

Thus, in 1970, the research work aimed at determining whether or not toxic levels of selenium are carcinogenic stood at one "yes" and one "no." There had been another report from Russia by Volgarev and Tscherkes (27) claiming that in one experiment 10 out of 23 male rats fed 4.3 ppm of selenium had tumors, and in another experiment five out of 19 animals fed 8.6 ppm of selenium showed tumors. This report should be entirely discounted because the Russian workers did not include any control groups of rats which received the basal diet without selenium. An attempt by the Russians to prove that selenium is carcinogenic was thwarted when in a third experiment, in which they included 200 rats fed the control diet, none of the animals in either the control or the selenium-fed groups showed any tumors or precancerous lesions (27).

In 1971 Schroeder and Mitchener (28) reported studies in which 418 Long-Evans strain rats were fed a diet containing whole

rye flour, dried skimmed milk, corn oil and iodized salt plus vitamins and trace minerals. One hundred five rats were fed the basal diet as controls. Sodium selenite and sodium selenate were given in the drinking water to 313 of the rats at a level of 2 mg Se per liter of water for a period of 1 year. The rats showed no signs of tumors after 1 year. At this time the levels of selenite and selenate in the drinking water were increased to 3 ppm of Se. Since most animals drink about 1.5 g of water per gram of dry food intake, this represented a level of approximately 4.5 mg of selenium per kilogram of diet (4.5 ppm). The experiment also was modified at 58 days by the substitution of selenate for selenite in the diet of the male rats because of a high mortality (50%) which had occurred in the selenite-fed males by 58 days of age.

The experiment was further complicated by an epidemic of virulent pneumonia when the rats were 21 months of age. The authors indicated that 38 males and 35 females (total 73 rats) receiving the control diet survived the pneumonia epidemic. Fifty percent of these were dead at 863 days of age. Of the 98 rats that had received selenate, 28 males and 47 females (total 75 rats) survived the pneumonia epidemic. These lived an average of 988 days before they reached 50% mortality.

Of the 73 control rats autopsied after 21 months of age, 20 had tumors, for an incidence of 27.4% in the controls. Thirty tumors were reported in the 75 selenate-fed rats autopsied, or a 40% incidence. In the selenite group only four rats were found to have tumors out of a total of 51 selenite-fed rats autopsied (approximately 8% incidence).[1]

These workers reported that the 8% incidence of tumors in the selenite group was not significantly lower than the 27.4% incidence in the controls, while they claimed that the 40% incidence in the selenate-fed rats represented a significant *increase* in tumors over the 27.4% incidence obtained with the controls.

In their discusion of this subject, Schroeder and Mitchener made much of the age of the rat as an important factor in cancer. They pointed out that only one tumor occurred before 18 months of age. They explained the discrepancy between their results and those of the Oregon workers on the basis that the Oregon workers' animals "did not survive to the tumor-bearing age of 24–39 months or longer, probably because the dose of selenium fed was at the toxic level (4.3 ppm or more in feed), whereas our dose of selenate was at a sub-toxic level."

The level of 3 ppm of selenium as selenate in the drinking water was not toxic to the Long-Evans strain of rats used by Schroeder and Mitchener. Indeed, it appears likely that the beneficial effect of the selenate on longevity of the rats may have been entirely responsible for the higher incidence of tumors observed in the selenate-fed rats as compared to the controls. As indicated above, the selenate-fed rats did not show 50% death from old age until 125 days *after* 50% of the control rats had died.

It is generally considered that 1 month in life-span of a rat is equivalent to approximately 30 months' life-span for man. The 988 days lived by 50% of the selenate-fed rats is equivalent to about 82 years in humans, while 50% of the control rats died at an age equivalent to about 72 years in man. It appears incongruous to point to a substance in the diet which has extended the life span from 72 years to 82 years and claim that this substance is a carcinogen because more individuals in the longer-living group died of cancer. In man and animals alike, it is well attested that if one lives long enough the chances of dying of cancer increase greatly.

Inhibitory effects of selenium on carcinogenesis. In 1949 Clayton and Baumann (29) found that the inclusion in a purified diet of 5 ppm of selenium as sodium selenite reduced the incidence of liver tumors in rats which had previously received the carcinogen *N'*-methyl-*p*-dimethylaminoazobenzene (*N'*-DAB) from 62% in ad libitum-fed controls and 93% in pair-fed controls to 31% in the rats which received selenium.

In 1956 Weisberger and Suhrland (30, 31) found that injection of 1 mg of selenocystine per kilogram of body weight per day for 14 days, following subcutaneous injection of Murphy's lymphosarcoma, reduced the average size of the tumors from an area of approximately 30 cm² in the con-

[1] These data were taken from table 4 of reference (28), after correcting an error in the second column, first two lines, where the numbers 75 and 73 appear to be reversed.

trol rats to only 8 cm³ in the rats receiving selenium.

Shamberger and Rudolph (32) in 1966 reported on the inhibitory effect of selenium on the cocarcinogenic effect of croton oil. Mice at 55 to 60 days of age were treated with 7,12-dimethylbenzanthracene (125 μg dissolved in acetone). After 21 days, five of these mice were painted five times weekly for 16 weeks with 0.25 ml of a mixture of 0.033% croton oil and 5 ppm of sodium selenide. At the end of 6 weeks there were nine tumors in the animals receiving selenium, whereas there were 132 tumors in the five comparable mice which were painted five times weekly with croton oil in the absence of selenium. Riley in 1968 (33) confirmed these results using the cocarcinogenic principle from croton oil, compound A_1.

Shamberger has recently extended his studies (34). After application of 0.25 ml of 0.03% benzo(*a*)pyrine in acetone for 27 weeks in ICR Swiss mice, it was found that 14 of the 35 mice receiving the control ration had cancers whereas only 8 of 33 mice showed cancers when the diet was supplemented with 1 ppm of sodium selenite.

Lack of correlation between selenium distribution in the USA and human cancer mortality. Because of the inhibitory effects of selenium against a variety of carcinogens in experimental animals, and the possibility that selenium at higher levels might actually be enhancing carcinogenesis, Shamberger and Willis of the Cleveland Clinic Foundation undertook a study (35) to determine whether or not a correlation could be obtained between the level of selenium in the soil, food and tissues of animals and humans, and the published incidence of human cancer mortality in these various areas. Allaway et al. (23) had determined the human blood selenium levels in 19 cities of the United States and had correlated these with the selenium content of the agricultural crops raised in the various areas in which these cities were located. These data were shown by Kubota and Allaway [8] also to correlate with the incidence of selenium deficiency and selenium excess in domestic animals. Shamberger and Willis arranged in descending order the Allaway data for the average selenium content of human blood in the 19 cities. They then recorded the cancer deaths per 100,000 population for each of these cities, as published in the bulletin on Vital Statistics of the United States for the year 1965. The city having the highest selenium blood level was Rapid City, South Dakota, with 0.256 ppm of selenium in the blood. This city showed the *lowest* incidence of cancer deaths—94 per 100,000 population. The city having the lowest average selenium blood level was Lima, Ohio, with 0.157 ppm of selenium. There were 188 deaths from cancer in 1965 per 100,000 population in Lima, Ohio.

Many factors other than selenium content of the blood undoubtedly figured in the cancer mortality in these various cities. Thus this correlation cannot be said to show that higher blood selenium levels *prevent* cancer. It is possible, however, to use these data as evidence that chronic blood selenium values at levels of approximately 0.25 ppm (which was the blood level found by Scott and Thompson (36) to represent the plateau blood level in chicks receiving dietary selenium ranging from 0.2 to 0.8 ppm) *do not increase the incidence of cancer* compared to populations where the selenium of the blood is 0.15 ppm (a level which in chicks and turkey poults verges upon a deficiency).

The work of Shamberger (34) indicates that the level of dietary selenium which had an inhibitory effect on carcinogenesis by croton oil and other chemical carcinogens is somewhat higher than the minimum nutritionally required level of selenium. Shamberger found that Torula yeast diets containing 1 ppm of sodium selenite (approximately 0.5 ppm Se) given to mice markedly decreased the number of skin tumors induced by 7,12-dimethylbenz(*a*)anthracene plus croton oil and benzo(*a*)pyramine. Torula diets containing 0.1 ppm of sodium selenite did not decrease tumor incidence.

In summary, selenium has been demonstrated to be a required nutrient. The nutritional requirement lies in the range of 0.1 to 0.3 ppm of selenium. Selenium also is an extremely toxic element. Levels of selenium in the range of 2 to 10 ppm produce a chronic toxicity while levels above 10 ppm produce drastic changes resulting in sudden death.

These studies suggest an inhibitory effect

[8] Kubota, J., and W. H. Allaway 1972. Unpublished results.

of selenium on cancer produced by various carcinogens. The evidence indicating a carcinogenic effect of high doses of selenium is open to question.

The Delaney amendment prohibits the addition to foods and feeds of anything that has been shown to be a carcinogen. The evidence presented in this report indicates a grim possibility. If the experiments reporting a protection against malignant tumors by low doses of selenium are conclusively confirmed, the consequences of restricting the use of selenium at nutritional levels may involve not only the death of untold thousands of chickens, turkeys and pigs from selenium deficiency, but also may be responsible for many deaths from cancer in the human population due to insufficient selenium in many areas of the United States to provide an inhibitory effect upon carcinogenesis.

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PRODUCTION OF LIQUID SHORTENING

Raymond O. Simmons, La Habra, Edward J. Reid, Brea, and Alfred F. Blankenship and Perry W. Morgan Jr., Fullerton, Calif., assignors to Hunt Foods and Industries, Inc., Fullerton, Calif., a corporation of Delaware

No Drawing. Continuation-in-part of application Ser. No. 381,557, July 9, 1964. This application Nov. 23, 1964, Ser. No. 413,344

14 Claims. (Cl. 99—118)

ABSTRACT OF THE DISCLOSURE

A liquid shortening is produced by hydrogenating a vegetable oil to an IV of 70-100, then cooling under controlled agitation to produce large-sized crystals, and separating the crystals.

The instant patent application is a continuation-in-part of our earlier application Ser. No. 381,557, filed July 9, 1964, and now abandoned, and entitled Liquid Shortening and Method of making same. This invention relates to the production of liquid shortenings which are especially adapted for use as pourable, frying or baking shortenings.

Shortenings produced for deep fat frying have been marketed by the shortening industry for many years. Initially these shortenings were a white, plastic, semi-solid mixture of a hard fatty component and a soft fatty component. During the manufacture of the foregoing product gas was commonly occluded to improve the workability of the shortening at ambient room temperatures and to produce a uniform white color. Before use, these products, being solid, required melting and because of their normal physical state were not possible to transfer by pumping. In the mid-1950's or the early 1960's pourable liquid shortenings were first marketed. The early products were solid suspensions of hydrogenated oils in liquid or in partially-hydrogenated liquids. The latter products have not been wholly acceptable to the housewife. However, the suspension-type product, because of its liquid nature, has advantages in the commercial trade primarily because it may be readily transferred by pumping. The suspension-type shortenings are characterized by a high linoleic acid content which limits or impairs the frying stability of the shortening and on prolonged storage there is a tendency for the suspended solids of such liquid products to settle out.

It is a principal object of the invention to provide an improved liquid shortening having an exceptionally low temperature fluidity and a process for its manufacture.

It is a still further object of the invention to provide an improved process for the manufacture of a liquid shortening characterized by generally improved low temperature properties.

It is another object of the invention to provide a fluid shortening for home or commercial use which is wholly liquid, containing no solid dispersion, and a method of manufacture of the product.

It is a still further object of the invention to provide a process for the manufacture of a liquid shortening characterized by substantially no solid formation upon prolonged storage at temperatures of approximately 60° F. (15.6° C.).

The method of the invention for producing the improved liquid shortening comprises initially selectively hydrogenating a vegetable oil characterized by a fatty acid composition having at least 45% linoleic acid at a temperature in the range of 100-250° C. to produce a partially hydrogenated material having an iodine value within the range of 70-100 and more often in the range of 80-90. Thereafter, the partially hydrogenated material is cooled from an elevated temperature to a fractionation

temperature within the general range of 5-25° C., preferably in the range of 13-17° C., with the cooling being carried on under conditions to obtain large-sized crystals which may be separated from the liquid component. Thereafter, the crystals are separated at the low fractionation temperature from the hydrogenated material to recover a liquid shortening product.

Certain vegetable oils such as soybean oil, cottonseed oil, safflower oil, and corn oil have been found to have fortuitous fatty acid makeups which permit the manufacture of a superior liquid shortening by the hydrogenating and crystal fractionating steps of the process of the invention. The vegetable oils suitable for use in the process of the invention have a fatty acid composition of at least 45% linoleic acid which is selectively hydrogenated principally to oleic acid. Preferably, the selective hydrogenation is carried on at a temperature within the more limited range of 160-185° C., which range of temperatures favors an increase in the trans isomer content of the fatty acids. The trans isomer form of the fatty acids is considerably more stable than the cis form at deep fat frying temperatures commonly used in large scale commercial establishments.

With proper selective hydrogenation, the polyunsaturated fatty acids of the soybean oil may be hydrogenated with a minimum of hydrogenation of oleic acid, a monosaturated material. Oleic acid is a particularly desirable constituent of the fluid shortening and hence it is the aim of the selective hydrogenation step of the process of the invention to hydrogenate the polyunsaturated acids, i.e., linoleic and linolenic acids without substantial hydrogenation of oleic acid occurring. Soybean oil, as an example, has a linoleic acid content of approximately 53% and linolenic acid content of roughly 7%. The selective hydrogenation step of the process of the invention is carried on to reduce the linoleic acid content to less than 15% and preferably about 8% or somewhat less. The linolenic acid content is desirably reduced to substantially zero. Linolenic acid is responsible for the characteristic odor of soybean oil and it is very important that it be substantially removed from the fluid shortening product. It has been established that linoleic acid, which contains two double bonds, if present in the fluid shortening in too large an amount, causes the shortening to oxidize too readily and generally adversely affects the stability of the oil, this being especially true under the conditions of commercial deep frying.

Cottonseed oil which may also be used in the process of the invention has typically a fatty acid composition containing 51.5% linoleic acid; corn oil 55% linoleic acid; and safflower oil 75% linoleic acid.

Nickel or other suitable catalysts are used in the hydrogenation of the vegetable oil with the hydrogenation being carried on to impart to the material an iodine value within the broad range of 70-100 and preferably within the more limited range of 80 or 83 to 85. An oil hydrogenated to an iodine value of 75 will have an oil component of approximately 25% in the preferred crystal fractionation range.

The separation of the liquid and solid fractions from the cooled partially hydrogenated material has proven to be a critical factor in the production of the liquid shortening of the invention. Too rapid cooling of the hydrogenated material from its elevated temperature above its crystal fractionation range to the fractionation temperature in the range of 5-25° C. results in the formation of very small crystals which have proven difficult, if not impossible, to separate. The use of a scraper-surface chiller of the Votator or Emulsorator type provides objectionable fast cooling leading to small crystal formation and it is best that the devices of this type be

avoided. However, a scraper-surface device may be used to quick chill the hydrogenated stock to a temperature above its crystal fractionation range without adverse effects with the rest of the chilling being carried on slowly and with agitation to the crystallization temperature. Such an arrangement will shorten the time of the production cycle.

Cooling of the hydrogenated material from its initial elevated temperature should be carried on slowly and with suitable agitation to the desired crystallization temperature. It has been mentioned that rapid chilling and agitation, such as that provided by a Votator, results in fine crystals which cannot be readily separated from the liquid portion. Similarly, cooling without agitation results in a solid mass characterized by excessively large crystals and no visible or poor crystal-liquid break. Air agitation, while it may be used, results in generally inferior products. Particularly satisfactory agitation may be had with a "gate type" impeller. With this means of agitation the crystals are discrete and large. The cooling is best carried on over a period of 1 to 16 hours and conditions should be such as to provide a phase having at least about 75% of the crystals 50 microns or larger in their smallest dimension and preferably within the range of 100 to 300 microns. Desirably, no more than 5% of the crystals should be over 1000 microns in maximum dimension and preferably no more than 10% of the crystals should be over 500 microns in size. The crystals may be separated from the oil by a filter press, basket centrifuge, or other devices. The term "crystals" is used herein to define single crystals and clusters of crystals.

The following is a report of one series of comparative experiments carried on to determine the effective agitation and cooling rate on crystal size in the fractionation of hydrogenated soybean oil. The starting stock used in each of the following experiments was a soybean oil hydrogenated at 180° C. to an iodine value (IV) of 85. Three methods of fractionation were employed as described in the examples below. Filtering where indicated in the following examples was accomplished by the following technique. A Büchner funnel with cotton duck filter cloth was used with a suction flask. A vacuum pump created a reduced pressure in the flask which was the driving force for pulling the liquid into the flask. The partial pressure was increased in stepwise increments from 5" to 28" of vacuum. Each reduced pressure was maintained until the liquid filtrage flow became dropwise.

Example 1

Fifteen hundred grams of the foregoing 85 IV soybean oil were heated to 60° C., placed in a glass jar, and stored in a 15.6° C. oven for approximately 100 hours. The stock required between 8 and 16 hours to reach 15.6° C. The consistency of the liquid-crystal mixture was very stiff, with poor liquid-crystal break (discreteness of crystals), and was nonpourable.

Example 2

In this example, two thousand grams of the same hydrogenated soybean oil were heated to 60° C., suspended in a temperature-controlled bath, and agitated with a large perforated paddle at 24 r.p.m. The bath temperature was 14.4–15° C. Three hours were required for the stock to reach the fractionation temperature of 15.6° C. The mixture was held with agitation at 15.6° C. for one hour. The liquid-crystal mixture was completely fluid, and exhibited a good break; that is, the crystals were well defined and uniformly suspended in the liquid and were readily separated in subsequent filtering.

Example 3

Two thousand grams of the same 85 IV soybean oil were melted and pumped through a scraped surface heat exchanger of the Emulsorator type. The stock was chilled to a temperature of 13.3° C. in less than three minutes. The quick chilling produced a small crystal formation which in a short time completely solidified.

Photomicrographs were taken of the materials produced in the three foregoing examples. It was shown that without agitation the crystals grow to a very large size having long thin tenacles extending from the crystals which likely are responsible for trapping and holding liquid oil. It is likely that the rate of cooling of Example 1 also significantly affected the crystal size because, with no agitation, the cooling rate was the slowest, which would also induce increased crystal size.

The photomicrographs of the liquid-crystal mixture obtained by the quick chilling in the scraped-surface heat exchanger of Example 3 show the crystal size to be exceedingly small. Again, it appears that the crystal size is a function of agitation and rate of cooling and both of these parameters were high in this case. The scraped surface chiller had an agitator speed of 600 r.p.m. With the presence of the many small crystals, a latticework was formed which prevented separation of the liquid and crystals.

In the method of Example 2 the photomicrographs showed fairly large crystals or clusters of crystals with relatively short tentacles and the amount of liquid entrapped or held by the crystals was low. The desired agitation for best performance of the process of the invention was obtained with gate paddle impellers or turbine-type agitators at low speeds. The range of speeds found useful varied from approximately 10 r.p.m. to 200 r.p.m. The preferred rate of cooling lies in the range of 5° F./hour to 50° F./hour.

There is set forth in Table I the results of fractionation studies carried on under varying fractionation conditions with differing soybean starting stocks. It will be seen that the hydrogenation temperature has a significant effect upon the percent of the desired trans isomer formed. The fractionation conditions employed have a definite bearing on the size of the oil fraction recovered.

TABLE I

Fractionation Condition			Starting Stock					Oil Portion				
Temp., ° F.	Agitator, R.P.M.	Hours at Temp.	Hydrogenation, Temp.	IV[1]	Solids at (° F.) 50	Solids at (° F.) 92	Melt Point	IV[1]	Percent Trans.	Solids at (° F.) 50	Solids at (° F.) 70	Percent Yield
60	40–45	3	120–130	82.6	20.0	0.5	30.9	59.1	31.8	0.2	.....	58.3
60	24	1	120–130	82.6	20.0	0.5	30.9	90.3	29.8	0.1	.....	50.6
45	24	3	120–130	82.6	20.0	0.5	30.9	88.5	33.1	12.0	0.7	70.0
40	40	3.5	160–165	80.8	29.8	0	29.7	85.4	26.5	8.1	0.2	50.4
60	24	1	160–165	81.6	29.8	0	29.7	85.3	37.0	4.7	0	47.0
60	24	2	160–165	80.6	29.8	0	29.7	88.7	36.9	1.4	0.4	49.0
60	24	4	160–165	80.9	29.8	1	29.5	85.7	37.3	0.7	0.2	49.3
60	24	1	160–165	80.8	29.8	0	29.7	84.4	36.0	10.5	0.2	54.0
60	24	1	160–165	81.5	28.5	0	29.7	85.9	38.6	0.2	0.1	49.0
60	24	1	160–165	83.9	25.4	0	29.6	88.4	35.3	0.1	.....	34.0
60	24	1	160–165	83.9	25.4	0	29.6	90.3	33.3	0	.....	47.3
60	24	1	160–165	75.2	45.9	3.4	35.6	91.9	46.2	29.9	0.6	32.4
60	24	1	160–165	75.2	45.9	3.4	35.6	91.4	47.6	31.6	4.0	25.4
60	32	1	160–165	81.1	30.5	0.2	30.2	83.7	39.4	7.1	.....	49.5
60	24	1	160–165	81.1	30.5	0.2	30.2	82.0	35.0	4.2	0.3	49.6
60	36	1	160–165	81.1	30.5	0.2	30.2	85.7	35.9	4.9	.....	48.0
60	48	1	160–165	81.1	30.5	0.2	30.2	85.9	35.1	5.5	0.2	49.2
60	24	1	160–165	81.9	25.4	0	29.8	87.3	36.4	11.7	.....	54.7

[1] Iodine value.

Example 4

This example demonstrates the relative yields obtainable with the processing of soybean, cottonseed, and safflower oils in accordance with the invention. Each of the three oils had two samples hydrogenated to the iodine values (I.V.) indicated in Table II. All hydrogenations were carried on at 180° C. with a nickel catalyst. The two soybean samples were hydrogenated respectively to iodine values of 80.8 and 83.9. The cottonseed samples were hydrogenated to iodine values of 82.6 and 84.8 and the safflower samples to iodine values respectively of 84.8 and 87.0. The samples of all three oils were processed in a like fashion with each hydrogenated stock being slowly cooled with agitation from 80° C. over a period of 3 hours to reach the fractionation temperature of 15.6° C. The cooled mixture was held with agitation at 15.6° C. for one hour prior to filtration accomplished as described above. The agitation was accomplished with a large perforated paddle at 24 r.p.m. All samples were completely fluid with the crystals being well defined and uniformly suspended in the liquid phase. There was no difference in filtration time or character of the separated solids. The crystals obtained were similar in microscopic appearance and size for the three different hydrogenated oils. The oil fraction percent yield for each of the samples is set forth in Table III where it is seen, for example, that the two soybean oil samples had percent oil fraction yields of 48.3 and 54.0. The safflower oil exhibited percent oil fraction yields of 50.7 and 54.7. The two cottonseed samples had percent yields of 57.1 and 64.5. Table III provides other fractionation data on the several examples.

TABLE II.—ANALYTICAL DATA ON VEGETABLE OILS HYDROGENATED AT 180° C.

Oil	Soybean		Cottonseed		Safflower	
I.V.	80.8	83.9	82.6	84.8	84.4	87.0
Solids:						
10° C.	29.5	26.4	21.6	16.3	21.1	28.8
21.1° C.	14.0	11.1	10.7	7.6	13.6	11.0
26.7° C.	6.5	4.9	7.6	3.0	6.8	3.8
33.3° C.	0	0	0.8	0.4	0	0
40° C.	0	0	0	0	0	0

TABLE III.—15.6 °C. FRACTIONATION DATA ON HYDROGENATED VEGETABLE OILS

	Starting Stock, Iodine Value	Oil Fraction			Solid Fraction					
		Percent Yield	IV	10° C. Solids	IV	Percent Solids At				
						10° C.	21.1° C.	26.7° C.	33.3° C.	40° C.
Stock Oil:										
Soybean	80.8	48.3	85.7	0.7	73.8	44.9	30.3	22.5	4.1	0
	83.9	54.0	88.4	0.1	76.9	41.9	26.0	20.8	3.4	0.3
Cottonseed	82.6	57.1	88.2	0.8	69.9	39.8	28.3	24.4	12.2	0.2
	84.8	64.5	91.0	0.3	73.0	35.2	25.1	21.8	11.3	0.4
Safflower	84.4	50.7	89.3	0.5	76.1	48.7	31.0	23.1	4.2	0.4
	87.0	54.7	91.7	0.4	79.0	44.6	28.4	19.6	2.1	0.3

Although exemplary embodiments of the invention have been disclosed herein for purposes of illustration, it will be understood that various changes, modifications, and substitutions may be incorporated in such embodiments without departing from the spirit of the invention as defined by the claims which follow.

We claim:

1. In a method of producing an improved liquid shortening, the steps comprising:

(1) selectively hydrogenating a vegetable oil characterized by a fatty acid composition having at least 45% linoleic acid at a temperature in the range of 100–250° C. to produce a partially hydrogenated material having an iodine value in the range of 70–100;

(2) cooling a quantity of the foregoing hydrogenated material from a liquid state to a temperature in the range of 5–25° C., said cooling being carried on with agitation to obtain large-sized, separable crystals; and

(3) separating the crystals from the hydrogenated material to recover a liquid shortening product.

2. A method in accordance with claim 1 wherein the vegetable oil is soybean oil.

3. A method in accordance with claim 1 wherein the vegetable oil is cottonseed oil.

4. A method in accordance with claim 1 wherein the vegetable oil is safflower oil.

5. In a method of producing an improved liquid shortening, the steps comprising:

(1) selectively hydrogenating a vegetable oil characterized by a fatty acid composition having at least 45% linoleic acid at a temperature in the range of 160–185° C. to produce a partially hydrogenated material having an iodine value within the range of 70–100 and characterized by an increase in trans isomer content of the fatty acids;

(2) cooling the partially hydrogenated material from a liquid state to a temperature in the range of 5–25° C., said cooling being carried on with agitation to obtain large-sized, separable crystals; and

(3) thereafter separating the crystals from the cooled partially hydrogenated material to recover an improved liquid shortening.

6. In a method of producing an improved liquid shortening, the steps comprising:

(1) selectively hydrogenating a vegetable oil characterized by a fatty acid composition having at least 45% linoleic acid at a temperature in the range of 160–185° C. to produce a partially hydrogenated material having an iodine value in the range of 80–90, said hydrogenated material having a linoleic acid content of less than 15% and an increased trans fatty acid content;

(2) cooling the foregoing partially hydrogenated material from a liquid state to a temperature in the range of 5–25° C., said cooling being carried on with agitation to obtain relatively large-sized, separable crystals; and

(3) thereafter separating the liquid component from the crystals of the partially hydrogenated material to recover an improved liquid shortening.

7. A method in accordance with claim 6 wherein the vegetable oil is soybean oil.

8. A method in accordance with claim 6 wherein the vegetable oil is cottonseed oil.

9. A method in accordance with claim 6 wherein the vegetable oil is safflower oil.

10. In a method of producing an improved liquid shortening, the steps comprising:

(1) selectively hydrogenating a vegetable oil characterized by a fatty acid composition having at least 45% linoleic acid at a temperature in the range of 160–185° C. to produce a partially hydrogenated material having an iodine value in the range of 80–85 and characterized by a linoleic acid content of less than 8% and being substantially free of linolenic acid, said partially hydrogenated material being further characterized by having a substantial increase in the percent of fatty acids having a trans form;

(2) cooling the hydrogenated material from a liquid state to a temperature in the range of 13–17° C., said cooling being carried on with agitation to obtain large-sized separable crystals; and

(3) thereafter separating the liquid component from the crystals of the cooled partially hydrogenated material to recover a liquid shortening.

11. In a method of producing an improved liquid shortening, the steps comprising:

(1) selectively hydrogenating a vegetable oil characterized by a fatty acid composition having at least 45% linoleic acid at a temperature in the range of 160–185° C. to produce a partially hydrogenated material having an iodine value in the range of 83–85 and characterized by a linoleic acid content of less than 8% and substantially no linolenic acid and further characterized by having a significant increase in the trans content of the fatty acids over that normally found in said oil;

(2) slowly cooling the foregoing hydrogenated material with agitation from an elevated temperature to a temperature in the range of 13–17° C., said cooling being carried on to obtain a crystal phase having at least about 75% of the crystals with a minimum dimension of not less than 50 microns; and

(3) thereafter separating the liquid component from the crystal phase to recover an improved liquid shortening.

12. A method as defined in claim 1 in which said agitation is a controlled mechanical agitation effected by moving rotating members at low speed through the hydrogenated material during the cooling thereof.

13. A method as defined in claim 1 in which the agitation is a controlled mechanical agitation effected by sweeping a gate-type impeller or turbine-type agitator through the hydrogenated material at a rate of about 10–200 r.p.m. during cooling of said hydrogenated material at a rate of about 5–50° F./hr.

14. In a method of producing an improved completely liquid shortening free of any dispersed solids at storage

temperatures above approximately 60° F., the steps comprising:

(1) selectively hydrogenating a vegetable oil characterized by a fatty acid composition having at least 45% linoleic acid at a temperature in the range of 160–185° C. to produce a partially hydrogenated material having an iodine value in the range of 80–85 and characterized by a significant increase in the trans isomer content of the fatty acid and further characterized by having a linoleic acid content of less than 8% and substantially no linolenic acid present; and

(2) cooling with agitation the foregoing partially hydrogenated material from an elevated temperature above its crystal fractionation range to a temperature in the range of about 13–17° C. to obtain large sized, separable crystals, said cooling being continued for a period of about 1–16 hours at a rate of about 5–50° F./hr. during agitation that is continued throughout the cooling, said agitation being effected by sweeping rotating members through the batch of partially hydrogenated material at a rate of about 10–200 r.p.m.

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MAURICE W. GREENSTEIN, *Primary Examiner.*

Removal of Chlorinated Pesticides From Crude Vegetable Oils by Simulated Commercial Processing Procedures

KEITH J. SMITH, PERCY B. POLEN,[1] D. M. DE VRIES[2] and FRANCIS B. COON,[3]
National Cottonseed Products Association, Inc., P.O. Box 12023, Memphis, Tennessee 38112

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Abstract

Crude soybean and cottonseed oil were processed using simulated commercial processing procedures to determine if oil processing would remove chlorinated pesticide contaminants of either natural or spiked origin. Two crude oil lots were spiked with endrin, DDT, DDE, aldrin, dieldrin, heptachlor and heptachlor epoxide before processing. Representative samples of crude oil and products following each processing step were analyzed for pesticide contamination. Results indicated that alkali-refining or subsequent bleaching did not reduce chlorinated pesticide contamination. Hydrogenation prior to deodorization reduced endrin contamination. Deodorization, with or without hydrogenation, eliminated chlorinated pesticides. The results of this study indicate that normal commercial processing of crude vegetable oils for human consumption effectively removes any chlorinated pesticides which may be present in crude oils. It is hypothesized that chlorinated pesticide removal is achieved by volatilization during deodorization, which is supported by known volatilization characteristics, similarity of behavior in pesticides studied, and absence of the pesticide or its conversion products in the finished oils, or both.

Introduction

CHLORINATED PESTICIDES ARE USED on many farm crops and are partially responsible for the increased agricultural productivity experienced in this country during the past few years. Concern by many has focused on possible food product contamination by the chlorinated pesticides resulting from misuse of chemicals, accidental product contamination or translocation from the soil to edible portions of the plant.

The specific objectives of the present study were to confirm previous reports that chlorinated hydrocarbons are removed from vegetable oils by processing procedures, that during oil processing endrin would act similarly to other closely related chlorinated pesticides, and that endrin residues are not detectable in the edible oil.

The basis for the study was a scientific communication by Gooding (1) and some limited studies conducted by Shell Chemical Company (2). Gooding spiked a vegetable oil with the following chlorinated pesticides at the indicated levels (ppm): aldrin, 1.0; chlordane, 1.0; DDT, 21.0; dieldrin, 1.0; heptachlor, 1.0; heptachlor epoxide, 1.0; Kelthane, 1.0; lindane, 15.0 and 30.0; methoxychlor, 42.0; sesone, 18.0; Strobane, 15.0; TDE or DDD, 21.0; and toxaphene, 21.0. The oil was alkali-refined, bleached and deodorized under pilot plant conditions. Standard GLC methods employing electron-capture and microcoulometric detectors were used to analyze for residue contamination. Results showed that deodorization caused removal of the pesticide residues to levels below detectability. These results were subsequently confirmed in plant scale tests in which the crude vegetable oil was found contaminated. It was also reported that a vegetable oil containing chlordane (2.8 ppm), DDT (0.5 ppm) and DDD (0.5 ppm) was subjected to hydrogenation during processing, whereupon the pesticide residues disappeared.

A series of U. S. Food and Drug Administration reports has been published on studies of pesticide residues in total diet composite samples which would represent the relative types and amounts of food consumed by 16- to 19-year-old boys during a 14-day period (3–8). The authors reported that the lower limits of sensitivity of the analytical procedures used were 0.01 ppm (1963) and 0.001 ppm (1964–67). Endrin was not detected in the composite food samples (3–5).

Survey results directly applicable to the present study have been reported on composite samples of oils, fats, shortening and other high-lipid diet ingredients. Williams (4) first reported the high-lipid composite sample contained low levels of several chlorinated pesticides. However, endrin was not qualitatively detected at a level of 0.001 ppm. These results were later confirmed (5–7). Duggan et al., (8) reported that endrin was also present in a composite sample of oils, fats and shortenings which may be presumed to be a mixture of animal and vegetable origin. This study, however, did not state the level found or the frequency of contamination in the composite samples analyzed.

These dietary surveys would indicate that processed, edible vegetable oils and oil products should be free of endrin contamination. Even though endrin was not included in Gooding's (1) research report, it would be logical to conclude that endrin would act similarly to related chlorinated pesticides. Each of those references, either directly or indirectly, provided evidence that endrin contamination is not likely to be present in the finished edible vegetable oils.

Experimental Procedures

A total of 10 crude oils, 5 soybean and 5 cottonseed lots were processed in a pilot plant (Votator Division, Chemetron Corporation, Louisville, Kentucky) designed to simulate commercial oil processing procedures. Eight samples of crude oil, suspected of pesticide contamination, were processed. To insure that the processing conditions would not produce any unknown artifacts which could be interpreted as pesticide contamination, a crude soybean oil (SBO) sample and a cottonseed oil (CSO) sample analyzed free of chlorinated pesticides were included as negative controls. All lots were alkali-refined, followed by bleaching and deodorization.

[1] Velsicol Chemical Corporation, 330 East Grand Avenue, Chicago, Illinois 60611.
[2] Shell Chemical Company, P. O. Box 813, Princeton, New Jersey 08540.
[3] Wisconsin Alumni Research Foundation, P. O. Box 2037, Madison, Wisconsin 53701.

(Andrews *et al.* 1960), presence of free radicals may cause alteration in protein quality (Roubal 1966), presence of high quantities of polyunsaturates in the diet under certain conditions cause undesirable effects such as growth retardation (Oldfield *et al.* 1963), and assimulation of polyunsaturates into the flesh of poultry which may cause development of undesirable flavors in the meat (Hardin *et al.* 1964). All of these effects may also be caused by consumption of unsaturated vegetable oils—e.g., the production of fishy flavor in the meat of poultry (Klose *et al.* 1951). With the greater quantity of natural antioxidants present in vegetable oils these effects are often less pronounced. The point has sometimes also been raised as to whether due to oxidative deterioration, an appreciable portion of the polyunsaturates may be altered or destroyed so as definitely to diminish their potential desirable nutritive properties.

These are all only potential effects. Whether or not they develop depends to a very large extent upon circumstances and the purpose for which this section is intended is to explore these circumstances.

The most important factor influencing the degree to which these potential alterations may develop is the degree and extent to which the polyunsaturates oxidize. This in turn is largely controlled by the use to which the oil is put.

Oils in Food for Human Consumption

Fresh Fish.—In fresh, unfrozen fish, the oil ordinarily oxidizes not at all or at the most to a very slight extent up until the time it becomes inedible from spoilage or other causes. This stability of the oil is dependent partly upon the fact that fresh fish, being highly perishable, is held at temperatures close to 0°C. and even so the storage life is limited to a very short period of days. The effect of low temperature and short storage time in any case does not permit extensive oxidation to take place. Of greater importance, however, is the effect cited by Stansby (1963) wherein there seems to be a competition for oxygen between bacteria and lipids such that the rate of oxidation of lipids is greatly repressed whenever aerobic bacterial spoilage is taking place. This results in the ordinary situation in peroxide values being in the range of 0 to 2, never ordinarily above 5 or at the most 10, in the lipid of fish oils in such fish up to the time when flavor alteration makes the fish of unacceptable quality. At such low levels of oxidation the peroxides are ordinarily at less than one per cent of the levels where they will *per se* cause any problems and free radical formation is far too low to cause any measurable alteration in protein quality. Other undesirable effects are also imperceptible.

Preserved Fish.—In frozen fish, storage temperatures are much less than in unfrozen, refrigerated fish but this desirable factor is more than offset

by the fact that bacterial action is arrested making fully available to the lipid any oxygen present. Usually the fish is stored in partially oxygen-impenetrable packaging, limiting access to air. Nevertheless, oxidation of oil in frozen fish after several months of storage generally somewhat exceeds that occurring in unfrozen fish. The levels of oxidation reached, however, are still very small with maximum peroxide numbers generally in the range of 5 to 10 with extreme values up to 25 occuring only with excessive storage periods well in excess of a year which rarely occur. Even these degrees of oxidation are far below levels required for any appreciable adverse nutritional effects.

