FISH OILS VOL III #104
COPIES OF ARTICLES CITED

023

Fish Oils-Volume 3 #104 12/5/73

VOLUME 3

GRAS MONOGRAPH SERIES

FISH OILS 023

(ARTICLES CITED IN TEXT)

prepared for

THE FOOD AND DRUG ADMINISTRATION
DEPARTMENT OF HEALTH, EDUCATION
AND WELFARE

DECEMBER 5, 1973

prepared by

Tracor Jitco, Inc.

Essential Fatty Acid-Deficient Rats: III. Distribution of Lipid Classes in Rat Testes After Feeding Partially Hydrogenated Oils

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ABSTRACT

Total and relative amounts of neutral lipids (NL) and phospholipids (PL) as well as the distribution of various lipid classes in these were determined in testes of rats fed different types of partially hydrogenated oils for 5,15 and 26 weeks. The dietary fats were partially hydrogenated arachis oil (HAO), partially hydrogenated soybean oil (HSO), partially hydrogenated herring oil (HHO) and, for comparison, arachis oil (AO). An additional series of animals was reared on a fat-free diet throughout the entire experiment. The total amount of NL is decreased by EFA deficiency parallel with the development of the degenerative changes of the spermatogenic tissue. The relative amounts of NL in the testis are not influenced by EFA deficiency during the first stages of degeneration. However, feeding of HHO for 26 weeks resulted in a marked decrease in NL. The total content of PL is directly related to tissue degeneration. This observation is supported by the data obtained after 5 weeks of feeding HHO and by the correspondence between the results found after 15 and 26 weeks on HAO and the fat-free diets, respectively. The relative amount of PL is less influenced by EFA deficiency, but severe degenerations as found for the group fed HHO are followed by decreases. The neutral lipids had three main fractions: triglycerides (TG), free fatty acids (FFA) and cholesterol (Chol). FFA was found to be the main fraction of NL after 5 weeks, whereas TG was the main component of NL after 15 and 26 weeks, especially in the animals with degenerated testes. The presence of the large quantities of FFA is discussed. Cholesterol was decreased markedly in the EFA deficient rats fed partially hydrogenated oils, but not in the fat-free reared groups. The variations in the PL distribution during the experiment were small with regard to the two main PL classes, the phosphatidylcholines and the phosphatidylethanolamines. The most remarkable change among the PL classes was an increase in the percentage of sphingomyelins when the spermatogenic degenerations developed.

INTRODUCTION

In a previous paper (1) were reported the growth, gross symptoms and histological changes in the testes and epididymides of rats fed partially hydrogenated arachis oil (HAO), partially hydrogenated soybean oil (HSO) and partially hydrogenated herring oil (HHO). Animals fed arachis oil (AO) were used as controls. The fatty acid pattern of the above mentioned oils, which were used as the sole dietary fat, has been studied in detail (2). Large individual differences were found in the fatty acid composition of the dietary fats.

Previous studies in this field have been made by Bieri and Prival (3), who described some of the lipid classes of testes from normal animals of different species. Holman and Hofstetter (4) studied the fatty acid composition of the lipids from bovine and porcine reproductive tissues. In the present paper we are reporting on the distribution of the various lipid classes from the testes of rats fed diets containing various partially hydrogenated oils, arachis oil or a fat-free diet.

EXPERIMENTAL PROCEDURES

The details of the animal experiment were described earlier (1). The dietary fat comprised 28% (by weight) of the total basal diet. After the animals were killed, both testes were removed immediately, weighed and frozen. One testis from each animal was used for histology (1) and the other for lipid analysis. In order to get enough material for all the analytical examinations it was decided to make two pools per group, each consisting of four testes. This was especially necessary in the cases of severely degenerated testes.

Homogenization and extraction were made simultaneously in a MSE homogenizer with $CHCl_3$:CH_3OH (2:1 v/v), according to Folch et al. (5). The purified extract was chromatographed on a column (2.6 cm i.d., length 10 cm), containing a mixture of 15 g silicic acid (Baker Analyzed Reagent, Deventer, Holland)

TABLE I

Total and Relative Weight of Neutral Lipids and Phospholipids of the Right Testes of the Rats[a]

Group no.	1	2	3	4	5
Diet characteristics[b]	Fat-free	28% HAO	28% HSO	28% AO	28% HHO
After 5 weeks on experimental diets					
NL total (mg)	50.3 ± 1.1	51.1 ± 2.1	65.1 ± 1.6	62.3 ± 0.9	27.5 ± 0.0
NL relative (mg/g)	9.3 ± 0.1	11.0 ± 0.5	10.4 ± 0.3	10.3 ± 0.1	12.3 ± 0.1
PL total (mg)	65.5 ± 2.5	48.3 ± 0.7	63.3 ± 0.3	64.3 ± 1.6	18.6 ± 0.8
PL relative (mg/g)	12.2 ± 0.3	10.4 ± 0.1	10.1 ± 0.0	10.6 ± 0.3	8.3 ± 0.4
After 15 weeks on experimental diets					
NL total (mg)	36.7 ± 4.3	30.2 ± 2.5	72.0 ± 6.6	77.4 ± 7.0	29.3 ± 0.9
NL relative (mg/g)	10.1 ± 0.8	10.3 ± 0.8	11.9 ± 0.9	12.5 ± 1.1	13.9 ± 0.1
PL total (mg)	42.3 ± 4.3	31.0 ± 0.7	75.4 ± 5.2	75.7 ± 4.2	17.4 ± 0.1
PL relative (mg/g)	11.6 ± 0.8	10.6 ± 0.3	12.4 ± 1.0	12.2 ± 0.7	8.2 ± 0.2
After 26 weeks on experimental diets					
NL total (mg)	29.8 ± 2.4	33.8 ± 0.0	81.4 ± 7.0	82.3 ± 0.7	27.0 ± 1.8
NL relative (mg/g)	9.6 ± 0.8	10.6 ± 0.0	10.6 ± 0.9	11.8 ± 0.0	7.7 ± 0.4
PL total (mg)	28.5 ± 2.0	30.3 ± 0.2	87.0 ± 0.5	85.9 ± 1.8	27.9 ± [c]
PL relative (mg/g)	9.2 ± 0.6	9.5 ± 0.1	11.3 ± 0.0	12.3 ± 0.2	7.8 ± [c]

[a]Standard deviations for two pools of four testes each are given.

[b]HAO, partially hydrogenated arachis oil; HSO, partially hydrogenated soybean oil; AO, Arachis oil; and HHO, partially hydrogenated herring oil.

[c]Only one quantitative determination.

and 5 g Hyflo supercel (Johns-Manville Co., New York), prewashed thoroughly with chloroform.

The neutral lipids (nonphospholipids, NL) were eluted with 150 ml of chloroform and the phospholipids (PL) with 150 ml of methanol. These two main fractions were concentrated on a rotating vacuum evaporator (Buchi, Switzerland) at a temperature of 40 C. Suitable aliquots were taken for quantitation by weight.

Lipid classes of the NL fraction were separated by TLC on Silica Gel G (Merck, Darmstadt, Germany) in a solvent system containing redistilled petroleum ether (bp 60-70 C, Chr. F. Petri, Copenhagen), diethyl ether (Ph.Nord., Chr. F. Petri, Copenhagen), and acetic acid (Merck, Darmstadt, Germany) in the ratio 70:30:1 (v/v/v).

Cholesterol and cholesterol esters were determined by the Liebermann-Burchard reaction as modified by Sperry and Webb (6). The scrapings from the TLC plate containing cholesterol and cholesterol esters were used directly for the measurements. Blank areas from the plates showed no color reaction. Cholesterol, purified via the dibromide (7) and cholesterol palmitate, purified by TLC, were used as standards. The triglycerides and the free fatty acids were determined by weighing. Elution from the Silica Gel G was made with three portions of wet diethyl ether, ether-water (98:2).