Canned fish, being stored in a vacuum, does not permit oxidation of the lipid so that no adverse changes take place.

Fish preserved by curing processes such as salting, smoking, and pickling are held at room temperature sometimes with fairly free access to air. When any of these products are stored for extensive period of time, increasing degrees of oxidation occur. Although peroxide levels can become higher in the oil of these types of products than in the others considered above, a built-in safety factor limiting consumption of excessively oxidized products is the flavor. Flavor alteration starts at very low levels of oxidation such that even in fresh fish with peroxide numbers in the range of 0 to 2 (and resulting carbonyl content—actual cause of the flavors—at almost indeterminable levels) a pronounced, perhaps unpleasant, flavor may be apparent. Long before oxidation reaches levels where undesirable nutritional properties would occur, the flavor has become so objectionable at least to the average taste in Western countries that the fish would never be eaten.

The question of whether the mere presence of highly polyunsaturated fatty acids from fish in human diets may result in adverse effects, even though no oxidation has taken place, remains to be considered. These effects, e.g., depression in growth and such conditions as encephalomacia have been observed only in farm animals or poultry in which fish oils have been used for long periods of time as a significant part of the daily diet and usually then only in the absence of adequate amounts of tocopherol. Animals living in a captive state may, in such cases, utilize fish oil as a constant portion, sometimes at a relatively high level, of their diet which then may bring about these adverse effects. Failure to ever note such conditions in humans doubtlessly is due to the far lower content of the total diet which fish plays. Even in countries such as Japan where fish may be a major source of animal protein and where Imaichi *et al.* (1965) have shown that human depot fat may contain significant proportions of typical C_{22} polyunsaturated fish oil fatty acids, there have been no indications of adverse effects of inclusion of these high levels of fish in the diet.

Stability of Fish Oil Polyunsaturation in Human Food.—The common processing methods used in preserving fish do not cause breakdown in the polyunsaturates. Thus Roubal (1963) was unable to detect any change in fatty acid content of tuna between the raw and uncooked state or after canning. The degree of oxidation present in refrigerated or otherwise preserved fish is so low that there is ordinarily no measurable change in degree of unsaturation as estimated by iodine number and there is no evidence for alteration in fatty acid content.

Summary.—In summary, the oil in the flesh of fish used for human consumption does not cause adverse nutritional problems. This is true whether the fish is freshly caught, or has been preserved. The lack of such changes is due, on the one hand, to the very slight degree of oxidation which occurs before flavor alteration renders the fish unfit for consumption, and, on the other hand, to the relatively low levels at which fish are used in human diets as compared to higher levels sometimes used with farm animals.

OILS IN FEED OF ANIMALS

Since the topic of nutritional effects of fish oils in the diet of animals is treated extensively in Chapter 24, it will be touched upon only briefly here.

Fish oil is fed to animals largely in the form of oil present in fish meal with the practice of adding fish oil as a feed supplement currently much less common than when fish-liver oils were the principal sources of vitamins A and D. During and immediately after the manufacture of fish meal, rapid, active oxidation of fish oil left in the meal takes place. This oxidation, probably by action of free radicals produced, causes some reduction in protein quality (Roubal 1966), but the loss cannot be severe as shown by the considerable literature on the superior protein quality of fish meal as well as the extensive commercial use of fish meal as a supplement to vegetable proteins.

The use of fish oils in the diets of animals and poultry is accompanied by several problems. Only as a result of relatively recent research are we reaching a point where these problems can be intelligently met. Fishy flavor may be imparted to the meat of animals fed excessive quantities of fish oil, but our present knowledge of limiting quantities to safe levels now permits control of this problem. Knowledge of the importance of the use of adequate antioxidants in farm animal diets (Oldfield *et al.* 1963) is beginning to solve problems formerly critical with respect to adverse effects caused by the polyunsaturates *per se*. The fact that fish oils while containing only small quantities of classical essential fatty acids are very high in growth-promoting fatty acids of the linolenic acid family is only recent-

ly being appreciated. Greater detail on these and other matters related to utilization of fish oils by animals, and in metabolism, will be found in Chapters 21 to 24.

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Food Science Pioneer Research Laboratory, Bureau of Commercial Fisheries, Seattle, Wash.

Nutritional Properties of Fish Oils

M. E. Stansby

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I. Introduction

Fish oils are much more complex in chemical structure than are other fats or oils. They contain many classes of lipids as well as a very diverse group of fatty acids ranging from saturated to highly polyunsaturated. Because of this varied composition, these oils are of considerable interest from the standpoint of biochemistry, metabolism, nutritional properties and in some cases pharmaceutical action.

Research on fish oils has lagged behind that on most other lipid materials. The very complexity of the fish lipids from the standpoints of difficulty of separation of components and the great instability of the polyunsaturates discouraged many of the earlier workers in the lipid field from carrying out any more than desultory investigations upon them. In the past 15 years or so, however, with introduction of many new techniques a considerable volume of research papers dealing with fish oils have appeared.

The purpose of this review paper is to discuss our knowledge of fish oils primarily from the viewpoint of nutritional properties. However, a considerable amount of background material will be presented with respect both to the chemistry of fish oils and to sources of fish oils. The chemistry will be discussed chiefly with regard to the many classes of lipids found in fish oils and with considerable emphasis on the diverse fatty acid make-up of the chief class, triglycerides. The topic of oxidation and stability will also be considered briefly. The source of fish oils will be discussed primarily with respect to the way

TABLE IV

Pesticide Residues Found in Fortified Cottonseed Oils at Various Stages of Processing[a]

	Refined oil	Bleached oil	Deodorized oil
	ppm		
Endrin	0.42	0.65	[b]
DDT	11.1	13.5	[c]
DDE	0.92	0.83	BDL[d]
Aldrin	0.55	0.65	BDL
Dieldrin	0.72	0.64	BDL
Heptachlor	0.56	0.63	BDL
Heptachlor epoxide	0.77	0.80	BDL

[a] Average pesticide value of two oils with three independent laboratory analytical values per oil.
[b] Values (CSO-1, 0.07; CSO-2, 0.02) were reported by one laboratory and the other two laboratories could not confirm the presence of a detectable level of residue.
[c] A value of 0.06 ppm was reported for CSO-2 by one laboratory and not confirmed by other laboratory analyses.
[d] BDL = Below detectable limits of analysis.

Endrin may be eliminated from the bleached oil during hydrogenation by adsorption onto the activated carbon contained in the catalyst, by reductive dehalogenation of the chlorinated hydrocarbon endrin molecule (12), or by volatilization of the molecule under the pressure-temperature conditions of hydrogenation. It would be unlikely that complete isomerization would occur at the hydrogenation temperature (275 F), since Phillips et al. (13) showed that isomerization occurred but at much higher temperatures (450 F). The analytical laboratories were unable to detect endrin rearrangement products or unknown chlorinated breakdown products in the hydrogenated oil.

The data indicate that the chlorinated hydrocarbons are removed from the neutral oil during deodorization by forced volatilization. Endrin or its isomerization products, or both, appeared to act in similar manner.

Evans (14) reported a preliminary study to assess the fate of endrin using radioactive carbon-labeled endrin. Laboratory deodorization showed that at low levels (under 3.5 ppm) 96% of the radioactivity was removed from the oil. Seventy-five per cent of the original activity was recovered in the condensate. It was stressed that these results were of preliminary nature.

Ott and Gunther (15) used forced volatilization of chlorinated pesticides (lindane, aldrin, dieldrin, DDD, DDE and heptachlor epoxide) for quantitative isolation from butterfat. The equipment described in their paper is essentially a small scale deodorizer; the temperature of the liquid material is essentially the same in both systems (550 F vs. 475 F for the pilot plant deodorizer). The only difference between systems is that one bubbles nitrogen and the other dry steam. The volatility of chlorinated pesticides was stressed as a possible source of error in pesticide measurements (16).

The foregoing references emphasize the volatility of chlorinated pesticides. It would appear that one could be completely justified in considering that chlorinated pesticides and their rearrangement or breakdown products behave in a similar fashion.

TABLE V

Average Endrin Content in Various Oil Fractions Obtained in Processing the Four Soybean Oils[a]

	Total endrin content
	mg
Crude oil	43.1
Soapstock oil loss	4.8
Refined oil	40.9
Bleached oil	36.5
Deodorized oil	BDL[b]

[a] Values are calculated from mean endrin analyses and oil fraction weights of the four soybean oils.
[b] BDL = Below detectable limits of analysis.

Therefore, if endrin does rearrange (13,17) at the temperature experienced in deodorization, it would not be of great concern since the conversion product would be volatilized.

A material balance for the pesticides was not established because of incomplete recovery of the deodorizer condensate. Rapid distillation of the chlorinated hydrocarbons and the lack of sophisticated collection equipment are two factors which probably prevented the complete recovery of the pesticide contaminants.

The overwhelming preponderance of evidence obtained in this study appears to justify fully the conclusion that vegetable oils processed for human consumption are free of chlorinated pesticide residues, including endrin. The data strongly support the hypothesis that if contamination of crude vegetable oils should occur from the use of pesticides during the growth period of the oilseeds, or artificially in handling of the seed or extracted oil, such pesticides will be removed through the pathway of volatilization during hydrogenation or deodorization, or both.

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M. E. Stansby

Misconceptions about Nutritional Properties of Fish Oils

INTRODUCTION

This chapter is presented as an introduction to this section of the book and with the aim to dispel certain possible misconceptions concerning fish oil nutritional properties. Fish oils contain a far wider spectrum of fatty acids than do other natural lipid sources (see Chapter 1). Just as ingested proteins require for maximum nutritional value a good balance of essential amino acids, in an analogous way lipids should contain a good balance of fatty acids which serve some useful function. Unfortunately our present stage of knowledge is incomplete with respect to which of the many fatty acids do perform useful functions under various states of normal and pathological conditions.

There are, however, indications that some fatty acids, not necessarily restricted to classical essential fatty acids (linoleic acid family), may perform such useful functions. For example, members of the linolenic acid family of fatty acids which occur in considerable proportions in fish oils both support growth and markedly lower serum cholesterol levels. On the other hand, presence of highly polyunsaturated fatty acids in fish oils has raised questions as to whether potentially undesirable effects may occur which stem from autoxidation leading both to the presence during oxidation of free radicals and to formation of certain oxidative intermediate and end-products. Some of these ideas will be examined briefly in this chapter.

HISTORICAL NOMENCLATURE DEVELOPMENT

Early research on essential fatty acids (EFA) overemphasized the curing of dermal symptoms even though recognizing that fatty acids of what are today described as of both the linoleic and of the linolenic acid families support growth. Knowledge of effects such as that on permeable membranes and cholesterol transport were completely lacking when EFA activity was first investigated. Since curing of dermal symptoms provided a specific assay procedure, the term EFA became linked more specifically to that aspect than to other effects of perhaps greater significance. Thus fatty acids of the linoleic acid family are now the only ones generally classified as having true EFA properties. If other effects, e.g., cholesterol depressant properties, had been discovered before dermal symptom curing properties, it is likely that we might today have a somewhat different con-

cept of what constitutes an EFA. Certainly from a practical nutritional standpoint, support of growth and even probably cholesterol depressant effects have greater practical importance than do the curing of dermal symptoms. These facts are often overlooked and it is not uncommon to find statements expressing surprise that fish oils, low in classical EFA content, are useful in stimulating growth or in depressing serum cholesterol levels.

Another way in which the historical development of nomenclature has reflected adversely on ideas concerning nutritive value of fish oils relates to the use of the term "polyunsaturates." This term, classifying all fatty acids with two or more double bonds per molecule together has tended to mask the fact that there is a marked difference in degree and type of polyunsaturation. Most vegetable oils contain a high percentage of dienes (primarily linoleic acid) sometimes exceeding 50%. Particularly with respect to effects on serum cholesterol levels, the widespread usage of the term polyunsaturates without consideration of degree or type of polyunsaturation tends to oversimplify the situation and thereby confuses the issue. Furthermore as has been pointed out by Bloch (1964), it is the fatty acids with three or more double bonds which possess multifunctional properties and hence are of greatest biochemical interest.

Development of these commonly used terms occurred when lipid research was restricted quite largely to the less complex animal and vegetable fats and oils. In such context, terms like EFA and polyunsaturates used in their limited sense resulted only to a minor extent in erroneous interpretation of their significance to nutritional applications. Now that research on the much more complex fish oils is getting under way and some small attention focused upon them, we find that these limited and imperfectly understood terms sometimes lead to misconceptions. Considering only the classical EFA content, limited to fatty acids of the linoleic acid family, one might predict fish oils to possess far less growth-promoting fatty acid content than tallow. An actual comparison of total growth-promoting fatty acid content (linolenic plus linoleic acid family fatty acids), however, shows fish oil to contain far more of these growth stimulants (see Chapter 24). Likewise, comparison of vegetable and fish oil polyunsaturate content would, if used as sole criteria, indicate the former to possess far greater cholesterol depressant activity when actually fish oils possess such activity to a greater extent.

OXIDATIVE PROPERTIES OF FISH OIL POLYUNSATURATES

Because of the high degree of polyunsaturation of fish oil fatty acids, there is a potential for several adverse nutritional effects to develop. Accumulation of peroxides in large quantities *per se* may result in toxicity

in which the diverse chemical entities found in fish originated and found their way into the fish as well as a consideration of the way these substances occur in different types of tissue or organs of the fish. Some brief treatment will be included of the chief sources of commercial fish oils and of their geographical distribution.

Nutritional properties will then be discussed first with respect to the various actions of the fatty acids, then with regard to the nutritional properties of other important classes of lipids.

II. Chemistry of Fish Oils

A. Introduction

The chemical composition of the *average* fish oil differs from that of most other naturally occurring oils and fats in having a greater variety of lipid compounds, in possessing larger quantities of fatty acids with chain length exceeding 18 carbons, in having a much greater proportion of highly polyunsaturated (more than 4 double bonds/molecule) fatty acids and in having polyunsaturates primarily of the $\omega 3$[1] rather than the more usual $\omega 6$. Most all fish oils possess these properties to a greater or lesser extent in common. There are, however, vast differences in composition of oils from different species of fish or even from oils of the same species of fish taken from different geographical areas. Such variations will be discussed in this section. There is another type of wide variation in fish oil composition which depends upon the part of the fish from which the oil is made. Not only is the liver oil, for example, quite different in composition from that of the oil of the flesh of the same fish, but also oil from different parts of the flesh (e.g. dark *versus* light muscle or depot fat *versus* cell constituents) may vary widely in chemical composition. Most of the discussion of oil within different parts of the fish will be given in the next section (Source of fish oil).

B. Classes of Lipids

All fish oils contain triglycerides and in many cases these are the only main components. With some species of fish and especially with fish

[1] The designation of the position of the first double bond related to the methyl end of the molecule by the ω numbering systems is used in this article.

liver oils, other classes of lipids such as hydrocarbons or ether linked compounds, e.g. alkoxyglycerides may be a major component.

1. Triglycerides and fatty acids

Fish body and liver oils have the polyenoic fatty acids preferentially located in the β position, yet in marine mammals the triglycerides of the blubber have the polyunsaturates primarily in the α position [BROCKERHOF and HOYLE, 1963]. The ethylenic bonds in fish oils occurring in the *cis* configuration are present as a methylene interrupted type (divinylmethane structure) [PRIVETT, 1956; KLENK, 1958].

The carbon chain length in fish oil fatty acids commonly exceeds C18, and a considerable portion of the fatty acids contain 20, 22 and to a limited extent, 24 carbon atoms. The total proportion of these long chain fatty acids usually amounts to $^1/_4$–$^1/_3$ of the total and sometimes approaches ½. This high incidence of long chain fatty acids contrasts sharply with the situation usual with most oils and fats of vegetable or animal origin where the proportion of fatty acids with more than 18 carbon atoms often is of only trace proportions, seldom exceeds 5% and is frequently less than 1% (especially in most vegetable oils).

The highly polyunsaturated fatty acid content of fish oils is far higher than that of any other common naturally occurring oils or fats. Most frequently these 5 and 6 double bonded fatty acids range in proportion of total fatty acids from 15–30%. They very seldom are less than 10% and occasionally range up to 50%. Five and six double bonded fatty acids in oils and fats of vegetable or animal origin seldom equal even 1%.

In addition to the highly polyunsaturated fatty acids fish oils contain moderate amounts of other polyunsaturates (2, 3 and 4 double bonds) and considerable quantities of both saturates and monoenes.

Another unusual feature of fish oils is the presence of relatively large amounts of odd carbon chain (mostly C15, 17 and 19) fatty acids. While these ordinarily total about 1–3%, with at least one species, mullet, they total 10% or more (GRUGER *et al.*, 1960). Odd chain fatty acids have been reported hitherto [HARTMAN, 1957] as occurring in triglycerides of the fat of ox, sheep and butter. The extent to which they occur in fats from these sources is considerably less, however, than that in the oils of some fish.

Nearly all of the polyunsaturates of fish oils ranging in chain length from C18–C22 are of the ω-3 type, i.e. they are of the linolenic acid family rather than of the linoleic acid family (ω-6 type) as occurs pre-

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Table I. Usual range of fatty acid contents of most common species of marine, freshwater fish and shellfish

Fatty acid No. C atoms	No. double bonds/molecule	Saltwater fish %	Freshwater fish %	Shellfish %
14	0	2 –8	2 –6	2 –6
15	0	0.3–7	0.3–1	0.5–3
16	0	10 –30	10 –20	15 –25
16	1	2 –11	7 –11	2 –11
16	2	0.5–2	0.5–1.5	0.5–2
17	0	0.7–2.5	0.5–1.5	0.5–2
18	0	2 –6	3 –4	4 –9
18	1	12 –28	18 –28	5 –18
18	2	1 –3	4 –6	0.5–2
18	3	0.5–1.2	3 –5	0.5–2
18	4	0.7–4	1 –2	0.5–5
19	0	0.5–2	—	—
20	1	1 –10	1 –3	2 –9
20	4	0.5–4	2 –4	1.5–5
20	5	6 –14	5 –7	9 –22
22	1	1.5–9	0.5–3	0.2–12
22	5	0.6–3	2.5–4	1 –8
22	6	8 –20	8 –20	11 –27
24	1	0.5–2	1 –4.4	0.5–1

dominantly in other oils and fats. The proportion of ω-6 fatty acids in fish oils is very small, under 10%, often below 5%. The oils of freshwater fish usually have somewhat higher proportions (around 10%) of ω-6 fatty acids than the marine oils.

The usual ranges of fatty acids occurring in the body oils of most species of fish is shown in table I. It is apparent that there is a considerable variation in the amount of any given fatty acid. For example the C20 monoene varies in saltwater species over a range of ten times and most fatty acids have a range spread of three times or more. Freshwater fish oils would appear to have somewhat greater proportions of 18:2 (linoleic acid) though still far less than vegetable oils and a lesser amount of the C20 pentaene than do the corresponding saltwater species. The oils of shellfish on the other hand appear to be unusually high in the C20 pentaene and of the C22 hexaene.

In table II are shown the fatty acid contents reported for oils made from different species of fish. Because as will be shown later, there is

a relatively wide variation in fatty acid content of different batches of oil made from the same species of fish and because the samples of fish from which the results are reported for in table II were not in any case large, these values are only very approximate. They can not be considered to be entirely representative of the fatty acid content of all oils made from the same species. This fact can indeed be seen by comparing values reported by two investigators for the fatty acid content of two batches of oil from Atlantic cod. The considerable difference in composition is most likely largely due to actual differences in composition of the different cod body oils employed. Nevertheless there is sufficient difference in fatty acid composition of oils made from different species to show certain trends. For example, pink salmon, which is well known to contain oil which is the most unstable of any of the species of salmon indeed as unstable as the oil of any fish has a very high content of 22:6 and 22:5 fatty acid (over 32%). Sablefish, on the other hand, widely known to contain oil which is highly stable, has only about 7% of these same highly polyunsaturated acids.

The fatty acids of the oil of any given species of fish are not of constant proportion from fish to fish, but vary depending upon the feed which the fish has been consuming as well as with other factors. Even large batches of many tons of commercial fish oil (such as menhaden) prepared at different times or places will vary considerably with respect to fatty acid composition. Table III gives fatty acid composition of several very large sized commercial batches of menhaden oil made at different plants. Menhaden oil made from fish in southern areas (e.g. Gulf of Mexico) has on the average lower iodine number than oil from fish caught farther north. The content of saturates is lower and of polyunsaturates higher in the menhaden oil made from fish caught in northern waters.

The fatty acid content of fish oils has been reviewed comprehensively by GRUGER [1967] and for further details the reader is referred to this reference.

2. Phospholipids

Our most complete knowledge of the nature of phospholipids which occur in fish concern those found in tuna. KATADA, ZAMA and IGARASHI [1960] and more recently SHUSTER, FROINES and OLCOTT [1964] have carried out extensive investigations with this species, the former using *Thunnus orientalis*, the latter albacore tuna, *Thunnus alalunga*. The predominant phospholipids occurring in these species and probably

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Table II. Fatty acid content of body[1] oils of certain species of fish and shellfish

Name of species		Fatty acid[2] content in percent																	
Common name	Scientific name	14:0	15:0	16:0	16:1	17:0	18:0	18:1	18:2	18:3	18:4	20:1	20:4	20:5	22:1	22:5	22:6	Literature Reference
Clam, Littleneck	Protothaca stiminea	3.2	0.8	23.8	9.6	1.3	5.4	10.8	1.4	1.6	3.0	3.5	1.7	10.0	2.6	1.7	14.5	GRUGER *et al.*, 1964
Cod, Atlantic	Gadus morhua	1.8	0.5	33.4	2.4	0.9	4.0	11.8	1.2	0.8	1.2	1.6	3.2	12.4	0.7	0.6	21.9	GRUGER *et al.*, 1964
Cod, Atlantic	Gadus morhua	0.4	0.1	24.1	1.1	0.1	3.4	7.8	0.3	0.1	0.1	1.2	3.7	17.6	1.1	0.9	37.5	ACKMAN, 1964b
Cod, Pacific	Gadus macrocephalus	1.0	+	18.2	3.6	NK	4.7	14.5	1.0	—[4]	NK	2.0[4]	—[3]	17.9	5.6[3]	1.9	29.2	SHIMMA, 1964
Cod Liver Oil	Gadus morhua	2.8	0.4	10.7	6.9	1.2	3.7	23.9	1.5	0.9	2.6	8.8	1.0	8.0	5.3	1.3	14.3	GRUGER *et al.*, 1964
Crab, Blue	Callinectes sapidus	2.2	0.9	15.2	11.2	1.9	7.2	17.6	1.9	1.2	0.6	1.9	4.1	13.4	1.5	1.1	11.0	GRUGER *et al.*, 1964
Haddock	Melanogrammus aeglefinus	1.1	0.5	22.7	4.7	1.5	5.3	15.1	2.5	0.3	NK	3.5	1.8	14.3	NK	0.7	24.3	BEARE, 1962
Haddock, Liver Oil	Gadus aeglefinus	2	+	16	6	1	3	30	1	+	1	4	1	15	1	1	14	KLENK, 1962
Halibut, Pacific	Hippoglossus stenolepsis	2.8	0.3	15.1	8.9	0.7	3.4	25.7	0.9	0.3	3.6	8.0	2.5	10.1	5.1	1.6	7.9	GRUGER *et al.*, 1964
Herring, Pacific	Clupea harengus pallasi	7.6	0.4	18.3	8.3	0.5	2.2	16.9	1.6	0.6	2.8	9.4	0.4	8.6	11.6	1.3	7.6	GRUGER *et al.*, 1964
Mackerel, Common	Scomber scrombrus	4.9	0.5	28.2	5.3	1.0	3.9	19.3	1.1	1.3	3.4	3.1	3.9	7.1	2.8	1.2	10.8	GRUGER *et al.*, 1964
Menhaden	Brevoortia tyrannus	8.0	0.5	28.9	7.9	1.0	4.0	13.4	1.1	0.9	1.9	0.9	1.2	10.2	1.7	1.6	12.8	GRUGER *et al.*, 1964
Mullet, Striped	Mugil cephalus	4.6	6.3	17.3	11.0	0.8	5.0	8.4	3.2	1.4	3.0	0.7	2.6	7.5	0.7	3.9	13.4	GRUGER *et al.*, 1960
Oyster, Pacific	Crassostrea gigas	2.7	0.9	21.4	4.6	1.4	4.0	8.5	1.2	1.6	4.3	+	1.9	21.5	2.6	1.0	20.2	GRUGER *et al.*, 1964
Perch, Ocean	Sebastes marinus	4.6	0.6	12.6	8.0	1.0	3.6	22.0	1.5	0.6	1.6	8.0	0.8	9.3	8.7	0.6	12.0	GRUGER *et al.*, 1964
Pilchard	Sardinops sagax; Sardinops caerulea	7.6	0.6	16.2	9.2	0.7	3.5	11.4	1.3	0.9	2.0	3.2	1.6	16.9	3.8	2.5	12.9	ACKMAN, 1964
Salmon, Chinook	Oncorhynchus tshawytscha	3.7	0.4	16.6	9.2	1.1	5.8	29.1	1.1	0.9	1.5	4.7	0.5	8.2	3.6	2.4	5.9	GRUGER *et al.*, 1964
Salmon, Chum	Oncorhynchus keta	2.2	0.6	17.0	4.1	1.1	3.2	21.4	2.0	1.0	2.0	5.4	0.9	6.7	9.4	2.3	16.1	GRUGER *et al.*, 1964
Salmon, Coho	Oncorhynchus kisutch	3.7	0.5	10.2	6.7	0.9	4.7	18.6	1.2	0.6	2.1	8.4	0.9	12.0	5.5	2.9	13.8	GRUGER *et al.*, 1964
Salmon, Pink	Oncorhynchus gorbuscha	3.4	1.0	10.2	5.0	1.6	4.4	17.6	1.6	1.1	2.9	4.0	0.7	13.5	3.5	3.1	18.9	GRUGER *et al.*, 1964
Scallop, Sea	Placopecten magellanicus	1.9	0.7	23.0	2.0	0.8	5.3	5.2	0.6	0.3	1.8	1.7	4.5	21.3	0.2	1.0	26.2	GRUGER *et al.*, 1964
Sole, Lemon	Microstomus kitt	4.3	0.7	16.5	14.4	1.4	2.4	12.2	0.3	2.0	1.6	3.9	4.0	11.9	+	10.6	7.0	OLLEY and DUNCAN, 1965
Trout, Rainbow	Salmo gairdneri	2.1	0.8	11.9	8.2	1.5	4.1	19.8	4.6	5.2	1.5	3.0	2.2	5.0	1.3	2.6	19.0	GRUGER *et al.*, 1964
Tuna, Albacore	Thunnus germo	3.7	1.0	29.3	6.3	1.2	6.1	16.6	0.7	0.6	2.2	2.7	1.2	6.5	2.0	0.8	17.6	ROUBAL, 1963
Tuna, Bluefin	Thunnus thynnus	4.5	0.6	22.1	2.8	0.8	6.1	21.7	0.8	+	0.9	6.3	1.0	6.4	5.4	1.4	17.1	ROUBAL, 1963
Tuna, Skipjack	Katsuwonus pelamis	7.0	1.0	24.0	6.3	1.1	3.0	16.2	2.1	1.2	0.5	2.0	3.0	13.2	+	1.5	17.3	ROUBAL, 1963
Tuna, Yellowfin	Neothunnus macropterus	2.6	0.6	27.1	4.4	2.1	7.5	17.8	0.9	0.4	+	1.1	3.6	4.6	+	1.3	22.2	ROUBAL, 1963
Whitefish, Lake	Coregonus clupeaformis	2.2	0.3	12.1	10.5	1.1	4.0	27.2	5.5	3.7	1.0	2.1	3.9	6.4	0.5	3.3	8.8	GRUGER *et al.*, 1964

[1] Two liver oils are included for comparison.
[2] The figure before the colon is chain length; that after it is the number of double bonds.
[3] Combined 20:4 and 22:1 acids.
[4] Combined 18:3 and 20:1 acids.

Table III. Variation in fatty acid content of large batch of menhaden oil made from fish caught in Gulf of Mexico compared to that from a large batch of fish caught in the North Atlantic

Fatty acid	% of fatty acid	
	Gulf of Mexico	North Atlantic
14:0	13.5	10.6
15:0 + 17:0	3.1	3.4
16:0	22.4	23.6
18:0	2.6	3.0
Total saturates	41.6	40.6
16:1	17.3	14.8
18:1	14.1	12.6
20:1	1.6	2.7
22:1	0.9	1.0
Total monoenes	33.9	31.1
18:2	0.9	1.2
18:3	0.4	1.0
18:4	1.1	0.9
20:4	0.8	0.8
20:5	13.5	13.1
22:5	1.4	1.4
22:6	3.3	6.5
Total polyunsaturates	21.4	24.9
Total hexaenes	3.3	6.5

in fish in general are lecithin (about half of the phospholipids) and cephalin (about ¼).

The range of fatty acids present in the lecithin and cephalin of albacore tuna as reported by SHUSTER *et al.* [1964] are shown in table IV. The ranges are for those occurring in four different fish which were analyzed. As will be noted a very high variation occurred from fish to fish. Palmitic and oleic were the chief saturated fatty acids occurring with the former being predominant in lecithin, the latter in cephalin. As with the triglycerides of fish, the C20:5 and 22:6 fatty acids were the principal polyunsaturates present in both lecithin and cephalin.

3. Hydrocarbons

Hydrocarbons occur in high quantity in the liver oil of several shark; about 90% of the liver oil of *Centrophorus uyato* [HELLER *et al.*, 1957]

Table IV. Principal fatty acids in albacore tuna lecithin and cephalin[1]

Fatty acid[2]	Content in cephalin %	Content in lecithin %
12:0	trace to 1.3	trace to 0.3
14:0	trace to 3.6	0–9.4
16:0	4.6–13.8	22.2–34.0
16:1	trace to 3.2	0–4.8
17:0	trace to 5.3	0–0.5
18:0	14.8–28.5	3.7–5.4
18:1	9.2–17.5	9.8–29.9
18:2	trace to 1.5	trace to 1.8
18:3	trace to 1.9	0–1.0
20:4	2.9–4.1	2.5–5.6
20:5	trace to 6.7	2.3–11.6
22:6	6.1–47.4	14.0–50.4
Total unsaturated	26.5–68.1	39.2–72.9

[1] Data from SHUSTER, FROINES and OLCOTT [1964].
[2] Figure before colon is carbon chain length; that after the colon is number of double bonds.

is squalene. Smaller quantities of pristane and zamene are also present in such oils [CHRISTENSEN and SORENSEN, 1951].

Body oils of fish may also contain hydrocarbons. Thus LAMBERTSEN and HOLMAN [1963] have shown that herring contains 0.05% hydrocarbons which were separated into 8 branched-chain and 21 straight-chain compounds. Among the branched chain hydrocarbons, pristane (70%) and squalene (13%) occurred as the principal components.

4. Ether-linked compounds

The presence in shark of alkoxydiglycerides yielding upon hydrolysis such glyceryl ethers as selachyl, chimyl and batyl alcohols has been known for a long time [TSUJIMOTO and TOYAMA, 1922; HEILBRON and OWENS, 1928]. Later KARNOVSKY, RAPSON and SCHWARTZ [1948] showed that these substances occurred also in many other shark body oils. The fatty acid content in both the ester- and ether-linked portion of dogfish flesh and liver oil has been determined by MALINS, WEKELL, and HOULE [1965]. A rather complete review of both the chemistry and nutritional properties of such ether-linked compounds has recently been made by SNYDER [in press].

(5) Wax esters

Wax esters in which a fatty acid ordinarily of a low degree of unsaturation is esterified to a fatty alcohol usually of 16 or 18 carbon chain length occur sometimes in very high proportions of both the body and liver oils of certain species of fish. Thus AGAWA, HIRAO and KOMORI [1953] found such waxes in the liver oil of a cod, and NEVENZEL, RODEGKER and MEAD [1965] found them in both the liver and flesh oils of the castor oil fish, *Ruvettus pretiosus*. In the latter case, the flesh oil was over 90% wax esters, the liver oil about 4%. The fatty acids were principally monoenes with 18:1, 20:1 and 22:1 accounting for 91% of the total.

C. Miscellaneous Components

Of the lipid components contained in fish not already mentioned, sterols are perhaps the most important. KRITCHEVSKY, TEPPER, DI TULLO and HOLMES [1967] have shown the presence of the following sterols in clams, scallops, and oysters: a C26 sterol, 22 dehydrocholesterol, cholesterol, brassicasterol, 24 methylene cholesterol and two different C29 sterols (one not present in oysters). Of these, cholesterol occurred in the greatest quantity and is also present in other fish and shellfish. The quantity occurring in several species is shown in table V. The amount present in fish is lower (under 100 mg/100 g wet fish) than in shellfish and crustaceans (usually 150–200 mg/100 g). The other sterols reported to be present in certain shellfish occur in considerably smaller amounts except for 24 methylene cholesterol (about 20 mg/100 g) and brassicasterol (about 15 mg/100 g).

Other components of fish lipids include vitamins and pigments. A discussion of pigments is beyond the scope of this review and vitamins will be treated only briefly. Vitamins A and D occur in widely varying amounts in the liver and body oils of fish [STANSBY and HALL, 1967] ranging from high values of up to 700,000 units vitamin A/g in halibut liver oil and up to 250,000 units vitamin D/g of certain tuna liver oils, down to values usually under 100 units/g in many body and some liver oils. For the average medium oil content fish however the vitamin A content per gram of wet flesh ranges from 10–100 units with an average of about 25 [STANSBY and OLCOTT, 1963]. Vitamin A and D, formerly obtained in the United States primarily from fish livers is now, because of the availability of the cheaper synthetic product, no longer manufactured here using fish as a source. In other

Table V. Quantity of cholesterol in certain species of fish and shellfish[1]

Species	Cholesterol content mg/100 g
Fish:	
Haddock	90
Pollock	75
Salmon	95
Shellfish and crustaceans:	
Clam	190
Crab	140
Lobster	170
Oyster	150
Scallop	175
Shrimp	200

[1] Data of KRITCHEVSKY, TEPPER, DITULLO and HOLMES [1967].

countries, e.g. Norway, Great Britain and Japan, fish livers are still an important source of Vitamin A [STANSBY, 1967a].

D. Oxidation

In this section of this review only a brief discussion of chemical oxidation of fish oils is presented. The reader is referred to Section III, Stability and Deterioration, in the book 'Fish Oils' [STANSBY, 1967a] for greater detail.

Fish oils, although differing chemically to a considerable extent from animal and vegetable oils, oxidize by the same general mechanism as for most lipids the theoretical basis for which was developed during the 1940's by FARMER [1946], BOLLAND [1946] and others. Fish oil fatty acids, however, have up to five methylene interrupted groups, where oxidation can take place. Compared to linoleic acid with only one or linolenic acid with two, many of the fish oil fatty acids are far more vulnerable to oxidation, and hence their rate of oxidation is far in excess of that of the fatty acids in most other oils.

With most polyunsaturates of fish oils of the ω-3 type the kind of oxidation products resulting may vary from those obtained from the more usual ω-6 variety.

Fish oils generally oxidize in a manner which results in a less distinct break in the induction curve and antioxidants are less effective

in prolonging the induction period than is the case with the same antioxidants applied to animal or vegetable fats and oils [LISTON, STANSBY and OLCOTT, 1963]. Oxidation in the oil of fish is very markedly affected by the presence of hematin compounds acting as prooxidants. The hematin compounds occur in fish primarily in the dark muscle and oxidation in this dark or red muscle may occur at a rate up to 100 times as rapid as takes place in the light muscle [BROWN, VENOLIA, TAPPEL, OLCOTT and STANSBY, 1957].

Fish oils with their high content of polyunsaturates can oxidize to a very great extent. Such extensive oxidation takes place, for example, when paint or other protective coatings containing fish oil oxidize. The extent of oxidation, however, which takes place in fish used for human food, and stored under ordinary handling conditions (e.g. in ice or frozen) is very small. The extent of oxidation in oil in iced fish is in fact almost imperceptible and there appears to be some relationship to the presence of growing bacteria in fish and reduction of oxidation rate [LISTON, STANSBY and OLCOTT, 1963]. When frozen fish is stored at usual temperatures, e.g. −20 °C, oxidation proceeds more rapidly, but the degree of oxidation at which obnoxious oxidative rancidity flavors develop—peroxide values of 5 or less—is at a very early stage of oxidation often still in the induction period as compared to the extreme oxidation occurring when fish oils thoroughly oxidize [STANSBY, 1967c].

The types of odors developing when a highly purified menhaden oil oxidizes have been described by STANSBY and JELLINEK [1965]. These follow through a pattern of first a loss of the initial normal, fresh, species characteristic flavor or odor to a particular 'fishy' type flavor or odor and later to the usual rancidity found in most oxidized oils. A more thorough discussion of odors and flavors in oxidizing fish oils has been given by STANSBY [1967b].

III. Sources of Fish Oil

A. Introduction

So far we have been considering primarily the chemistry of fish oils. In this section will be discussed just how the fish oils and their component fatty acids arise and where they occur within the flesh of the commercial fish. Some brief treatment will be also given to the subject of commercial fish oils.

B. Oil in Fish

1. Food chain in the sea

The many unique lipids found in fish come unaltered from the plankton and other feed of the fish, by metabolism in the fish of components of the fish's diet or as a combination of both processes. There is good evidence that the latter combination process prevails in most instances. In this section the transfer of lipid substances from the fish's feed to the fish will be examined briefly.

Early work by LOVERN [1935, 1951] and others compared the fatty acid composition of the oil in the fish with that in its feed. It was shown, for example, that when the fatty acid composition of the feed showed a seasonal variation, the fatty acids in the fish changed in the same way. In more recent work, KLENK and EBERHAGEN [1962a, b] have isolated, from plankton, polyunsaturated fatty acids identical with those found in fish. Fish have also been fed definite fatty acids and the effect on deposited fat observed. Thus KELLY, REISER and HOOD [1958a, b] showed that feeding of linseed oil, cottonseed oil, or herring oil would influence the fat of a freshwater fish to resemble that of the fat ingested and that the long chain polyunsaturated fatty acids originally present in the fish all but disappeared if they did not occur in the oil fed. KAYAMA, TSUCHIAYA, and MEAD [1963a] showed that by feeding a phytoplankton, *Chetoceros simplex* to a crustacean, *Artemia salina* as the only feed, the fatty acids of the fat of the crustaceans resembled those of the phytoplankton. When, however, these crustaceans were fed as the sole food of guppies *Lebistes reticulatus*, although the fatty acid pattern resembled that of the fat of the original phytoplankton to a small degree, the resemblance was far less than in the former case.

2. Metabolism in fish

Our knowledge of metabolism in fish has lagged behind that of such work with other species. It was not until BROWN and TAPPEL [1959] carried out work with carp liver mitochondria that it was confirmed that the fatty acid co-enzyme A derivatives of fish are oxidized making use of the same mechanism of β oxidation as elsewhere and making use of the same cofactors-Cytochrome C, ATP, Mg^{++} and a tricarboxylic cycle member. These workers also showed that this mechanism readily oxidized the long chain polyunsaturates such as docosahexaenoic acid.

The inability of fish in common with other organisms to synthesize the essential fatty acids was demonstrated by KLENK and KREMER [1960] in experiments in which polyunsaturates were formed during incubation of fish liver slices with acetate-1-^{14}C. In other work by NICOLAIDES and WOODALL [1962] it was demonstrated that chinook salmon maintained from the time of hatching on a completely linoleic acid-free diet developed deficiency symptoms and showed a lowered gain in weight. Fish, however, can readily change linoleic and linolenic acid, supplied in the diet to other members of the ω-6 and ω-3 polyunsaturate families of fatty acids respectively as was shown in such work as that of MEAD, KAYAMA and REISER [1960] and of KAYAMA *et al.* [1963b]. These and similar experiments resulted in a proposal for conversion pathways of polyunsaturates in fish by KAYAMA and TSUCHIYA [1962] such as is shown in figure 1. In this figure chain elongation is shown as long arrows which cut across the horizontal lines. The short arrows represent desaturation. The solid arrows show reactions already demonstrated to occur in fish and the dashed lines steps which are assumed to occur.

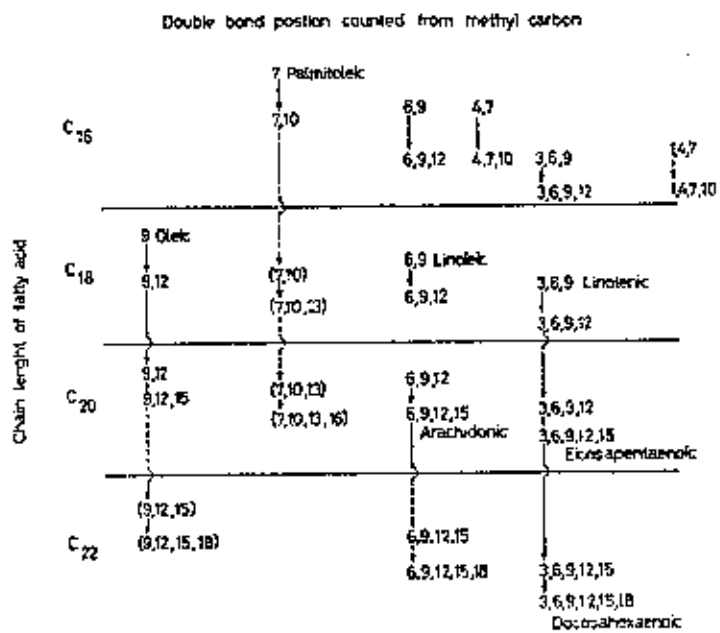

Fig. 1. Possible conversion pathways of polyunsaturated acids in fish.

Much research remains to be done to bring our knowledge of metabolism in fish abreast of that for other organisms. In addition to gaps in knowledge in pathway conversions shown by the dashed lines in figure 1, almost nothing is known about the mechanism for the formation of esters such as glycerides, phospholipids or waxes. Our knowledge of the function of lipids in fish is also very incomplete. In such cases as the presence of up to 90% in the flesh of *Cristivomer namaycush siscowet* (Siscowet trout occurring in Lake Superior) [THURSTON, 1962] and of large wax ester deposits in certain species of fish [NEVENZEL, RODEGKER and MEAD, 1965] the lipids must act in some way other than as a mere energy source. MEAD and KAYAMA [1967] have suggested that in these instances the lipids may represent a means of controlling buoyancy in lieu of employment by the fish of an air bladder.

3. Environmental effects

The effect of feed on fatty acids deposited in fish has already been mentioned. Certain other environmental effects occur. It has long been known that the geographical area or the season wherein the fish is caught effects fatty acids deposited in the tissue. These effects in addition to being influenced by different types of feed are often largely a matter of effects of water temperature [LOVERN, 1942; SWAIN, 1953]. It has been long observed in commercial fisheries that a species like menhaden taken from both warm water and from cold waters produces oil of a higher iodine value, the colder the water from which the fish is taken. These observations receive confirmation from a number of experiments. FARKAS and HERODEK [1964] showed that as the environmental temperature decreased, the content of C20 and C22 fatty acids in freshwater crustacean plankton increased. KAYAMA, TSUCHIYA and MEAD [1963a] found that guppies raised in 17 °C water contained a higher proportion of C22 fatty acids than did those raised in 24 °C water. KNIPPRATH and MEAD [1965] likewise found an increase in 22:6 and other polyunsaturated fatty acids in rainbow trout after they were transferred from water at about 12 °C to water at about 5 °C. The fatty acid change occurred in oil from both the flesh and from composite organs (liver, heart, and spleen). JOHNSTON and ROOTS [1964] found a similar effect for polyunsaturated fatty acids from the brain of goldfish held in water at different temperatures. On the other hand, REISER *et al.* [1963] were unable to find any significant change in the pattern of fatty acids in mullet or goldfish raised in waters at

13 °C and 23 °C. In this experiment, however, the fish were first depleted of long chain fatty acids and then fed with linoleic and linolenic acids.

4. Cellular *versus* depot fat

The lipids of fish occur primarily as phospholipids associated with cells as non-depot fats to an extent of about 0.7%. This type of lipid scarcely ever exceeds 1% [LOVERN, 1962]. The fish may or may not contain, in addition, depot fats which ordinarily consist of triglycerides and where phospholipids are absent or at most occur in very small quantity. Fish like cod and haddock with 1% or less total oil have little or no triglyceride with substantially all the lipid occurring as non-depot fat. Species of fish like herring or salmon with often 10% or even considerably more total oil have a large proportion of their lipid occurring as triglycerides.

5. Light *versus* red muscle

The oil is divided in fish between light and dark (or red) muscle. Ordinarily, but not invariably, the oil content of the dark flesh is higher (sometimes ten times or more) than in the light flesh. The dark muscle (usually of a dark red color) occurs predominantly as a 'v' shaped strip beneath the lateral line, but also in other areas, to a lesser extent, such as along the dorsal region.

BARETS [1961] has given a comprehensive discussion of the anatomy and function of the red and light muscle in fish. The red muscle is described as 'slow' muscle because it is believed to function as a means of propelling the fish through the water during slow, steady swimming. The light muscle sometimes referred to as 'rapid' muscle is believed to function primarily where bursts of speed of short duration are needed. GEORGE [1962] points out that the red muscle, provided with a relatively large supply of mitochondria, hematin compounds and enzyme systems such as lipase and succinase dehydrogenase, acts primarily to aerobically convert fat into energy. The white muscle, on the other hand, with limited mitochondria and associated lipid converting enzyme systems operates largely as a converter of carbohydrate (glycogen) into energy. These ideas correlate well with observations on migrating fish such as salmon which are provided with an extensive dark muscle system and which live exclusively off from stored depot fats during extended spawning migrations. More dormant species such as bottomfish like cod, on the other hand, do not store much depot fat, do not go on long spawning migrations

where stored fat is needed as an energy source, and do not have very extensive dark muscle systems.

The red muscle was once thought of as being exclusively an organ-like liver for carrying out some metabolic functions but having no muscular activity purpose [BRAEKKAN, 1956]. As shown by BUTTKUS [1963] red muscle contracts during rigor actually to an extent three times as great as does white muscle. There would seem to be no doubt that the red muscle does possess important function with respect to muscular activity in the fish as well as carrying out important metabolic activity particularly as the most efficient site for conversion of lipid to energy.

6. Oil in flesh *versus* organs

In addition to its occurrence in differing amounts in the flesh of fish, oil also occurs in certain organs particularly in the liver in highly variable proportions. In fish like cod, where almost all of the flesh oil occurs in form of phospholipids associated with the cells, the liver is large (about 10% of body weight), is very oily (up to 80%) and consists largely of triglycerides. In species of fish like salmon or herring which contain a preponderance of their flesh lipids as triglycerides occurring in depot fats, the livers are small (1–3% of body weight) and relatively non-oily (under 5%).

Table VI. Types of fish based on oil and protein content of the flesh

Category	Type of fish	Prototype Species	Moisture %	Protein %	Oil %	Ash %
A	Low oil-high protein (under 5%) (15–20%)	Pacific cod *Gadus macrocephalus*	81.5	17.9	0.6	1.6
B	Medium oil-high protein (5–15%) (15–20%)	Mackerel *Scomber scombrus*	67.5	18	13	1.5
C	High oil-low protein (over 15%) (under 15%)	Siscowet lake trout *Cristivomer namaycush siscowet*	52.5	11.3	36.0	0.53
D	Low oil-very high protein (under 5%) (over 20%)	Skipjack tuna[1] *Katsuwonus pelamis*	72.4	26.2	0.7	1.5
E	Low oil-low protein (under 5%) (under 15%)	Butter clams *Saxidomus nuttall*	83.0	13.3	1.3	1.9

[1] Analysis of light meat only. This part is the only one that is ordinarily canned.

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7. Oil content of different species of fish

The oil content of the flesh of fish, as has been previously noted, varies over very wide limits from minimum values of about 0.6% up to maximum values of about 90%. Not only does this wide variability occur from species to species, but also the oil content of different fish of a single species may vary over a range of ten times or more. It is not, therefore, possible to present any data on average oil content of the different species of fish which would have much significance.

STANSBY [1962] has proposed a system for classifying composition of the flesh which is based upon a purely arbitrary consideration of oil and protein content. The 5 categories in this classification system are shown in table VI.

Oil content ranges in categories A, B and C are (A) under 5%, (B) 5–15%, and (C) over 15%. Most fish come under categories A or B. Category A includes both those species like bottomfish (e.g. cod) which possess little or no depot fat, and a number of species which have only modest quantities of depot fats. Category B includes most species which contain fairly high quantities of depot fats. Since many of these species vary widely in oil content from season to season, they may at one season or another have so little oil as to drop down temporarily to category A or so much as to occasionally lap over into category C. Most crustaceans and shellfish (except shrimp) with very low protein content and also low oil content fall into category E and a few special species with very high protein as well as low oil contents like tuna and halibut fall into category D. Some of the most important species of fish are classified by this system in table VII.

The range of oil contents in the livers of some species of fish are listed in table VIII. Note by comparison with table VII how fish with low oil content in the flesh have high oil content in the liver and vice versa and that the relative size of the livers is also generally related inversely to the oil content of the flesh.

C. Commercial Fish Oils

1. Introduction

Fish oils are manufactured in widely scattered areas of the world from in some instances whole fish, in others, from trimmings from cannery or fillet operations. The oil may be used as a component of food, as an animal feed, or for a variety of industrial purposes. In this section is

Table VII. Types of composition of the flesh of some important species of fish

Species	Category	Remarks
Anchovies	B	Some caught at certain seasons or in certain localities may fall in category C
Carp	A	Some caught at certain seasons or in certain localities may fall in category B
Cod	A	
Flounder	A	
Haddock	A	
Hake	A	
Halibut	D	
Herring	B	Some caught at certain seasons or in certain localities may fall in category C
Mackerel	B	Some caught at certain seasons or in certain localities may fall in category C
Mullet	A	Borders on category B
Ocean perch	A	
Pollack	A	
Rockfishes	A	
Salmon	B	Some species of salmon, chum (Oncorhynchus keta), Atlantic (Salmo salar), and pink (Oncorhynchus gorbuscha) may border on category A
Sardines	B	Some caught at certain seasons and in certain localities may be category C
Tuna	D	Albacore tuna (Germo alalunga) when caught in certain areas is in category B. Bluefin tuna (Thunnus thynnus) borders on category B
Whiting	A	
Clams	E	
Crabs	A	
Oysters	E	
Scallops	A	
Shrimp	A	

Category A = oil < 5%, protein 15–20%; category B = oil 5–15%, protein 15–20%; category C = oil > 15%, protein < 15%; category D = oil < 5%, protein > 20%; and category E = oil < 5%, protein < 15%.

presented a brief description of the methods used in manufacturing such commercial fish oil followed by a short description of some of the different varieties of fish oil and their use and method of preparation in different geographical areas.

2. Manufacture of commercial fish oils

Most fish oil is manufactured in an operation which produces both fish oil and a dried protein product, fish meal, which is used primarily

Table VIII. Oil content and liver size of the livers of certain species of fish

Species	Liver Ocean from which caught	Proportion of round weight %	Oil content %
Cod	Atlantic	7	20–60
Herring	Pacific	2	5–25
Menhaden	Atlantic	2	5–20
Rockfish	Pacific	1.5	5–25
Sablefish	Pacific	2	10–25
Salmon, chum	Pacific	2	2–6
Salmon, king	Pacific	2	4–8
Salmon, pink	Pacific	2	4–6
Salmon, silver	Pacific	2	4–6
Salmon, sockeye	Pacific	2	5–8
Shark, dogfish	Pacific	10	50–72
Shark, sleeper	Pacific	12	40–55
Shark, soupfin	Pacific	10	55–72
Swordfish	Atlantic	2	8–35
Tuna, albacore	Pacific	2	7–20

as a poultry feed supplement. By far the most common manufacturing procedure, known as the wet rendering process, involves cooking the wet fish to coagulate protein and to release moisture and oil followed by pressing away the oil and water, separating the oil from the water by centrifuging and drying of the solid phase to produce the meal. Usually the aqueous phase containing dissolved protein, amino acids, and vitamins is concentrated to produce condensed fish solubles (also used in poultry feed). The crude oil is generally given a 'polishing' in a high speed centrifuge to remove small amounts of water or proteinacious material.

Small quantities of oil and (meal) are produced by the dry rendering method in which the fish is cooked and dried in one operation, followed by pressing of the oils from the meal. Very small quantities of fish oil are currently produced by a solvent process. Such a method may in the future form the basis of producing large quantities of fish protein concentrate (FPC). If this develops into a large scale operation larger quantities of oil may be produced in the future by such a method. Oil produced by either the dry rendering or by the solvent extraction processes are generally of darker color than when produced by the wet

rendering method, and they then require additional and special refining procedures. Oil made as a part of an FPC operation unlike that by most other processes meets US Governmental standards for use as a food product for human consumption. For a more complete description of these processes see PIGOTT [1967].

Fish liver oils are manufactured by cooking the livers immediately after catching of the fish with water and centrifuging the oil from the water. For highest quality of medicinal oil the livers may be cooked aboard the fishing vessel. Low oil content livers which often contain very high vitamin A content usually give an insufficient yield by a simple cooking process and they are, therefore, frequently cooked with dilute alkali to partially solublize the protein, followed by centrifuging away the oil. AUBE [1967] gives much greater detail concerning methods of producing fish liver oils.

Fish oils are usually refined by some additional processing steps which are used to remove free fatty acid, stearine, and odor, and to improve color of the oil [CHANG, 1967]. Stearine which consists of some of the most saturated fatty acid that may be solid at relatively high temperatures and might separate out when the fish oil is chilled, are removed by 'winterizing' i.e. by cooling the oil to about 0–1.5°C and, while cold, pressing away the solid stearine. Free fatty acids are neutralized with either a sodium hydroxide or sodium carbonate solution. Color is bleached using special clays or activated carbon. Bleaching also reduces odor and flavor. Hydrogenation removes all odor and flavor, but, of course, converts the fish oil to a more nearly fully saturated, solid form. This process is used when the fish oil is to be incorporated into edible margarine or shortening and it has been described by HANNEWIJK [1967].

3. Anchovy

The anchovy fishery of Peru extending also into Chile, produces the raw material for the world's largest production of fish oil (and fish meal). This large industry is of relatively recent origin, the production having increased by a factor of over 15 since 1958. Anchovy oil is one of the most highly unsaturated of important commercial fish oils and it has an especially high content of eicosopentaenoic acid. The oil is manufactured in relatively new plants established during the past decade. The usual wet-rendering process is used throughout this industry and alkali refining and bleaching are carried out at several of the manufacturing plants. Most of the anchovy oil is exported to

Europe and other areas and it is largely used (after hydrogenation) in margarine and shortening. In recent years an increasingly larger amount is being hydrogenated in Peru for domestic consumption for human use. The anchovy fish oil industry has been described in detail by SANCHEZ TORRES [1967].

4. Menhaden

The menhaden industry is the basis for by far the largest fish oil production in the United States. In some recent years, the catch of menhaden has approximated the catch of all other species combined. The fishery is carried out off the Atlantic Coast from New York southward to Florida and around into the Gulf of Mexico. Important production areas include coast areas in New Jersey, Delaware, Virginia, North Carolina, Florida, Mississippi, Louisiana and Texas. Within recent years there has been a relatively larger proportion of the production taking place in the southern segment of the industry in the Gulf of Mexico with fish in the northern areas becoming relatively scarce and with a downward trend in total production of the entire industry.

Menhaden oil is produced entirely by the conventional wet rendering process. Details concerning production methods and of the menhaden industry in general have been described by LEE [1963] and by JUNE [1963] respectively. More than half the oil is exported to Europe for hydrogenation and use in margarine and shortening (use of commercial fish oil as ordinarily currently produced in the United States for human food applications even after hydrogenation is not permitted by Federal authorities in this country). The remainder finds application in protective coatings and for a wide variety of other industrial uses [FINEBERG and JOHANSON, 1967].

5. Herring

Herring oil is produced in widely separated areas of the world. Relatively large production takes place in Norway and Iceland with smaller production in Sweden, Denmark, West Germany, Canada (largely British Columbia) and to a small extent in the United States (Alaska and Maine, the latter only by use of trimmings from sardine (herring canneries).

Herring oil is of somewhat lower iodine number (unsaturation) than is menhaden or anchovy and contains a relatively large proportion of monoenes.

6. Other oils

Fish liver oils are no longer produced to any significant extent in North America and South Africa because of competition from synthetic vitamin A. Cod liver oil is still an important commodity in such European countries as Norway, Iceland and Great Britain [SAND, 1967]. Liver oil is produced in Japan from cod, pollack and shark liver to a considerable extent [SONE, 1967].

Pilchard oil was once a very important commodity produced on the West Coast of North America (California, Washington and British Columbia) its production at one time exceeding that of menhaden. Because of disappearance of pilchard in this area this industry has ceased. Pilchard oil production is of an important magnitude in South Africa as is also that of Maasbanker oil [DREOSTI, 1967].

In Japan, in addition to fish liver oil, there is a sizable production of saury oil with lesser quantities of squid oil, mackerel oil, and sardine oil [SONE, 1967].

7. Uses of commercial fish oils

The various uses of fish oils have been described in considerable detail by FINEBERG and JOHANSON [1967], DE SESA [1967], HANNEWIJK [1967], and DYER [1967]. In this brief description, the uses of menhaden oil which are reasonably typical of world-wide uses of fish oils will be chiefly emphasized.

A preponderant part of all fish oils is used for hydrogenation whereby the unique chemical characteristics of fish oils are destroyed in the process of changing them over into a more saturated form. Hydrogenated fish oil is used in substantial quantity in shortening and margarine in northern European countries and to lesser extents in Canada, South America, Japan, in fact in most other countries except the United States. Nevertheless about half of the menhaden oil (all of which is produced in the United States) is exported for hydrogenation and used abroad in edible shortenings and margarine. About 15% of the menhaden oil is used directly without hydrogenation in domestic poultry and animal feeds.

A most important use of menhaden (and other) oils, second only to use in food and feeds is as a drying oil for use in protective coatings. Originally fish oil was added directly to paint or varnish. During drying, strong fishy odors developed which restricted use of these coatings for uses such as for painting the outside of buildings. Today much of the menhaden oil destined for the domestic protective coating

field is first converted into another chemical form which eliminates many of these odor problems. For example alkyd resins are prepared by reaction of the menhaden oil with phthalic anhydride and the alkyd then used in the paint. In other cases a maleated oil is produced by reaction of the menhaden oil with maleic anhydride or a co-polymer is produced by reaction with cyclopentadiene. These various derivatives improve the characteristics of fish oil not only from the odor stand-point but also aid in preventing undesirable yellowing which sometimes limits use of fish oils in varnish or other coatings.

Usually a little over 10% of menhaden oil is used in preparation of lubricants. Soaps of menhaden oil such as those of lithium, barium, sodium, aluminum, or lead are prepared and added to other lubricating oils to form a grease. Another 10% or somewhat less of the menhaden oil production goes into the making of fatty acids. The long carbon chain (C20 and C22) of many fish oil fatty acids impart special qualities not found in other fatty acids. The fatty acids may be used as such or employed as a starting material for preparation of a wide variety of other chemical derivatives.

Derivatives of fish oils are used in a very wide range of miscellaneous industrial applications including in leather tanning, ore flotation, printing inks, glazing and caulking compounds, water repellents, core oils in foundries, and as corrosion inhibitors.

IV. Nutritional Properties

A. Introduction

Under this heading will be considered various effects of fish oils and their component constituents when ingested or otherwise applied to animals including humans. Metabolism of fish oils, already treated briefly with respect to metabolism in fish will be included in a more general way along with nutritional properties. This latter topic will also include pharmaceutical and related effects. Most of the discussion will relate to fatty acids, the principal chemical component of fish oils. Briefer mention will be made of other classes of components such as ether linked compounds and phospholipids.

B. Fatty Acids

1. Essential fatty acids (EFA) in fish oils

Before we consider the situation with respect to the EFA role of

certain fish oil fatty acids we should clarify the meaning of EFA for the purpose of this discussion. As has been pointed out by STANSBY [1967c] the historical development of our concepts of EFA was based upon research with animal or vegetable oil, and certain aspects of the role of fish oils with respect to EFA concepts do not lend themselves to proper interpretation unless we clearly understand this situation. When BURR and BURR [1929] first enunciated their ideas on EFA, the important function of these acids was restricted to their effects in supporting growth and in curing of dermal symptoms. In the years immediately following this discovery the curing of the dermal symptoms became the basis for the assay procedure used to measure EFA content. We know now, of course, that this assay procedure measures primarily fatty acids of the ω-6 series (linoleic acid family), but that fatty acids of the ω-3 series (linolenic acid family) although unable to cure the dermal symptoms completely not only do support growth but also possess certain other important functions such as transportation of cholesterol. For the purpose of discussion in this article, we will, therefore, consider that fatty acids of the linoleic acid family to be 'classical EFA' but recognizing that fatty acids of the linolenic acid family display certain EFA properties and may be considered to be EFA's perhaps in a more limited sense.

PRIVETT, PUSCH, HOLMAN and LUNDBERG [1960] showed that tuna, herring, and menhaden oil cured dermal symptoms in rats (and that these oils therefore contained classical EFA) when included in the diet at moderately high concentrations (10%) but that they were relatively ineffective at lower concentrations [PRIVETT, AAES-JØRGENSEN, HOLMAN and LUNDBERG, 1959]. The amount of linoleic acid family of fatty acids was estimated from these experiments to be about 2% in these fish oils. Present day knowledge of the fatty acid composition of the oils of fish [GRUGER, 1967] indicates that this figure of 2% is probably not far from the correct value for the content of linoleic acid family of fatty acids of these commercial fish oils. Body oils of marine fish may average a little higher perhaps about 3% and with some species as high as 6%; the content of linoleic family of fatty acids in the oil of freshwater fish is somewhat higher, perhaps in the range of 5–9%.

2. Growth effects

In the experiments of PRIVETT *et al.* [1960] the rats fed for 16 weeks on a fat-free diet followed by supplementation for 12 weeks with 10%

Table IX. Supplementing effect on growth of rats of addition of 10% menhaden, tuna, herring, or corn oil to a fat-free diet [DATA of PRIVETT, PUSCH, HOLMAN and LUNDBERG, 1960]

Supplement	Initial weight after 16 weeks on fat-free diet	Final weight after 12 further weeks on supplemented diet	Weight gain grams
None (fat free)	232±8	252±8.2	20
10% menhaden oil	237±7.6	354±15.4	117
10% tuna oil	219±15.2	329±16.8	110
10 herring oil	235±11.9	354±15.1	119
10% corn oil	223±4	340±8.1	117

menhaden, tuna, or herring oil showed growth response at least equal to that of the positive control where corn oil was the supplementing oil (see table IX). The final weights of animals on all of these additives after 28 weeks averaged 344 g as compared to only 252 g for the animals on the negative control with no fat in the diet.

An effect of feeding of linoleic and linolenic acid simultaneously upon metabolism rates of these two families of fatty acids was indicated by work of KLENK and OETTE [1960], MARCO, MACHLIN, EMERY and GORDON [1961] and MACHLIN [1962]. A series of experiments by MOHRHAUER and HOLMAN [1963a, b] and summarized by HOLMAN [1964] showed that when the ratio of linolenic to linoleic acid increased, the conversion of linoleic to arachidonic acid was progressively inhibited. HOLMAN [1964] also points out that this is a more general situation such that fatty acids of any one family repress the metabolism of another. Thus oleate fed with linoleate will also repress the conversion of the latter to arachidonate. Even saturated fatty acids repress the metabolism of unsaturated ones. These experiments dealing only with linolenic acid so far as ω-3 fatty acids are concerned is not directly applicable to fish oils which are very low in linolenic acid itself although very high in fatty acids of the linolenic acid family.

More recent research, however, indicates that this repression of metabolism of linoleic to arachidonic acid occurs not only when linolenic acid itself is the inhibitor, but also when other members of the linolenic acid family including the typical ω-3 fatty acids common in fish oils are present in considerable quantity. Thus BRENNER and

PELUFFO [1966] using *in vitro* experiments involving incubation of labeled fatty acids with adenosine triphosphate, reduced nicotinamide adenine dinucleotide, reduced nicotinamide adenine dinucleotide phosphate, magnesium chloride, coenzyme A, glutathione, nicotinamide, sodium fluoride, and sodium cyanide in oxygen at pH 7 and 35°C showed that docosahexaenoic acid decreased the percentage of desaturation of linoleic acid. Likewise MOHRHAUER, CHRISTIANSEN, GAN, DEUBIG and HOLMAN [1967] showed by similar means that chain elongation is inhibited by both eicosapentaenoic acid (ω-3) and docosahexaenoic acid (ω-3).

3. Effects of feeding fish oils

A large part of our evidence concerning nutritional properties of fish oils is based on more practical feeding tests in which farm animals or poultry were employed as test animals. A vast literature reviewed by KARRICK [1967] documents such experiments. In much of this type of work it soon became apparent that when fish oil was fed in a proportion of the total feed beyond some small limit, problems of imparting of a fishy taste to the meat or eggs of the product from the animal resulted. This and other considerations have limited the use of fish oils in animal feeds to a considerable extent.

a) Digestibility, adsorption, and metabolizable energy

The coefficient of digestibility for fish oil when consumed by many animals [ARTMAN, 1964; DEUEL, 1954; LEOSCHKE, 1959; REDER, 1942] has been reported to be high. STEENBOCK, IRWIN and WEBER [1936] reported that halibut liver oil and cod liver oil were absorbed more rapidly than were corn oil, lard, or partially hydrogenated fats. These results were based upon experiments employing rats. Metabolizable energy of fish oil is generally reported to be relatively high, e.g. 3700 cal/lb for menhaden oil for chicks as compared to 2900 cal/lb for tallow [RENNER and HILL, 1958].

b) Fish oil in poultry feed

Insofar as growth only is concerned, fish oils have been found to give excellent growth in quantities up to 12% [ARTMAN, 1964] and mixtures in the ratio of 1:1 of tallow and fish oil increased the utilization of the former. Both growth and feed utilization efficiency have been reported to be improved by use of fish oil in the diet of poultry by many workers, e.g. EDWARDS *et al.* [1961, 1962, 1963].

Chicken flesh very readily takes on an objectionable fishy flavor when the birds are fed fish oils [CARRICK and HAUGE, 1926; DANSKY, 1962; HARDIN, MILLIGAN and SIDWELL, 1964]. Safe levels of fish oil in the diet low enough to prevent this problem are probably no higher than 1% of the diet. Turkeys are even more susceptible than are chickens and fishy flavor has been noted in turkey flesh in cases where as little as 0.4% fish oil occurred in the birds' diet [KLOSE *et al.*, 1952]. These flavor problems can usually be alleviated to a considerable extent by removing the birds from a diet containing fish oil some time before slaughter [KARRICK, 1967]. Stability of frozen poultry flesh toward oxidative deterioration in cold storage can also be decreased by including excessively large amounts (e.g. 2%) of fish oil in the feed [KLOSE *et al.*, 1953; SCHREIBER *et al.*, 1947].

When fish oils are included in the diet of hens, egg production efficiency improves [EDSON, 1932]. Fishy flavor is not ordinarily imparted to the eggs as it would be to the flesh of the fowl. Thus VENDELL and PUTNAM [1945] found no fishy flavor whatever in eggs from hens which had been consuming for 30 days a strong-smelling, low grade sardine oil at levels up to 14 times the recommended amount.

c) Fish oil in swine feed

Both texture and flavor or pork can be adversely altered if the swine consume a diet with excessively high proportions of fish oil. The depot fat readily takes on the chemical characteristics of the fish oil [ELLIS and ISBELL, 1926] and a yellow discoloration may also result [BROWN, 1931]. Restrictions of fish oil content in the ration to 0.5% has been reported to eliminate all these problems [KARRICK, 1967]. Elimination of fish oil from the pigs' rations 30 days before slaughter is also reported to be an effective remedy [FRASER, STOTHART and GUTTERIDGE, 1934]. OLDFIELD and ANGLEMIER [1957] also reported improvement in pork color, flavor and odor from pigs fed polymerized fish oil but at the expense of growth efficiency.

d) Fish oil in cattle fodder

Fish oil when fed to cattle can result in the same effects as when included in swine rations, but the effects are usually considerably less [BEARD, SCHULTZ and CULBERTSON, 1935; DAVIS and MAYNARD, 1938; NICHOLSON *et al.*, 1962]. Muscular dystrophy can result if too high a quantity of fish oil is fed to calves, and this effect can be controlled by inclusion of supplementary tocopherol [BLAXTER *et al.*, 1953a, b, c].

Fish oil fed to cows lowers the fat content of the milk as well as increasing the degree of unsaturation of the milk fat [BROWN and SUTTON, 1931]. These changes result from alteration in the pattern of fatty acids in the rumen which is brought about by incorporation of fish oil in the feed.

Acetic acid and butyric acid in the rumen decrease and propionic acid increases [SHAW and ENSON, 1959].

e) Fish oil in the diet of other animals

Herring oil fed to goats alters the butter fat content in a manner similar to that which occurs when it is fed to cows [GRAHAM and CUPPS, 1938]. Mink, although not fed fish oils directly, often are fed fresh fish which contain fish oil. As with feeding of other unsaturated oil to mink, oil in fish can cause steatitis unless an adequate supplement of tocopherol is included [LALOR, LEOSCHKE and ELVEHJEM, 1951]. Cats, if fed a diet consisting almost exclusively of fish can also develop steatitis. Here again the solution is to provide an adequate quantity of tocopherol [GERSHOFF and NORKIN, 1962].

4. Polyunsaturates effects in animal feeds and human food

We have seen, in a previous section, that fish oils with their relatively high content of polyunsaturated fatty acids are highly unstable, subject to rapid oxidation. There has also been, in other discussions included in the foregoing material, suggestion that in some instances the presence of the polyunsaturates or their oxidation metabolites may raise nutritional problems. The purpose of this section of the review is to consider further what these effects are and under what circumstances they may be of serious consequence.

Of the two types of effects of polyunsaturates, those involving oxidation and those relating to the nature of the unoxidized condition, the former will be discussed first. We must first have a clear distinction between the varying extents of oxidation which fish oils can reach under different methods of handling of either the oil itself or the oil in fish flesh. The oil in fresh or frozen fish may oxidize during storage resulting in flavors described as 'fishy' or rancid [STANSBY, 1967b]. When such fish reach a point of marginal acceptability so far as human food is concerned with a somewhat pronounced unpleasant flavor, the extent of oxidation is exceedingly minute. Only a small fraction of 1% of the oil has actually been oxidized, no loss whatever in fatty acid content can be measured, nor is there any detectable drop in

iodine value. This situation occurs because the carbonyl compounds resulting from oxidation of oil and which cause the unpleasant rancid flavor are so exceedingly flavorful that a mere 0.01% or so will cause exceedingly unpleasant flavors in fish. Peroxide values of fish which correspond to definite rancidity may be in the range of 0–2 or when the flavor is pronounced and exceedingly objectional usually no higher than 5–10, and such levels of oxidation can never cause any adverse nutritional problems [STANSBY, 1967c].

Protection of oils in fresh or frozen fish destined for human consumption is well provided both by the fact that the fish is generally shielded from direct contact with oxygen from the air by being provided with a barrier such as packaging material and by storing at a low temperature where the chemical oxidation reaction is retarded. Of even greater significance is the built-in factor of flavor such that any sample of fish in which oxidation had taken place to an extent capable of impairing nutritive value would have resulted in a flavor so disagreeable that at least in Western nations, it would have been rejected on the basis of its exceedingly unpalatable flavor.

With animal feed, especially fish meal, conditions for oxidation are far more favorable than with fish stored for human consumption. The fish meal is stored in burlap or paper bags with relatively free access to air. The meal is held without refrigeration and during storage oxidation occurs at such a rate that the temperature of the meal rises from the heat produced by the oxidation to levels which sometimes even creates a fire hazard. Under these storage conditions, a much later stage of oxidation is reached. Iodine values may fall from 150 to 80 or less. A considerable portion of the fatty acids of the oil is destroyed. Even if there were no toxicity caused by the end products of oxidation, the energy value and the growth stimulating value of the fatty acids are lost when these are destroyed.

When oils oxidize, two types of products with possible adverse nutritional effects which form are peroxides and carbonyls. High levels of peroxides cause intestinal irritation, diarrhea, and weight loss [ANDREWS *et al.*, 1960]. In their experiments, soyabean oil with high peroxide values (over 1000) when fed to rats caused them to lose weight. When the peroxide value of the oil was lower the effect diminished until, at peroxide values of 100, no difference in weight gain was apparent from that of the unoxidized oil, and no other toxicity symptoms occurred.

Fish oil or fish oil in fish meal seldom has peroxide values as high

as 100. The peroxides of fatty acids of fish oils are so unstable that, instead of accumulating, they usually break down almost as fast as they form resulting usually in relatively low peroxide values.

KANEDA, SAKAI, ISHII and ARAI [1955] showed that when fish oils oxidize it is the peroxides, not the carbonyls, which cause toxic effects. GROOT and KLEIN OBBINK [1953] showed that oxidized cod liver oil when fed to rats resulted in impaired growth rate only when peroxide values exceeded 50. RASHEED, OLDFIELD, KAUFMES and SINNHUBER [1963] showed that oxidized menhaden oil fed to rats gave toxic symptoms when peroxide values exceeded 125. CARPENTER, LEA and PARR [1963] found no retarding of growth of chicks when herring oil with a peroxide value of 142 was fed at a 6% level in the diet.

A potentially more serious condition may, under certain circumstances, occur as a result not of the end products of the oxidation process itself but rather from the effect of the oxidation upon other components of the diet during storage before consumption as a result of free radical action. These types of effects will now be considered. ROUBAL [1966] has shown that such free radical damage can occur to proteins, amino acids, or enzymes. The level of peroxide in the oxidizing lipids in this work was relatively high (peroxide value of 220). At these levels of oxidation, protein damage was quite severe. Evidence that such type of damage occurs in commercial fish meal is also shown indirectly by experiments of CARPENTER, LEA and PARR [1963] where fish meal was used in which the oil was badly oxidized and which gave poor growth and yet when the extracted oil was fed no retardment in growth occurred. It was shown that oxidizing of the lipid had made some of the amino acids of the protein unavailable.

These effects, where free radicals formed during oxidation of lipids and damaged the proteins, would never be apparent at the very low levels of oxidation which go on in fish being handled fresh or frozen for human consumption. As was seen in an earlier section, oxidation in such a case results in peroxide values of about 10, usually much less. At such low levels of oxidation free radical damage to protein would be insignificant.