The PL fraction was separated into classes by TLC on Silica Gel H (Merck, Darmstadt) suspended in 1 mM Na_2CO_3, according to Skipski (8).

The separation of testis phospholipids was performed in a solvent system of chloroform-methanol-water (65:25:4 v/v/v) according to Wagner et al. (9). In this system, the phospholipids present interfered less with each other than in other systems tried. The only disadvantage is that phosphatidylserine (PS) is not completely resolved from phosphatidylcholine (PC). However, the PS fraction was found to be a minor fraction (3-5%) in testis phospholipids compared to PC. This is in accordance with the findings of Bieri and Andrews (10) and Davis et al. (11).

The TLC was performed in a saturated chamber, migration 15-16 cm. Spots were visualized with iodine vapor. After disappearance of the iodine color, the fractions were scraped off and phosphate determined according to Bartlett (12). It was found that direct measurement of phosphate in the TLC scrapings gave reproducible results, when the plates were prewashed by developing them in the solvent system used for PL separation. Reactivation of the plate was necessary before spotting the phospholipid mixture. The analytical procedure was checked by PC and PE standards.

RESULTS AND DISCUSSION

Neutral Lipids

Total and relative amounts of NL in a pool of four testes are given in Table I.

TABLE II

Distribution of Neutral Lipid Fractions From Rat Testes as Per Cent of Total Neutral Lipids[a]

Group no.	1	2	3	4	5
Diet characteristics[b]	Fat-free	28% HAO	28% HSO	28% AO	28% HHO
After 5 weeks on experimental diets					
Cholesterol	15.6 ± 0.1	15.7 ± 2.2	12.6 ± 0.0	14.0 ± 0.4	11.9 ± 2.1
Free fatty acids	51.4 ± 0.1	57.2 ± 1.0	56.4 ± 0.1	56.0 ± 0.4	51.2 ± 0.7
Triglycerides[c]	31.9 ± 0.1	25.9 ± 1.4	30.1 ± 0.2	28.8 ± 0.2	35.2 ± 2.9
Cholesterol esters[d]	1.0 ± 0.1	1.2 ± 0.1	1.0 ± 0.1	1.1 ± 0.2	1.4 ± 0.1
After 15 weeks on experimental diets					
Cholesterol	18.6 ± 1.0	12.0 ± 0.1	16.3 ± 1.8	11.8 ± 0.3	11.0 ± 0.3
Free fatty acids	35.1 ± 3.4	36.1 ± 3.6	41.2 ± 3.7	29.5 ± 1.4	29.3 ± 1.3
Triglycerides[c]	44.4 ± 3.4	50.9 ± 3.8	41.4 ± 5.2	57.9 ± 1.4	58.7 ± 1.7
Cholesterol esters[d]	1.6 ± 0.3	1.0 ± 0.1	1.1 ± 0.2	0.9 ± 0.1	1.1 ± 0.1
After 26 weeks on experimental diets					
Cholesterol	15.8 ± 0.7	8.3 ± 3.4	16.3 ± 0.5	12.1 ± 1.1	7.9 ± 1.0
Free fatty acids	27.6 ± 3.4	35.2 ± 7.9	46.0 ± 1.7	37.7 ± 1.7	30.4 ± 9.0
Triglycerides[c]	54.7 ± 4.6	55.6 ± 4.0	36.3 ± 1.2	49.3 ± 2.8	60.2 ± 7.7
Cholesterol esters[d]	1.9 ± 0.5	0.9 ± 0.4	1.4 ± 0.1	0.9 ± 0.1	1.4 ± 0.2

[a]Standard deviation for two pools of four testes each are given.

[b]For explanation of abbreviations, see Table I.

[c]This fraction also includes diacylglyceryl ethers. Neutral plasmalogens were only found in trace amounts, reduced by GLC.

[d]Cholesterol esters plus small amounts of nonidentified, nonpolar lipids.

Already after five weeks of experimental feeding, it is evident that the animals fed HHO are very low in total NL compared to those receiving EFA (Groups 3 and 4). However, the relative amount of NL in the HHO group seems to be even higher than for the animals receiving EFA. A suggestive explanation of this observation may be that the HHO animals have very little, if any, visible depot fat, i.e., all dietary fat is, generally speaking, metabolized. However, some triglycerides are still present in the very small testes resulting in a high relative value. This NL may, in part, be structural NL, but may be, in part, a result of accumulated NL because of slow transfer to PL of the rather unusual fatty acids present in the partially hydrogenated herring oil (2). A somewhat similar tendency is observed in the animals fed HAO (Table I). The relative weight of NL was lowest for the fat-free groups. This may be due to mobilization of NL for PL synthesis.

After 15 weeks of experiment, a remarkable decrease in total NL was observed in the rats on the fat-free diet or with 28% HAO (Table I). Simultaneously, the absolute weight of the testes, especially in the HAO group, decreased markedly.

After 26 weeks on the experimental diets, the absolute amount of NL in the testes was at the same level and very low in all three EFA-deficient groups of animals (Table I). The relative amount of NL was very low in the animals fed HHO or a fat-free diet. The absolute weight of the testes from the HHO animals was increasing from the 5th through the 25th week of experiment. The data (Table I) clearly indicate that this increase in absolute weight is not due to increased deposition of NL. The histological studies of the left testis from all of the animals (1) showed severe degenerative changes of the spermatogenic tissue after a short feeding period with HHO. A similar picture was observed later with the HAO diet, and finally with the fat-free diet. It may thus be concluded that the relative amount of NL in the testes is not necessarily affected to any marked degree by EFA deficiency, whereas the total amount of NL and the weight of the testes give valuable information about the functional status of the spermatogenic tissue. Whether the fatty acid profile of the NL compounds may further enlarge our understanding of these problems will be the subject of forthcoming papers.

Phospholipids

Values for total and relative amounts of phospholipids in testes of rats fed different dietary fats for 5, 15 and 26 weeks, respectively, are shown in Table I.

Five weeks of feeding HHO caused a very drastic decrease in the total as well as in the relative content of PL in the testes. Feeding of HAO reduced the total content of PL in the testes to some extent, whereas the relative

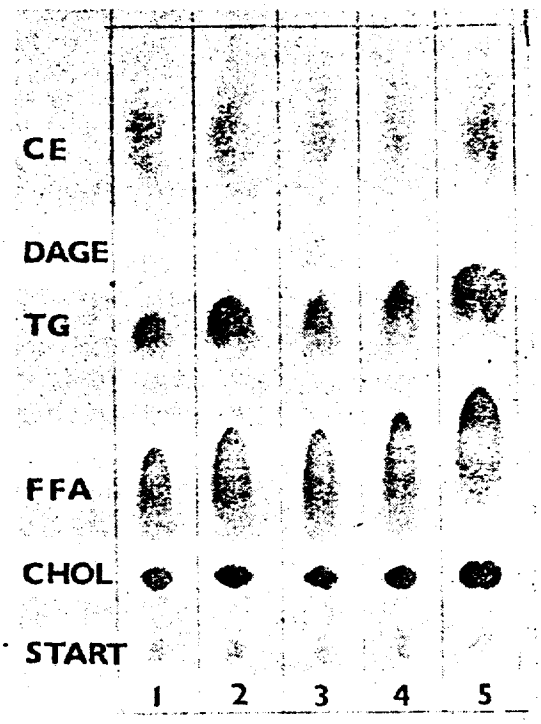

FIG. 1. Distribution of neutral lipids in testes from rats after five weeks of experimental feeding. CE, cholesterol esters; DAGE, diacylglycerol ethers; TG, triglycerides; FFA, free fatty acids; CHOL, cholesterol. The figures correspond to the various group numbers in the tables.

amount of PL was not affected (Table I). This rather short experimental period did not influence the content of PL in the testes of the fat-free reared animals, but the relative amount was increased even compared to the EFA-supplemented animals.