The second main type of potentially adverse effect of polyunsaturates involves *in vivo* effects which may occur when the unoxidized polyunsaturate is fed to animals. Adverse effects of this type have been observed with animals fed diets which consisted either almost exclusively of fish or where highly polyunsaturated concentrates from fish oil were fed in considerable quantity. Thus encephalomacia has been

observed in chicks fed either large quantities of unsaturated vegetable or fish oils without adequate tocopherol supplementation [DAM, 1944] or fed concentrates of fish oil polyunsaturates [MILLER *et al.*, 1964]. The incidence in mink of yellow-fat disease [LALOR *et al.*, 1951] and of steatitis in cats [GERSHOFF and NORKIN, 1962], both mentioned previously, are other examples of this effect. In such cases these conditions can be remedied by adding an adequate tocopherol supplement to the diet. Effects like these have never been noted in the case of fish consumed by humans. Even in areas of the world where fish is eaten in relatively large quantity by most of the population, there is no evidence of such conditions. In Japan, for example, IMAICHI *et al.* [1965] show that as a result of eating very large quantities of fish, some members of the population lay down in their depot fat significant amounts of C22 fatty acids. Such individuals, however, fail to develop conditions mentioned above. In the case of the animals which live on diets fed to them, it is very easy for them to get so much fish that tocopherol content is inadequate, a condition not met with in human diets where much other food always is included.

5. Cholesterol depressant effect

a) Introduction

The idea that cholesterol is somehow connected with heart disease is not a new one. In 1843 VOGEL, noting that cholesterol was present in relatively large amounts in the arteries of atherosclerotic victims, proposed this cholesterol accumulation as a cause for the disease and as early as 1838 LECANU had shown that cholesterol occurred in human blood. Work continued during the 19th Century, VIRCHOW [1856] suggesting that atherosclerosis occurred when cholesterol precipitated out as a solid layer in the arteries.

As early as 1908 IGNATOWSKI was able to induce atherosclerosis in rabbits by feeding them a diet high in meat and dairy products. At this stage, it was believed that the cholesterol arose entirely from its content in ingested food, and it was not until the work of RITTENBERG and SCHÖNHEIMER [1937] and BLOCH and RITTENBERG [1942] that it was realized that much of the cholesterol was formed by metabolism within the body.

Although a connection between heart disease, cholesterol, and high fat in the diet was suggested much earlier [WIDAL *et al.*, 1913] experience during World War II in which enforced dietary changes caused noticeable changes in the incidence of atherosclerosis [MALMROS, 1950] gave impetus to research on diet and heart disea... initially much work was done connecting dietary habits with atherosclerosis incidence, and the relationship between high dietary fat content and high blood cholesterol levels with accompanying low incidence of heart disease levels received much attention [KEYS, 1952]. KINSELL *et al.* [1952, 1953, 1954] were the first to relate the type of fat (i.e. saturated *versus* polyunsaturated) to serum cholesterol levels.

b) Effect of fish oil on serum cholesterol levels

Soon after it was noted that the type of fat or oil in the diet was related to the serum cholesterol levels, the effect of fish oils was studied by several workers on both man [BRONTE-STEWART *et al.*, 1956; MALMROS and WIGAND, 1957; AHRENS *et al.*, 1959] and animals [PEIFER *et al.*, 1960; HOWE and BOSSHARDT, 1962a, b; KAHN *et al.*, 1963a, b]. Results of some of this work on dietary fish oils compared to dietary oil of vegetable origin is shown in table X.

It is apparent that for each species, man, rat, chick and mouse, fish oils lowered serum cholesterol to a greater extent than did corn oil or linoleate (corn oil is predominately linoleate). The extent of lowering of the serum cholesterol level in these experiments was about twice as great for the fish oil as for the corn oil or linoleate for man and for the rat and ranged up to 12 times as great in the chick.

Figure 2 from the work of PEIFER *et al.* [1960] depicts the changes in serum cholesterol levels in rats fed diets in which one-third of the fat (the other two-thirds was tallow) consisted of palmitate, oleate, linoleate, linolenate, tuna oil or menhaden oil. There was no decrease of serum cholesterol levels when palmitate or oleate were fed for 5 weeks. The other fat additives lowered serum cholesterol levels, with the lowering caused by linoleate being considerably less than that for the fish oils or linolenate. Of particular interest is the fact that the two fish oils showed a lowering effect immediately after they were included in the diet. After 2 weeks serum cholesterol had dropped significantly in rats on the fish oil diets but was either unchanged or actually increased for rats on the other diets. This more *rapid* lowering of serum cholesterol levels seems to be specific to fish oil and has been consistently noted.

The quantity of fish oil needed to effect a given decrease in serum cholesterol level is considerably less than that of corn or other vegetable oils. HOWE and BOSSHARDT [1962a, b] found a cod liver oil concentrate to be ten times as effective, weight for weight, as corn oil

Table X. Comparative effects of fish oil with corn oil or linoleate on serum cholesterol depression in man and animals. Data of PEIFER [1967]

Species	Reference	Test lipid	Proportion of test lipid to total dietary lipid %	Test period weeks	Lowering of serum cholesterol %
Man	AHRENS *et al.*, 1959	corn oil	100	6	—29
		menhaden oil	100	8	—57
Rat	PEIFER *et al.*, 1960	linoleate	33.3	4	—31
		menhaden oil	33.3	4	—51
		tuna oil	33.3	4	—59
Chicken	KAHN *et al.*, 1963	corn oil	50.0	1	— 5
		cod-liver oil	50.0	1	—63
Mouse	HOWE and BOSSHARDT, 1962	linoleate	100	1.7	— 4
		corn oil	100	1.7	— 4
		cod-liver oil	100	1.7	—26

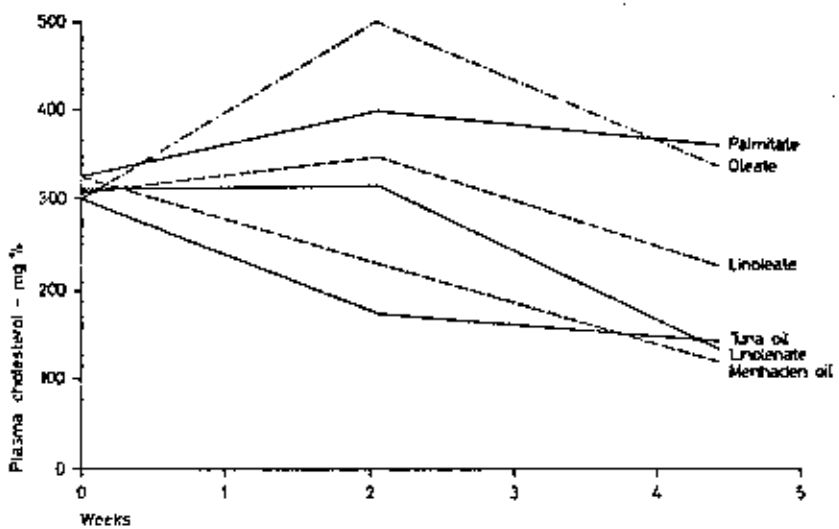

Fig. 2. The effects of feeding specific fatty acids and fish oils on the plasma cholesterol levels of hypercholesteremic rats (PEIFER, 1960).

in lowering serum cholesterol in mice. KINGSBURY *et al.* [1961] found that less than ¼ as much cod liver oil was as effective in lowering serum cholesterol in man as the 40% level of vegetable oils ordinarily employed [KINSELL *et al.*, 1954]. PEIFER *et al.*, 1965; PEIFER, 1966, have obtained significant lowering of serum cholesterol in the rat when less than 4% of the fat calories were supplemented as fish oil.

Cholesterol depressant effects have been noted for oils prepared from a wide variety of species of fish as shown in table XI. The effect is found in oils from the whole fish, from fish liver oils, and from fish body oils.

Not only is serum cholesterol brought to a lower level by use of fish oil than by vegetable oils, but also the same holds true for the lowering of plasma triglycerides which, with some individuals may be abnormally high when their serum cholesterol level is normal [ALBRINK and MANN, 1959]. In one case, AHRENS *et al.*, [1959] found that with an initial plasma triglyceride level of 810 mg/100 ml, corn oil intake resulted in a minimum value of 486. When this treatment was followed by menhaden oil intake, the value fell to 230, but when ingestion of corn oil was resumed, the value returned to 435.

The results so far discussed have been obtained with fish oil being consumed by man or animals. Presumably similar results would be expected if the oil were fed in the form of fish flesh which contains oil. In order to confirm this point, PEIFER *et al.* [1962] fed rats freeze-dried flesh of menhaden, coho (silver) salmon, mullet and ocean perch. In another part of the experiment oil extracted from these species and to another group of animals, the oil-free fish tissue was fed. Results of these experiments are given in table XII. Three of the species used were moderately oily. The fourth species, ocean perch, contains only a few percent oil, and it was not, therefore, possible to get as much oil into the diet as with the other species. In spite of the fact that when the ocean perch flesh which supplied only 2.5% of fish oil to the diet (as compared to about twice this amount for the other species) quite as great a cholesterol depressant effect occurred. All the species showed very nearly the same cholesterol depressant activity whether the flesh containing the oil or the oil in the extracted form was fed to the rats. Feeding of the fat-free flesh, however, resulted in either no change or a small increase in serum cholesterol levels.

Somewhat analogous but less comprehensive experiments involving consumption of fish by man have been carried out by HARLOW and MORTON [1962] and by IMAICHI *et al.* [1963]. Although data from these

Table XI. Species of fish for which cholesterol depressant effect has been demonstrated

Species	Source of oil	Reference
cod	liver	HAUGE-NICOLAYSEN, 1959; HOWE and BOSSHARDT, 1962a, b; KAHN *et al.*, 1963a; KINGSBURY *et al.*, 1961
cuttlefish	liver	KANEDA and ALFIN-SLATER, 1963
dogfish	liver	PEIFER *et al.*, 1965; WOOD *et al.*, 1961
halibut	liver	WOOD *et al.*, 1961
menhaden	whole fish	AHRENS *et al.*, 1959; PEIFER *et al.*, 1960, 1962, 1965
mullet	body	PEIFER *et al.*, 1962
ocean perch	body	PEIFER *et al.*, 1962
pilchard	body	BRONTE-STEWART *et al.*, 1956
salmon (coho)	body	PEIFER *et al.*, 1962
sardine	body	MILLER *et al.*, 1962
seal	body	BRONTE-STEWART *et al.*, 1956
tuna	whole fish	PEIFER *et al.*, 1960
whale	body	MALMROS and WIGAND, 1957

Table XII. Cholesterol depressant effect of feeding to rats whole fish, oil-free whole fish, and the extracted oil. Data of PEIFER *et al.* [1962]

Fishery product in diet	Fish oil in diet %	Fish oil polyunsaturates in diet %	Change in serum lipids %
None (tallow control)	0	0	—
menhaden flesh	4.9	13	—51
silver salmon flesh	5.3	12	—62
mullet flesh	3.8	7	—49
ocean perch flesh	2.5	5	—61
menhaden extracted oil	4.9	13	—56
silver salmon extracted oil	5.3	12	—68
mullet extracted oil	3.8	7	—48
ocean perch extracted oil	2.5	5	—63
menhaden oil-free flesh	0.0	0	0
silver salmon oil-free flesh	0.0	0	+20
mullet oil-free flesh	0.0	0	+12
ocean perch oil-free flesh	0.0	0	+9

tests are very limited, the trend seems to be the same as in the animal experiments of PEIFER *et al.* [1962] just described.

c) Mechanism for cholesterol depression action

There has, in the past, been much confusion as to just what types of polyunsaturates possess the cholesterol depressant activity and how this relates to presence of saturated fatty acids. The picture is still far from clear. In much of the literature we see use of the terminology introduced by KEYS *et al.* [1957] in which the ratio of polyunsaturated fatty acids (P) to that of the saturated fatty acids (S), expressed as P/S is used as a measure of the effectiveness of an oil. Although this ratio is still used today it is not always applicable especially in the case of fish oils. Fish oils contain a high proportion of saturated fatty acids, the percentage sometimes exceeding 40 and frequently the proportion of saturated acids exceeds that of the polyunsaturates. This would give a P/S ratio of less than 1 which is very low as compared to vegetable oils where often more than half of all the fatty acids are linoleic resulting in P/S far above unity. If the P/S ratio were a valid measure of cholesterol depressant activity of oils, we would have to predict that fish oils were far inferior to all vegetable oils. Yet we have just seen that on the contrary, fish oils are superior to vegetable oils in this respect. The discrepancy, of course, lies in the fact that when we add up all the polyunsaturates we make no distinction between dienes with only two double bonds like linoleic acid common in vegetable oils and 4, 5 and 6 double-bonded fatty acids such as are so prevalent in fish oils.

It was postulated earlier that it is the classical EFA which possess the cholesterol depressant activity. That this can not be the case is obvious from the fact that fish oils possessing much less content of classical EFA than other polyunsaturated oils, nevertheless have the greatest cholesterol depressant activity. Moreover HAUGE and NICOLAYSEN [1959] showed that when young rats were placed on a special diet which both depleted their supply of EFA and raised their serum cholesterol level, either cod-liver oil or linolenate resulted in greater depression of serum cholesterol levels than did linoleate.

The degree of total unsaturation such as might be measured as iodine value has sometimes been considered to be an indication of cholesterol depressant activity of an oil. This measure is also quite unreliable. PEIFER *et al.* [1965] fed to rats a series of fractionated menhaden oil ethyl esters as well as corn oil, margarine, butter, and tallow.

Two soybean oil samples were hydrogenated and then deodorized. Two cottonseed oil samples were fortified prior to refining with commonly used chlorinated hydrocarbons. The fortification levels for endrin, DDE, aldrin, dieldrin, heptachlor and heptachlor epoxide were 1.0 ppm and for DDT, 21.0 ppm.

Representative samples of crude oil, oil following each processing step and the by-products of processing were obtained, aliquoted and distributed to three analytical laboratories (Shell, Velsicol and Wisconsin Alumni Research Foundation) for pesticide analyses.

filter to remove the adsorptive clay. The refined-bleached oil was weighed and sampled.

Two samples of soybean oil were hydrogenated. After the hydrogenation vessel was charged with oil and the residual air removed by vacuum (50 mm Hg abs), the oil temperature was increased (230–250 F) to dehydrate the oil. The vacuum was broken with hydrogen. Nickel was used to catalyze the reaction (0.05%). During hydrogenation, the hydrogen pressure was maintained at 40 psig and the temperature at 275 F. After the desired iodine

ns of tallow-test lipid mixtures. From Peifer, Lundberg, Ishio and Warmanen [1965]

Marga-rine	Corn oil	MO-1	MO-2	MO-3	MO-4	MO-5
		4.1	5.2		1.9	
21.2	21.3	36.7	22.8	18.8	17.4	17.0
18.0	17.7	18.7	17.3	19.0	17.3	16.6
1.1	1.1	2.8	8.5	2.2	2.3	1.1
46.0	36.9	29.0	37.7	44.2	28.4	27.4
		+			1.9	1.7
3.5	17.4	0.8	1.1	1.3	1.4	2.5
0.5	0.5	0.2	0.3	0.2	0.8	0.2
				4.6		
					1.1	
					0.4	2.7
					0.4	0.3
					2.6	+
		0.5	+	1.9	9.3	15.7
					2.4	1.1
		+	?	+	3.7	6.9
3.4	15.5	0.8	—	0.8	2.1	0.7
0.1	0.4	0.2	—	0.4	0.5	0.1
		0.9	—	1.1	4.5	6.2
		1.2	—	2.6	9.5	16.2
		0.4	—	1.8	5.6	9.7
95.6	81.1	98	98.3	95	73.1	66.7
4.4	18.9	2	1.7	5	26.9	33.3
90.3	94.9	92.8	92.9	92.2	91.3	93.2
80	124	49	93	127	267	353

ed (S) and monounsaturated (O) acids present in 100 g of dietary amount of total polyunsaturated acids found in the same amount of ese values are based on the results obtained by GLC. rent diets. All calculations were based on a 95% fatty acid composi-ty acid composition for the ethyl esters.

the menhaden oil methyl esters and other test le XIII. The most saturated fraction of the men-1, with an iodine value of only 49 gave equivalent t effect as the corn oil with an iodine value of 124. ter fraction MO-4 with iodine value of 266 gave cholesterol depressant effect than did fraction e value of 353. These results (table XIV) show

clearly that total unsaturation as measured by iodine value is not a measure of cholesterol depressant effect of an oil.

It might appear that several different polyunsaturated fatty acids were involved or even some monoenes or saturates with perhaps some of these acting as synergists to increase the activity of certain polyunsaturates [Peifer, 1967].

Another possible explanation for the superior cholesterol depressant effect of fish oil fatty acids may be in the preponderance of ω-3 fatty acids present in these oils. If such fatty acids are somehow more effective in cholesterol transport than the more usual ω-6 types, such a fact could account for the greater effectiveness of fish oils without resorting to any reference to degree of unsaturation. Well over 90% of the polyunsaturates of fish oil are ordinarily of the ω-3 variety whereas in most edible vegetable oils the percentage is usually well under 5%. We have no specific data, however, which lends itself to testing of such a hypothesis.

Certainly much work employing purified fatty acids found in fish oil is needed before this point can be clarified. A bottleneck to carrying out such research is the fact that so many different fatty acids, several of them unavailable commercially, are present in fish oils. Until, however, such experiments are carried out, our picture of the mechanism of the cholesterol depressant activity of fish oils will remain obscure.

d) Fatty acid pattern change in tissues

When hypercholesterolemic animals are fed diets containing different types of fat, not only are there changes in serum cholesterol levels but also changes take place in the fatty acid patterns in the different tissues. Table XV shows the tissue fatty acid pattern in the heart tissue of hypercholesterolemic rats after 1 month on diets containing different fats [Peifer *et al.*, 1965]. Serum cholesterol levels fell from the initial value of about 300 mg/100 ml to values in the range of 140–175 when fish oil or linolenate were fed and to 238 when linoleate was used. At the same time the hexaene and pentaene contents rose in all heart tissues. Initial hexaene content of heart tissue in rats having normal serum cholesterol level is, according to Peifer *et al.* [1965], not higher than 0.3 mg/g of heart tissue. Even the hypercholesterolemic rats on tallow or linoleate had more than double this amount (0.7). Rats on the fish oil or linoleate diets had seven to seventeen times this much hexaenoic acid in the heart tissue. A similar pattern change took place for

the pentaenoic acids. Build-up of hexaenoic acid in the heart tissue increased from diet to diet as terminal serum cholesterol level decreased.

Since the hexaenoic acid content of the heart tissue showed a marked increase for rats on diets (tallow, linoleate) which contained very little of this polyunsaturate in the oil being fed (actually only trace

Table XIV. Relative effects of menhaden oil fractions[1] and of other oils and fats on plasma cholesterol levels of the rat

Treatments	I.V. of supplements	Total PUFA in dietary[2] fat mix %	Plasma lipids, mg/100 ml		
			TC	Pl	TC/Pl
Tallow	41	1.5	524	145	3.6
Butter	44	2.2	499	151	3.3
Margarine	80	4.4	401	142	2.8
Corn oil	124	18.9	297	123	2.4
Men. oil Fr.					
MO-1	49	2.0	255	126	2.0
MO-2	93	1.7	206	113	1.8
MO-3	127	5.0	142	125	1.2
MO-4	266	26.9	112	77	1.5
MO-5	353	33.3	160	89	1.8

[1] PEIFER *et al.* [1965].
[2] The diets contained 10% fat, 1/3 of which was the indicated test lipids and 2/3 was tallow. The test lipids had widely different iodine values (I.V.) and the test lipid-tallow mixtures contained polyunsaturated fatty acids (PUFA) ranging from 1.5–33.3% of the total fat calories.
I.V. = Iodine value TC = Total cholesterol Pl = Phospholipids

Table XV. Change in heart tissue fatty acids resulting from feeding hypercholesterolemic[1] rats different kinds of fat for 1 month. Data of PEIFER *et al.* [1965]

Oil fed	Fatty acid-content in mg/g of tissue					Final serum cholesterol level mg/100 ml
	Hexaenoic acids	Pentaenoic acids	Tetraenes	Trienes	Dienes	
Tuna oil	4.5	1.0	2.5	0	0.7	140
Menhaden oil	5.1	1.8	3.5	0	0.9	169
Linolenate	2.1	1.8	2.2	0.6	0.6	175
Linoleate	0.7	0.8	5.0	0	3.3	238
Tallow	0.7	0.3	2.1	0.7	1.3	344

[1] Initial serum cholesterol value about 300 mg/100 ml.

amounts too small for ordinary GLC analysis to detect, see table XIII), it would appear that there is a need for such polyunsaturates in hypercholesterolemic animals and that the body mobilizes them from wherever they are available. With diets containing fatty acids of the linolenic acid family (as with fish oils) the highly polyunsaturated fatty acids (C22:6, C20:5) are already present or can be readily metabolized from linolenic acid itself.

These results might suggest that serum cholesterol depression, while easily possible with dietary fats containing predominately dienes and tetraenes of the ω-6 family is more efficient with a wider combination of fatty acids including ω-3 types such as occurs in fish oils. Much more research is needed to further elucidate what takes place in such cases.

6. ω-3 fatty acids

A paper has recently appeared [BERNSOHN and STEPHANIDES, 1967] linking ω-3 fatty acids and more specifically docosahexaenoic acid with multiple sclerosis. These authors show that in areas of the world where ω-3 fatty acids occur in some quantity in the diet such as where much fish is eaten or, where certain seed products containing linolenic acid are eaten, multiple sclerosis is almost unknown. The disease is most common in areas where very little fatty acids of the ω-3 type are found in the diet. Furthermore KISHIMOTO *et al.* [1967] have found that the human brain which normally contains considerable 22:6 ω-3 fatty acids, for subjects who have had multiple sclerosis contains less than half the normal amount of this fatty acid. In their experiment, they found that the 22:6 ω-3 fatty acid content in the average normal appearing white matter of the brains of several multiple sclerosis victims averaged 1.5% of total lipids as compared to an average value from control (non-multiple sclerosis patients) of 3.5%.

BERNSOHN and STEPHANIDES [1967] have developed the theory that lack of docosahexaenoic acid or its precursor, linolenic acid in the diet is one factor in development of multiple sclerosis. They recognize, of course, that even in areas of high incidence of multiple sclerosis and of low intake of ω-3 fatty acids, most individuals do not contract multiple sclerosis. They hypothesize, therefore, that in those individuals in such geographic areas who do develop the disease, in addition to the lack of a safe quantity of ω-3 fatty acids in the diet, some other factors such as faulty enzyme system for conversion of linolenic acid to docosahexaenoic acid are present. In the presence of

adequate dietary ω-3 fatty acids, these other factors would not be sufficient to allow the disease to develop. This theory is receiving attention in laboratories of BERNSOHN and STEPHANIDES as well as elsewhere. Still to be determined is whether ω-3 fatty acid supplementation is effective only at a very early age while the brain is developing or to what extent it is important with older indivuals. Also evidence is being sought as to whether supplementation of diets of multiple sclerosis patients with ω-3 fatty acids can reverse the trend of the disease.

Needless to say, should these findings be positive, the importance of fish in the diet as a source of ω-3 fatty acids and indeed of its high content of the effective component, docosahexaenoic acid, would be much greater than presently recognized. Fish oil fatty acids are usually about 30–40% of the ω-3 type whereas vegetable oils contain usually under 2%. Furthermore the ω-3 fatty acids of animal and vegetable fats and oils are only precursors for the apparently desired docosahexaenoic fatty acid and none of this acid is present in edible oils or fats other than fish oils where the content approximates 10% of the total fish oils. Thus consumption of only a very small quantity of fish could provide docosahexaenoic acid in substantial quantity without need for its perhaps less efficient metabolism from other ω-3 precursors. Thus the old adage 'fish is a brain food' may be not a mere superstition but rather a recognition of a relationship between docosahexaenoic acid and its requirement in the brain.

7. Discussion and summary

Fish oil fatty acids, as we have seen, possess nutritional properties in common with fatty acids from other sources both in providing metabolizable energy and as growth stimulants. Although fish oil polyunsaturated fatty acids in common with any of the ω-3 type lack certain EFA properties as exemplified by inability for complete curing of dermal symptoms, there is some indication that these same ω-3 fatty acids may, of themselves, possess other functions which are lacking in the ω-6 series.

The antagonistic effect of one fatty acid family upon another has been mentioned. The effect of the ω-3 and ω-6 families upon each other may be of concern when fish oils, high in ω-3 fatty acids are used in animal feed or for human food together with other food which contains predominantly members of the ω-6 fatty acid family. This effect of depressing the conversion of fatty acids of one family is

dependent upon the presence of large excesses of members of the other family. In almost every case of use of fish in either animal feed or for human consumption there will be a large excess of ω-6 fatty acids from other components of the diet. Insofar as animal feed is concerned, the amount of fish used is nearly always greatly limited by the need to prevent imparting of a fishy flavor to the meat or eggs so that oil or fat from non-fish source with ω-6 type of fatty acids predominates. Likewise in any human diet, at least in Western countries, ω-6 type fat or oils always greatly exceeds in quantity that from fish sources. This means that under normal conditions there will be no critical suppression of conversion of linoleic to arachidonic acid by inclusion in the diet of fish containing ω-3 type fatty acids. What will happen is that the excess of ω-6 fatty type acids in the diet will decrease the conversion of what little linolenic acid is present to docosahexaenoic acid and other related ω-3 fatty acids so that there will be less of these formed than would have been if fish oils or linolenic acid were the sole type of oil or fat present. It is here, of course, that the beneficial effect of fish oils show up. If indeed the higher members of the ω-3 fatty acid family such as docosahexaenoic acid are needed for some important function, they already occur preformed in fish oils and it is unnecessary for them to be metabolized from linolenic acid. Thus inclusion of fish or fish oils in a diet insures the presence of the higher members of the ω-3 family for whatever benefit they may impart.

Some preliminary evidence indicating nutritional effects of fish oils beyond those found in other oils or fats has been presented. These deal with properties concerned with superior cholesterol depressant action and beneficial effects for maintaining normal levels of docosahexaenoic acid in the brain and other tissues with a possible relationship to multiple sclerosis or other conditions. These effects need a great deal more documentation and elucidation before they can be accepted as being firmly established.

Certain potentially harmful effects of fish oils based upon their high content of polyunsaturates have been considered. It appears that under normal conditions there is no possibility that accumulation of oxidation products of harmful effect can be a cause for concern in either human or animal food. The *in vivo* effect of the presence of unoxidized polyunsaturates in a diet while of no concern at the level fish is included in our diet, may be potentially hazardous for captive animals if fish oils predominate in the diet. This effect, however, is readily avoided by adequate tocopherol supplementation.

Fish thus has a place in the diet of animals and man. Future research may further substantiate the role of fish (at present only partially documented) as a source of ω-3 fatty acids which may well play a more important nutritional role than previously suspected.

C. Miscellaneous Classes of Lipids

1. Introduction

Nearly all of the well documented evidence concerning nutritive value and related pharmaceutical activity of fish oils (aside from vitamin properties only being incidentally mentioned in this review) deal with fatty acid components. Nevertheless a considerable body of literature exists in which claims are made as to beneficial effects of fish oils or their components and which are believed by their proponents to be due either to specific classes of components of fish oils other than fatty acids or to unidentified components. In this section, this type of material will be covered. Since in most instances there is uncertainty as to precisely which class of compound may be responsible for a given action, to facilitate discussion the treatment will be made according to observed effects rather than by classes of compounds. Two principal types of activity have been extensively reported. One of these, dealing with many healing effects ranging from curing of wounds and burns to tuberculosis alleviating action has a very extensive literature. A second quite different and more recent field covers work on reticuloendothelial stimulation with application to phagocytosis.

2. Healing effects

a) Wound and burns

For many years fish oils have been reputed to effect rapid healing of burns and wounds. It is, of course, unquestionable that the pain from a fresh burn can be immediately and effectively alleviated by keeping air away and that this can be readily achieved by applying a solid fat such as petrolatum or butter or a liquid oil such as mineral oil or a fish oil. The question which, however, has perhaps never been resolved is whether use of any of these products actually promotes better healing.

The early literature contains many papers indicating a healing property of vitamin A for wounds [LOLTAN, 1935; SANDOR, 1936; PROTO, 1936] when applied topically to the wound or when taken orally [LAUBER, 1936a, b]. Oddly enough those investigators who reported

effective healing by topical application usually found no effect for dietary supplementation and vice versa. Other investigators in these early periods could find no effect of vitamin A on wound healing or they even found an inhibiting effect [LAMBERT and ROCHOLL, 1935]. Along with investigations on vitamin A promotion of wound healing are, of course, several papers in which fish liver oils was the source of the vitamin A [DRIGALSKI, 1934; LESSER, 1950]. Other workers who believed fish liver oils promoted wound healing believed that this result was achieved because of a bacteriostatic action of other components of the oil such as oxidation products like peroxides and aldehydes [SPANHOFF and JACOBSEN, 1950] or even certain fatty acids [SOLOMIDES, 1949].

The occurrence in many fish liver and body oils of alkoxydiglycerides which yield upon hydrolysis such glyceryl ethers as chimyl, selachyl, and batyl alcohols has more recently been suggested as the component of fish oils primarily responsible for wound healing [BODMAN and MAISIN, 1958]. These workers found that such compounds were greatly effective in promoting the healing of deep-seated lesions caused by irradiation therapy. They developed a theory based upon the fact that the glycerol ethers and plasmalogens are found in bovine and human tissues in foetus or in tissues the first few days after birth [HARTREE, 1964] in much higher concentration than at later stages. They predicate that in prenatal cell development, glyceryl ethers play a role in cell development before triglycerides are present and that gradually the role of the glycerol ethers is taken over by triglycerides. From these considerations they explain their findings that deep-seated wounds are more rapidly healed by topical application of the glyceryl ethers which would facilitate regeneration of new cells. BODMAN and MAISIN [1958] found no healing effect of glyceryl ethers on burns; in fact, if anything, topical application resulted in a slight impeding of curing of burns.

Fish oils are currently added to several commercial ointments for the purpose of increasing healing of wounds, burns and skin irritation. Because of the conflicting evidence on the role of fish oils used topically to promote healing, the US Bureau of Commercial Fisheries and the Dermatology Department of Mayo Clinic carried out a series of experiments in which various fish oil preparations were applied to hairless mice which had been given wounds or burns under controlled conditions [STANSBY, ZOLLMAN and WINKELMANN, 1967]. Treatments used consisted of topical application of (1) a high vitamin A fish liver

oil, (2) a typical fish body oil with negligible vitamin A content, (3) a fish body oil to which glyceryl ethers had been added, (4) a commercially available ointment containing cod-liver oil, (5) a laboratory prepared cod-liver oil ointment and (6) a mineral oil control. The length of time required to effect curing of the wound or burn was measured for each treatment. No significant difference was observed in the healing time for any treatment of either the wounds or burns except that in most cases the use of any oil including mineral oil gave small improvement in healing time over use of no treatment at all. It was observed that during the healing process, the fish oil treated samples in many cases seemed to result in a cleaner lesion which gave the impression of a more rapid healing. The elapsed time of healing (point where scabs fell off of wound or burn) was not, however, any shorter than that for the control animals.

b) Irradiation damage

Workers at the Radiumhemmet, Karolinska Institute, Stockholm, have carried out extensive work on the use of glyceryl ethers taken orally in connection with irradiation treatment. This work is described in considerable depth by BROHULT [1963]. In early research the workers in this group found that certain bone marrow fractions (which contain alkoxydiglycerides) when given orally to children suffering from leukemia resulted in an increase in white cell and thrombocyte count. The unsaponifiable fat portion of the bone marrow preparation gave the best results, leading to the belief that glyceryl ethers might be the principal active components. In subsequent work alkoxydiglyceride concentrates, prepared from fish liver oils (dogfish and Greenland shark) were employed. When these were fed to patients suffering from irradiation leucopenia, the same protection against drastic drop in white cell and thrombocyte count occurred as those with leukemia patients treated similarly. This type of effects has not only been observed over years of research by this group, but also has been confirmed by workers at other laboratories [LINMAN, 1958; SUKI and GROLMAN, 1960]. Tests have been run using both glyceryl ether concentrates prepared from fish liver oils and with synthetic batyl alcohol.

Contrary to these results are those of CARLSON [1966] in whose work little or no significant hematopoietic effect could be demonstrated when chimyl, selachyl, or batyl alcohols were fed to dogs or rats at dose levels of 6 mg/kg of body weight and a slight, marginally

significant such effect was obtained when doses were used of up to 2400 mg/kg of body weight. Similar negative results with rats have been reported by SNYDER [in press].

3. Effects with miscellaneous diseases

A disease of cattle, bracken poisoning, is said to be alleviated by feeding batyl alcohol [EVANS, EVANS, EDWARDS and THOMAS, 1957]. Since this condition caused by the cattle having consumed a type of fern results in damage to bone marrow similar to that encountered with irradiation, there would appear to be a relationship between the action of glyceryl ethers in these two conditions. On the other hand DALTON [1964] was unable to reproduce such results for bracken poisoning treatment with glyceryl ethers.

Fish oils, especially cod liver oil, have been reported to have favorable properties in connection with curing of tuberculosis [GETZ, 1955]. He has indicated [GETZ, 1960] that faulty vitamin A metabolism in tubercular patients could be offset by natural vitamin A concentrates which, he believes, also contain fragments of the vitamin A molecule of an unidentified nature more readily utilized by such patients. Along quite another vein, EMMERIE, ENGEL and KLIP [1952] report a fraction isolated from cod liver oil (containing batyl alcohol) which inhibits the growth of tubercle bacilli.

CALCAGNA [1935] reports on the use by natives of South America of certain fish oils in the treatment of leprosy.

CHALMERS, WOOD and SHAW [1966] describe a means of treatment of inflammatory diseases such as rheumatoid arthritis or gouty arthritis with fish liver oil concentrates of glyceryl ethers. Their supporting evidence is based upon rat feeding tests with the anti-inflammatory action measured by the granuloma pouch test of ROBERTS and NEZAMIS [1957].

4. Cosmetics

Fish oils and derivatives obtained from them are sometimes used in cosmetics. The most commonly used substance of this kind is squalane. It is the hydrogenated derivative of squalene, the unsaturated hydrocarbon which occurs not only in abundance in the liver oils of several shark but is also an important constituent of surface lipids of the skin [ROSENTHALL, 1964]. Originally the squalene as it occurs in the shark liver oil was used directly in the cosmetic cream but it was found that due to instability against oxidation, the unsaturated squalene soon

developed undesirable oxidative products. This lead to the present practice of use of the hydrogenated product, squalane.

Fish oils or fish oil fatty acids have sometimes been used directly in cosmetics with the belief that some of the unsaturated fatty acids or in other instances the content of vitamin A were of value [GOLDRICK, 1966]. In recent years there has been a vogue for incorporation of turtle oil in cosmetic creams.

5. Reticuloendothelial stimulation

Effects of a different kind which have been reported to be influenced by fish oil components relate to the enhancement of the reticuloendothelial action which results in an increase in the phagocytic effect. HELLER, PASTERNAK, RANSOM and HELLER [1963] reported the isolation of a lipid fraction from the liver oil of certain sharks which acts as a stimulating agent for reticuloendothelial systems. The lipid fraction, which has not been identified, is extracted with ethanol, ether, and hexane and is separated and concentrated by various solvent and chromatographic procedures. The activity of this lipid concentrate was evaluated by measurement of stimulation of phagocytosis by the carbon clearance technique. An increase of 50% survival of mice treated with this fraction over controls was obtained when mice were injected with *Salmonella typhimurium*. RINGLE, HERNDON and BULLIS [1966] have also tested this same lipid fraction from shark for its resistance to shock but with negative results.

D. Discussion and Summary

The role of fish lipids other than fatty acids is even less clear than is the case with polyunsaturated fatty acids. Such effects as that of a reticuloendothelial stimulant by some unknown component of shark liver is still very obscure and will require a great deal more evidence both in replicating the effect itself and in learning what classes of compounds are involved and how they operate.

The case for desirable effects in using alkoxydiglycerides or their hydrolysis products, glyceryl ethers, is somewhat better established although still not universally accepted. On the one hand, the fact that several quite distinct groups of workers sometimes working independently and without knowledge of what other workers had found came up with the same type of action of glyceryl ethers certainly must carry some weight.

On the other hand much of the earlier work was based upon inadequate number of animals or patients used in the research or use of inadequate controls. As more thorough clinical or animal work involving more adequate numbers of tests and controls are used these effects are being indicated to be less important and in some cases are being completely disproved. A great deal more research will have to be carried out before we can be certain which of the various effects which have been described have a genuine basis in fact and warrant a practical application of fish oils for various purposes.

V. General Summary and Conclusions

In this review, the chemical and nutritional properties of fish oils have been considered in some detail with some background information included on sources of fish oils.

The highly diverse fish oil fatty acids varying from fully saturated ones making up about one-third of the total to polyunsaturates ranging in amount upward from one-quarter to nearly one-half have been discussed. The unique characteristics of the fish oil fatty acids including presence sometimes of substantial quantities having odd carbon chain length, the presence of both very long carbon chain—up to C24—and of very highly polyunsaturated acids with up to six double bonds/molecule as well as the predominance of ω-3 rather than the usual ω-6 family of fatty acids have been mentioned.

Brief discussion has been given of such topics as the origin of fatty acids in fish either via food chain in the sea or during metabolism in the fish; occurrence of fish oils in the flesh *versus* in certain organs, in cells *versus* in depot fats and in light *versus* dark muscle; differences in amounts and type of oil in different species of fish; commercial production and uses of principal industrial fish oils especially from the South American anchovy, American menhaden and various herring industries.

The nutritional properties of fish oil fatty acids have been examined in some detail. Special attention has been paid to the unique predominance of the ω-3 family of fatty acids in fish oils. Relationships between the different families of fatty acids in metabolism have been considered. It has been indicated that incorporation of moderate amounts of fish oils in diets does not upset the important conversion of linoleic to arachidonic acid yet insures by reason of the presence of preformed

docosahexaenoic and other ω-3 family fatty acids that these will be available when and if needed.