Fifteen weeks of experiment caused a very pronounced decrease in the absolute content of PL compared to the results after 5 weeks in the rats fed HAO and the fat-free diet, respectively. This decrease in PL is paralleled by a pronounced decrease in the weight of the testes in these groups.

After 26 weeks of experimental feeding, a further decrease was noticed in the total amount of PL in the testes of the animals fed a fat-free or a 28% HAO diet (Table I). The relative amounts of PL were also reduced.

It should be noticed that after 26 weeks the total amount of PL increased somewhat when the HHO diet was used. This is also indicated by an increase in the weight of the testes. However, the absolute and the relative amounts of PL in all of the deficient groups are now of the same order and significantly lower than in the EFA-supplemented groups.

The present data thus indicate that degeneration of the testes tissue caused by EFA deficiency (1) can be stressed by the type of dietary fat. It is paralleled by decrease amounts of total PL and NL. The relation between these facts seems well established, especially by the findings after 5 weeks of feeding HHO and the correspondence between the developments after 15 and 26 weeks on HAO and the fat-free diet, respectively. The relative amount of PL is decreased in severely degenerated animals. However, the relative content of NL does not parallel the increase in degeneration.

Distribution of Neutral Lipid Classes in Rat Testes

The results of fractionation of the NL into lipid classes are given in Table II and Figure 1.

In qualitative TLC, a distinct spot of diacylglyceryl ethers (DAGE) was seen in all groups throughout the experiment. In preparative work, i.e., quantitation of lipid classes and preparation of methyl esters for GLC, this fraction was not completely resolved and therefore was taken together with the triglycerides (TG).

Five weeks of experimental feeding gave nearly the same NL distribution in all groups examined (Table II). Surprisingly enough, the main component in the NL fraction was free fatty acids, which represented more than half of the neutral lipids.

A large amount of free fatty acids has previously been found in milt from herring. Here, 13-14% of the total milt oil were free fatty acids (13). Holman and Hofstetter (4) reported 10% free fatty acids in the total lipids of beef testes, whereas the content in pork was only 0.5%. Therefore, it is possible that the amount of free fatty acids present in the testis NL fraction may play a role in the male reproductive organs of some species.

After 15 weeks of experiment, the distribution of lipid classes in the NL fraction was different from that observed after 5 weeks. Now, the main component was the triglycerides, except for the rats on HSO, where nearly equal amounts of triglycerides and free fatty acids were found. Simultaneously, the amount of free fatty acids decreased considerably.

Extension of the experiment to 26 weeks further underlines the changed distribution of the main components of NL observed already after 15 weeks. The triglycerides are now the dominant component, except in the HSO group. Further, the cholesterol content in the HAO and HHO groups is significantly lower than that of the EFA-supplemented groups.

The reversal of the proportion of free fatty acids and triglycerides during the 26 weeks of

experiment could be the result of tissue changes related to physiological circumstances in connection with sexual maturity. However, the findings of Davis et al. (11) on changes in lipid composition of the maturing rat testis are not in agreement with our findings. They did not find free fatty acids at all in the testes. Davis et al. fed rats a Purina laboratory chow (fat per cent is not given), whereas our diets contained 28% of specific oils in a semisynthetic basal diet. Thus, these dietary differences may influence the results substantially. Oshima and Carpenter (14) used commercial rat pellets containing about 5.5% crude lipid, which was rather high in linoleic acid and contained cholesterol. They found free cholesterol and triglycerides to be the predominant classes of NL of prepubertal and adult rat testes. Only trace amounts were found of materials with R_f values corresponding to free fatty acids and free aldehydes. They stress that in order to avoid possible contamination of testes lipid with adipose tissue the tunica albuginea of the testis was removed. This coat was not removed in our experiments, but the epididymides and its fat pad was removed very carefully. We feel that discrepancies between their data and ours are not related to this latter fact.

In the present experiments: (a) only very small values were found for diglycerides and monoglycerides; (b) lysophospholipids, were not present in appreciable amounts; (c) free fatty acids in great amounts occurred in the NL fraction from all three experimental periods; and (d) storage of NL extracts for several months at -20 C did not increase the free fatty acid content markedly. The presence of the great amounts of free fatty acids have also led to the idea that they could be artifact, i.e., they were liberated during storage or analytical procedures. Davis et al. (11) and Oshima and Carpenter (14) extracted the testes immediately after killing the animals. We have stored the testes at -20 C for several months before extraction and analysis, because of the large number of animals killed at various times during a maximum experimental period of 26 weeks. Therefore, we cannot exclude the long storage as a partial explanation of the high values found for free fatty acids, although we did not find any intermediates, e.g., mono- and diglycerides or lysophosphatides.

Distribution of Phospholipid Classes in Rat Testes

Distribution of the total PL fraction from column chromatography gave seven separate phospholipid classes (Fig. 2). The results of

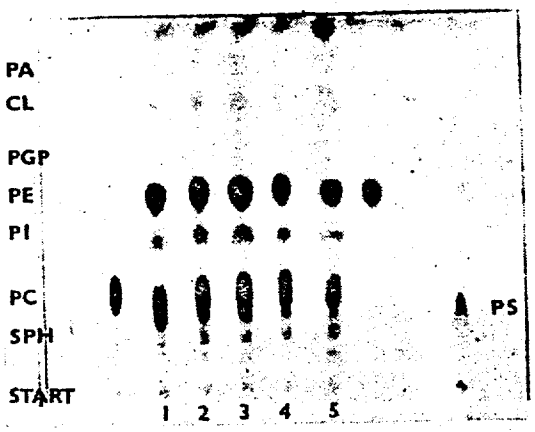

FIG. 2. Distribution of phospholipids in rat testes after five weeks of experimental feeding. CL, cardiolipin; PA, phosphatidic acid; PE, phosphatidylethanolamines; PGP, polyglycerophosphatidic acid; PI, phosphatidylinositols; PC, phosphatidylcholines; SPH, sphingomyelins; PS, phosphatidylserines. The figures correspond to the various group numbers, the remaining samples were standards.

these studies are given as percentage lipid phosphorus (Table III).

After five weeks of experimental feeding, it was seen that the group fed HHO had an extremely high amount of sphingomyelin (SPHM) compared to all the other groups. In the latter groups, minor differences only were observed in SPHM. In the HHO group, the high value for SPHM resulted in somewhat lower values for PE.

The relative amount of SPHM (mg/g tissue) in the HHO group was 1.2 (Tables I and III), whereas the values for the other groups ranged from 0.7 to 0.9. This indicates a real increase in the SPHM in the testes of HHO animals.

The results obtained after 15 weeks of experiment (Table III) showed a pronounced increase of the SPHM content in the testes from the rats fed HAO and the fat-free diet, compared to the data after 5 weeks. In these cases the increase seems primarily to be compensated by decreases in the minor PL constituents. The relative content of SPHM in these two groups of animals was 1.2 (mg/g tissue). This is in accordance with an increasing degree of degeneration of the spermatogenic tissue (1) and similar to the picture seen in the HHO rats already after five weeks. After 15 weeks, the SPHM content was 1.6 mg/g tissue for these latter animals.