The use of fish oils in feeds for different animals has been described as well as for special pharmaceutical uses such as for cholesterol depressant action. The superior activity of fish oil for this purpose as compared to the use of various vegetable oil polyunsaturates has been pointed out.

Finally a number of effects of non-fatty acid components of fish oils which have been claimed to be important have been discussed. These include use of such components as glyceryl ethers and hydrocarbons for a variety of purposes. It has been pointed out that these purported effects—wound healing, radiation damage alleviation, reticuloendothelial stimulation, etc.—need a great deal more research before they can be firmly established and generally accepted.

VI. Summary

Fish oil fatty acids differ from those in other fats and oils especially with regard to being more polyunsaturated, being largely members of the ω-3 rather than the more usual ω-6 family. These properties result in some nutritional effects which differ at least in extent from the effects of fatty acids from other sources. Evidence is beginning to accumulate to indicate that docosahexaenoic acid, the end metabolite of the ω-3 family has desirable nutritional effects not previously recognized.

Glyceryl ethers and some other non-fatty acid components of fish oils are reported to have special nutritional properties, but much more evidence is needed before these claims can be fully substantiated.

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Author's address: Mr. M.E. STANSBY, Food Science Pioneer Research Laboratory, Bureau of Commercial Fisheries, *Seattle*, Wash. 98 102 (USA).

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DDT RESIDUE LEVELS IN SOME U.S. FISHERY PRODUCTS AND THE EFFECTIVENESS OF SOME TREATMENTS IN REDUCING THEM

by

V.F. STOUT, F.L. BEEZHOLD and C.R. HOULE
U.S. Department of Commerce
National Oceanic and Atmospheric Administration
National Marine Fisheries Service
Technological Laboratory
Seattle, Washington 98102, U.S.A.

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1 INTRODUCTION

Because of the extensive use of DDT since World War II, the ease with which it disperses, and the stability of the chemical, it has become a widespread pollutant of our environment. For this reason, the National Marine Fisheries Service[1/], which is concerned with the wholesomeness of fishery products, has been determining the concentration of total DDT (that is, of DDT and its metabolites, TDE and DDE) in food fish and shellfish, in fish protein concentrate, in the products from industrial fish, and in edible oils. Methods for reducing the DDT content have been examined.

2 FOOD FISH AND SHELLFISH

The aim of our work on food fish and shellfish was to determine the residue levels in the edible portion, and in those species with high residue levels to attempt to reduce the levels to meet the U.S. Food and Drug Administration interim tolerance for edible flesh, 5 ppm on a wet-weight basis. Both marine and freshwater species were studied.

2.1 Marine species

2.1.1 Monitoring

Several species (Table 1) of marine fish and shellfish collected from the open waters of the Pacific Ocean and from the coastal waters of the Northeast Pacific Ocean were examined. Although all species shown are edible, several are used primarily for industrial or bait purposes or are not fished commercially. The findings are conveniently considered in relation to the geographic areas of Washington and Oregon (Stout, 1968, for some of the data) and of southern California.

None of the samples from the Pacific Ocean or Washington and Oregon contained more than 0.5 ppm total DDT. These residues of less than 10% of the current FDA limit for fish constitute some of the lowest levels found for fish in the United States. DDT usage depends on the intensity of farming, the weather, and the density of the population. Washington and Oregon have a short growing season, a winter cold enough to kill many pests, and a relatively low population density. Thus, relatively little DDT has been used in these areas, so the marine life contains little DDT.

In southern California, farming is intensive and the growing season is long. There are as many as three crops a year. Also, the density of population is high, and the winter mild. It is not surprising, therefore, that some of the fish from this area contain excessive residues of total DDT. In our study of species from this area we gave particular attention to jack mackerel, inasmuch as it has been involved in FDA action.

During the fall of 1969, the FDA seized jack mackerel from southern California because some samples contained more than 5 ppm total DDT. Since that time, high levels of residues have been found in the livers and edible portion of other species of fish taken from Santa Monica Bay, an area not fished by commercial fishermen. The livers from one sample, for example, contained 1,026 ppm total DDT; fillets of the same fish contained 57 ppm. Although the relation between residues in the livers and flesh was not constant, the concentration of residues in the liver was always greater than that in the flesh. In fish with low levels of DDT, such as those from the coastal waters of Washington, the ratio of DDT in the liver to that in the flesh was greater than 100. When the concentration of DDT was high, the ratio decreased in an irregular, unpredictable way to as low as 5. Generally, it was in the range of 20 to 50.

1/ Formerly the Bureau of Commercial Fisheries

Table I. Species of marine fish and shellfish collected both on the high seas and off the coast of the Northeast Pacific and examined for the presence of DDT and its metabolites

Common name	Scientific name	Location[c]
Dungeness crab	Cancer magister	NW
Pink shrimp	Pandalus jordani	NW
Pacific herring	Clupea harengus pallasi	NW[d]
Northern anchovy	Engraulis mordax	NW[d]
Coho salmon	Oncorhynchus kisutch	NW[ab]
Sockeye salmon	Oncorhynchus nerka	High seas[ab]
Chinook salmon	Oncorhynchus tshawytscha	NW[ab]
Pacific cod	Gadus macrocephalus	NW
Pacific hake	Merluccius productus	NW[d]
Jack mackerel	Trachurus symmetricus	Cal
White croaker	Genyonemus lineatus	Cal[b]
Albacore	Thunnus alalunga	NW[ab]
California scorpionfish	Scorpaena guttata	Cal[bd]
Pacific ocean perch	Sebastodes alutus	NW
Starry rockfish	Sebastodes constellatus	Cal[b]
Vermillion rockfish	Sebastodes miniatus	Cal[b]
Blue rockfish	Sebastodes mystinus	Cal[b]
Bocaccio	Sebastodes paucispinis	Cal[b]
Rosy rockfish	Sebastodes rosaceus	Cal[b]
Treefish	Sebastodes serriceps	Cal[bd]
Yellowtail rockfish	Sebastodes flavidus	NW
Sablefish	Anoplopoma fimbria	NW[ab],Cal[b]
Rex sole	Glyptocephalus zachirus	NW
Dover sole	Microstomus pacificus	NW,Cal[b]
English sole	Parophrys vetulus	NW,Cal[b]
Starry flounder	Platichthys stellatus	NW
Sand sole	Psettichthys melanostictus	NW

[a] Only the livers of these fishes were analyzed.
[b] Office of the Secretary, U.S. Department of Interior, Laboratory, Sabine Island, Gulf Breeze, Florida analyzed the livers. For the relation between residues in the livers and the edible flesh, see the text.
[c] NW is Pacific northwest area of the United States--i.e., Washington, Oregon, and occasionally British Columbia, Canada; Cal is California, High seas is lat. 45-55° N, long. 155-177° W, that is, south of the Aleutian Is.
[d] Industrial or bait species or not fished commercially.

2.1.2 Attempts to lower residue levels

Several experiments were done with jack mackerel to determine if simple precanning process alterations would reduce significantly the level of DDT in the flesh. Relevant to this work were the findings of Ritchey (1969, and references therein) who studied the effects of cooking methods on DDT residues in chickens fed diets containing DDT. He and his coworkers found that the reduction in the residue levels paralleled somewhat the loss of fat by rendering. Frying and pressure cooking were most successful; baking effected very little change even though the fat content was reduced. The greatest reduction of total residues was 38% on a dry-weight basis or 17% on a wet-weight basis, which is the basis used by the FDA. In most cases of high residues, a greater reduction would be needed to provide a practical solution. For example, flesh found to contain 10 ppm (wet) might after cooking contain only 8 ppm (wet), but it

would still be unacceptable according to the current tolerance. Fish presumably would act in a similar way to chicken. Our prospects for success, in reducing DDT level in fish, therefore, were few.

Nonetheless, we studied the effects of cooking time and temperature on residues of DDT in the flesh of jack mackerel. After being placed in the can these fish are commonly cooked for about 15 min at about 95° C. Following this, salt is added, the cans are sealed and given a sterilizing cook. We altered precook time from 15 to 60 min and temperature of precook from 85° to 100° C. No reduction in residues in the drained fish was observed. In fact, as the cooking time was increased, the level of DDT on a wet-weight basis increased, because of the reduction in the moisture content of the fish. Similarly, soaking the jack mackerel in water overnight at 3° C and precooking them in upright cans or in inverted cans to permit drainage during heating did not reduce the residues, but instead increased them slightly even after we corrected for the loss of water. Heat may possibly free DDT that is bound to protein in the raw fish (the control) and that is not extractable in the analytical procedure.

Three experiments were carried out. In each experiment fish were divided into lots of equal numbers of fish. These lots were 19 to 52 fish taken at random from large landings. A later study in which 25 fish from a large landing were analyzed individually showed the tremendous variation in DDT, oil, moisture, and size among individuals from a supposedly homogeneous lot. The concentrations of total DDT in the flesh ranged from 2.1 to 17.4 ppm; oil content ranged from 1.1 to 6.3%; and moisture content from 71.3 to 76.7%. The weight ranged from 123.8 to 386.7 g.

Because of this wide variation, ground, well mixed jack mackerel were used in subsequent work. No reduction of DDT residues resulted from precooking the ground flesh in water or 1.5% NaCl brine.

These experiments show that no simple means exist for decreasing the residues of DDT in jack mackerel and still producing the normal canned product. As has been found with chicken, DDT is too closely associated with muscle components to be removed by conventional processing procedures.

The level of DDT in jack mackerel dropped early in 1970, so fishing was started again, but the threat of excessively high levels still continues. A cyclic rise and fall related to the season of the year or to the age of the fish may be suggested. In the fall, fish could be accumulating residues applied throughout the agricultural growing season. We found that those fall-caught fish having high residues were also large and therefore older; a relation between age and residue content would not be surprising. In support of this, we found little residue in a sample of small fish caught in December. Large fish caught one month later contained residues in excess of 5.0 ppm. Jack mackerel caught in late winter and early spring contained low levels of DDT. In June, the fleet was still catching small fish containing low levels of DDT.

Interpreting these findings on a seasonal basis alone is difficult. During the winter season, for example, the agricultural application of DDT is low but heavy winter rain would cause major runoffs into marine waters.

The data for the 25 fish analyzed individually showed some correlation between size and residue levels. That only the largest fish in an admittedly small sample had high residue levels suggests that older or faster growing fish accumulate more DDT. Additional work is needed to relate season and age of fish to DDT residue content.

2.2 Freshwater species

In the Spring of 1969 the occurrence of excessive residues of DDT in freshwater fish was brought to the attention of the general public by the seizure of Lake Michigan coho salmon containing up to 20 ppm DDT and up to 0.32 ppm dieldrin: the maximum permissible content of dieldrin in the edible portion is 0.30 ppm.

At about the same time excessive levels of DDT residues were being reported in chub (*Coregonus hoyi*).

The seriousness of the DDT residue problem in freshwater fish was shown by the data of the National Pesticide Monitoring Program of the U.S. Bureau of Sport Fisheries and Wildlife (Henderson, 1969). In 12 of 44 lakes and rivers sampled, some or all of the fish contained more than 5 ppm DDT, and in 8 of these locations, the mean for all fish examined exceeded the tolerance level.

Work has been in progress at the National Marine Fisheries Service Laboratory at Ann Arbor, Michigan, to study methods of reducing the residues in local fish. A partial solution has been found for salmon (Reinert, 1969). Analyses of various portions showed (Table II) that DDT is concentrated in areas of high fat content. Removal of the skin and the fatty deposits in the dorsal median, lateral line, and belly flap areas of fish with high residues would therefore reduce the contamination substantially.

Table II. Distribution of DDT in Lake Michigan coho salmon[a]

Sample	Total DDE + DDT[b] (ppm)
Whole fish	12.2
Whole eviscerated steak	14.9
Flesh (muscle)	5.7
Dorsal median fat	62.8
Laterial line fat	34.3
Abdominal adipose	92.3
Belly fat	69.7

[a] Reinert, 1969
[b] TDE is not found in significant amounts in Great Lakes fish.

The situation with other fish is less readily improved. Trimming is practical only in a product of high value such as salmon. Furthermore, in many fatty fish such as chub, residues are so evenly distributed that trimming would be ineffective. Cooking chub, for 15 min at 96° C in open cans filled with boiling water or 1.5% NaCl brine or with no added liquid caused little reduction in the level of residues. The drained liquid portion of the product contained little oil. Only if substantial rendering of fat occurred during processing would any removal of pesticides be anticipated. Extended high temperature cooking might well be successful in reducing the residues, but the product would probably be unpalatable.

3 FISH PROTEIN CONCENTRATE

Studies (Stout, MS) on the development of methods for production of fish protein concentrate (FPC) required information about the fate of the pesticides during processing. The lipophilicity of organochlorine pesticides suggested that DDT would be concentrated into the byproduct oil. Table III indicates the validity of this inference by showing that FPC made from Pacific herring both by the isopropyl alcohol extraction method (Bureau of Commercial Fisheries Technological Laboratory, College Park, Maryland, 1966) and by the aqueous fractionation method (Spinelli, 1968) contained a barely detectable amount of DDT. The largest concentration of pesticide occurred in the oil portion. Phospholipids are potent solubilizing agents for organic molecules, which may explain the concentration of the pesticides in Miscella 1 of the isopropyl alcohol extraction method. Fortunately, because of the fractionation of the DDT into Miscella 1, the edible-oil fraction contains a very low level of DDT, especially after being washed with water, which tends to remove any of the suspended phospholipids and associated compounds.

Table III. Fate of pesticide residues during FPC production

Sample	Total DDT (ppm) Isopropyl alcohol extraction	Aqueous fractionation
Whole raw Pacific herring	0.097	0.097
Separated raw herring[a]	0.084	0.084
FPC	<0.002	0.006
Oil		1.12
Miscella 1[b]	1.75	
Miscellas 2 + 3[c]	0.33	
Miscellas 2 + 3, washed with H_2O	0.28	

[a] Skin and bones removed.
[b] Mainly phospholipids, not usually considered edible.
[c] Triglycerides, potentially for food use.

4 INDUSTRIAL FISHERY PRODUCTS

Fish meal and it accompanying oil are used in poultry feeds. The residues in meal contribute to the pesticide burden imposed on poultry and contribute to the load in foods derived from the animals.

Residue levels in menhaden (Brevoortia tyrannus and B. patronus) meal and oil are shown in Table IV. These data were obtained in 1969. As we found with FPC, the meal contains less than one-tenth as much DDT as does the oil. Because meal contains up to about 15% oil, processes that reduce the oil content would also reduce the pesticide content. The Food and Drug Administration tolerance of 5 ppm for DDT residues in the edible portion of fish flesh does not apply to fish meal or oil.

Table IV. Range of pesticide residue levels in menhaden oil and meal in 1969

States	No. of samples	Total DDT (ppm) Oil	Meal
Middle Atlantic	6	4.4-9.4	0.29-0.61
Chesapeake	2	2.8-4.0	0.21-0.25
South Atlantic	12	2.1-7.2	0.10-0.32
Gulf of Mexico	2	2.2-2.3	0.10-0.11

The concentration of pesticides in oil suggests that removal of DDT from fish oil may become necessary. Deodorization of oil in the refining process of edible vegetable oil (Saha, 1970; Smith, 1968) removes almost all of the chlorinated hydrocarbon pesticides originally present. Unfortunately deodorization is too expensive for animal feed products so we tried to find simple methods to reduce the residue content of fish oil. Washing with cold or hot water, steam distillation at atmospheric pressure, or partitioning with 55 and 90% (V/v) aqueous isopropyl alcohol, however, effected no change in the residue level.

5 PESTICIDES IN EDIBLE OILS

Most of the work on the elimination of chlorinated pesticides has been done with vegetable oils. Fish oils, which are of primary interest in this paper, differ markedly in chemical makeup from vegetable oils, so the problems encountered with fish oil are different. Accordingly, in this section, we first consider the pertinent work that has been done with vegetable oils and then the work we have done with fish oils.

5.1 Elimination from vegetable oils

Recent studies by Smith et al. (1968) on soybean and cottonseed oils and by Saha, Nielsen, and Sumner (1970) on rapeseed oil have demonstrated that vacuum steam deodorization at 6 mm Hg pressure and at temperatures in the range of 230-260° C will remove as much as 95% of the chlorinated pesticide content. Other commercial processing techniques such as solvent extraction, steam refining, alkali refining, bleaching, and hydrogenation were reported to have little effect in eliminating chlorinated pesticides in these vegetable oils. The mechanism of the removal of chlorinated pesticides was indicated to be a forced volatilization of the pesticides in the presence of steam at the high temperatures used in the deodorization process.

5.1.1 High temperature requirements

The minimum temperature (i.e., 230° C) deemed essential for the removal of chlorinated pesticides from vegetable oils by vacuum steam deodorization coincides with the maximum temperature used in the United States for deodorization of fish oils. In the United States, fish oils are deodorized at 190-230° C, whereas in Europe they are deodorized at 170-190° C (Chang, 1967). Fish oil is not presently used in food products in the United States, but it is used in margarine and other edible fat products in Europe. The lower deodorization temperature limits in Europe are probably much more desirable for critical applications, as in foods. Owing to the more unsaturated nature of fish oils in comparison with vegetable oils, high temperature processing of the former leads to a greater degree of thermal degradation and polymerization.

5.1.2 Steam distillation

Vacuum steam distillation is used in the processing of fats and oils for two basically different purposes. One purpose is to remove off flavors and odors after an oil has been hydrogenated. The second--and less common purpose--is to remove free fatty acids. In this application, it is called "steam refining" and is used on degummed oils. In the present work, we have used both applications in our attempts to gain insight into the best ways of removing chlorinated pesticide residues from fish oils.

5.2 Removal from fish oils

5.2.1 Vacuum steam refining

Table V presents data on the effectiveness of vacuum steam refining of two crude bleached Pacific herring oils containing closely similar concentrations of chlorinated pesticides. The two oils were subjected to the same conditions during the steam refining except for the temperature of distillation. From the data, the effectiveness of the removal of the chlorinated pesticides does not appear to depend on the temperature in the range studied. In both cases, as shown in Table V, p,p'-DDT was eliminated to below the limit of detection.

Although the rate of removal of pesticide residues is more rapid at the higher temperature, the quality of the fish oil is undoubtedly better retained at the lower temperature.

5.2.2 Hydrogenation and deodorization

In a preliminary experiment, to obtain artificially high levels of both p,p'-DDT and p,p'-DDE, we added these pesticides to a bleached menhaden oil. By hydrogenation, we then eliminated 90% of the p,p'-DDT present. The p,p'-DDE was not found to decrease to such a degree. The mechanism of this decrease in the concentration of p,p'-DDT during hydrogenation was not investigated by us. The phenomenon had previously been observed by Gooding (1966).

Table V. Partial removal of pesticide residues from two bleached[a] Pacific herring oils by steam refining

Distillation temperature[b]	Pesticide	Pesticides (ppm) Original oil	Refined oil	Condensate fraction
190 to 200° C	p,p'-DDE	0.73	0.47	40.3
	p,p'-TDE	0.59	0.53	21.3
	p,p'-DDT	0.37	BDL[c]	BDL
	Total	1.69	1.00	61.6
230 to 240° C	p,p'-DDE	0.82	0.49	34.1
	p,p'-TDE	0.70	0.62	24.5
	p,p'-DDT	0.44	BDL	BDL
	Total	1.96	1.11	58.6

[a] Bleached with activated clay (4% weight of oil) for 30 min at 8 mm Hg and 80° C.
[b] Deodorized for 3 hr at 8 mm Hg with 9 wt % steam based on the wt of the oil.
[c] BDL: below detectable limit.

The pesticide p,p'-DDE, a product of the natural breakdown of p,p'-DDT, is often the chlorinated pesticide found in highest concentrations in marine animals. Table VI presents data on the effectiveness of removal of p,p'-DDE from menhaden oil containing added amounts of this substance. The table shows that the combination of hydrogenation and deodorization is effective in reducing the level of this pesticide. The deodorization process, did not, under the conditions used, reduce the pesticide level in menhaden oil at as high a rate nor to as great a degree as Smith et al. (1968) and Saha, Nielsen, and Sumner (1970) found with vegetable oils.

Table VI. Partial removal of artificially high residues of p,p'-DDE from bleached menhaden oils by hydrogenation and vacuum steam deodorization

Sample	Residues of p,p'-DDE (ppm) Trial 1	Trial 2	Trial 3
Original	63.1	23.4	12.0
Hydrogenated oil[a]	51.8	21.9	10.5
Deodorized oil[b]	28.7	16.8	8.2

[a] Melting point: 42° C.
[b] Deodorized for 3 hours at 12 mm Hg and 190-200° C with 10 wt % steam based on the wt of the oil.

The major difference in the application of deodorization to the removal of pesticides from fish oils, compared with work of these authors on vegetable oils, is the lower temperature that is nearly mandatory in work on fish oils to prevent thermal degradation. Because of this requirement for a lower temperature, the duration of the deodorization must be extended if a substantial reduction in the pesticide level is to be accomplished.

6 SUMMARY

The residue levels of DDT and its metabolites were examined in fish from waters off the Pacific Coast of the United States. The effects of simple alterations in the pre-

canning process for jack mackerel were studied. The fate of pesticides during production of fish protein concentrate was followed. Pesticide levels in menhaden meal and oil from both Atlantic and Gulf of Mexico fisheries were studied. Methods for lowering the DDT content of fish oil were examined.

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THE BIOLOGICAL VALUE OF OILS AND FATS

IV. THE RATE OF INTESTINAL ABSORPTION

H. J. THOMASSEN
Unilever Research Laboratory, Zwijndrecht, The Netherlands

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INTRODUCTION

Earlier experiments established that the increase in weight of young rats is dependent on the type of the dietary fat (Thomassen, '55a). These investigations further revealed that the different growth action of the fats and oils investigated is actually due to a different food intake.

It is conceivable that food intake and growth are correlated with the rate at which the fat in question is absorbed. The literature in this field is very restricted, however. Steenbock et al. ('36) established that halibut- and cod-liver oil are absorbed significantly more rapidly than lard and maize oil. In addition, a number of fats and oils could be arranged in the order of decreasing rates of absorption as follows: linseed oil, olive oil, whale oil, soya-bean oil, groundnut oil, rancid lard, cottonseed oil, coconut fat, and palm oil. Deuel et al. ('40) observed no differences in the rates of absorption of cottonseed oil, butterfat, and coconut fat; they found, however, that rapeseed oil was absorbed at a much lower rate. In later investigations carried out in Deuel's laboratory it could be shown that maize oil (Bavetta and Deuel, '42) and lard (Crockett and Deuel, '47) possessed the same rate of absorption. In addition, Bhalerao et al. ('47) found that sesame oil, coconut fat, and butterfat are absorbed at the same rate, and safflower, groundnut and cottonseed oil slightly, although not significantly, slower.

In the present investigation the rate of absorption of a series of 18 different natural oils and fats, of vegetable and animal origin, has been studied.

EXPERIMENTAL PART

For the determination of the absorption rate a modification of Deuel's method (Deuel et al., '39, '40) was used. Male Wistar rats weighing 150 to 250 gm and fed a Sherman diet (⅔ wheat, ⅓ milk powder and salts), were fasted for 48 hours (drinking water was available during this period) the fat to be investigated then being administered by stomach-tube. After a certain test period the fat remaining in the gastro-intestinal tract was determined. Since, according to Deuel et al. ('40, '41) more constant results are achieved when the amount of absorbed fat is expressed per unit of body surface, a dosage of 400 mg of fat per 100 cm² surface was administered in all cases. The body surface was calculated from Lee's formula (Lee, '29). For the determination of the fat content in the gastrointestinal tract the animal was decapitated, the abdomen opened, and the contents of stomach, small and large intestine separated by means of ligatures. Subsequently, each of these three parts was flushed twice with 20 ml of diethyl ether, using a syringe with blunt needle, care being taken to ensure that all solid material in the tract was removed. The three extracts obtained in this way were dried overnight with Na_2SO_4, and filtered. The residue was washed with 100 to 125 ml of ether, the ether evaporated, and the residue weighed. Blank determinations carried out with fasted animals to which no fat had been administered, showed that an average correction of 5, 8 and 17 mg of fat must be applied to the values found for stomach, small and large intestine respectively.

As a criterion for comparing the fats and oils investigated the *A*(bsorption) *T*(ime)$_{50}$ was employed, i.e. the time after which 50% of the administered fat has disappeared from the entire gastrointestinal tract. This value was determined as follows: in two groups, each of 6 animals, the contents of the gastrointestinal tract were analyzed three and 6 hours respectively after administering the fat. From these values it was calculated at what time 50% of the dosage would have disappeared from the gastrointestinal tract. The value so obtained (it varied from 5 to 11 hours) was applied to a third series of 6 animals.

When calculating AT_{50} it was supposed that, at any rate within the scope of the relevant observations, a linear relationship exists between time and the percentage of fat absorbed. AT_{50} was calculated as follows: $AT_{50} = \bar{x} + \frac{50 - \bar{y}}{b}$,

in which

x = absorption time = time interval between administering the fat and the analysis (in mins.);

y = percentage of fat recovered in the gastrointestinal tract after absorption time x;

$\bar{x}$ = the mean of all x values;

$\bar{y}$ = the mean of all y values;

$$b = \text{regression-coefficient of } y \text{ upon } x = \frac{\Sigma xy - \frac{\Sigma x \cdot \Sigma y}{n}}{\Sigma x^2 - \frac{(\Sigma x)^2}{n}},$$

in which n = number of observations.

The 95% confidence interval of the AT_{50} value is:

$$\bar{x} + c \cdot \frac{50 - \bar{y}}{b} \pm t_{(n-2)} \cdot s_{AT_{50}},$$

in which

$s_{AT_{50}}$ = standard deviation of the AT_{50}-value;

$t_{(n-2)}$ = critical value from Student-distribution for $P_s = 0.05$;

c = correction factor for skewness = $b^2 / (b^2 - t^2_{(n-2)} \cdot s_b^2)$, in which s_b = standard deviation of the regression coefficient.

In the calculation of AT_{50} the values for the entire gastrointestinal tract have been employed. Actually, however, separate data for each of the three parts — stomach, small and large intestine — are available.

RESULTS AND DISCUSSION

The amounts of fat recovered in the entire gastrointestinal tract after administration of 400 mg per 100 cm² of body surface are shown in table 1 as percentages of the dosage. These

figures are for absorption times of three and 6 hours, as well as the time in which (as estimated from the absorption values after three and 6 hours), 50% of the fat would have been absorbed (d hours). Following the latter percentages the corresponding (d hours)-value is shown in parentheses. Each percentage represents a mean of 6 determinations, with the exception of the values for rapeseed oil after 8 and 9 hours, which are based on 17 and 18 determinations respectively.

TABLE 1

Percentages of the fats recovered in the gastrointestinal tract, 3, 6 and d hours after administering 4½ mg 10 cm³, their AT_{50}-values and their relative order of growth-promoting ability[1]

CLASS	TYPE OF FAT	PERCENTAGE OF FAT RECOVERED AFTER			AT_{50}-VALUES (MINS)		ORDER OF GROWTH-PROMOTING ABILITY
		3 h	6 h	d h	mean	95% limits	
I	Butterfat	63	41	55 (5)	355	272–349	2
II	Maize oil	81	47	49 (6)	350	329–378	7
	Cottonseed oil	75	49	49 (6)	352	328–385	4
	Beef tallow	78	47	56 (6)	354	327–394	5
	Coconut fat	73	48	59 (6)	354	310–436	13
	Soyabean oil	77	47	53 (6)	359	321–429	8
	Sunflower oil	83	48	69 (6)	362	333–409	11
	Groundnut oil	69	48	50 (6)	365	318–487	9
	Olive oil	82	48	61 (6)	369	325–418	3
III	Sesame oil	79	60	54 (6)	392	349–486	14
	Lard	74	50	58 (6)	396	343–532	1
	Palm fat	79	62	56 (7) 31 (8½)	416	392–445	10
	Whale oil	79	59	47 (7½)	426	373–525	15
IV	Shea butter	76	64	40 (9½)	477	427–548	6
	Herring oil	79	60	52 (8)	487	423–619	16
V	Rapeseed oil	86	63	63 (8) 49 (9)	558	510–608	17
	Poppyseed oil	83	70	41 (11)	560	500–646	12
	Kupokseed oil	82	69	46 (10½)	588	523–690	18

[1] AT_{50} is the number of minutes after which 50% of the fat administered had disappeared from the gastrointestinal tract; d hours is the time at which, according to an estimation based upon the absorption values after three and 6 hours, 50% of the fat would have been absorbed.

In table 1 the AT_{50} values (= number of minutes after which 50% of the fat administered disappears from the gastrointestinal tract) calculated from these percentages, are also recorded. In addition, values are given for the 95%-interval representing the limits between which lie 95% of the AT_{50} values of the respective fats. From these statistical data the fats under investigation can be arranged in 5 classes of decreasing rates of absorption, although it is not certain whether sesame oil and lard belong to group II or to group III or form a separate group. The influence of these oils and fats on the increase in weight of new-born male rats is known from previous investigations (Thomasson, '55a). On the basis of these findings the fats have been arranged according to decreasing growth-action; these rank numbers are given in the last column of table 1. It appears that a correlation exists between the rate of absorption (AT_{50} values) and the growth-action ($\varepsilon = 0.62$ with $P < 0.01$).[1]

Such a correlation suggests that the divergent growth-action of various fats and oils [which, as previously shown (Thomasson, '55), is in turn determined by the food intake], might be due to the rate at which these fats are absorbed. Some doubt regarding this supposition is justified, however, as certain fats do not conform to it. The growth-action of lard, shea butter, olive oil and poppyseed oil for example, is too favorable in respect of their relative slow rate of absorption, whereas coconut fat and maize oil have a poorer growth-action than would be expected from the favorable rates of absorption of these fats. However, rapid growth and a correspondingly rapid absorption need not necessarily be considered as favorable. It has been shown (Thomasson, '55b) that longevity on butterfat-containing diets is less than when this fat is replaced by rapeseed oil, in spite of the fact that with the former fat the growth rate and the rate of absorption

[1] This correlation was tested by numbering the fats investigated according to decreasing growth-promoting ability and according to decreasing rate of absorption, and applying the method of rank correlation to these two series of values (Dixon and Massey, '51).

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are considerably greater. The reason why fats have different rates of absorption is unknown. It is plausible to suppose the rate of absorption is determined by the rate at which fats are hydrolyzed by lipase in the intestinal lumen. An investigation into the rate of hydrolysis of various types of fat by pancreas lipase is at present being carried out at this laboratory.

The mean amounts of fat recovered in the gastrointestinal tract after three hours varied from 63 to 86% of the dose administered (table 1). After 6 hours these values were from 41 to 70%, and after an absorption time of 8½–11 hours (= d hours) the percentage was between 31 and 63.

The distribution of these widely varying amounts of fat over stomach, small and large intestine is shown in table 2. In this table the amount recovered in the entire gastrointestinal tract is taken as 100% (this value actually varies from 31 to 86% of the dosage administered), and the amounts present in stomach, small and large intestine are shown as percentages of the amount recovered.

At the foot of table 2 the mean values found after three, 6 and d hours are given. Although these values show a certain constancy, they suggest that the percentage of fat in the stomach decreases on extending the absorption time (after three, 6 and d hours, averaging 74.9, 73.1 and 70.0% respectively) whereas the percentage of fat in the large intestine shows the opposite tendency (after three, 6 and d hours, averaging 2.2, 5.1 and 7.3% respectively). Application of the sign test (Dixon and Massey, '51) reveals that the differences in fat contents of the large intestine are, in fact, statistically significant after three and 6 hours, and after three and d hours. This is not the case, however, for the values found for the stomach, so that there is no conclusive evidence that the percentage of fat in the stomach decreases with an increase in the time of absorption.

Contrary to the differences observed in the percentage of fat present in the stomach and the large intestine, that of the small intestine is notably constant throughout the test period.

The mean values after three, 6 and d hours are 23.0, 21.9 and 22.7% respectively. This homogeneity prompted the calculation of the mean percentage from all the 56 observations: 22.5% with a standard error of 0.945%. Thus, this value

TABLE 2

Percentage distribution, over stomach, small and large intestine, of the recovered fat

TYPE OF FAT	DURATION OF ABSORPTION: 3 HOURS			DURATION OF ABSORPTION: 6 HOURS			DURATION OF ABSORPTION: D HOURS[1]			d
	stomach	intestine small	intestine large	stomach	intestine small	intestine large	stomach	intestine small	intestine large	
Butterfat	74	25	2	77	21	2	58	35	7	5
Maize oil	78	20	2	65	27	8	66	32	2	6
Cottonseed oil	84	16	0	74	24	2	68	28	4	6
Beef tallow	85	14	1	85	15	0	87	10	3	6
Coconut fat	74	22	4	75	19	6	63	27	10	6
Soya-bean oil	62	33	5	58	38	4	66	28	6	6
Sunflower oil	81	17	2	77	19	4	74	23	3	6
Groundnut oil	69	31	0	78	20	2	64	29	7	6
Olive oil	67	31	2	72	23	5	79	16	5	6
Sesame oil	70	29	1	67	29	4	77	20	3	6
Lard	78	22	0	73	21	6	82	14	4	6
Palm fat	87	10	3	77	19	4	58 71	32 19	10 10	8½ 7
Whale oil	72	25	3	76	21	3	72	21	7	7½
Shea butter	60	20	5	60	22	18	50	35	15	9½
Herring oil	71	29	0	60	34	6	71	25	4	8
Rapeseed oil	70	23	7	75	18	7	73 58	15 19	12 23	8 9
Poppyseed oil	76	22	2	80	14	6	85	11	4	11
Kapokseed oil	84	16	0	86	10	4	78	15	7	10½
Mean	74.9	23.0	2.2	73.1	21.9	5.1	70.0	22.7	7.3	

[1] Time at which, according to an estimation based upon the absorption values after three and 6 hours, 50% of the fat would have been absorbed.

seems to be independent of the time of absorption and, independent, therefore of the total amount of fat present in the gastrointestinal tract. Probably the body has a mechanism at its disposal which attempts to maintain the percentage of fat in the small intestine at a constant level, namely 22.5% of

the total amount of fat in the gastrointestinal tract. Similarly, the fat contents of the stomach and the large intestine, although involving greater variations, also show a certain constancy, about 73 and 5%, respectively. Finally it is noteworthy that the distribution of the fat over stomach, small and large intestine seems to be independent of the nature of the fat administered.

Some practical objections can be raised to the dosage of 400 mg/100 cm² as applied in these experiments. In the case of fats which are absorbed at a slow rate, the AT_{50} value is especially unfavorable from a point of view of the normal

TABLE 3

AT_{50} values of butterfat and rapeseed oil related to various dosages

DOSAGE mg/100 cm²	AT_{50} VALUES (MINS.)	
	Butterfat	Rapeseed oil
100	194	258
200	294	336
300	370	442
400	395	558

[1] AT_{50} is the number of minutes after which 50% of the fat administered had disappeared from the gastrointestinal tract.

working hours in the laboratory. Consequently, a lower dosage is to be preferred. Table 3 records the AT_{50} values at various dosages of two fats: butterfat, which is rapidly absorbed and rapeseed oil as an example of an oil having a very low absorption rate. The table shows that on administering 200 mg/100 cm² of the slowly absorbed rapeseed oil the AT_{50} value is approximately 6 hours. This period is undoubtedly more convenient and for further investigations this dosage is therefore recommended.

SUMMARY

The rates of absorption of 18 natural oils and fats of vegetable and animal origin have been studied. It appeared that

these oils and fats could be divided into 5 groups according to a decreasing rate of absorption:

1. Butterfat.
2. Maize oil, cottonseed oil, beef tallow, coconut fat, soyabean oil, sunflower oil, groundnut oil and olive oil.
3. Sesame oil, lard, palm fat and whale oil.
4. Shea butter and herring oil.
5. Rapeseed oil, poppyseed oil and kapokseed oil.

A significant correlation appeared to exist between the rate of absorption and the growth-action of the oils and fats.

The fat recovered three to 11 hours after the administration of 400 mg per 100 cm² of body surface appeared to be distributed over stomach, small and large intestine with a certain constancy, averaging 73, 22.5 and 5% respectively.

The percentage of fat in the small intestine, seems to be independent of the absorption time and consequently of the amount of fat in the tract; that in the stomach showed a tendency to decrease while that in the large intestine increased when absorption time was extended.

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PROCEEDINGS
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Human Nutrition

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Nutritional evaluation of hydrogenated fats

H. J. Thomassen, J. J. Gottenbos, J. Kloser and R. O. Vles, *Unilever Research Laboratory, Vlaardingen, Holland*

In continuation of previous work, oils hardened in different ways were investigated by means of a biological method analogous to that applied to the evaluation of the safety of food additives and defined as a 'sub-acute oral toxicity study' (Thomassen & Gottenbos, 1957; Gottenbos & Thomassen, 1965; de Jongh & Beerthuis, 1965).

Experimental details

Groups of twenty-four male and twenty-four female rats consumed during 12 weeks adequate diets in which 54% of the calories came from experimental fat. Moreover, 6% of the calories derived from sunflower-seed oil in order to cover the requirement for essential fatty acids.

The fatty acid compositions of the dietary fats including the sunflower-seed oil are shown in Table 1.

As control, unhardened soya-bean oil was used, the effect of which was compared with that of five industrially hardened fats and with that of one naturally hardened fat, butterfat. Soya-bean oil is rich in linoleic acid (55%). The three hardened

Table 1. *Percentage fatty acid composition of dietary fats*

Type of experimental fat	Fatty acids: Saturated	Monoene: *cis*	Monoene: *trans*	Diene: *cis-cis*	Diene: *trans*
Soya-bean oil (control)	15	21	0	55	0
Hydrogenated soya-bean oil (1·5)	21	12	51	6	1
Hydrogenated soya-bean oil (1·15)	16	27	1	19	19
Hydrogenated soya-bean oil (1·10)	15	22	14	41	7
Hydrogenated linseed oil	16	29	49	6	16
Hydrogenated olive oil	21	39	29	6	1
Butterfat	53	34	8	7	3

soya-bean oils were prepared by hydrogenation in different ways, which is reflected in the differences in their fatty acid compositions. Hardened linseed oil and hardened olive oil were used as representatives of oils originally rich in linolenic acid and oleic acid respectively.

Food intake

The mean food intake per animal during the 12 weeks was 910 g for the males and 745 g for the females. This represented 305 and 245 g of dietary fat, respectively. There appeared to be hardly any differences between the experimental groups.

The *trans*-acid intake, on the other hand, varied considerably for the different groups. Apart from the group given unhardened soya-bean oil containing no *trans*-acids, it was lowest in the groups that received butterfat, hardened soya-bean oil 1·15 or 1·10 (25–60 g/12 weeks), moderate in the group given hardened olive oil (70–95 g) and highest in the group that received hardened linseed oil and hardened soya-bean oil 1·5 (130–175 g/12 weeks).

Body-weight

The mean gain in weight in 12 weeks was 258 g for the males and 137 g for the females; no significant differences were found between the experimental groups.

Faecal fat

In all groups the amount of fat excreted in the faeces was less than 5% of that eaten, being within the limits of normal excretion. Some differences seemed to exist between the groups, and there was a positive correlation between the consumption of *trans*-acids and the percentage of fat excreted in the faeces.

Water consumption

The mean water consumption (mg/day per cm² body surface) of males and females was 6·6 and 5·8 mg respectively. No significant differences between the groups appeared to exist, except that the females given hardened linseed oil drank less water. However, it is doubtful whether this finding could be reproduced.

Rectal temperature

The means were 38·8° for the males and 39·0° for the females. There were no significant differences between the experimental groups.

Kidney function

18 h concentration test. The specific gravity of the urine after the animals had been deprived of water for 18 h was for males and females 1·067 and 1·068 respectively. No disturbances of the concentration capacity of the kidneys occurred in any of the experimental groups.

Urine aspartate transaminase. The values varied between 11 and 23 Wróblewski units/ml (La Due, Wróblewski & Karmen, 1954), being higher for males than for females. Under pathological conditions the level is increased. However, the values found for the groups given hardened oils were generally lower than those of the controls.

Liver function

Serum alanine transaminase. The serums appeared to contain an average of 20 Wróblewski units/ml (Wróblewski & La Due, 1956) and there were no indications that one of the groups differed from the other.

Alkaline phosphatase. The values were between 2 and 3·5 m-mol/l. and no differences occurred between the experimental groups. Males showed higher levels than females.

Blood

Haemoglobin. The mean content was about 16 mg/100 ml. There were no differences between the groups.

Haematocrit. The values were constant with means per group of 50 or 51 %.

Non-electrolyte haemolysis. The time in which 75% haemolysis occurred in a saline medium varied between 39 and 49 sec. The values for males were higher than those for females. Although no significant differences between the groups were found, the values for the groups given industrially hardened fats were in general somewhat lower than those for the controls. The biological significance of this observation may become clear in future research.

White blood cells. Neither total nor differential counts indicated pathological changes, except that values for the total white cell counts for the group given hardened soya-bean oil I.50 and for the butterfat group were slightly higher than for the controls. The number of white cells was distinctly higher in males than in females. The percentage of lymphocytes was high and between narrow limits, *89–93%*.

Coagulation. This was determined by means of a thromboelastograph and exclusively in males. The *r* values (time after which the coagulation begins) seemed to be longer in animals fed on hardened oils than in controls, although the differences were not significant.

Platelet adhesiveness. The method of Hellem, Ødegaard & Stålberg (1963) (without addition of ADP) was used. Some indication was found that the platelets of the females fed on hardened soya-bean oils were more adhesive than those of the controls. However, the number of animals tested was limited (three to four per group) and the difference was not significant and not found in the males.

Post-mortem examination

Autopsies were carried out on all rats. The weights of heart, liver, kidneys, adrenals, spleen and testes were determined. Moreover, a histological investigation was carried out. None of these studies provided evidence of any unfavourable effect of the fats used.

The composition of body fat at the end of the experiment was also determined. Highly significant correlations appeared to exist between the saturated fatty acids, monoenoic, polyenoic acids and *trans*-acids, respectively, of the dietary fats and the body fats.

Summary

On applying several biological criteria, including food intake, body-weight, fat content of faeces, serum cholesterol, rectal temperature, kidney function

(concentration test, aspartate transaminase content), liver function (alanine transaminase content, alkaline phosphatase content), blood (Hb, haematocrit, haemolysis, white blood cells, coagulation, platelet adhesiveness), post-mortem examinations and body fat composition, no indications were obtained that a 12-week feeding period with high amounts of hydrogenated fats of extremely divergent composition, induces abnormal changes in rats.

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Nutritional aspects of processing and chemical additives on fats and oils

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J. P. K. VAN DER STEUR
Unilever N. V., Rotterdam, The Netherlands

Oils and fats are important human foods. They not only provide a concentrated form of the energy that people need to do their daily job, but they also include certain factors which are essential to life and which the human body does not synthesise. We have to ensure, therefore, that man's diet includes adequate amounts of fats, containing enough of the essential constituents.

Some oils and fats can be consumed in the form in which they are recovered from their natural sources. These include olive oil which is obtained by crushing ripe olives at the place of cultivation, and milk fat which is separated from fresh milk by centrifuging and is converted into its ultimate form as butter or ghee by churning and kneading and gentle heating. These products can be made in an appetising form, however, only if they are obtained fresh from the raw materials. The are available only in limited quantities. The same applies to beef tallow and lard which if freshly rendered, can be used without refining. But only a limited proportion of the two latter fats can be obtained in this way.

In most cases the raw materials for edible oils and fats are obtained in countries far away and the oils and fats cannot always be produced locally from the seeds or fruits owing to economic conditions. In cases where this is done—for instance with plantation palm oil—the taste of the fat prevents it from being used for direct consumption by the pampered Westerner. Moreover the quality of the product would deteriorate during transport over long distances. Coconut fat that could be obtained from fresh nuts in tropical countries has an attractive flavour but is not to everyone's taste.

In the vast majority of cases the oils and fats obtained from oil seeds and fruits contain substances that make them unsuitable for human consumption in that form, mainly because of their flavour or odour or appearance. Before becoming commercial products for human consumption, these oils and fats first have to be purified in order to remove the off-flavours, together with the colouring matter and free fatty acids.

On the other hand many of them have also to be processed to impart the required consistency to them. In some countries oil is used in the kitchen for the preparation of food. Other countries are more accustomed to using fats with a solid consistency, such as butter or margarine, or hard fats such as lard and beef tallow. Endeavours must therefore be made to process the raw materials so that they also will comply with the consumers' requirements regarding consistency.

It is the task of the oils and fats industry to process the raw materials made available to it by the world economy so as to make edible products which will guarantee a good diet and will suit living habits in different countries.

The main constituents of oils and fats are the triglycerides, esters of glycerol with a great variety of fatty acids. Many fatty acids can be synthesised by the human body; some, however, such as the essential fatty acids (cis-cis linoleic acid, arachidonic acid, etc.) cannot. Oils and fats also contain much smaller quantities of other constituents which are saponifiable and contain fatty acids as part of their molecules, such as phosphatides and waxes. In the natural oils and fats there are, moreover, unsaponifiable constituents such as hydrocarbons, e.g. squalene, sterols, e.g. phytosterols and cholesterol, and—in some of them fat-soluble vitamins. Many oils and fats, especially the vegetable ones, contain vitamin E (tocopherol) which is practically all conserved during processing. Some contain vitamin A or carotene.

RAW MATERIALS

On the whole, oils and fats are obtained from animal fat tissue, from the flesh of oil fruits or from seeds. These cannot always be processed while fresh, and storage may lead to deterioration. This may be caused by bacteria and insects or by the effects of moisture, high temperature and atmospheric oxygen. These factors may in turn accelerate the effect upon the fat in fatty tissues of the fat-splitting and oxidising enzymes, the lipases and lipoxydases, which such tissue always contains. The result is that free fatty acids are formed on the one hand and mono- and diglycerides on the other, and further on some oxidation may take place. The better the raw materials are handled, the better the

quality of the crude oil will be and consequently of the refined oil.

Let us now discuss the main operations applied by the oils and fats industry one by one and see how far they influence the nutritive values of oils and fats.

PROCESSING

In order to separate the fat and to convert it into an edible form, we find that some of the following chemical and physical operations are necessary: *a)* separation from tissues, fruit or seed; *b)* degumming; *c)* neutralisation; *d)* bleaching; *e)* deodorising. Besides this the oils and fats industry applies other operations to make the fats more suitable for the purposes for which they are used. These are: *f)* hardening; *g)* esterification; *h)* mixing, cooling, kneading and—for special products such as margarine—emulsifying.

Separation of oils and fats. Before oils and fats are separated the tissue and the seeds must be finely divided in order that the fat released from the cells can run off properly. This is followed by heating which, in the case of animal tissue, already releases a substantial part of the fat. This is the process applied, for instance, in melting out beef tallow, lard and whale oil. Some fat remains which is absorbed in the cell walls and can later be separated by pressing or centrifuging. The same applies to the flesh of fruit, for instance palm fruit.

In the case of seeds, the fat is often more difficult to remove. After crushing, the material is heated and most of the fat is removed by pressing at high pressure and temperatures of about 100°C.

Besides this process based on heating and pressing, extraction is often used to obtain a higher yield of oil. The extractant used nowadays is almost always a low-boiling petroleum fraction: usually technically pure hexane with a boiling point of about 65°C. Solvents used in the past, such as trichlorethylene, which has the advantage of being noninflammable, have been completely abandoned. The reason for this is that it brings into solution many more substances (nonfats) from the seeds which cause trouble during refining. Moreover trichlorethylene when brought into contact with water very easily forms hydrochloric acid, which may corrode the steel of the apparatus.

For economic reasons a combination of expelling and extraction is often applied. A fairly high oil content remains in the pressed seed which is then crushed again and solvent-extracted. After extraction a meal is left with a low oil content (e.g. 1.0%) and a solution of oil in solvent, the miscella. From this miscella the solvent is distilled off and finally volatilised by blowing through steam.

It has sometimes been thought that the solvent might contain higher aromates having a toxic effect. Extraction solvent is so well fractionated, however, that there will certainly be no higher fractions in it. After refining the edible oil is completely free from solvent. This has been proved, inter alia, by using extracting agents containing radioactive tracers. After the solvent had been distilled off and after neutralisation, washing and drying under vacuum at 90°C, all the radioactivity had vanished.

Degumming. When the oils and fats are removed from their natural sources (tissues, seeds) some constituents other than triglycerides are also partly dissolved in the oil. In many cases it is useful to remove such substances, e.g. phospholipids, before further refining is commenced. This applies particularly to soybean oil, for instance, in which quantities of phospholipids amounting to several per cent are often found after extraction from the beans. These can easily be removed by swelling the phospholipids with small quantities of water, after which they can be separated by centrifuging. This product is the well-known soya-lecithin. For degumming, chemical agents are also often used, for instance sodium chloride solutions, phosphoric acid, citric acid, acetic acid, acetic acid anhydride, hydrochloric acid, etc. It has been found that many of these substances are separated more easily under the influence of these agents. This degumming usually takes place at temperatures below 100°C. The agents do not appear in the degummed oil and the nutritional value is not affected by this operation.

The phosphatides that are removed may be important food constituents. Their presence makes an adequate refining into tasteless fats impossible, but the lecithin may be purified separately and subsequently resadded to margarine to improve the frying properties. It is also used to improve the viscosity of chocolate in manufacture.

Neutralisation. As I have already mentioned, some splitting has occurred in the oils and fats through storing the seed or the fatty tissue under the influence of moisture and lipases, as a result of which free fatty acids and mono- and diglycerides have been formed from the triglycerides originally present. Although in many cases i.e. with long-chain acids (C_{16} and higher) these free acids do not adversely affect the taste of the oil, it is advisable to remove them, as they affect refining of the oil and the use of the final product (low smoke point).

The fatty acids combined with caustic soda in solution to form soaps are removed as an aqueous phase that settles at the bottom of the neutraliser vessel and can be drawn off or removed by centrifuging. The alkaline aqueous phase also contains part of the colouring matter and other unwanted substances. This is an additional advantage of the neutralisation process as the latter might adversely influence the taste of the oils and fats in further processing. Remains of the soap phase are removed as well as possible by washing with water. This entire operation which is carried out at temperatures below 100°C definitely does not remove any integral constituent of the diet.

Bleaching. After the fatty acids have been neutralised we have to remove as much as possible of the colouring material that is still present. Strongly coloured oils are difficult to make tasteless. At the same time the bleaching

process eliminates the soap residues left in the oil after neutralisation and washing with water.

This bleaching is done by treating the oils and fats under vacuum with bleaching earth at a temperature of 90°-100°C. Some fats, such as coconut- and palm-kernel oil, are treated with small quantities of activated carbon in addition to bleaching earth. The decolouring is for the greater part a purely adsorptive action at the surface of bleaching earth and activated carbon.

In addition, however, slight changes may occur in unsaturated oils and fats under the influence of the bleaching earth. The extent to which this takes place depends partly upon the past history of the crude materials.

Mitchell and Kraybill (8) first observed that under the influence of bleaching earth characteristic changes occur in the ultraviolet spectra of edible oils. Absorption bands develop at 268 mμ when the oil contains linoleic acid and 268, 300 and 316 mμ when linolenic acid is also present. These absorption bands indicate the presence of conjugated trienes or tetraenes, which are formed only when the oil has been previously oxidised. The trienes are formed from primary hydroxy fatty acids or hydroperoxides by the splitting off of water under the influence of bleaching earth. The absolute concentration of these trienes and tetraenes is however very small, viz. about 0.1%.

Polymerisation may also occur under the influence of bleaching earth, especially in oils with a high unsaturated fatty acid content. The normal test on polymers in oil (Hügel test) is negative, but more sensitive methods for detecting dimers have taught us that while the content in crude oil is 0–0.01% it goes up to 0.01–0.08% in neutralised and bleached soybean oil, to 0.02% in sunflowerseed oil, to 0.03% in cottonseed oil and to 0.02% in rapeseed oil.

Nevertheless these reactions occur to such a small extent and the compounds are of such a kind that no influence on the food value is to be expected from them.

Deodorising. After gums, fatty acids and colouring material have been removed, it is necessary to eliminate the off-odours and flavours from the crude oils and fats. The usual method that has been applied for many years is steaming under vacuum. The oil is pumped into vacuum containers, heated to 160°-230°C (normally 180°C) under a constant stream of superheated steam which entrains the odoriferous substances. This method can be effected batchwise, semicontinuously or continuously and its big advantage is that treatment under high vacuum prevents oxidation, which might lead again again to deterioration of the refined oil. The steaming time and the quantity of steam passed through are determined by the taste, the operation being continued until the fat is tasteless.

During the deodorisation process some chemical reactions can take place under the influence of steam and high temperature. Some saponification will occur through which free fatty acids will be formed that are distilled off. In addition to this, heating may also cause polymerisation. The same sensitive methods as mentioned before have shown that steaming has only very little effect on the formation of polymers.

Deodorisation of sunflowerseed oil at 240°C, a temperature which is not applied normally (180°C) increased the dimer content from 0.02% to 0.07% and 0.08%. With soybean oil the dimer content when deodorised at 170°C increased from 0.03% to 0.08% and at 235°C from 0.05% to 0.10%. In groundnut oil and hardened groundnut oil the dimer content was increased by bleaching and deodorisation from 0.01% to 0.06%.

Experiments so far have not indicated even the slightest effect upon the growth of rats attributable to these extremely small quantities of dimers. Only if polymerisation is increased up to contents of 10% and 20% in the treated oil is growth affected, because the highly polymerised portions are no longer digested and pass out of the body again without having been used.

Considering all the processes we have discussed so far, i.e. degumming, neutralising, bleaching and deodorising, we can say that the fat that is obtained has in all likelihood the same nutritional value as the starting product, but has become more palatable. As to the latter, our views will hardly differ. To elucidate what I have said regarding nutritional value, let me mention comparative experiments we made with rats.

These animals had been fed diets with a high content (50–70 cal.%) of an unrefined fat; as controls served groups which were fed comparable amounts of the same, but refined fats. According to Thomasson these diets with an extremely high fat content give an excellent opportunity of detecting even slight differences in growth stimulation between various fats. At the levels normally used in human diets these differences will be much smaller, or even vanish (cf. table 1).

A number of other oils and fats are still being investigated. The results of these tests will be known in September 1960.

Hydrogenation. In addition to the treatments outlined above, hydrogenation methods are also applied in the edible oil industry in order to meet the requirement that for satisfactory usability many fats must have a solid consistency. Of course it is possible to use solid fats, but these are often in short supply. Moreover such products frequently do not have the required melting

TABLE 1. *Growth on Crude and Refined Fats in Rat Tests*

	Growth of Animals				
	1 wk.	2 wk.	3 wk.	4 wk.	5 and 6 wk.
Crude olive oil, 50 cal.%	26.3	64.9	101.4	135.3	
Refined olive oil, 50 cal.%	26.9	65.8	102.9	140.7	
Butterfat, 70 cal.%	15.1	41.3	78.8	119.2	160.2
Refined butterfat, 70 cal.%	15.6	40.1	72.1	109.9	148.9

behaviour and consistency to come up to the climatic conditions of the country in which they are used. For this reason, it is frequently necessary to harden oils, and sometimes, although to a smaller extent, also solid fats, in order to impart the desired consistency to them. In addition, fish oil and whale oil, which as such are unsuitable for human consumption, must be hardened. In this process the highly unsaturated fatty acids which can give an unpleasant fishy taste, are converted into dietary fats containing fatty acids of satisfactory taste. Of the total quantity of oils and fats used in the edible oil industry about 90% is subjected to this treatment.

Before they can be hardened, the crude oils and fats are first neutralised to remove fatty acids and mucilage which unfavourably affect the hardening. The mucilage and other catalyst poisons, possibly sulphur compounds, could inactivate the catalyst, while fatty acids are able to dissolve the nickel present in the catalyst.

After the addition of an amount of suitable nickel catalyst, the product is hardened at temperatures from 100° to 200°C, while hydrogen is passed through, until it has required the desired properties. By the nature of the catalyst and the hardening method, the properties of the final product can, within certain limits, be controlled. The height of the melting point of the fat and its dilatation values at different temperatures are generally taken as criteria for the final result.

As a result of this hardening, which in the case of edible oils and fats only removes part of the unsaturation, various changes take place in the fatty acid molecules. A part of the double bonds in the fatty acids is saturated. At the same time a shifting of double bonds in the molecule occurs, accompanied by a transformation of a part of these double bonds from the cis- into the trans-form.

Hardened fats have been investigated in order to find out in what way their nutritive value is related to that of unhardened fats.

During hardening a minute amount of nickel is dissolved; but by careful refining (neutralisation, bleaching, deodorisation) of the hardened fat, the dissolved nickel is again removed. As a result, hardened fats contain extremely small amounts of nickel (1–4 μg/kg), much lower than are found in many natural products (100–300 μg/kg).

From investigations by Aaes Jörgensen *et al.* (11–5) and by Thomasson (11, p. 446 *et seq.*) it has appeared that on feeding rats diets containing a high percentage of hardened fats, there is some slight decrease in growth rate as compared with the unhardened products. However, Thomasson has further shown that diets containing hardened oils are completely satisfactory and support normal growth subject to the presence of 3–4% of linoleic acid in the diet. He has further shown that for satisfactory growth it is necessary to have a proper balance between saturated fatty acids with 12–18 carbon atoms and linoleic acid.

It has been investigated whether the trans-fatty acids formed from natural cis-fatty acids have identical nutritive values as the original acids. Sherman *et al.* (9) have published in 1949 some work on the influence of elaidic acid compared with oleic acid and petroselinelaidic acid compared with petroselinic acid. The cis-acids gave some better growth and a higher arachidonic acid content in the body fat. Different results were obtained by Thomasson (11, pp. 471, 472), who has compared a fatty acid having a cis-double bond (oleic acid) and the corresponding fatty acid having a trans-double bond (elaidic acid). It was found that even a very high dosage of elaidic acid gave normal growth, and no indications were obtained that the depot-fat formation and the combustion proceeded differently from those of normal oleic acid. In addition the growth on feeding elaidic acid was not changed by the addition of linoleic acid. As is generally known, trans-fatty acids are also to be found in butter and beef fat.

Thomasson established that on admixture of unhardened and hardened oils, a growth was obtained which in most cases exceeded that of the original non-hardened oil. This assures us that no harmful substances are formed during the hardening process.

From the results of numerous investigations it is at present assumed that a high content of unsaturated compounds may have a blood-cholesterol lowering effect. This is actually a fact which has to be taken into account. For this reason it would seem advisable to see to it that during the hardening the content of unsaturated fatty acids decreases as little as possible. The thesis that in this respect the essential fatty acids are important, now appears to be incorrect. Various oils (whale oil, fish oils and rapeseed oil) which have a low content of essential fatty acids are, according to Ahrens, Brock, Malmros and Thomasson, more effective in bringing about a decrease in the blood-cholesterol content than oils having a very high content of essential fatty acids.

Moreover Thomasson is investigating the influence of various fats and fatty acids on the blood-cholesterol content by means of a rat-screening test. He has found that by proper hardening of various oils and fats, marine oils, cottonseed oil, soybean oil, etc., provided this process is not continued to too high melting points (not in excess of 35°C), there is very little effect on the cholesterol-lowering action. In addition it was found that not only the degree and the nature of unsaturation is important, but other factors may play a role as well. According to Thomasson it is probable that the longer the chain length of the unsaturated fatty acid, the greater the effect will be on blood-cholesterol lowering. The experiments are only in the initial stage and will need confirmation on human panels.

In all the discussions on the influence of specific fats on growth, blood-cholesterol, etc., it must, however, be kept in mind that man seldom consumes a single fat. Indeed, we eat much fat in such products as fish, meat, cereals, etc. This invisible fat comprises 40% of the total fat intake in some Western countries and even 70% of the fat intake in certain underdeveloped co-

ries. Thus the human diet generally contains a mixture of many kinds of fat, which individually may have a specific action. This, however, will be modified when in admixture with other fats.

It is perhaps useful in conclusion to consider the contribution that hardening has made to the fat industry. As I have observed already, the advantage of hardening is that with the prevailing shortage of solid fats, a product suitable for certain climatic conditions can also readily be made from oils. Next, the decrease in the degree of unsaturation imparts better keeping qualities to the product. When the degree of unsaturation is higher, the fat is usually more susceptible to oxidation. This means a shorter shelf-life and increase in the chance that oxidised fatty materials which are not always harmless will be consumed. It is important, therefore, to find a compromise between these various factors.

Esterification. To improve the consistency, and to impart other desired properties to margarine, shortenings, and similar products, interesterification is frequently applied. In this process a fat or a mixture of fats is treated with a catalyst which is able to rearrange the fatty acid-radicals in the glyceride molecules via a transition stage, in such a way that a random esterified product is obtained. In addition, by application of so-called directed interesterification, high-melting triglycerides formed at low temperature can be made to crystallise, thus effecting a fractionation at the same time. In this operation catalysts are employed (sodium, sodium glycerate, sodium methylate and sodium ethylate) at temperatures varying from 50° to 150°C. Conditional for this operation is that it is carried out with a completely dry fat, as otherwise the catalyst is split by the water present, before the interesterification reaction has taken place. In this operation, the fatty acids undergo no change whatever. Only their position on the glycerin molecules changes. These conversions are comparable with the processes going on in the animal body during fat metabolism, when partial splitting off of fatty acids and subsequent re-synthesis to fats of frequently quite different compositions take place.

In addition to interesterification it is also possible to re-esterify fatty acids as such or a mixture of fatty acids and glycerides to a triglyceride. If in this process the starting products are fatty acids originating from natural fats either of sufficient purity or which have been purified, for instance by distillation *in vacuo* of the relevant fatty acids, no objections can be raised to this procedure on physiological grounds. These acids can then be re-esterified with glycerin in various ways, under the influence of catalysts. This process was formerly carried out at high temperatures, while using, inter alia, tin as a catalyst. Now it is, however, also possible to carry out this esterification at much lower temperatures.

The interesterification process does not affect the nutritive value of a fat. This is especially clear from the results of relevant experiments. Thomasson carried out tests with rats; the growth of the animals was determined after administration of diets containing high percentages of triglycerides in their original state by the side of the fatty acids made from these triglycerides and then re-esterified. The results can be seen in table 2. No significant growth difference could be found.

TABLE 2. *Growth of Rats Fed on Diets Containing Natural Triglycerides and Re-esterified Glycerides Made From the Same Fatty Acids*

	Calorie Percentage Fat in Diet	Growth
		after 3 wk
Hardened groundnut oil	72	41.5 g
Fatty acids re-esterified	72	39.0 g
Low melting fraction f.a. re-esterified	72	44.5 g
High melting fraction f.a. re-esterified	72	41.0 g
		after 4 wk
Groundnut oil	50	131 g
Groundnut fatty acid distilled re-esterified	50	123 g

Physical treatments. In addition to the methods discussed so far, and which are all to a greater or lesser extent based on chemical reactions, various physical treatments are likewise employed in the edible oil industry. First among these is the mixing of various oils and fats as is current practice in the edible fat industry. In the margarine industry, for instance, oils, solid natural fats and hardened oils and fats are mixed until a composition is obtained which has a satisfactory consistency for the climate and the use for which the product is intended. The same applies to nonwater containing edible fats. This mixing of various fats is not only advantageous from the point of view of use, but it may also favourably affect the nutritive value of the mixture.

From the work by Thomasson we have learnt that on feeding pure fats in diets with a high fat content to rats, a fluctuating growth is observed in the test animals. The greatest growth-action is found in the case of fats such as lard, butterfat, olive oil, cottonseed oil, and sheafat. Beef tallow, maize oil, soybean oil, groundnut oil, and palm oil, at a level of 70 cal.%, give a slightly less marked growth, while other fats such as sunflower-seed oil, coconut fat, sesame oil, whale oil, herring oil, and rapeseed oil, when amounts of 50 to cal.% are contained in the diets, give even less good growth (10). On mixing various fats these differences can be levelled out, and so give a product which has a more uniform growth action. This brings me to the point I made earlier when I stressed the nutritional advantages of diets containing mixed fats. The range of both visible and invisible fats in our diet normally ensures this.

It is clear that the physical process of mixing introduces no changes at all in the nutritive properties of each of the component fats. The same applies to other physical treatments which are used in industry, such as the emulsification process in the margarine industry where skimmed milk is emulsified in a fat phase, the cooling of fat mixtures on cooling drums or in votators, the sub-

sequent kneading of the cooled fat in various types of kneaders.

ADDITIVES

In the foregoing pages the additives used during the processing of oils and fats and their possible influence on these raw materials have been described in great detail. Now some attention will be paid to those additives which are added to the refined fat or fat mixture.

Vitamin. Low concentrations of fat-soluble vitamins are found in oils and fats. As already mentioned in most vegetable fats vitamin E is present, of which only a small portion is lost during refining. In animal fats only a minute amount of this vitamin is present. Beef tallow contains small amounts of vitamin A; butterfat contains a larger amount of vitamin A; a large amount of carotene is found in palm oil. Vitamin D occurs in butter in very small amounts.

During refining of fats and oils the greater part of the carotene and of the vitamin A is lost. It would be possible to retain part of the carotene in palm oil by application of a special refining technique, but the taste of palm oil so treated becomes poor due to the presence of decomposition products originating from carotene and similar colorants. Therefore if palm oil is used in large amounts, it has to be bleached. It is possible to recover the carotene contained in palm oil, and suitable processes have been worked out. Currently produced synthetic β-carotene, however, is completely pure and its price is not any higher than that obtained from palm oil by applying the methods known at present.

To meet the vitamins A and D requirements of the population, synthetic vitamin A and synthetic vitamin D are added to a number of fat products, inter alia margarine. The amount of added vitamin D must not be too high, for instance *ca.* 300 g, to prevent the danger of giving older people an over dose of this vitamin.

Flavouring substances. Attempts are being made to impart an attractive taste to a number of oils and fat products, notably margarine. This is done by the addition of synthetic flavouring substances. For a long time past diacetyl has been used for this purpose; at present also lactones and other flavouring substances are employed. Physiological tests have shown that these substances, even when added in very large amounts to the diet, are harmless for the animal organism. This has been proved in short-term experiments and in tests covering the entire life-span of rats fed these substances. At a later date it has moreover appeared that these substances are identical to the flavouring substances found in butter.

Anti-oxidants. To improve the shelf-life of edible oils fat-soluble anti-oxidants could be used. As a rule it can be said that these substances have little effect on vegetable oils, but a pronounced action on being added to animal oils and fats. These latter products contain only small amounts of natural anti-oxidants, so that they can be markedly improved by the addition of natural tocopherol or such synthetic anti-oxidants as gallates (propyl, octyl and dodecyl), nordihydroguaiaretic acid (NDGA), butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT). Vegetable oils and fats contain natural antioxidants, inter alia tocopherol. Especially γ-tocopherol is active as such at room temperature. It has appeared that during refining and hardening of vegetable oils only a small part (10–20%) of the tocopherol is converted. Only in those cases when a vegetable fat must be used in edible products which have to be stored for very long periods of time, e.g. biscuits for export, it will be advantageous to add an anti-oxidant to a vegetable fat.

The above-mentioned anti-oxidants have all been tested biologically, and found to be harmless. It is not necessary here to go into details concerning all the experiments which have been carried out, but they form the basis on which the use of these anti-oxidants has been sanctioned in various countries.

Anti-microbial substances. In some water-containing fat products, such as for instance margarine, the use of preservatives is allowed in many countries. These products have the advantage that even in nonsalted margarine a large amount of milk can be used, without the danger of rapid deterioration. As such especially benzoic acid and sorbic acid are being used. These two acids, as has been proved by biological investigations, can be considered harmless to health. Sorbic acid, however, because it is decomposed in the normal way in the body, is somewhat preferred to benzoic acid.

Colouring matter. The colorants currently used in oils and fats are generally synthetic carotene, palm oil and annatto. In a number of countries fat-soluble aniline-dyes are still allowed, but their use is gradually abandoned, mainly because to fat-soluble aniline-dyes a more unfavourable effect on the animal organism is ascribed than to water-soluble aniline-dyes.

Synthetic carotene which is identical to natural β-carotene (pro-vitamin A) has been discussed higher up. Annatto, the coluring constituent of which, bixine, is present on the outside of the annatto seeds, can be used without any harmful effects on health (6, 7). The annatto seeds are procured in Central America where they are used on a large scale in the preparation of food. The colouring material is dissolved in oil, and this oil can be added to edible fats.

Emulsifiers. The most important emulsifiers used in the oil and fat industry are: MONO/DIGLYCERIDES. At low temperature these substances have the tendency to retain the water phase finely dispersed in the fat phase, which is especially advantageous in the margarine industry. These mono- and diglycerides are preferably prepared from hardened fat, because in that case they have better action than when mono- and diglycerides from lower-melting fats are added. From the work by Frazer and other research-workers it has appeared that in man, on consuming fats and oils, part of the triglycerides is broken down to mono- and diglycerides. Moreover, mono- and diglycerides are present in

natural and also in refined oils and fats. Consequently, they form normal constituents of natural oils and fats and normal degradation products of fat metabolism. Their value as foodstuffs is consequently the same as of triglycerides.

LECITHIN. As already mentioned under desliming, a phosphatide is obtained from a number of oils, notably soybean oil, during this treatment. After purification, this phosphatide can be used to improve the frying properties of products such as margarine. Moreover, lecithin is a substance known to have favourable physiological effects.

SCIENTIFIC RESEARCH

The oils and fats industry, like many other industries, originally developed almost entirely along empirical lines. Some processes, such as neutralising and deodorising, were originally applied in a comparatively primitive fashion, but grew up through their practical application during the course of years.

The process of hardening, however, had a somewhat different starting point. This process is in fact based on scientific research and was initially due to the work of Sabatier and Senderens who hydrogenated unsaturated compounds in the gaseous state using catalysts. Their method was later applied by Normann to liquid oils and fats. The hardening process and the catalysts employed were further improved, mainly by trial and error methods, but nevertheless with striking results.

It is only in the last few decades that research laboratories in the modern sense of the term have begun to play an important part in the industry. Scientific means were sought to study the effectiveness of various processes and for the development of means to improve them. A desire for perfection ensued and progressive advancement has lead to the present high state of development.

A stage has now been reached at which further improvement of the various processes employed is only likely to be achieved through more fundamental work. The direction in which further improvements can be sought can only be seen when the fundamental processes involved are better understood. In hardening, e.g. x-ray, work is being undertaken to determine the crystal size in nickel catalysts, which in turn should provide a better insight into the mode of action of the catalysts. New techniques, such as spectrophotometry, gas chromatography and mass spectrometry, etc., are being generally applied in the oils and fats field and have already cast new light on many of the problems of the chemistry and physical nature of these substances.

From this account it must be abundantly evident that in addition to chemical and physical research an important position is occupied by biological research. Great attention is being devoted to the possible physiological influence of dietary oils and fats. Much of this research is being undertaken in cooperation with working groups in universities, but it is yet too early to say what the consequences might be. It is certain, however, that in this way industry can make an important contribution to the development of science and to public health.

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Effects of the Dietary Carbohydrate–Fat Combination on Changes in Lipid Metabolism Induced in Rats by Amino Acid Imbalance [1,2]

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LOUISE WILLIAMS AND CATHERINE CARROLL
Home Economics Department, Agricultural Experiment Station, University of Arkansas, Fayetteville, Arkansas 72701

ABSTRACT A study was conducted to evaluate interrelated effects of type of carbohydrate and fatty acid content of fat in the diet on lipid accumulation in liver of rats fed imbalanced protein diets, deficient in threonine. Regular and high oleic safflower oils were each combined with fructose and with glucose in both balanced and imbalanced protein diets. Rats were fed for 3 weeks. Rats fed fructose-imbalanced rations accumulated more liver lipid than rats fed glucose-imbalanced rations. The only significant differences attributable to the type of safflower oil fed were in fatty acid content of liver and adipose lipids. Liver fatty acids from all rats fed imbalanced rations had a pattern of reduced 18:0, increased 16:1 and 18:1, and a higher 18:2/20:4 ratio as compared with those from rats fed a balanced ration. These differences were accentuated by fructose. This pattern existed with either oil, although the absolute percentage of each fatty acid was a reflection of the dietary oil. J. Nutr. *103*: 991–998, 1973.

INDEXING KEY WORDS carbohydrate · fatty acids · threonine deficient · fatty liver

Rats fed low protein diets deficient in essential amino acids develop fatty livers (1, 2). The accumulated lipid consists mainly of triglycerides (3), and has been attributed to increased fatty acid synthesis (3, 4). Sidransky and Clark (5) suggested that in rats fed imbalanced protein (deficient in threonine or valine) the excess hepatic lipids are derived from dietary carbohydrates, and not from ingested fats or amino acids. Fructose has also been found to stimulate fatty acid synthesis of triglycerides by enhancing formation of α-glycerophosphate needed to provide the glycerol moiety.

We found that rats fed fructose had more total liver lipid than rats fed glucose in low protein diets containing 5% of hydrogenated coconut oil (HCO) [3] but not 5% of corn oil (CO) (8). However, rats fed CO in comparable threonine-deficient diets had more liver lipid if the diet contained fructose than if it contained glucose. Thus the combination of carbohydrate and fat in the diet apparently influences the response to threonine deficiency.

Experiments have shown that chain length, saturation (9), and partial hydrogenation of fatty acids (10) can influence the amount of liver lipid deposition in threonine-deficient rats. The chemical composition of the fat source apparently makes a difference in liver lipid accumulation. Since different "families" of fatty acids are metabolized by separate pathways (11), we wanted to see how the relative amounts of oleic and linoleic acids would influence the lipid metabolism in rats fed glucose or fructose in threonine-deficient diets. Dietary fats were regular safflower oil (SO) and high oleic safflower oil (HiO). These oils contain less than 10% of saturated acids, and there is an almost complete reversal in the percentages of linoleic and oleic acids. SO contains 79% of linoleic and 12% of oleic acids; HiO contains 15% of linoleic and 80% of oleic acids. Fatty acid analyses

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[3] Unpublished data.

of liver and fat pad lipids were made to see if the fatty acid patterns would indicate possible reasons for liver lipid accumulation.

METHODS

Experimental design, animals, and diets. Male weanling rats of the Sprague-Dawley strain[4] were housed individually in wire mesh-bottom cages in an air-conditioned animal room. They were fed experimental diets ad libitum for 3 weeks and had access to water at all times. Body weights and food intakes were recorded three times per week.

All diets contained the following (%): protein, 9; salts,[5] 4; vitamin mixture,[6] 0.25; choline chloride, 0.2; fat, 5 (regular safflower or high oleic safflower oil[7]); carbohydrate, 75 (glucose or fructose); and nonnutritive bulk[8] in quantities to make the ration provide 380 kcal per 100 g ration. The protein was vitamin-free casein supplemented (at the expense of casein) with 0.3 g methionine and 0.1 g tryptophan per 100 g ration for amino acid-imbalanced diets, and further supplemented with 0.36 g DL-threonine per 100 g ration for amino acid-balanced diets.