After 26 weeks on the experimental diets, the SPHM fraction increased further in the groups on HAO and the fat-free diet. Thus, at this stage, with total degeneration of the

TABLE III

Distribution of Phospholipids (Weight Per Cent of Phosphorus) in Testes of Rats

Group no.	1	2	3	4	5
Diet characteristics[a]	Fat-free	28% HAO	28% HSO	28% AO	28% HHO
After 5 weeks on experimental diets					
SPHM[d]	5.7 ± 1.1[b]	8.4 ± 0.4	7.7 ± 0.7	6.2 ± 0.1	15.2 ± 2.1
PC + (PS)	51.2 ± 0.6	49.5 ± 2.1	46.5 ± 0.5	49.8 ± 0.8	45.6 ± 1.2
PI	2.8 ± 0.5	4.1 ± 0.2	3.5 ± 0.5	4.7 ± 0.2	4.1 ± 0.0
PE + PE-plasmalogen	33.4 ± 2.1	33.9 ± 1.4	38.3 ± 1.0	34.1 ± 0.0	29.8 ± 1.1
PGP	3.6 ± 0.3	2.0 ± 0.1	1.6 ± 1.5	2.5 ± 0.1	3.0 ± 0.1
CL	3.1 ± 0.5	2.1 ± 0.1	2.6 ± 0.1	2.0 ± 0.4	1.7 ± 0.0
PA	0.4 ± 0.4	Trace	Trace	1.1 ± 1.0	Trace
After 15 weeks on experimental diet					
SPHM	10.5 ± 1.5	11.0 ± 1.0	8.5 ± 0.5	9.0 ± 1.0	19.0 ± 3.0
PC + (PS)	54.8 ± 0.8	51.5 ± 1.5	50.0 ± 4.0	54.0 ± 1.0	43.5 ± 4.5
PI	0.4 ± 0.3	Trace	2.5 ± 0.5	Trace	Trace
PE + PE-plasmalogen	32.9 ± 0.9	33.0 ± 3.0	36.5 ± 2.5	35.0 ± 0.0	34.0 ± 1.0
PGP	Trace	Trace	Trace	Trace	Trace
CL	1.3 ± 0.6	1.5 ± 0.5	2.0 ± 0.0	2.0 ± 0.0	3.5 ± 0.5
PA	0	2[c]	0.5 ± 0.5	0	0
After 26 weeks on experimental diet					
SPHM	13.5 ± 0.5	17.0 ± 1.0	9.0 ± 0.0	8.5 ± 0.5	15.8 ± 0.5
PC + (PS)	51.5 ± 2.5	48.0 ± 1.0	49.0 ± 0.0	51.0 ± 1.0	45.5 ± 1.1
PI	0	Trace	2.5 ± 0.5	2.5 ± 0.5	3.0 ± 0.3
PE + PE-plasmalogen	32.0 ± 4.0	32.0 ± 2.0	34.5 ± 0.5	33.0 ± 1.0	34.4 ± 0.0
PGP	Trace	Trace	3.0 ± 0.0	2.5 ± 0.5	Trace
CL	3.0 ± 1.0	3.0 ± 0.0	2.0 ± 0.0	2.5 ± 0.5	1.4 ± 0.0
PA	0	Trace	0	Trace	0

[a]For explanation of abbreviations, see Table I.

[b]Standard deviations for two pools of four testes each.

[c]Only one determination.

[d]SPHM, sphingomyelin; PC + (PS), phosphatidylcholine + phosphatidylserine; PI, phosphatidylinositol; PE, phosphatidylethanolamine; PGP, polyglycerophosphatide; CL, cardiolipin; and PA, phosphatidic acid.

spermatogenic tissue (1), the SPHM values (expressed as wt % P) were about the same in all the EFA-deficient animals and significantly higher than in the EFA-supplemented animals. It should be noticed also that, throughout the experiment, the changes (calculated as per cent of phosphorus) were very small in the two major PL groups, PL and PE. Bieri and Andrews (10) found a similar relative increase in SPHM content when they compared normal and vitamin E-deficient rat testes.

In the EFA-deficient animals, the mg SPHM/g tissue was 1.2, 1.6 and 1.2 for the fat-free, the HAO and the HHO fed rats, respectively; thus indicating a real increase in SPHM with increasing degeneration of the spermatogenic tissue. The value of 1.2 for the HHO testes, which were degenerated already at an early stage of the experiment, may be related to increasing amounts of connective tissue resulting in a small increase in absolute weight of these testes (1).

The changes in the amounts of the minor phosphatides were less clear cut, but it should be mentioned that long time feeding with EFA-deficient diets decreased the content of polyglycerophosphatidic acid (PGP) and perhaps also of phosphatidylinositols (PI), whereas cardiolipin (CL) was always present in small amounts only.

From the distribution of PL (Table III) and the total amounts of PL (Table I) it appears that EFA deficiency decreased PC and PE known as structural elements of membranes more severely than the SPHM. A similar comparison of the relative amounts (mg/g tissue) of PC and PE revealed a slight decrease, whereas SPHM increased somewhat. The explanation of this difference may be that the pathways followed in biosynthesis of SPHM are different from those followed in the synthesis of glycerophospholipids and, apparently, less EFA-dependent. This may be due to the fatty acid composition of the SPHM, which is much less unsaturated than that of the glycerophosphatides. Feeding of isomers, geometrical as well as positional, as found in partially hydrogenated oils, may greatly restrict the building up of glycerophosphatides containing two long chain fatty acids in very close steric position.

Although the sphingomyelin molecule in its steric form has a resemblance to PC, it is obvious that the molecular position of one of the long chains, namely the sphingosine, is fixed, i.e., only one fatty acid has to be fitted into the structure. Furthermore, the fatty acid pool in EFA-deficient animals has a large content of monoenes and saturated acids which are the main fatty acids in normal sphingomyelins, whereas a high degree of unsaturated fatty acids is necessary in the glycerophosphatides.

The fatty acid composition of the NL and the PL of the generated and normal testes of rats will be reported later.

ACKNOWLEDGMENT

Miss Britta Offenberg gave technical assistance in these experiments.

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[Received November 18, 1968]

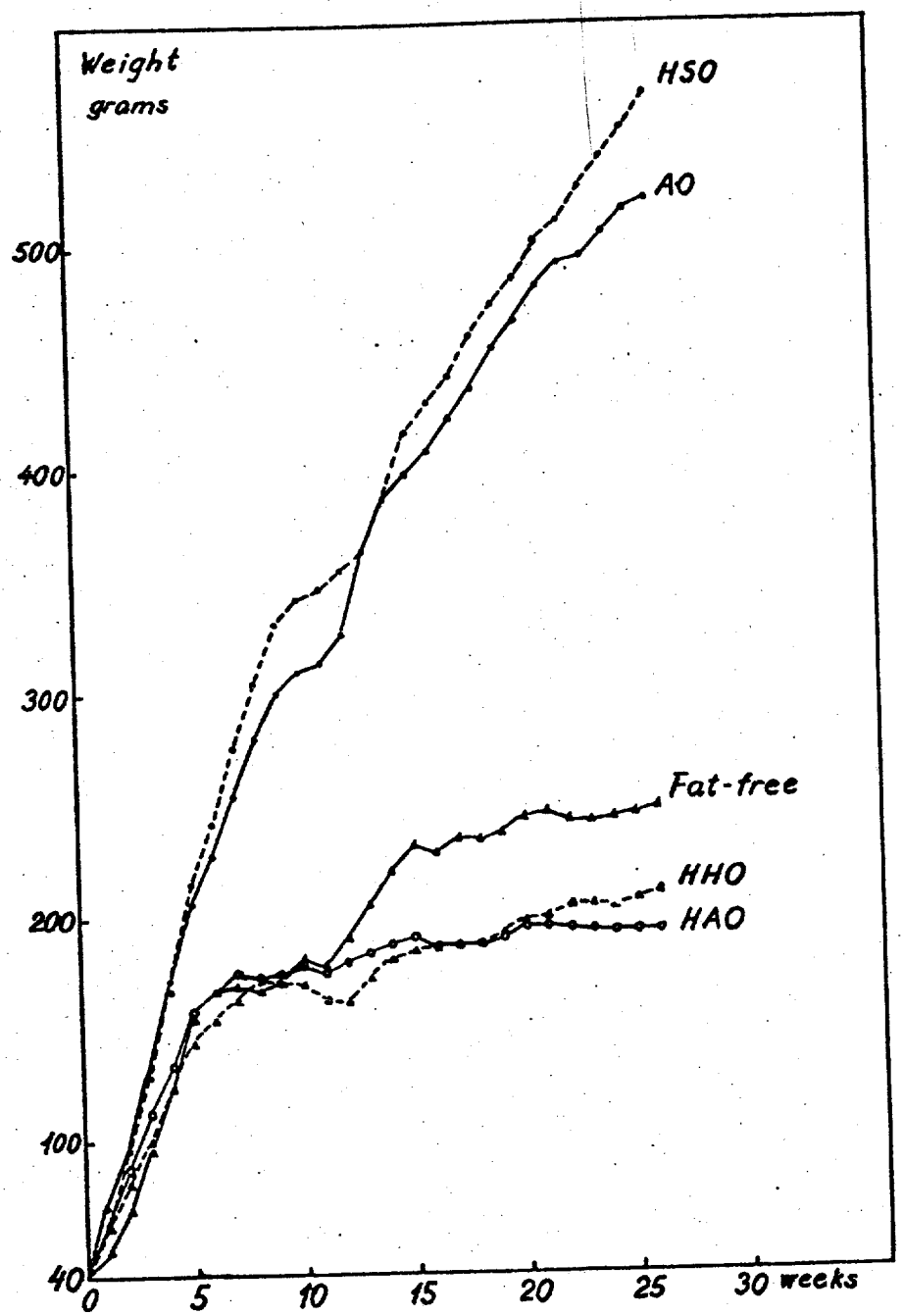

FIG. 1. Average growth rate of male rats fed on a diet with 28% partially hydrogenated soybean oil (HSO), 28% partially hydrogenated herring oil (HHO), 28% partially hydrogenated arachis oil (HAO), 28% arachis oil (AO), or a fat-free diet (Fat-free).

sumption during both periods was observed in the two groups fed AO or HSO (Table II). These animals also grew the most (Fig. 1). The weight increase in these animals was to a great extent due to very large quantities of depot fat.

The caloric intake per square meter surface during both testing periods (Table II) was greatest for the rats fed HAO or HHO, respectively. At the end of the experiment, a somewhat similar picture was obtained with the animals on

TABLE II

Scaliness, Pigmentation and Food Consumption Measurement

Group no.	Diet characteristics	Scaliness[a]		Pigment[b]		Food intake/animal/day					
						Average 8th-9th week			Average 24th-25th week		
		9th week	26th week	9th week	26th week	grams	calories[c]	calories/ sq.m/surface[d]	grams	calories[c]	Calories sq.m/surface[d]
1	Fat-free	1.8	1.2	0	0	11.0	41.4	1186	13.1	49.3	1105
2	Partially hydrogenated arachis oil (HAO)	1.7	1.5	0	0.7	10.0	51.6	1453	9.3	48.0	1270
3	Partially hydrogenated soybean oil (HSO)	0	0	1.7	2.4	12.6	65.0	1195	14.2	73.3	959
4	Arachis oil (AO)	0	0	1.7	2.1	11.2	57.8	1135	11.2	60.9	841
5	Partially hydrogenated herring oil (HHO)	1.5	1.1	0	0.6	9.7	50.1	1446	9.9	51.1	1281

[a]Dermal symptoms were scored with a range of 0 to 3 each for tail, forelegs, hindlegs and dandruff, and the average of these four scores per animal was calculated for the group.

[b]Yellow-brown pigment on the back of the animals was scored 0 to 3.

[c]Calculated by assuming that carbohydrate and protein yield 4 Cal/g, and fat 9 Cal/g.

[d]The surface area is calculated by the formula: surface area (sq.m) = $11.63 \sqrt[3]{w^2}$, where w is weight in grams (17,18).

008

the fat-free diet (Table II). These data compared to those obtained with animals fed AO or HSO illustrate the poor caloric efficiency in the EFA-deficient animals. This corroborates the findings of previous experiments from this laboratory (8,9). The reduced caloric efficiency may possibly be explained, at least to some extent, as an uncoupling of the oxidative phosphorylation process (10-15). It is tempting to speculate that the present results may partially be explained as an effect of the fatty acid pattern of the dietary partially hydrogenated oils. Thus, the affinity for isomeric unsaturated fatty acids, e.g., in the build up of lipoproteins, may be much less than for EFA, and for the isomeric acids synthesized in EFA-deficiency. The incorporation of isomeric unsaturated fatty acids into lipoproteins may result in drastic changes in the physico-chemical characteristics of membranes and enzyme moieties, thereby directly influencing formation of normal cells, e.g., in spermatogenesis, or indirectly by disturbing the normal course of oxidative phosphorylation. However, the rather similar data obtained after long-term experiments with animals fed on the fat-free diet indicate that, apart from a possible specific role of dietary isomeric unsaturated fatty acids, a similar effect eventually results from the disappearance of EFA, which favors the biosynthesis of large quantities of positional isomers of unsaturated fatty acids.

Testes: Weight and Histology

On gross examination, no differences in size were observed between the left and the right testes from the same animal in any of the experimental groups.

After five weeks of experiments, the absolute and relative weights of the left testis from the animals fed HHO were significantly lower than those of any of the other groups (Table III). Histological examination of the right testis from the former animals revealed an almost complete degeneration of the spermatogenic tissue (Table III).

The fat-free diet and that with HAO also caused a remarkably lower absolute weight of the testis after five weeks compared to that of the EFA supplemented animals (Table III).

During the following 10 weeks the absolute and the relative weights of the testis decreased in the animals fed no fat or 28% HAO (Table III). The decrease in relative weight is to some extent explained by a weight increase in the animals on the fat-free diet (Group 1, Fig. 1), whereas the animals fed 28% HAO hardly gained in weight during this period (5th-15th week, Fig. 1). The decrease in testis weight

TABLE III

Testes: Average Weight of the Left Testis and Histological Examinations of the Right Testis and Epididymis of the Rats

Group no.	Diet characteristics	Left testis: weight (g)						Average degree of degeneration of spermatogenic tissue of right testis and epididymis[a]		
		5 weeks		15 weeks		26 weeks				
		absolute	relat.[b]	absolute	relat.[b]	absolute	relat.[b]	5 weeks	15 weeks	26 weeks
1	Fat-free	1.08 ± 0.04	0.70	0.94 ± 0.07	0.42	0.69 ± 0.04	0.28	1.0	2.5	3.9
2	Partially hydrogenated arachis oil	0.93 ± 0.04	0.61	0.70 ± 0.07	0.39	0.64 ± 0.05	0.33	1.6	4.0	4.9
3	Partially hydrogenated soybean oil	1.25 ± 0.03	0.63	1.51 ± 0.06	0.39	1.54 ± 0.04	0.28	0	0	0
4	Arachis oil	1.22 ± 0.06	0.62	1.55 ± 0.05	0.43	1.55 ± 0.07	0.30	0	0	0
5	Partially hydrogenated herring oil	0.49 ± 0.04	0.34	0.59 ± 0.03	0.35	0.76 ± 0.04	0.41	4.7	5	5

[a]Assessed from a scale graduated from 0 (no degeneration) to 5 (total degeneration) (3).
[b]Percentage of body weight.

occurred simultaneously with an increasing degree of degeneration of the spermatogenic tissue. These developments are further underlined by the findings that a much more severe degeneration of the spermatogenic tissue occurred in the animals on HAO than in those on the fat-free diet (Table III).

During the following experimental period (15th through 25th week), there was practically no change in body weight of the animals fed the fat-free or the HAO diets. However, the absolute and relative weights of the testis of these animals decreased and the degenerative changes of the spermatogenic tissue increased.