The study was conducted in two parts: one for analysis of total liver, and the other for isolation and analysis of liver mitochondria. Diets, feeding procedures, and duration of feeding periods were identical for parts 1 and 2. Regular and high oleic safflower oils were each combined with fructose and with glucose in both balanced and imbalanced protein diets, making a total of eight dietary groups. In part 1 there were eight rats per group, and in part 2 there were six rats per group.

Analytical procedures. In part 1, gross composition of liver and fatty acid composition of liver and epididymal fat pad lipid were determined by methods described previously (12).

In part 2 the excised liver was plunged into partially frozen 0.25 M sucrose solution, blotted dry, weighed, and homogenized with an equal volume of 0.25 M sucrose. The liver mitochondria were then separated by the method of Hogeboom et al. (13) in a refrigerated centrifuge.[9] Lipid was extracted from the mitochondria with chloroform-methanol (2:1) and washed according to the method of Folch et al. (14). The samples were evaporated under nitrogen to approximately 1 ml and fatty acids methylated and assayed as described previously (12).

Standard errors of the means were calculated for all data. Student's *t* test was used as a measure of significance.

RESULTS AND DISCUSSION

Both carbohydrate source and amino acid imbalance affected the growth rate of rats. In part 1, rats fed fructose gained less weight than rats fed glucose, and rats fed fructose-imbalanced diets gained less than rats fed fructose-balanced diets. In part 2, growth was depressed in rats fed imbalanced rations containing either carbohydrate (table 1). Harper et al. (15) found food intake was rapidly depressed in rats fed an amino acid-imbalanced diet, and suggested that the depressed food intake might be a primary factor in limiting growth. Only one of the four groups fed imbalanced diets in part 1, and two of the four groups fed imbalanced diets in part 2 had significantly decreased food intake. The food efficiency ratio (weight gain/100 g ration) indicated that rats fed imbalanced protein in diets containing SO did not utilize calories as well for growth as did those fed balanced diets (table 1).

Rats fed fructose in balanced or imbalanced diets had larger livers per 100 g body weight than rats fed corresponding glucose diets (table 2). The larger livers contained significantly more nitrogen, and more lipid and glycogen. The differences in lipid were significant when dietary fat was HiO, and the differences in glycogen were significant when dietary fat was SO.

Threonine imbalance also resulted in liver lipid accumulation, and when the im-

[4] Obtained from Hormone Assay Laboratories, Inc., Chicago.

[5] Wesson, L. S. 1932 A modification of the Osborne-Mendel salt mixture containing only inorganic constituents. Science 75: 339.

[6] Vitamin mixture contained the following: (mg/kg of ration) thiamine-HCl, 8.0; riboflavin, 6.0; pyridoxine, 4.0; Ca pantothenate, 40.0; niacin, 50.0; inositol, 200.0; folic acid, 4.0; vitamin B_{12}, 0.04; biotin, 0.2; vitamin A powder (20,000 IU/g), 100.0; calciferol (850,000 IU/g), 1.6; dl-α-tocopherol (250 IU/g), 800; and menadione, 8.8. Individual vitamins obtained from Nutritional Biochemicals Corp., Cleveland, Ohio.

[7] Oils obtained from Pacific Vegetable Oil Corp., Richmond, Calif.

[8] Alphacel. Nutritional Biochemicals Corp., Cleveland, Ohio.

[9] Sorvall. Model RC-2-B.

TABLE 1

Weight gain, food intake and food efficiency ratios from rats fed diets containing different carbohydrate–fat combinations and balanced or imbalanced protein for 3 weeks

	Glucose diets		Fructose diets	
	Bal[1]	Imbal	Bal	Imbal
Regular safflower oil				
g weight gain				
Part 1[2]	76± 3[3]	67± 3	60±2[b]	51±2[b,y]
Part 2	72± 3	54± 5[y]	63±2[a]	54±2[x]
g food intake				
Part 1	240± 5	227± 7	195±5[b]	184±5[b]
Part 2	243± 1	207±11[x]	205±6[b]	207±7
g weight gain/100 g ration				
Part 1	32± 1	29± 1[x]	31±1	28±1[y]
Part 2	30± 1	26± 1[x]	31±1	26±1[y]
High oleic safflower oil				
g weight gain				
Part 1	78± 2	70± 4	67±2[b,x]	49±2[b,y]
Part 2	74± 3	56± 4[y]	68±3	55±2[x]
g food intake				
Part 1	243± 9	231± 7	207±5[b]	185±4[b,x]
Part 2	247±11	206±10[x]	210±9[a]	195±4
g weight gain/100 g ration				
Part 1	32± 1	30± 1	32±1	26±1[b,y]
Part 2	30± 1	28± 1	32±1	29±1

[1] Abbreviations: Bal equals 9% protein supplemented with threonine; Imbal equals 9% protein without threonine supplementation. [2] Part 1 had eight rats per group; Part 2 had six rats per group. [3] SEM. [a, b] Significantly different ($P < 0.05$, 0.01, respectively) from corresponding group fed glucose. [x, y] Significantly different ($P < 0.05$, 0.01, respectively) from corresponding group fed protein supplemented with threonine. [x, y] Significantly different ($P < 0.05$, 0.01, respectively) from corresponding group fed safflower oil.

balanced ration contained fructose, the accumulation of lipid was greater than when it contained glucose. These two nutritional factors, fructose and threonine deficiency, appear to have a synergistic effect. Both fructose (16) and threonine imbalance (3, 4) have been found to increase fatty acid synthesis and triglyceride accumulation in the liver.

The source of dietary fat appeared to have no direct effect on liver lipid deposition, as the amount of lipid in livers of groups fed diets containing HiO was not significantly different from that of corresponding groups fed diets containing SO. However, HiO apparently enhanced the effect of fructose, in that increases attributable to fructose were greater when the source of fat was HiO than when it was SO.

There was at least 30% less ($P < 0.01$) lipid deposited in fat pads of rats fed fructose than in those of rats fed glucose (table 2). Also, fat pad lipid from all rats fed fructose contained lower percentages of palmitic (16:0) and palmitoleic (16:1) acids, lower ratios of 16:0/18:0, and higher percentages of oleic (18:1) and linoleic (18:2) acids than that from rats fed corresponding glucose diets (table 3). Fatty acid patterns were also affected by dietary fat, but very little by threonine deficiency. Oleic acid accumulated in the fat pads of all rats fed HiO to approximately 50% of the total fatty acids, whereas linoleic acid accounted for only 25 to 30% of the total fatty acids in fat pad lipid of rats fed SO. The only significant differences attributable to threonine deficiency were in rats fed

TABLE 2

Relative liver weight and composition of livers from rats fed diets containing different carbohydrate-fat combinations and balanced or imbalanced protein

Dietary fat	Glucose diets		Fructose diets	
	Bal[1]	Imbal	Bal	Imbal
	g liver/100 g body weight			
SO[2]	4.48± 0.11[3]	4.52± 0.33	6.03± 0.12[b]	6.05± 0.10[b]
HiO	4.54± 0.08	4.50± 0.08	5.97± 0.19[b]	5.65± 0.30[b]
	mg liver nitrogen/100 g body weight			
SO	106 ± 2	106 ± 3	128 ± 3[b]	126 ± 3[a]
HiO	108 ± 2	102 ± 3	129 ± 2[b]	124 ± 2[b]
	mg liver lipid/100 mg liver nitrogen			
SO	135 ± 5	210 ±20[y]	149 ± 5	250 ±20[y]
HiO	139 ± 4	217 ±17[y]	172 ±12[a]	309 ±30[a,y]
	mg liver glycogen/100 mg liver nitrogen			
SO	402 ±29	364 ±27	493 ±30[a]	507 ±20[b]
HiO	364 ±47	396 ±47	458 ±44	553 ±82
	mg fat pad lipid/100 g body weight			
SO	681 ±60	742 ±59	475 ±31[b]	458 ±22[b]
HiO	717 ±20	846 ±72	418 ±30[b]	454 ±28[b]

[1] Bal equals 9% protein supplemented with threonine; Imbal equals 9% protein without threonine supplementation. [2] SO equals regular safflower oil; HiO equals high-oleic safflower oil. [3] Avs ± SEM, eight rats per group. [a,b] Significantly different ($P < 0.05$, 0.01, respectively) from corresponding group fed glucose. [x,y] Significantly different ($P < 0.05$, 0.01, respectively) from corresponding group fed protein supplemented with threonine. [a,b] Significantly different ($P < 0.05$, 0.01, respectively) from corresponding group fed safflower oil.

rations containing fructose. Fat pad lipid from rats fed the imbalanced, fructose-SO diet had a smaller 16:0/18:0 ratio and a larger percentage of 18:2 than that from any other dietary group.

The relative amounts of fatty acids in total liver lipids are shown in table 4. Fructose affected fatty acid patterns of liver lipid differently than those of fat pad lipid, in that the percentage of stearic acid (18:0) was consistently decreased and the ratio of 16:0/18:0 was increased directly as the amount of lipid increased. Liver lipid from rats fed fructose also contained larger percentages of monounsaturated acids (except 16:1 in deficient, HiO group), smaller percentages of polyunsaturated fatty acids, and higher 18:2/20:4 ratios than from rats fed glucose.

Effects of fructose and threonine imbalance on fatty acid patterns of liver lipid were qualitatively similar, except with respect to linoleic acid in rats fed SO diets; and in most cases, effects of the two factors were additive. Liver lipid from rats fed threonine-deficient diets had a distinctive fatty acid pattern. There were smaller percentages of 18:0 and higher 16:0/18:0 ratios, larger percentages of monounsaturated fatty acids (except 16:1 in the F-HiO group), and depression of 20:4 with resultant higher ratios of 18:2/20:4 than in liver lipid from rats fed balanced protein diets. The percentage of 18:2 in liver lipid increased with omission of threonine from SO diets. This is in agreement with data reported by Viviani et al. (17) from rats fed lysine- and threonine-deficient diets. They stated that this indicated a decreased transformation of linoleic acid into arachidonic acid. This supposition was also made by Rogers (18) from findings made after feeding low protein (4% casein) diets. Liver lipids from rats with the fattest livers (deficient F-SO and F-HiO groups) contained the lowest percentages of 18:0 and 20:4.

The increase in monounsaturated fatty acids resulting from feeding HiO (80% oleic acid) was not only a reflection of the

TABLE 3

Fatty acid composition of fat pad lipids from rats fed diets containing different carbohydrate-fat combinations and balanced or imbalanced protein

Fatty acid	Glucose diets		Fructose diets	
	Bal[1]	Imbal	Bal	Imbal
	%	%	%	%
Regular safflower oil				
Saturated				
14:0	2.3±0.1[2]	2.3±0.1	2.4±0.1	2.2±0.1
16:0	33.8±0.5	33.3±0.8	30.7±0.5[b]	29.5±0.3[b,a]
18:0	3.1±0.1	3.2±0.2	3.3±0.1	4.0±0.1[b,y]
Monounsaturated				
16:1	10.3±0.4	10.5±0.5	8.3±0.3[b]	7.7±0.2[b]
18:1	25.0±0.4	25.2±0.4	27.2±0.3[b]	26.5±0.2[b]
Polyunsaturated				
18:2	25.6±0.9	25.6±1.0	27.7±0.7	30.1±0.5[b,a]
High oleic safflower oil				
Saturated				
14:0	2.2±0.1	2.2±0.1	2.0±0.1[a,a]	2.0±0.1
16:0	29.4±0.5[a]	29.3±0.6[a]	24.6±0.5[b,a]	25.7±0.3[b,a]
18:0	2.8±0.1	2.9±0.2	2.8±0.1[a]	2.9±0.1[a]
Monounsaturated				
16:1	10.7±0.3	11.3±0.5	7.8±0.3[b]	8.3±0.3[b]
18:1	48.8±0.6[a]	48.7±0.6[a]	55.0±0.5[b,a]	54.0±0.5[b,a,a]
Polyunsaturated				
18:2	6.1±0.2[a]	5.7±0.3[a]	7.2±0.4[a,a]	7.1±0.3[b,a]

[1] Abbreviations: Bal equals 9% protein supplemented with threonine; Imbal equals 9% protein without threonine supplementation. [2] Average, eight rats per group. [a,b] Significantly different ($P < 0.05$, 0.01, respectively) from corresponding group fed glucose. [a,y] Significantly different ($P < 0.05$, 0.01, respectively) from corresponding group fed protein supplemented with threonine. [a,a] Significantly different ($P < 0.05$, 0.01, respectively) from corresponding group fed safflower oil.

oleic acid in the dietary oil, but also included an increase in palmitoleic acid. The accumulation of linoleic acid was not as great in livers of rats fed SO (79% linoleic acid) as was the accumulation of oleic acid in livers of rats fed HiO. Oleic acid accounted for approximately 35 to 40% of total fatty acids in rats fed diets containing HiO. Linoleic acid accounted for 16 to 22% of total liver fatty acids in rats fed diets containing SO. Mohrhauer et al. (19) found that metabolism of 18:1ω9 (oleic acid) to 20:3ω9 was inhibited 50% by the addition of approximately 0.6% of calories as 18:2ω6 (linoleic acid). They found inhibition of linoleic acid metabolism by oleic acid to be of much smaller magnitude. This might explain the greater accumulation of 18:1 in lipid of rats fed HiO diets than of 18:2 in lipid of rats fed SO diets. Linoleic acid accounted for approximately 1.4% of calories in the HiO diet, nearly twice the amount reported to be inhibitory to conversion of 18:1 to 20:3.

Fatty acid content of lipid extracted from the liver mitochondria in Part 2 are given in table 5. In groups fed balanced diets, influences of carbohydrate source on fatty acid patterns were less pronounced than in total liver lipid. However, threonine deficiency considerably altered the proportions of fatty acids in liver mitochondria of rats fed diets containing either carbohydrate. The proportion of palmitic acid was increased and that of stearic acid decreased (increasing the 16:0/18:0 ratio), the monounsaturated fatty acids were increased, and the 18:2/20:4 ratio was increased. This is in agreement with the findings of Rogers (18). In threonine deficient groups, especially when the fat

TABLE 4

Fatty acid composition of total liver lipid from rats fed diets containing different carbohydrate–fat combinations and balanced or imbalanced protein

Fatty acid	Glucose diets		Fructose diets	
	Bal[1]	Imbal	Bal	Imbal
	%	%	%	%
Regular safflower oil				
Saturated				
14:0	1.1±0.1[2]	1.4±0.1	1.6±0.1[b]	1.7±0.1[b]
16:0	28.9±0.7	29.9±0.9	31.3±0.6[a]	30.0±0.5
18:0	13.7±0.5	10.5±0.6[y]	11.4±0.6[b]	7.9±0.5[b,y]
Monounsaturated				
16:1	3.2±0.3	4.3±0.2[y]	4.4±0.3[b]	5.4±0.4
18:1	18.3±0.9	21.6±0.8[x]	24.8±0.8[b]	28.3±0.8[b,y]
Polyunsaturated				
18:2	19.2±1.0	21.6±1.2	15.7±1.1[a]	19.6±0.9[x]
20:4	15.8±0.9	10.8±0.8[y]	11.0±0.6[b]	7.1±0.5[b,y]
High oleic safflower oil				
Saturated				
14:0	1.6±0.2	1.8±0.2	1.8±0.2	1.7±0.1
16:0	29.4±0.6	32.7±0.8[y,a]	30.2±0.6	32.1±0.8
18:0	13.1±0.7	9.7±0.7[y]	9.6±0.2[b,c]	7.3±0.6[a,y]
Monounsaturated				
16:1	4.8±0.2[a]	6.8±0.2[y,a]	6.6±0.3[b,a]	7.3±0.6[c]
18:1	34.1±1.1[a]	36.8±1.3[a]	39.4±0.9[b,a]	43.0±0.9[b,a,c]
Polyunsaturated				
18:2	5.8±0.4[a]	4.5±0.3[x,a]	4.6±0.3[a,a]	3.8±0.4[c]
20:4	11.0±0.6[a]	7.3±0.6[y,a]	8.0±0.6[b,a]	5.2±0.7[a,y,c]

[1] Abbreviations: Bal equals 9% protein supplemented with threonine; Imbal equals 9% protein without threonine supplementation. [2] Ave ± stat, eight rats per group. [a,b] Significantly different ($P < 0.05$, 0.01, respectively) from corresponding group fed glucose. [x,y] Significantly different ($P < 0.05$, 0.01, respectively) from corresponding group fed protein supplemented with threonine. [c] Significantly different ($P < 0.05$, 0.01, respectively) from corresponding group fed safflower oil.

source was HiO, substituting fructose for glucose greatly enhanced effects of threonine deficiency, resulting in highly significant differences between deficient groups fed fructose and those fed glucose. The most striking synergistic effects of fructose and threonine deficiency were increased percentages of 18:1 and decreased percentages of 20:4 and longer-chain polyunsaturated fatty acids in groups fed diets containing either fat; and, in the HiO group only, significantly increased 16:0 and decreased 18:0, resulting in a very high 16:0/18:0 ratio (3:1).

Significant differences in fatty acid patterns of mitochondrial lipids are of special interest, because these lipids consist mainly of phospholipids (structural and functional), and would presumably include little of the accumulated triglycerides of the fatty liver (20). Both total lipid and mitochondrial lipid from the most fatty livers (from rats fed threonine-deficient fructose rations) contained the lowest proportions of 18:0 and 20:4, fatty acids which frequently occur together in phospholipids (21). In mitochondrial lipids of the deficient HiO–fructose group, the percentage of 20:4 was only half that of the corresponding glucose group. Thus it seems possible that the combined effects of dietary fructose and amino acid imbalance on

TABLE 5

Fatty acid composition of liver mitochondrial lipid from rats fed diets containing different carbohydrate–fat combinations and balanced or imbalanced protein

Fatty acid	Glucose diets		Fructose diets	
	Bal[1]	Imbal	Bal	Imbal
	%	%	%	%
Regular safflower oil				
Saturated				
16:0	20.8±0.3[a]	22.9±0.8[a]	20.4±0.9	24.4±0.9[a]
18:0	16.0±0.4	12.0±1.1[x]	14.5±0.2[b]	9.4±0.8[y]
Monounsaturated				
16:1	2.1±0.2	3.4±0.4[x]	3.0±0.2[b]	4.1±0.3[x]
18:1	10.3±0.5	14.9±1.6[a]	13.5±0.5[b]	23.9±1.6[b,y]
Polyunsaturated				
18:2	16.9±0.5	21.1±0.7[y]	17.8±0.4	20.7±0.6[y]
X[4]	2.4±0.2	2.5±0.2	2.6±0.1	2.0±0.2
20:4	22.6±0.3	17.1±1.8[x]	21.8±0.8	11.3±1.5[a,y]
Y[5]	8.7±0.5	5.8±0.9[x]	6.3±0.5[b]	3.3±0.4[a,y]
High oleic safflower oil				
Saturated				
16:0	20.9±0.7	23.2±1.1	21.3±0.6	26.8±0.6[a,y]
18:0	15.1±0.8	12.0±1.4	13.7±1.0	8.9±0.6[a,y]
Monounsaturated				
16:1	3.4±0.3[a]	4.6±0.5[a]	4.5±0.4[a]	6.5±0.3[a,y,z]
18:1	20.7±1.6[a]	27.3±3.2[a]	25.1±1.5[b]	37.9±1.8[a,y,z]
Polyunsaturated				
18:2	8.7±0.6	8.2±0.5[a]	8.8±0.4[a]	6.0±0.4[a,y,z]
X[4]	4.5±0.5[a]	2.7±0.3[x]	3.6±0.2[a]	2.0±0.2[y]
20:4	20.6±1.1	16.1±2.0	18.2±0.9[a]	8.9±0.8[a,y]
Y[5]	5.6±0.4[a]	4.8±0.9	4.3±0.2[a,z]	1.9±0.1[a,y,z]

[1] Abbreviations: Bal equals 9% protein supplemented with threonine; Imbal equals 9% protein without threonine supplementation. [2] Ave ± SEM, six rats per group. [a,b] Significantly different ($P < 0.05$, 0.01, respectively) from corresponding group fed glucose. [x,y] Significantly different ($P < 0.05$, 0.01, respectively) from corresponding group fed protein supplemented with threonine. [z] Significantly different ($P < 0.05$, 0.01, respectively) from corresponding group fed safflower oil. [4] X = unidentified peaks eluted between 18:2 and 20:4. [5] Y = unidentified peaks eluted after 20:4.

fatty acid metabolism could have modified phospholipid composition.

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EFFECTS OF SOY OIL ON EXPERIMENTAL ATHEROMATOSIS OF THE AORTA AND ON CERTAIN ENZYMES IN THE VASCULAR WALL IN RABBITS*

T. Zemplenyi, Z. Lojda, D. Grafnetter, J. Fodor, V.Felt

Institute for Blood Circulation Diseases, Praha-Krc, director, Prof. MUDr. K. Weber ---Embryological Institute, Charles University, Praha, director, Prof. MUDr. Z. Frankenberger

The effect of diet on atherosclerosis is one of the most urgent problems in research on this disease. In 1952 Kinsell et al verified that some vegetable oils decrease cholesterolemia in man. Harding and Stare (1954), Bronte-Stewart et al (1955) and other authors arrived at the same conclusions. Malmros and Wigand (1955, 1957), Beveridge et al (1956), Sinclair (1958), Ahrens et al (1957) and Keys et al (1957) pointed out the significance of unsaturated, that is, superfluous fatty acids resulting from vegetable oils.

It is of course necessary to call attention to the fact that, according to Beveridge et al (1957) and others, the sterol content of vegetable oils is also important from this point of view. The fact is that Peterson already indicated in 1951 and 1952 that the addition of mixed soy sterols and refined sitosterols into the feed of chickens lowers their plasmatic and liver cholesterol and also decreases the level of atherosclerosis. Pollak (1953) achieved

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*During this period this oil was used in persons in the Institute for Research in Human Nutrition in Praha (viz Vavrikova 1958). Dedicated to Prof. Dr. Kl. Weber on his 70th birthday.

similar results in rabbits with raw soy oil while Dreisbach et al (1953) and Clarcson et al (1956) reached negative conclusions.Soy oil contains above all sitosterol gama.

Other authors used sitosterol beta contained for example in cottonseed oil. Beher et al (1956), Heptinstall and Porter (1957) refer to the favorable effect of sitosterol beta in rabbits. Best et al (1954), Sachs and Weston (1956), Farquhar et al (1956) and others obtained favorable results also in humans.

Soy oil is also interesting because it contains a great deal of lecithin. Lecithin given perorally (Keston et al (1942)and long-term intravenous infusion of lecithin (Friedmann et al, 1957, Felt et al, 1958) lowers the level of cholesterol atheromatosis in rabbits. Morrison writes about the favorable action of lecithin during its peroral application in humans also (1953).

For the reasons cited above we considered it proper to test the effect of raw unrefined soy oil from which the undesirable taste and smell had been extracted (supplied by the national enterprise SOJA) on the development and regression of cholesterol atheromatosis of the aorta in rabbits. At the same time we were interested in the question whether the lipolytic and esterolytic capability and other enzymatic properties of the aorta change along with the development of atheromatosis and whether the effect of soy oil will also appear in this direction. Our previous study (Zemplenyi and Grafnetter, 1958a, b, 1959a; Zemplenyi, Lojda, Grafnetter, 1958,1959) in fact admit the possibility that the lipolytic and esterolytic activity of the aorta belongs to the factors affecting the accumulation of lipids in the blood vessel. If this is so then it can be expected that the effect

on the level of experimental atheromatosis of the aorta could change even these enzymatic properties of the vascular wall.

Method

In the beginning of the experiment a total of 33 rabbits (males) approximately six months old divided into four groups were used. The effect on the development of atheromatosis was observed in the animals in groups I, II while in groups III, IV regression of atheromatosis was observed.

Group I of rabbits (9 animals) was used in the long-term experiment from October 5, 1957 to March 19, 1958. We fed the animals every day 0.5g of cholesterol dissolved in 5g of margarine mixed with potato and oats groats so that each rabbit received 120 g of this mixture daily; also alfalfa, barley and water ad libitum. From January 31, 1958 the daily dose of cholesterol was increased to 1 g. The animals were kept in individual cages and the consumption of the diet was checked daily.

Group II of 9 animals received cholesterol dissolved in 5 ml of soy oil. The rest of the conditions for the experiment were the same as for group I.

Group III of 9 animals took part in the test from October 5, 1957 to July 3, 1958. It was fed cholesterol dissolved in margarine for twenty days more than group I (until April 9, 1958). From that time the animals were fed the basic diet without the addition of margarine and cholesterol until May 15, 1958 when 20 ml of soy oil was added daily to the diet, until July 3, 1958. Of this group two rabbits died during the course of the experiment.

Group IV of 6 animals was kept under the same conditions as group III but from April 9, 1958 until they were killed (by air embolism) this group was only on a basic diet. At the end of this experiment only four rabbits were left from these animals.

We used soy oil from refined soya from which objectionable taste and odor had been removed prepared by a new method in the national enterprise SOJA. According to information from the experimental and inspection institute of the food industry in Prague its iodine number was 131.8 to 133.3; Reichert-Meissl number under 1; percentage of free fatty acids 0.24 to 0.29. (The iodine number of the currently used margarine is 54 to 79, Reichert-Meissl number 189 to 210, percentage of free fatty acids 0.05 to 0.2.) The analysis, made by eng. Pokorny at the College for Chemical Technology, Department of Food Inspection, showed that the soy oil used contained 42.8% oleic, 39.7 linoleic, 7.9 linolenic acids and 9.6% saturated fatty acids.*

The cholesterol in the serum for the rabbits was fixed according to Abel. Atheromatosis of the aorta was evaluated by a graduated scale from 1 to 5 degrees (viz Fodor et al, 1958).

In determining the lipolytic activity (Zemplenyi, Grafnetter 1958a,b) it is necessary , in principle, to incubate the human lipemic serum with a finely dissected specimen of the aorta carefully cleaned from the periaortal tissue and adventitia. We determine the degree of lipolysis by determining the quantity of split-off non-esterified fatty acids. (See Table 3). For the histochemical analysis sections were provided on the freezing microtome from a non-fixed

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*We thank Dr. Vavrikova for furnishing some of the cited values.

aorta (for evidence of hydrogenase of succinic acid) and from the aorta after a four-hour fixation with calcium-chloride formol according to Baker (1944). (Sections 15 μ thick.) We fixed other samples with cooled-off acetone, poured it into paraffin by the standard method (Lojda 1958) and sliced it to 10 μ. We verified the non-specific esterase by an azocopulating reaction (Lojda 1958) with substrata alpha naphthylacetate, alpha naphthylpropionate and alpha naphthylbutyrate, and also naphthol-AS-acetate. Tetrazofluoroborate O-dianisidine, or diazofluoroborate 5-chlor-2-toluidine was used as a copulating agent. We also used the indoxylacetate method according to Holt and Witherse (for instructions see Pearse 1953) and Gomori's method with Tween 80 as a substrata (instructions by Gomori 1952). We verified alkaline and acid phosphatase with azocopulative reactions in our own modification (Lojda 1958) with alpha naphthylphosphate as a substrata and diazofluoroborate 4-benzamido-2,5-dimethyloxylaniline, or tetrazofluoroborate o-dianisidine as copulative agents with pH 9.2 or 5.9. The system of hydrogenase of succinic acid was verified according to Farber and Louvier (1956) with tetrazolium blue in a nitrogen atmosphere and according to Rosy and Velard (1954) with neotetrazolium with cyanide blocking of cytochromoxydase.

Results

A. Cholesterolemia and atheromatosis of the aorta

In Table 1 the values of cholesterolemia and levels of atherosclerosis in rabbits which were fed soy oil as a preventive (group number II) are arranged in comparison with the group on cholesterol and margarine. It is evident that soy oil in the dosage used did not have an effect on the resulting atheromatosis. The difference in the

average degree of atheromatous changes (3.55 against 3.33) is not statistically significant. The average level of cholesterol in the blood was on both January 9, 1958 and February 24, 1958 lower in the rabbits on soy oil but the statistical analysis again did not show a significant difference.

In Table 2 the results on the regression of atherosclerosis are shown. In view of the fact that the basic condition in such an arrangement of the test should be that before interruption of the cholesterol feeding the degree of the achieved atheromatosis should be approximately equal in all animals, it was necessary subsequently to eliminate from group III rabbits number 122 and 123 in which for unascertainable reasons cholesterol did not increase. The results do not show the difference in the degree of atherosclerosis between the group of rabbits with the soy oil and with margarine. As far as cholesterolemia is concerned the individual variability in the animals of both groups is striking. However a tendency to a greater decrease under the effect of soy oil was not seen.

B. Lipolytic activity of the aorta

The values of lipolytic activity (applied to damp weight) and the degrees of atheromatosis in rabbits fed cholesterol dissolved in margarine (group I) or in soy oil (group II) are shown in Table 3. In this table the values in 9 normal rabbits on the basic diet are shown.

The lipolytic activity of the aorta rises with the degree of atheromatous changes. (Viz Zemplenyi and Grafnetter 1958c, 1959b). This rise is striking especially in group I in the values listed in the table. The statistical analysis shows that in this group

there is a close linear dependence between the degree of atheromatosis and the lipolytic activity of the aorta expressed by a high value of the coefficient of correlation ($r=0.9493$), with a possibility of error less than 0.001. The regression straight line is in this case of the form $y= -1.73 + 2.257x$. In group II the coefficient of correlation is lower ($r= 0.6704$), however there is no significant difference between the regressive straight line in this group ($y= 1.03 + 1.375x$) and between the regressive straight line of the preceding group. (The test of significance conducted both for the direction of the straight lines and for the section on axis y.)

This then means that a soy oil diet did not affect the increase of the activity of the lipolytic enzymatic system during the course of the cholesterol fattening.

C. Histochemia of some enzymes of the aorta

The localization of esterase proved by the azocopulative method and the indoxylitic method was basically the same in the normal animals through the entire aorta. The aorta with the naphthyl-acetate gave the most intensive reaction. The reaction weakened with the increasing number of carbons in the chain of fatty acid from which the naphthol was esterified and to achieve equal intensity further incubation was necessary. Even with the use of naphthol-AS-acetate as a substrata the aortas gave a weaker reaction.

In a normal rabbit (Illustration 1) the reaction is negative in the intima, in the media the reaction is positive in the muscular cells between the elastic membranes. The reaction becomes stronger in the outward direction. The adventitial fibrocytes also give a positive reaction.

The reaction to the alkaline phosphatase is negative in the intima and the media. The reaction to the acid phosphatase is mildly positive in the media in the muscular cells. It cannot be said from the reactions made whether the intima also reacts normally.

Both reactions used on the system of dehydrogenase of succinic acid showed grains of formazine in the media between the elastic membranes (in the muscular cells).

In the atheromatous aorta of the rabbit in addition to discovery of lipophagy a reaction was also discovered in the intima to non-specific esterase. This reaction is very intensive in the beginning and we can see it in the entire extent of the fattened intima (Illustration 2). We cannot say whether it is localized also in addition to the lipophagy but we are now studying exact localization with an improved technique allowing for an accurate localization also on the cellular level. As soon as the atheromatous plate becomes enlarged and cholesterol is discovered in it/in a histochemically proven quantity, the reaction is localized only in some places. (Closely under the endothelium and in the depth near the media.) In the interior of the plate there is a reaction in the macrophages grouped in some places into larger nests (Illustration 3).

It is known that the reaction to non-specific esterase with naphthylacetate also proves cholinesterase. In this connection we call attention to an interesting finding of positively reacting cholinenergetic nerve fibers working their way from the subintimal plexus to the atheromatous plate. Reaction in the media remains the same as in the control animals.

In the atheromatous plate there is also a reaction to alkaline phosphatase (Illustration 4) whereas in the controls the reaction is negative in the intima and the media. Also the reaction to acid phosphatase is strongly positive in the atheromatous plate, especially in the macrophage (Illustration 5).

We succeeded also in proving the presence of a system of hydrogenase of succinic acid in the atheromatous plate.

From the histochemical side we did not find any difference between the animals on a soy oil diet and the animals that were not fed soy oil.

Discussion

Our results show that under our experimental conditions the soy oil used did not have an effect on the development of experimental atheromatosis in the aorta of rabbits and also the recession of atheromatous changes was not hastened with the application of this preparation.

Favorable results can be expected especially for the content of sitosterol and for the content of unsaturated fatty acids. in soy oil.

In the introduction we stated that reports regarding the use of sitosterol in experimental and clinical atherosclerosis are not clear. It seems that they will have an effect on cholesterolemia (by decreasing the resorption of cholesterol from the intestine) only with a very large dose. In man a large dosage creates great difficulties and for this reason a number of distinguished researchers completely abandonned the use of sitosterol in treating atherosclerosis. (Viz group discussion in June 1953.) The amount of sitosterol

given under our experimental conditions obviously was not so great as to have an effect on cholesterolemia or the degree of atheromatosis of the aorta.

As far as unsaturated fatty acids are concerned it has been clearly proven that a diet rich in unsaturated fats decreases cholesterolemia in man. However, thus far there is no clear evidence that this decrease goes hand in hand with the withdrawal of atherosclerotic changes. Long-term research aimed in this direction is being conducted in various research centers. (For example, in Sweden and Denmark, according to information given personally by Prof. Malmrose.)

Our negative results in producing cholesterolemia and morphological changes in rabbits are well in agreement with recent reports in the literature. Thus, for example, Lambert et al (1958) did not find differences in the degree of cholesterol experimental atheromatosis in rabbits on a diet of hydrogenated fat. Steiner et al (1959) also could not bring about cholesterol atheromatosis in rabbits with corn oil. Merrill (1959) even found a gradual effect of linoleic acid and corn oil on atheromatous changes. The results of Stamler et al (1959) were also negative in chickens with cholesterol atherosclerosis. For example Diller et al (1957) did not bring about cholesterolemia in rats with a diet containing fatty acids with various degrees of saturation.

In the light of our negative experiences and those abroad with cholesterol atheromatosis - in comparison with human disorders - it seems to be an important contribution for the study of atherosclerosis that in rabbits it is necessary to bring about atherosclerotic changes also with feeding pure saturated (hydrogenated) fats without

an admixture of cholesterol (Lambert et al 1958 and others). However, for the creation of these vascular changes it is necessary to actively resist feeding a diet containing a high content of unsaturated fatty acids. For this reason it is possible that this pattern of atherosclerosis is closer to the human disorder than cholesterol atheromatosis.

Our results further show that there was no difference between rabbits on cholesterol feed given margarine or soy oil even in the enzymatic properties of the aorta especially in its lipolytic and esterolyic capabilities.

As we have called attention to it before (Zemplenyi, Grafnetter 1958c, 1959b, Lojda, Zemplenyi 1958), during the course of development of rabbit atheromatosis a functional adaptation of the lipolytic and esterolytic enzymatic systems occurs. An increase in non-specific esterase in the intima of atheromatous aortas, as is seen from this study, occurs chiefly in macrophages. This is in agreement with previous evidence of increased esterase conducted by Gomori's method with Tween (Gomori 1946, McMillan et al 1954, Narpozzi 1957). Our findings are, however, more explicit and we have more exact localization. With this we consider especially noteworthy the fact that in all the large plates, with an accumulation of a larger amount of cholesterol, the activity is lower in contrast to other peripheries.

The products of fatty metabolism can be partially oxidized on the spot evidence of which is given by the presence of dehydrogenase of succinic acid in the fattened intima. We must consider the alkali and acid phosphatase in the plates also as adaptive enzymes encroaching probably on the metabolism of phospho-lipids

and to proteosynthesis. The fact is that it is known that in atheromatous aortas there are demonstrable changes in the proteosynthesis (Kricman and Bavina, 1953) and Zilversmit et al (1954) showed with the aid of radioisotopes that phospholipids are synthetisized in the atheromatous aortas of rabbits five times more rapidly than in normal aortas.

Our research then has uncovered several new aspects of the enzymatic properties of atheromatous rabbit aortas. However they do not show the special effect of soy oil even in this direction.

We wish to thank J. Hajkova, J. Dvorakova and E. Prochazkova for technical assistance and eng. M. Bartonicek for statistical preparation of the results.

Table 1. Effect of soy oil on the development of atheromatosis of the aorta and on cholesterolemia.

Tab. 1. Působení sójového oleje na vývoj ateromatózy aorty a na cholesterolémii.

1 Skupina	4 Králík čís.	5 Cholesterolémie v mg% 4. 9. 1957	9. 1. 1958	24. 2. 1958	6 Stupeň ateromatózy
2 I (Cholesterol + margarin)	109	98	725	1080	5
	107	79	473	920	4,5
	106	97	380,5	1156	2,5
	103	95	787	1104	4,5
	104	69	181,5	387,2	3,5
	102	77	1032	1180	4
	105	94	72,0	234	2
	101	90	832	1280	3,5
	108	103	684	784	5
3 II (Cholesterol + sójový olej)	114	84,5	852	1216	5
	110	102	430	1052	4
	7/0	109	185,2	392	2,5
	59/0	88	577	1090	4,5
	100	56	320	724	3
	9	—	521	489	3
	18	—	175	292	2
	3	—	1160	1090	5
	10	—	580	980	3

1. Group, 2. I (Cholesterol + margarine, 3. II (Cholesterol + soy oil)

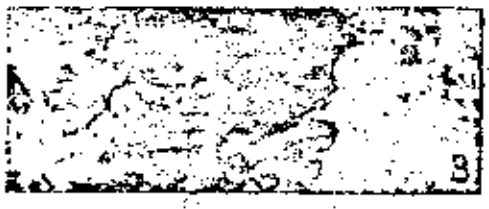
4. Rabbit number, 5. Cholesterolemia in mg%, 6. Degree of atheromatosis

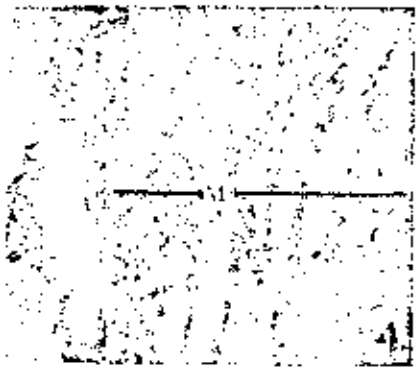
Table 2. Effect of soy oil on the regression of atheromatosis of the aorta and on cholesterolemia.