The HHO had only half the amount of *trans* acids observed in HAO (Table I). The former oil, however, contained about equal amounts of *trans* $C_{18:1}$, $C_{20:1}$ and $C_{22:1}$ and smaller amounts of *trans* dienes and trienes, whereas HAO contained practically all of its *trans* acids as elaidic acid. The partially hydrogenated oils also contained varying but rather small amounts of positional isomers (6). Whether the effects observed may, in part be ascribed to either the geometric or the positional isomers, or a combination of the two types of isomeric fatty acids is the subject of further studies.

The present findings raise two questions. First, the appropriateness of talking about degeneration of the spermatogenic tissue after five weeks of feeding 28% HHO to these young animals. Maybe the degeneration is merely a dietary effect resulting in a delay in the development of the spermatogenic tissue during the period of sexual maturation. In this case, it must be concluded from the results obtained during the last 21 weeks of the experiments (Table III) that the spermatogenic tissue never did develop in these animals. Davis et al. (16) have reported that changes in lipid composition occur in the rat testes at the same time as the appearance and maturation of the spermatids. Second, during the experimental period a slight increase in the absolute and relative weights of the testis was observed in these animals (Table III). Whether this weight increase concerns the deposition of lipids or their compositional changes will be shown by analytical work which is in progress.

It should be pointed out that the relative weights of the testes of Groups 1, 2, 3 and 4 (Table III), after 26 weeks of experiment, are almost equal, although the degenerative changes of those from Groups 1 and 2 are very severe, while the testes from Groups 3 and 4 are normal.

The present experiments underline the fact that partially hydrogenated oils devoid of EFA, as the sole dietary fat of rats, stress the EFA deficiency. Further, partially hydrogenated marine oils apparently have a stronger effect in this respect than partially hydrogenated vegetable oils and fat-free diets. The reduced caloric efficiency and the tissue degenerations described can possibly be explained as membrane changes or reduced enzymatic activity, or both, due to changes in lipoprotein structures.

ACKNOWLEDGMENT

Mrs. E. Kortegaard and Miss B. Rasmussen gave technical assistance in the histological work.

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[Received November 18, 1968]

LIPIDS 4: 6, 501-506, 515-521

Essential Fatty Acid-Deficient Rats: I. Growth and Testes Development

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ABSTRACT

Partially hydrogenated oils as the sole dietary fat enhances the development of essential fatty acid (EFA) deficiency in young rats. Partially hydrogenated herring oil (HHO) caused total impairment of the spermatogenic tissue after five weeks of experiment, while partially hydrogenated arachis oil (HAO) caused severe degeneration of this tissue in 15 weeks. A fat-free diet caused degeneration in 26 weeks. In the dietary fats, the total content of *trans* acids, calculated as elaidic acid, was 47% and 23% in HAO and HHO, respectively. Further, varying amounts of different positional isomeric fatty acids were also present in the partially hydrogenated oils. Besides the specific tissue changes, poor growth, poor feed efficiency and skin signs characteristic of EFA deficiency were noticed. On the other hand, partially hydrogenated soybean oil (HSO) as the sole dietary fat kept the animals normal in all respects. This oil still contained 32% linoleic acid; the total content of *trans* acids amounted to 11%, calculated as elaidic acid.

INTRODUCTION

Essential fatty acid (EFA) deficiency in weanling male rats causes very severe impairment of the spermatogenic tissue after about 24 weeks on a fat-free diet (1-3). It is our experience that diets containing partially hydrogenated vegetable oils as the sole dietary fat enhance the degeneration of the spermatogenic tissue as compared to fat-free diets (3-5). It should be mentioned that all the effects of feeding partially hydrogenated oils are prevented by the presence of small amounts of essential fatty acids. In the present studies, we are reporting on experiments comparing the effects on growth and testes development of weanling male rats fed on partially hydrogenated vegetable oils or a partially hydrogenated herring oil. Thus, the fatty acid spectrum in the experimental dietary fats is quite different. This fact may be of interest in case the fatty acid pattern as such, or the isomeric unsaturated fatty acids, are of importance for the degenerative changes in the spermatogenic tissue in EFA deficiency. Details on the fatty acid composition of the dietary fats and of the different lipid classes of the testes will be given in forthcoming publications (6,7).

EXPERIMENTAL PROCEDURES

One hundred and fifty weanling male rats were divided into five groups of 30 animals. Each of these groups was further divided into three subgroups of 10 animals. One subgroup from each of the main experimental groups was killed after 5, 15 and 26 weeks, respectively. Group 1 received a fat-free diet of the following composition: Vitamin Test Casein (Genatosan Ltd., Loughborough, England), 20%; sucrose, 74%; vitamin mixture [0.5 g of the vitamin mixture consisted of: biotin, 0.05 mg; folic acid, 0.2 mg; para-aminobenzoic acid, 1 mg; riboflavin, 1.5 mg; pyridoxine, HCl, 1.5 mg; thiamin, HCl, 1.5 mg; inositol, 5 mg; niacinamide, 5 mg; calcium pantothenate, 5 mg; ascorbic acid, 10 mg; tocopherol acetate, 5 mg (as Ephynal acetate. F. Hoffmann-La Roche & Co.); vitamin K, 0.5 mg (as Synkavit, F. Hoffmann-La Roche & Co.); vitamin B_{12}, 0.003 mg (as Bendogen, GEA); vitamin A, 1000 IU, and vitamin D_3, 100 IU (as Rovimix AD_3, 50/5, F. Hoffmann-La Roche & Co.); and sucrose, 500 mg], 0.5%; salt mixture [100 g of the salt mixture contained: NaCl, 6 g; $Mg(HCO_3)_2$, 5 g; HCl, 7 g; K_2HPO_4, 17 g; NaH_2PO_4, 2 H_2O, 10 g; $Ca(H_2PO_4)_2$, 2 H_2O, 17 g; Ca-lactate, 5 H_2O, 36 g; ferric citrate, 2 g. The following trace elements were added per 100 g of salt mixture: $Ca(IO_3)_2$, 6 H_2O, 5.81 mg; zinc carbonate, basic (56% Zn), 72 mg; $MnCO_3$ (47.8% Mn), 42 mg, NaF, 40 mg; $NaMoO_4$, 2 H_2O, 5 mg; $Cr_2(SO_4)_3$, 15 H_2O, 1.3 mg; SeO_2, 0.32 mg. In total: 185.43 mg per 100 g of salt mixture.] 5.0%; and choline chloride, 0.5%. Groups 2, 3, 4 and 5 received the fat-free diet in which 28 wt % of sucrose was replaced by 28 wt % of partially hydrogenated arachis oil (HAO) (Aarhus Oliefabrik Inc., Aarhus, Denmark), partially hydrogenated soybean oil (HSO) (Aarhus Oliefabrik Inc.), normally refined arachis oil (AO) (Aarhus Oliefabrik Inc.) or partially hydrogenated herring oil (HHO) (Dansk Sojakagefabrik Inc., Copenhagen, Denmark), respectively.

Diets and water were provided ad libitum. The animals were weighed and inspected week-

TABLE I

Fatty Acids of Dietary Oils (Wt %)

Fatty acids, per cent of oil	Partially hydrogenated arachis oil (HAO)	Partially hydrogenated soybean oil (HSO)	Arachis oil (AO)	Partially hydrogenated herring oil (HHO)
Saturated	29.3	19.8	19.1	41.9
Monoenoic	67.0[a]	41.4[b]	50.9[c]	44.8[d]
Polyenoic	3.3	39.0	29.9	13.2
$C_{18:2}$, ω6 Linoleic acid	0.8	31.8	28.8	0.6
$C_{18:3}$, ω3 Linolenic acid	—	3.4	1.1	—
Total trans acids (as Elaidic acid)	47	10.7	0	22.9

[a]$C_{16:1}$, 1%; $C_{18:1}$, 64.8%; $C_{20:1}$, 1.2%.
[b]$C_{16:1}$, 0.6%; $C_{18:1}$, 40.8%.
[c]$C_{16:1}$, 0.3%; $C_{18:1}$, 48.5%; $C_{20:1}$, 1.7%.
[d]$C_{16:1}$, 5.6%; $C_{18:1}$, 13.0%; $C_{20:1}$, 11.3%; $C_{22:1}$, 14.1%.

ly. On autopsy, after 5, 15 and 26 weeks, respectively, the left testis was removed, weighed and frozen for lipid analysis (6,7), while the right testis and epididymis were fixed in 4% formaldehyde for histological examinations.

RESULTS AND DISCUSSION

Dietary Fats

The dietary fats were all commercial products. However, the partially hydrogenated soybean oil was hydrogenated by means of a special technique, which primarily eliminated the high content of trienoic acid from the original oil. Gas chromatographic analyses of all the dietary fats are published in detail in a following publication (6).

A summary of the composition of the dietary fats is shown in Table I. The greatest discrepancies in composition are between the HHO and the other three dietary fats. HHO has a large content of different monoenoic fatty acids, especially $C_{20:1}$ and $C_{22:1}$ (6). In the HAO, the unsaturated fatty acid picture is dominated by $C_{18:1}$. The HSO and the AO contain about equal amounts of mono- and dienoic acids.

In the partially hydrogenated oils the contents of *trans* acids are considerable. The amount of these fatty acids in HSO is half that of HHO and one quarter that of HAO. The *trans* acid in HAO and HSO is practically all elaidic acid. In HHO, the *trans* acids comprise about equal amounts of $C_{18:1}$, $C_{20:1}$ and $C_{22:1}$. Further, several other isomers are also present, e.g., *cis*, *trans* $C_{20:2}$ and $C_{22:2}$, and *cis,cis,trans* 20:3 and 22:3. The content of conjugated fatty acids appears negligible in all four experimental fats.

Growth Rates and Skin Signs

On the EFA-deficient diets, growth rates began to level off after five to seven weeks, i.e., Groups 1, 2, and 5 (Fig. 1). The animals on the fat-free diet resumed growth during the 11th through the 14th week, and then again leveled off at a somewhat higher plateau. In contrast, the animals fed HAO or HHO gained very little weight from the 8th through the 25th week. The former of the two fats had twice as much *trans* acids as the latter (Table I), and both were very low in linoleic acid. The HSO contained even more essential fatty acid than the AO (Table I). The animals fed on the HSO diet grew slightly more than the controls fed AO. These results indicate that a possible stressing effect of partially hydrogenated dietary oils was eliminated or counteracted by the presence of EFA.

The scaliness of the feet, tail and skin did not develop to any great extent (Table II). The humidity in the animal quarters varied from 30-40% during the last two months. The yellow-brown pigment on the back of the animals developed normally in the non-EFA-deficient animals, Groups 3 and 4, but did not, or practically not, occur in the EFA-deficient rats (Table II).

Food Consumption

Measurement of food intake in the various groups was carried out through the 9th and 10th week, and again through the 24th and 25th week (Table II). The highest caloric con-

43. FATS AND OILS: PROPERTIES AND COMPOSITION

Values are typical rather than average, and frequently were derived from specific analyses for particular samples (especially the constituent fatty acids). Extreme variations may occur, depending on a number of variables such as source, treatment, and age of the fat or oil.

Part I. Physical and Chemical Properties

Specific Gravity was calculated at the specified temperature and referred to water at the same temperature, unless otherwise indicated. Refractive Index was measured at 40°C, unless otherwise specified, using the D-line of sodium (589 nm). Data in brackets refer to the column heading in brackets.

	Fat or Oil	Source (Synonym)	Melting [Solidification] Point, °C	Specific Gravity 1/ [Temp, °C]	Refractive Index [Temp, °C]	Iodine Value	Saponification Value
			Land Animals				
1	Butterfat	*Bos taurus*	32.2	0.911 [40] 2/	1.4548	36.1	227
2	Depot fat	*Homo sapiens*	[15]	0.918 [15]	1.4602	67.6	196.2
3	Lard oil	*Sus scrofa*	[30.5]	0.919 [15]	1.4615	58.6	198.5
4	Neat's-foot oil	*Bos taurus*	.....	0.910 [25]	1.464 [25]	69-76	190-199
5	Tallow, beef	*Bos taurus*	.....	.....	.....	49.5	197
6	mutton	*Ovis aries*	[42.0]	0.945 [15]	1.4565	40	194
			Marine Animals				
7	Cod-liver oil	*Gadus morhua*	.....	0.925 [25]	1.481 [25]	165	186
8	Herring oil	*Clupea harengus*	.....	0.900 [60]	1.4610 [60]	140	192
9	Menhaden oil	*Brevoortia tyrannus*	.....	0.903 [60]	1.4645 [60]	170	191
10	Sardine oil	*Sardinops caerulea*	.....	0.905 [60]	1.4660 [60]	185	193
11	Sperm oil, body	*Physeter catodon (P. macrocephalus)*	.....	.....	.....	76-88	122-130
12	head	*Physeter catodon (P. macrocephalus)*	.....	.....	.....	70	140-144
13	Whale oil	*Balaena mysticetus*	.....	0.892 [60]	1.460 [60]	120	195
			Plants				
14	Babassu oil	*Attalea funifera*	22-26	0.893 [60] 3/	1.443 [60]	15.5	247
15	Castor oil	*Ricinus communis*	[−18.0]	0.961 [15]	1.4770	85.5	180.3
16	Cocoa butter	*Theobroma cacao*	34.1	0.964 [15]	1.4568	36.5	195
17	Coconut oil	*Cocos nucifera*	25.1	0.924 [15]	1.4493	10.4	257
18	Corn oil	*Zea mays*	[−20.0]	0.922 [15]	1.4734	122.6	190
19	Cottonseed oil	*Gossypium hirsutum*	[−1.0]	0.917 [25]	1.4735	105.7	194.3
20	Linseed oil	*Linum usitatissimum*	[−24.0]	0.938 [15]	1.4782 [25]	178.7	190.3
21	Mustard oil	*Brassica hirta*	.....	0.914 [15]	1.475	102	174
22	Neem oil	*Melia azedarach*	−3	0.917 [15]	1.4615	71	194.5
23	Niger-seed oil	*Guizotia abyssinica*	.....	0.925 [15]	1.471	128.5	190
24	Oiticica oil	*Licania rigida*	.....	0.974 [25]	1.514 [25]	140-180	190.5
25	Olive oil	*Olea europaea sativa*	[−6.0]	0.918 [15]	1.4679	81.1	192
26	Palm oil	*Elaeis guineensis*	35.0	0.915 [15]	1.4578	54.2	199.1
27	Palm-kernel oil	*Elaeis guineensis*	24.1	0.923 [15]	1.4569	37.0	250
28	Peanut oil	*Arachis hypogaea*	[3.0]	0.914 [15]	1.4691	93.4	192.1
29	Perilla oil	*Perilla frutescens*	.....	0.935 [15] 3/	1.481 [25]	195	192
30	Poppy-seed oil	*Papaver somniferum*	[−15]	0.925 [15]	1.4685	135	197.5
31	Rapeseed oil	*Brassica campestris*	[−10]	0.915 [15]	1.4706	98.6	174.7
32	Safflower oil	*Carthamus tinctorius*	.....	0.900 [60] 3/	1.462 [60]	145	192
33	Sesame oil	*Sesamum indicum*	[−6.0]	0.919 [25]	1.4646	106.6	191.5

1/ Unless otherwise indicated. 2/ Referred to water at 15°C. 3/ Density, measured at the specified temperature.

continued

43. FATS AND OILS: PROPERTIES AND COMPOSITION

Part I. Physical and Chemical Properties

	Fat or Oil	Source (Synonym)	Melting [Solidification] Point, °C	Specific Gravity [Temp, °C]	Refractive Index [Temp, °C]	Iodine Value	Saponification Value
34	Soybean oil	*Glycine max (G. soja)*	[−16.0]	0.927 [15]	1.4729	130.0	190.6
35	Sunflower-seed oil	*Helianthus annuus*	[−17.0]	0.923 [15]	1.4694	125.5	188.7
36	Tung oil	*Aleurites fordii*	[−2.5]	0.934 [15]	1.5174 [25]	168.2	193.1
37	Wheat-germ oil	*Triticum aestivum*	.....	0.929 [25]	1.4745	125	174.5

Contributor: Geyer, Robert P.