Tab. 2. Působení sojového oleje na regresi ateromatózy aorty a na cholesterolémii.

1 Skupina	4 Králík čís.	5 Cholesterolémie v mg % 4. 9. 1957	9. 1. 1958	24. 2. 1958	25. 3. 1958	17. 4. 1958	15. 5. 1958	11. 6. 1958	3. 7. 1958	6 Stupeň ateromatózy
III 2	8/0	86,0	870,0	1320,0	444,0	166	41,4	27,2	20,9	2,5
(Cholesterol +	10/0	47,0	1042,0	1172,0	1700,0	765	186,0	121,0	46,3	5,0
margarin do	59/0	88,0	235,0	338,0	1822,0	260	60,0	18,0	21,5	1,0
9. 1. 1958,	122,0	62,0	28,5	22,0	31,5	39	21,4	18,0	12,2	4,0
sójový olej od	123,0	64,6	147,5	524,0	1406,0	548	208,0	404,0	—	4,0
15. 5. 1958)	113,0	67,5	32,0	71,6	—	28	50,0	14,5	—	2,0
	120,0	90,0	688,0	1202,0	1160,0	542	109,6	57,0	50,0	5,0
IV 3	75/7	56,0	812,0	—	903,0	493	1111,5	476,0	257,5	4,0
(Cholesterol +	5/0	73,6	141,2	1152,0	642,0	246	170,0	35,2	27,0	1,0
margarin do	4/0	66,0	1028,0	824,0	544,0	220	101,5	107,5	32,5	4,5
9. 4. 1958)	117	77,0	702,0		1040,0	808	648,0	305,0	103,3	3,5

1. Group, 2. III (Cholesterol + margarine to 4/9/1958, soy oil from 5/15/1958), 3. IV (Cholesterol + margarine to 4/9/1958), 4. Rabbit number, 5. Cholesterolemia in mg%, 6. Degree of atheromatosis





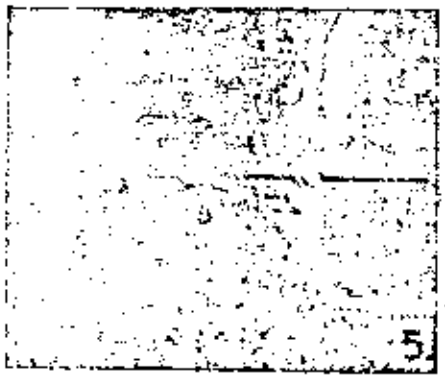


Illustration 1. Non-specific esterase in the aorta of normal rabbits. Positive reaction is evident in the muscular cells of the outer part of the media. Reaction in the intima is practically negative. In the adventitia fibrocytes react weakly. Azocopulative reaction. Magnification about 170 times. I = intima, M= media, A = adventitia.

Illustration 2. Non-specific esterase in the aorta of a rabbit with experimental atheromatosis. Strong reaction in the intima. In comparison with the intima the reaction in the media is weaker. Indoxyl method. Magnification about 80 times.

Illustration 3. Non-specific esterase in the atheromatous aorta of a rabbit. More advanced stage. Reaction is evident on the surface and on the bottom of the atheromatous plate. Inside the plate nests

of macrophages. Azocopulative reaction. Magnification about 170 times.

Illustration 4. Alkaline phosphatase in the atheromatous aorta of a rabbit. The positively reacting elements of the fattened intima can be seen. Azocopulative reaction. Magnification about 170 times.

Illustration 5. Acid phosphatase in the atheromatous aorta of a rabbit. Intensively positive reaction in the macrophages of the plate, weaker reaction in the media in the muscular cells. Azocopulative reaction. Magnification about 170 times.

Table 3. Lipolytic activity of the aorta (in micromoles of non-esterified fatty acids per gram of moist tissue) and the degree of atheromatosis.

Tab. 3. Lipolytická aktivita aorty (v mikromolech nesterifikovaných mastných kyselin na gram vlhké tkáně) a stupeň aterosklerózy.

1 Králíci krmení cholesterolem a sójovým olejem (skup. II)		4 Králíci krmení cholesterolem a margarinem (skup. I)		7 Králíci na základní stravě	
2 lipolyt. akt.	3 stupeň atero	5 lipolyt. akt.	6 stupeň atero	8 lipolyt. akt.	9 stupeň atero
2,68	3	3,22	2,5	2,94	0
3,36	3	3,84	2	2,70	0
5,20	2,5	5,52	3,5	3,92	0
5,50	2	6,06	3,5	4,69	0
5,62	3	7,24	4	5,00	0
6,12	4	7,92	4,5	5,04	0
6,12	5	9,00	5	5,62	0
8,76	4,5	11,06	5	5,74	0
9,80	5			5,84	0

1. Rabbits fed cholesterol and soy oil (group II), 2. lipolytic activity, 3. degree of atheromatosis, 4. Rabbits fed cholesterol and margarine (group I), 5. lipolytic activity, 6. degree of atheromatosis, 7. Rabbits on basic diet, 8. lipolytic activity, 9. degree of atheromatosis

Conditions for the experiment: Finely cut, accurately weighed sample of aorta was incubated under constant movement in the incubating vessels 150 min. at 37°C with 2 ml of substrata. As a substrata male lipemic serum was used diluted 1:1 with a phosphate stabilizer.pH 7.38. The released fatty acids determined by Dole's method.

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T. Z., Praha-Krč, Budějovická 830

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PŮSOBENÍ SÓJOVÉHO OLEJE NA EXPERIMENTÁLNÍ ATEROMATÓZU AORTY A NA NĚKTERÉ ENZYMY CÉVNÍ STĚNY U KRÁLÍKA*)

T. Zemplényi, Z. Lojda, D. Grafnetter, J. Fodor, V. Felt

Ústav pro choroby oběhu krevního, Praha-Krč, přednosta prof. MUDr. K. Weber — Embryologický ústav KU v Praze, přednosta prof. MUDr. Z. Frankenberger

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Dietní ovlivnění aterosklerózy je jedním z nejaktuálnějších problémů ve výzkumu této nemoci. Kinsell a spol. r. 1952 zjistili, že některé rostlinné oleje snižují u člověka cholesterolémii. Ke stejným závěrům dospěli i Hardinge a Stare (1954), Bronte-Stewart a spol. (1955) a jiní autoři. Malmros a Wigand (1955, 1957), Beveridge a spol. (1956), Sinclair (1956), Ahrens a spol. (1957) a Keys a spol. (1957) ukázali význam nenasycených, respektive nezbytných mastných kyselin při účinku rostlinných olejů.

Je ovšem nutné upozornit na to, že podle Beveridge a spol. (1957) a jiných je z tohoto hlediska důležitý i sterolový obsah rostlinných olejů. Peterson totiž ukázal již r. 1951 a 1952, že přídavek smíšených sójových sterolů a čištěných sitosterolů ke stravě snižuje u kuřat plazmatický a jaterní cholesterol a snižuje i stupeň aterosklerózy. U králíků dosáhl podobného efektu se surovým sójovým olejem Pollak (1953), kdežto Dreisbach a spol. (1953) a Clarcson a spol. (1956) dospěli k negativním závěrům. Sójový olej obsahuje především sitosterol gama.

Jiní autoři použili sitosterolu beta, obsaženého např. v bavlníkovém a klíčkovém oleji. Beher a spol. (1956), Heptinstall a Porter (1957) referují o příznivém účinku sitosterolu beta u králíků. Best a spol. (1954), Sachs a Weston (1956), Farquhar a spol. (1956) a jiní mají příznivé výsledky i u lidí.

Sójový olej je zajímavý i proto, že obsahuje hodně lecithinu. Perorálně podávaný lecithin (Keston a spol. 1952) a dlouhodobá nitrožilní infúze lecithinu (Friedman a spol. 1957, Felt a spol. 1958) snižuje u králíků stupeň cholesterolové ateromatózy. Morrison píše o příznivém působení lecithinu při perorální aplikaci i u lidí (1958).

Z uvedených důvodů jsme považovali za správné vyzkoušet účinek surového nerafinovaného a odhořčeného sójového oleje (dodaného národním podnikem SOJA) na vývoj a na regresi cholesterolové ateromatózy aorty u králíka.*) Současně nás zajímala otázka, zda se mění lipolytická a esterolytická schopnost a jiné enzymatické vlastnosti aorty paralelně s vývojem ateromatózy a zda se projeví vliv sójového oleje i v tomto směru. Naše dřívější práce (Zemplényi a Grafnetter, 1958a, b, 1959a; Zemplényi, Lojda, Grafnetter, 1958, 1959) totiž připouštějí možnost, že lipolytická a esterolytická aktivita aorty patří k faktorům ovlivňujícím hromadění lipidů v cévě. Jeli tomu tak, lze očekávat, že ovlivnění stupně experimentální ateromatózy aorty by mohlo změnit i tyto enzymatické vlastnosti cévní stěny.

*) V téže době byla použita tohoto oleje u lidí v Ústavu pro výzkum výživy lidu v Praze (viz Vavříková 1958).

Věnováno prof. dr. Kl. Weberovi k 70. narozeninám

Metoda

Na začátku pokusu bylo použito celkem 33 králíků (samců) přibližně 6 měsíců starých, rozdělených do 4 skupin. U zvířat skupiny I, II bylo sledováno ovlivnění vývoje ateromatózy, u skupiny III, IV byla sledována regrese ateromatózy.

I. skupina králíků (9 zvířat) byla v chronickém pokusu od 5. 10. 1957 do 19. 3. 1958. Zvířata jsme denně krmili 0,5 g cholesterolu rozpuštěným v 5 g margarínu, smíšeného s bramborem a ovesným šrotem, takže každý králík obdržel 120 g této směsi denně; dále vojtěšku, ječmen a vodu ad libitum. Od 31. 1. 1958 byla denní dávka cholesterolu zvýšena na 1 g. Zvířata byla chována v individuálních klecích, spotřeba diety byla denně kontrolována.

II. skupina 9 zvířat dostávala cholesterol rozpuštěný v 5 ml sójového oleje. Ostatní pokusné podmínky byly stejné jako u skupiny I.

III. skupina 9 zvířat byla v pokusu od 5. 10. 1957 do 3. 7. 1958. Byla krmena cholesterolem rozpuštěným v margarínu o 20 dní déle než skupina I. (do 9. 4. 1958). Od této doby byla zvířata krmena základní stravou bez přísady margarínu a cholesterolu, a to až do 15. 5. 1958, kdy bylo ke stravě přidáváno denně 20 ml sójového oleje. (Až do 3. 7. 1958.) Z této skupiny uhynuli během pokusu 2 králíci.

IV. skupina 6 zvířat byla chována za stejných podmínek jako skupina III., avšak od 9. 4. 1958 až do zabití (vzduchovou embolií) byla pouze na základní stravě. Ke konci pokusu zůstali z těchto zvířat pouze 4 králíci.

Použili jsme sójového oleje z čištěné a odhořčené sóje, připraveného novým způsobem v nár. podniku SOJA. Podle údajů zkušebního a kontrolního ústavu potravinářského průmyslu v Praze bylo jeho jódové číslo 131,8 až 133,3; číslo Reichertovo-Maislovo pod 1; procento volných mastných kyselin 0,24 až 0,29. (Jódové číslo běžně užívaného margarínu je 54 až 79, Reichertovo-Maislovo číslo 189 až 210, procento volných mastných kyselin 0,05 až 0,2.) Rozbor, provedený Ing. Pokorným na Vysoké škole chem. technol. — katedře kontr. potravin, ukázal, že používaný sójový olej obsahoval 42,6 % olejové, 39,7 % linolové, 7,9 % linolenové kyseliny a 9,6 % nasycených mastných kyselin.*)

Cholesterol v séru králíků byl stanoven podle Abela. Ateromatóza aorty byla posuzována odstupňováním od 1 do 5 stupňů (viz Fodor a spol. 1958).

Při stanovení lipolytické aktivity (Zemplényi, Grafnetter 1958a, b) jde v zásadě o inkubaci lidského lipemického séra s jemně rozkouskovaným vzorkem aorty, pečlivě očištěném od periaortální tkáně a

*) Děkujeme dr. Vavříkové za sdělení některých uvedených hodnot.

Tab. 1. Působení sojového oleje na vývoj ateromatózy aorty a na cholesterolémii.

Skupina	Králík čís.	Cholesterolémie v mg % 4. 9. 1957	9. 1. 1958	24. 2. 1958	Stupeň ateromatózy
I (Cholesterol + margarín)	109	98	725	1080	5
	107	79	473	920	4,5
	100	97	380,5	1150	2,5
	103	95	787	1164	4,5
	104	60	181,5	387,2	3,5
	102	77	1082	1180	4
	105	94	72,9	234	2
	101	90	039	1280	3,5
	108	103	684	784	5
II (Cholesterol + sójový olej)	114	84,5	852	1216	5
	110	102	430	1052	4
	7/0	108	185,2	302	2,5
	53/0	88	577	1090	4,5
	100	50	320	724	3
	9	—	521	489	3
	12	—	175	202	2
	3	—	1160	1000	5
	10	—	539	900	3

adventicie. Stupeň lipolýzy určujeme stanovením množství odštěpených neesterifikovaných mastných kyselin. (Viz tab. 3.) Pro histochemické studium byly pořízeny řezy na zmrazovacím mikrotomu z nafixované aorty (k pro průkaz dehydrogenázy kyseliny jantarové) a z aorty po čtyřhodinové fixaci kalciumchloridovým formolem podle Bakera [1944]. (Řezy síly 15 μ.) Jiné vzorky jsme fixovali vychlazeným acetonem, zalili do parafinu standardním způsobem (Lojda 1958) a krájeli na 10 μ. Nespecifickou esterázu jsme prokazovali azokopulační reakcí (Lojda 1958) se substráty alfa naftylacetátem, alfa naftylpropionátem a alfa naftylbutyrátem, jakož i naftol-AS-acetátem. Jako kopulačních činidel bylo použito tetrazofluoroborátu o-dianisidinu, popř. diazofluoroborátu 5-chlor-2-toluidinu. Dále jsme použili indoxylacetátové metody podle Holta a Withorse (předpis viz Pearse 1953) a Gomoriho metody s Tweenem 80 jako substrátem (předpis Gomori 1952). Alkalickou a kyselou fosfatázu jsme prokazovali azokopulačními reakcemi ve vlastní modifikaci (Lojda 1958) s alfa naftylfosfátem jako substrátem a diazofluoroborátem 4-benzamido-2,5-dimethyloxyanilinu, respektive tetrazofluoroborátem o-dianisidinu jako kopulačními činidly při pH 9,2, respektive 5,9. Systém dehydrogenázy kyseliny jantarové byl prokazován podle Farbera a Louviera [1956] s tetrazoliovou modří v dusíkové atmosféře a podle Rosy a Velarda [1954] s neotetrazoliem s kyanidovým blokováním cytochromoxydázy.

Výsledky

A. *Cholesterolémie a ateromatóza aorty*

V tab. 1 jsou sestaveny hodnoty cholesterolémie a stupně ateroskterózy u králíků, kterým byl podáván sójový olej preventivně (skup. č. II) ve srovnání se skupinou na cholesterolu a margarinu. Je zřejmé, že sójový olej v použitém dávkování neovlivnil výslednou ateromatózu. Rozdíl v průměrném stupni ateromatózních změn (3,55 proti 3,83) není statisticky významný. Průměrná hladina cholesterolu v krvi byla jak dne 9. 1. 1958, tak i dne 24. 2. 1958 nižší u králíků na sójovém oleji, avšak statistický rozbor opět neukázal signifikantní rozdíl.

V tab. 2 jsou sestaveny výsledky týkající se regrese aterosklerózy. Vzhledem k tomu, že základním požadavkem při takovém uspořádání pokusu má být, aby před přerušením cholesterolového krmení byl stupeň dosažené ateromatózy přibližně stejný u všech zvířat, bylo nutné dodatečně vyřadit ze skupiny III králíky č. 122 a 113, u kterých z nezjištěných příčin nedošlo k vzestupu cholesterolému. Výsledky neukazují rozdíl ve stupni ateroskterózy mezi skupinou králíků se sójovým olejem a s margarinem. Pokud jde o cholesterolémii, je nápadná individuální variabilita u zvířat obou skupin. Není však vidět tendenci k většímu poklesu pod vlivem sójového oleje.

B. *Lipolytická aktivita aorty*

V tab. 3 jsou sestaveny hodnoty lipolytické aktivity (vztahované na vlhkou váhu) a stupně ateromatózy u králíků krmených cholesterolem rozpuštěným v margarinu (skup. I), respektive v sójovém oleji (skup. II). V téže tabulce jsou uvedeny hodnoty u 9 normálních králíků na základní stravě.

Se stupněm ateromatózních změn stoupá lipolytická aktivita aorty. (Viz Zemplényi a Grafnetter 1958c, 1959b.) Toto stoupání je u hodnot uvedených v tabulce nápadné zejména u skupiny I. Statistický

Tab. 2. Působení sójového oleje na regresi ateromatózy aorty a na cholesterolémii.

Skupina	Králík čís.	Cholesterolémie v mg % 4. 9. 1957	9. 1. 1958	24. 2. 1958	25. 3. 1958	17. 4. 1958	15. 5. 1958	11. 6. 1958	3. 7. 1958	Stupeň ateromatózy
III (Cholesterol + margarín do 9. 1. 1958, sójový olej od 15. 3. 1958)	8/0	80,0	870,0	1320,0	414,0	166	41,4	27,2	20,0	2,5
	10/0	47,0	1042,0	1172,0	1700,0	705	186,0	121,0	46,5	5,0
	50/0	88,0	235,0	338,0	1822,0	260	60,0	18,0	21,5	1,0
	122,0	62,0	28,5	22,0	31,5	30	21,4	18,0	12,2	4,0
	123,0	84,5	147,5	524,0	1400,0	548	208,0	404,0	—	4,0
	113,0	67,5	32,0	71,6	—	28	50,0	14,5	—	2,0
	120,0	90,0	688,0	1202,0	1160,0	542	108,8	57,0	50,0	5,0
IV (Cholesterol + margarín do 9. 4. 1958)	75/7	56,0	812,0	—	908,0	493	1111,5	470,0	257,5	4,0
	5/0	73,6	144,2	1152,0	612,0	246	110,0	38,2	27,0	1,0
	4/0	66,0	1028,0	824,0	514,0	220	101,5	107,5	32,5	4,5
	117	77,0	792,0		1050,0	808	818,0	307,0	103,3	3,5

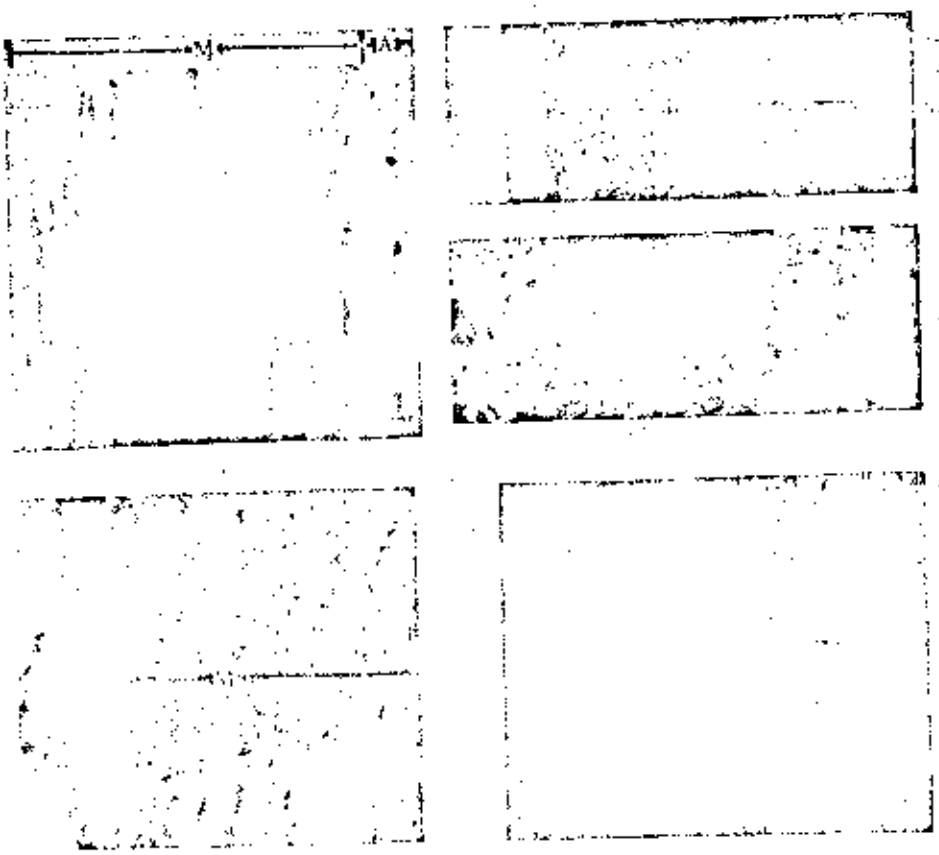
rozbor ukazuje, že v této skupině je těsná lineární závislost mezi stupněm ateromatózy a lipolytickou aktivitou aorty vyjádřena vysokou hodnotou koeficientu korelace (r = 0,9493), při pravděpodobnosti omylu menší než 0,001. Regresní přímka je v tomto případě tvaru y = — 1,73 + 2,257 x. Ve II. skupině je koeficient korelace nižší (r = 0,6704), avšak není signifikantního rozdílu mezi regresní přímkou v této skupině (y = 1,03 + 1,375 x) a mezi regresní přímkou předcházející skupiny. (Test významnosti proveden jak pro směrnice přímek, tak i pro úsek na ose y.)

To tedy znamená, že krmení sójovým olejem neovlivnilo vzestup aktivity lipolytického enzymatického systému během cholesterolového žiru.

C. Histochemie některých enzymů aorty

Lokalizace esterázy, prokazované azokopulační metodou i indoxylovou metodou, byla u normálních zvířat v průběhu celé aorty v podstatě stejná. Nejintenzivnější reakci dávala aorta s naftylacetátem. Se stoupajícím počtem uhlíků v řetězci mastné kyseliny, s níž byl naftol esterifikován, se reakce zeslabovala a k dosažení stejné intenzity byla nutná delší inkubace. Slabší reakci dávaly aorty i s použitím naftol-AS-acetátu jako substrátu.

U normálního králíka (obr. 1) je reakce v intimě negativní, v médii je pozitivní reakce ve svalových buňkách mezi elastickými blankami.



Obr. 1. Nespecifická esteráza v aortě normálního králíka. Pozitivní reakce je patrna ve svalových buňkách zevní části médie. Reakce v intimě je prakticky negativní. V adventicii reagují slabě fibrocyty. Azokopulační reakce. Zvětšení asi 170krát. I = intima, M = média, A = adventicia. Obr. 2. Nespecifická esteráza v aortě králíka s experimentální ateromatózou. Silná reakce v intimě. Ve srovnání s intimou je reakce v médii slabší. Indoxylová metoda. Zvětšení asi 80krát. — Obr. 3. Nespecifická esteráza v ateromatózní aortě králíka. Pokročilejší stadium. Reakce je patrna na povrchu a na spodině ateromatózního plátu. Uvnitř plátu reagují hnízda makrofágů. Azokopulační reakce. Zvětšení asi 170krát. — Obr. 4. Alkalická fosfatáza v ateromatózní králičí aortě. Je vidět pozitivně reagující elementy ztluštělé intimy. Azokopulační reakce. Zvětšení asi 170krát. — Obr. 5. Kyselá fosfatáza v ateromatózní aortě králíka. Intenzivně pozitivní reakce v makrofázích plátu, slabší reakce v médii ve svalových buňkách. Azokopulační reakce. Zvětšení asi 170krát.

Směrem zevním se reakce zesiluje. Také adventiciální fibrocyty dávají pozitivní reakci.

Reakce na alkalickou fosfatázu je v intimě i médii negativní. Reakce na kyselou fosfatázu je slabě pozitivní v médii ve svalových buňkách. Z provedených reakcí nelze říci, zda reaguje normálně i intima.

Obě použité reakce na systém dehydrogenázy kyseliny jantarové ukázaly zrna formazinu v médii mezi elastickými membránami (ve svalových buňkách).

V ateromatózní králičí aortě se zároveň s objevením lipofágů objevuje v intimě také reakce na nespecifickou esterázu. Tato reakce je zpočátku velmi intenzivní a můžeme ji vidět v celém rozsahu ztluštělé intimy (obr. 3). Zdá se [illegible]

vána i mimo lipofágy, nemůžeme říci, ale přesnou lokalizaci studujeme nyní zlepšenou technikou dovolující přesnou lokalizaci i na buněčné úrovni. Jakmile se ateromatózní plát zvětší a objeví se v něm v histochemicky prokazatelném množství cholesterol, je reakce lokalizována pouze na některých místech. (Těsně pod endotelem a v hloubce při médii.) Uvnitř plátu je reakce v makrofázích seskupených na některých místech do větších hnízd (obr. 3).

Je známo, že reakce na nespecifickou esterázu s naftylacetátem prokazuje také cholinesterázu. V této souvislosti upozorňujeme na zajímavý nález pozitivně reagujících cholinergických vláken nervových, pronikajících ze subintimální pleteně do ateromatózního plátu. Reakce v médii zůstává stejná jako u kontrolních zvířat.

V ateromatózním plátu se objeví také reakce na alkalickou fosfatázu (obr. 4), kdežto u kontrol je reakce v intimě i médii negativní. Rovněž reakce na kyselou fosfatázu je silně pozitivní v ateromatózním plátu, hlavně v makrofázích (obr. 5).

V ateromatózním plátu se nám podařilo prokázat i přítomnost systému dehydrogenázy kyseliny jantarové.

Po histochemické stránce jsme nenalezli rozdílů mezi zvířaty krmenými sójovým olejem a zvířaty, která sójovým olejem krmena nebyla.

Rozprava

Naše výsledky ukazují, že použitý sójový olej nedovedl za našich pokusných podmínek ovlivnit vývoj experimentální ateromatózy králičí aorty a rovněž ústup ateromatózních změn nebyl urychlen při aplikaci tohoto přípravku.

Příznivý účinek by bylo možno očekávat především po obsah sitosterolů a pro obsah nenasycených mastných kyselin v sójovém oleji.

V úvodu jsme uvedli, že zprávy o použití sitosterolu při experimentální i klinické ateroskleróze nejsou jednoznačné. Zdá se, že dovedou ovlivnit cholesterolémii (snížením resorpce cholesterolu ze střeva) až při velmi vysokém dávkování. U člověka vysoké dávkování naráží na veliké obtíže, a proto řada významných badatelů zcela upustila od použití sitosterolu při léčení ateroskleózy. (Viz skupinová diskuse v červnu 1958.) Množství sitosterolu dodaného za našich experimentálních podmínek zřejmě nebylo natolik vysoká, aby ovlivnilo cholesterolémii anebo stupeň ateromatózy aorty.

Pokud jde o nenasycené mastné kyseliny, je dnes jasně prokázáno, že dieta bohatá na nenasycené tuky snižuje cholesterolémii u člověka. Nejsou však dosud známy jednoznačné doklady o tom, že by toto snížení šlo ruku v ruce s ústupem aterosklerotických změn. Dlouhodobé pokusy zaměřené tímto směrem jsou v běhu na různých pracovištích. (Např. ve Švédsku a Dánsku, podle osobního sdělení prof. Malmroso.)

Naše negativní výsledky s ovlivněním cholesterolémie a morfologických změn u králíka jsou v dobrém souladu s čerstvými zprávami z literatury. Tak např. Lambert a spol. (1958) nenašli rozdíly ve stupni cholesterolové experimentální ateromatózy u králíků krmených hydrogenovaným tukem

Tab. 3. Lipolytická aktivita aorty (v mikromolech neesterifikovaných mastných kyselin na gram vlhké tkáně) a stupeň ateromatózy.

Králíci krmeni cholesterolem a sójovým olejem (skup. II)		Králíci krmeni cholesterolem a margarínem (skup. I)		Králíci na základní stravě	
lipolyt. akt.	stup. atero	lipolyt. akt.	stup. atero	lipolyt. akt.	stup. atero
2,08	3	3,22	2,5	2,08	0
3,36	3	3,84	2	2,70	0
5,20	2,5	5,52	3,5	3,92	0
5,50	2	6,06	3,5	4,60	0
6,02	3	7,24	4	5,00	0
6,12	4	7,92	4,5	5,04	0
6,12	5	9,00	5	5,62	0
8,76	4,5	11,06	5	5,74	0
9,80	5			5,84	0

Podmínky pokusu: Jemně rozkouskovaný, přesně zvážený vzorek aorty byl inkubován za králího pohybu inkubačních nádobek 150 min. při 37° C a 2 ml substrátu. Jako substrátu bylo použito mužského lipemického séra zředěného 1 : 1 fosfátovým ústojem při 7,38. Uvolněné mastné kyseliny stanoveny Dolcovou metodou.

anebo světlicovým olejem. Rovněž Steiner a spol. (1959) nedovedli ovlivnit králičí cholesterolovou ateromatózu kukuřičným anebo světlicovým olejem. Merrill (1959) dokonce nachází stupňující vliv linolové kyseliny a kukuřičného oleje na ateromatózní změny. Negativní jsou i výsledky Stamlerovy a spol. (1959) u kuřat s cholesterolovou aterosklerózou. U krys neovlivnil např. Dillor a spol. (1957) cholesterolémii dietou obsahující mastné kyseliny o různém stupni nasycení.

Ve světle našich i cizích negativních zkušeností s cholesterolovou ateromatózou — ve srovnání s lidským onemocněním — zdá se být důležitým přínosem pro studium aterosklerózy, že u králíků lze vyvolat aterosklerotické změny i krmením pouhými nasycenými (hydrogenovanými) tuky bez příměsi cholesterolu (Lambert a spol. 1958 a jiní). Avšak vzniku těchto cévních změn lze účinně čelit krmením dietou obsahující vysoký podíl nenasycených mastných kyselin. Je proto možné, že tento model aterosklerózy je bližší lidskému onemocnění než cholesterolová ateromatóza.

Naše výsledky ukazují dále, že nebylo rozdílů mezi králíky na cholesterolovém žíru, krmenými margarínem anebo sójovým olejem, ani v enzymatických vlastnostech aorty, zejména v její lipolytické a esterolytické schopnosti.

Jak jsme již dříve upozornili (Zemplényi, Grafnetter 1958c, 1959b, Lojda, Zemplényi 1958), dochází během vývoje králičí ateromatózy k funkční adaptaci lipolytických i esterolytických enzymatických systémů. Zmnožení nespecifické esterázy v intimě ateromatózních aort, jak je vidět z této práce, je hlavně v makrofázích. To je ve shodě s dřívějším průkazem zmnožené esterázy, provedeným Gomoriho metodou s Tweenem (Gomori 1946, McMillan a spol. 1954, Narpozzi 1957); naše nálezy jsou však výraznější a máme přesnější lokalizaci. Za zvlášť pozoruhodné považujeme při tom to, že ve velkých plátech, s nahromaděním většího množství choleste-

rolu, je na rozdíl od jiných okrsků aktivita nízká.

Produkty tukového metabolismu mohou být částečně oxydovány na místě, jak o tom svědčí přítomnost dehydrogenázy kyseliny jantarové ve ztluštělé intimě. Alkalickou a kyselou fosfatázu v plátech lze považovat rovněž za adaptační enzymy, zasahující pravděpodobně do metabolismu fosfolipidů a do proteosyntézy. Je totiž známo, že v ateromatozních aortách jsou prokazatelné změny v proteosyntéze (Kriemann a Bavina, 1953) a Zilversmit a spol. pomocí radioizotopů ukázali [1954], že fosfolipidy jsou v ateromatózních aortách králíků syntetizovány 5krát rychleji než v normálních aortách.

Naše studie odkrývají tedy několik nových aspektů enzymatických vlastností ateromatózní králičí aorty, avšak neukazují speciální působení sójového oleje ani v tomto směru.

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Souhrn

1. Byl studován účinek sójového oleje v potravě na ateromatózu aorty u králíčích samců na cholesterolovém žíru. Sójový olej nijak neovlivnil ani rozvoj, ani regresi ateromatózy aorty ve srovnání se skupinou kontrolních zvířat.

2. Studium lipolytické aktivity a některých histochemicky prokazatelných enzymů aorty rovněž neprokázalo žádný účinek diety obsahující sójový olej na změny enzymatické aktivity spojené s rozvojem ateromatózy.

3. V normálních králičích aortách je histochemická reakce na nespecifickou esterázu pozitivní především ve svalových buňkách média mezi elastickými blankami. V ateromatózních aortách se objevuje pozitivní reakce ve ztluštělé intimě, a to hlavně v makrofázích. Ve velkých plátech je však reakce slabší. V ateromatozní aortě je v intimě pozitivní i reakce na alkalickou fosfatázu, na kyselou fosfatázu (v normálních aortách v médii) a na dehydrogenázu kyseliny jantarové (normálně ve svalových buňkách v médii). Reakce v médii zůstávají v ateromatózních aortách nezměněny.

Выводы

Земленки Т., Лойда З., Графнеттер Д., Фодор Й., Фелт В.: **Действие соевого масла на экспериментальный атероматоз аорты и на некоторые энзимы сосудистой стенки у кроликов**

1. Производилось исследование влияния соевого масла на атероматоз аорты у кроликов, кормленных пищей, содержащей большое количество холестерола. По сравнению с контрольной группой животных соевое масло не оказывало никакого влияния на развитие, или на регресс атероматоза аорты.

2. Результаты изучения липолитической активности и некоторых энзимов аорты, подающихся выявлению при помощи гистохимического исследования, также показывают, что диета, содержащая соевое масло, не оказывает никакого влияния на изменения активности энзимов, находящейся в связи с развитием атероматоза.

3. В нормальных аортах кроликов является гистохимическая реакция на неспецифическую эстеразу положительной главным образом в мышечных клетках t. media между эластическими оболочками. В атероматозных аортах реакция является положительной в утолщенной t. intima главным образом в макрофагах. В больших пластинках реакция является более слабой. В t. intima атероматозных аорт бывает положительной также и реакция на щелочную фосфатазу, на кислую фосфатазу (в нормальной аорте в t. media) и на дегидрогеназу янтарной кислоты (нормально только в мышечных клетках t. media). Реакции в t. media атероматозных аорт остаются без изменений.

Summary

Zemplényi T., Lojda Z., Grafnetter D., Fodor J., Felt V.: **The Effect of Soya Oil on Experimental Atheromatosis of the Aorta and Some Enzymes of the Arterial Wall in Rabbits**

1. The effect of soya oil in the diet on atheromatosis of the aorta in male rabbits on a cholesterol diet was investigated. The soya oil did not influence the development or regression of the atheromatosis of the aorta as compared with a control group of animals.

2. Investigations of the lipolytic activity and some histochemically detectable enzymes of the aorta did not suggest any effect of the diet containing soya oil, on changes of the enzyme activity associated with the development of atheromatosis.

3. In normal rabbit aortae the histochemical reaction to non-specific esterase is positive particularly in the muscle cells of the media between the elastic membranes. In atheromatous aortae a positive reaction was observed in the thickened intima, particularly in the macrophages. In extensive plaques the reaction is, however, weaker. In the atheromatous aorta the reaction for alkaline phosphatase and acid phosphatase is positive in the intima (in normal aortae in the media) and for succinic dehydrogenase also in the intima (normally in muscle cells of the media). Reactions in the media remain unchanged in atheromatous aortae.

Résumé

Zemplényi T., Lojda Z., Grafnetter D., Fodor J., Felt V.: **Effet de l'huile de soya sur l'athéromatose expérimentale de l'aorte et sur certains enzymes des parois vasculaires chez les lapins**

1. Les auteurs ont étudié l'effet de l'huile de soja dans la nourriture sur l'athéromatose de l'aorte chez des lapins mâles, nourris au cholestérol. L'huile de soya n'a notoirement influencé ni l'évolution ni la régression de l'athéromatose de l'aorte en comparaison avec le groupe des animaux de contrôle.

2. L'étude de l'activité lipolytique et de certains enzymes de l'aorte décelables histochimiquement n'a également accusé aucun effet du régime comprenant de l'huile de soya sur les changements de l'activité enzymatique liés à l'évolution de l'athéromatose.

3. Dans les aortes de lapins normaux, la réaction histochimique à l'estérase non-spécifique est avant tout positive dans les cellules musculaires de la tunique moyenne entre les cellules élastiques. Dans les athéromatoses de l'aorte, une réaction positive se manifeste dans la tunique intime épaissie, et ce, surtout en macrophages. Dans les grandes coupes, la réaction est plus faible. Dans l'aorte athéromateuse, dans la tunique intime sont également positives les réactions à la phosphatase alcaline, à la phosphatase acide (dans les aorte normales, dans la tunique moyenne) et à la déhydrogénase d'acide succinique (normalement dans les cellules musculaires de la tunique moyenne). Les réactions de la tunique moyenne dans les aortes athéromateuses restent inchangées.

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T. Z., Praha-Krč, Budějovická 800

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