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Part II. Fatty Acid and Sterol Composition

	Component (Synonym)	Concentration g/100 g total fatty acids[1/]
	Butterfat (Bovine)	
1	Butyric acid	3.6
2	Caproic acid	2.0
3	Caprylic acid	0.5
4	Capric acid	2.3
5	Lauric acid	2.5
6	Myristic acid	11.1
7	12-Methylmyristic (12-Methyltetradecanoic) acid	0.4
8	*n*-Pentadecanoic acid	1.0
9	Palmitic acid	29.0
10	Margaric acid	1.0
11	Stearic acid	9.2
12	Arachidic acid	2.4
13	Decenoic acid	0.1
14	Dodecenoic acid	0.1
15	Tetradecenoic acid	0.9
16	Palmitoleic acid	4.6
17	Oleic acid	26.7
18	Linoleic acid	3.6
19	Gadoleic + erucic acids	1.4
20	Sterols, total	0.37%
	Depot Fat (Human)	
21	Myristic acid	2.7
22	Palmitic acid	24.0
23	Stearic acid	8.4
24	Palmitoleic acid	5
25	Oleic acid	46.9
26	Linoleic acid	10.2
27	Gadoleic + erucic acids	2.5
	Lard Oil (Swine)	
28	Myristic acid	1.3
29	Palmitic acid	28.3
30	Stearic acid	11.9
31	Tetradecenoic acid	0.2
32	Palmitoleic acid	2.7
33	Oleic acid	47.5
34	Linoleic acid	6
35	Gadoleic + erucic acids	2.1
36	Sterols, total	0.12%
	Neat's-Foot Oil	
37	Myristic acid	0.7
38	Palmitic acid	16.9
39	Stearic acid	2.7
40	Arachidic acid	0.1
41	Tetradecenoic acid	1.2
42	Palmitoleic acid	9.4
43	Oleic acid	64.4
44	Linoleic acid	2.3
45	Linolenic acid	0.7
46	Eicosapolyenoic + docosapolyenoic acids	1.6
	Beef Tallow	
47	Myristic acid	6.3
48	Palmitic acid	27.4
49	Stearic acid	14.1
50	Oleic acid	49.6
51	Linoleic acid	2.5
52	Sterols, total	0.11%
	Mutton Tallow	
53	Myristic acid	4.6
54	Palmitic acid	24.6
55	Stearic acid	30.5
56	Oleic acid	36.0
57	Linoleic acid	4.3
58	Sterols, total	0.07%
	Cod-Liver Oil	
59	Myristic acid	5.8
60	Palmitic acid	8.4
61	Stearic acid	0.6
62	Palmitoleic acid	20.0

[1/] Unless otherwise specified.

continued

43. FATS AND OILS: PROPERTIES AND COMPOSITION

Part II. Fatty Acid and Sterol Composition

	Component (Synonym)	Concentration g/100 g total fatty acids 1/
63	Oleic + linoleic acids	29.1
64	Eicosapolyenoic acid	25.4
65	Docosapolyenoic acid	9.6
66	Sterols, total	0.48%
	Herring Oil	
67	Myristic acid	7.3
68	Palmitic acid	13.0
69	Stearic acid	Trace
70	Palmitoleic acid	4.9
71	Linolenic acid	20.7
72	Eicosapolyenoic acid	30.1
73	Docosapolyenoic acid	23.2
	Menhaden Oil	
74	Myristic acid	5.9
75	Palmitic acid	16.3
76	Stearic acid	0.6
77	Arachidic acid	0.6
78	Behenic acid	0.8
79	Palmitoleic acid	15.5
80	Linolenic acid	29.6
81	Eicosapolyenoic acid	19.0
82	Docosapolyenoic acid	11.7
	Sardine Oil	
83	Myristic acid	5.1
84	Palmitic acid	14.6
85	Stearic acid	3.2
86	Tetradecenoic acid	Trace
87	Tetradecapolyenoic acid	15.4
88	Palmitoleic acid	11.8
89	Oleic + linoleic acids	17.8
90	Eicosapolyenoic acid	18.1
91	Docosapolyenoic acid	14.0
	Sperm Oil (Body)	
92	Lauric acid	1
93	Myristic acid	5
94	Palmitic acid	6.5
95	Tetradecenoic acid	4
96	Palmitoleic acid	26.5
97	Oleic acid	37
98	Linoleic acid	0
99	Gadoleic acid	19
100	Docosapolyenoic acid	1
	Sperm Oil (Head)	
101	Capric acid	3.5
102	Lauric acid	16
103	Myristic acid	14
104	Palmitic acid	8
105	Stearic acid	2
106	Dodecenoic acid	4
107	Tetradecenoic acid	14
108	Palmitoleic acid	15
109	Oleic acid	17
110	Linoleic acid	0
111	Gadoleic acid	6.5
	Whale Oil	
112	Lauric acid	0.2
113	Myristic acid	9.3
114	Palmitic acid	15.6
115	Stearic acid	2.8
116	Tetradecenoic acid	2.5
117	Palmitoleic acid	14.4
118	Oleic acid	35.2
119	Eicosapolyenoic acid	13.6
120	Docosapolyenoic acid	5.9
121	Tetracosapolyenoic acid	0.2
	Babassu Oil	
122	Caproic acid	0.2
123	Caprylic acid	4.8
124	Capric acid	6.6
125	Lauric acid	44.1
126	Myristic acid	15.4
127	Palmitic acid	8.5
128	Stearic acid	2.7
129	Arachidic acid	0.2
130	Oleic acid	16.1
131	Linoleic acid	1.4
	Castor Oil	
132	Lauric + myristic + palmitic + stearic acids	2.4
133	Oleic acid	7.4
134	Linoleic acid	3.1
135	Ricinoleic acid	87
136	Sterols, total	0.5%
	Cocoa Butter	
137	Palmitic acid	24.4
138	Stearic acid	35.4
139	Oleic acid	38.1
140	Linoleic acid	2.1
141	Sterols, total	0.19%
	Coconut Oil	
142	Caproic acid	0.8
143	Caprylic acid	5.4
144	Capric acid	8.4
145	Lauric acid	45.4
146	Myristic acid	18.0
147	Palmitic acid	10.5
148	Stearic acid	2.3
149	Arachidic + behenic + lignoceric acids	0.4
150	Palmitoleic acid	0.4
151	Oleic acid	7.5
152	Linoleic acid	Trace
153	Sterols, total	0.1%
	Corn Oil	
154	Myristic acid	1.4
155	Palmitic acid	10.2
156	Stearic acid	3.0
157	Palmitoleic acid	1.5
158	Oleic acid	49.6
159	Linoleic acid	34.3
160	Sterols, total	0.79%
	Cottonseed Oil	
161	Myristic acid	1.4
162	Palmitic acid	23.4
163	Stearic acid	1.1
164	Arachidic acid	1.3
165	Palmitoleic acid	2.0
166	Oleic acid	22.9
167	Linoleic acid	47.8

1/ Unless otherwise specified.

continued

43. FATS AND OILS: PROPERTIES AND COMPOSITION

Part II. Fatty Acid and Sterol Composition

	Component (Synonym)	Concentration g/100 g total fatty acids[1]
168	Sterols, total	0.29%
	Linseed Oil	
169	Palmitic acid	6.3
170	Stearic acid	2.5
171	Arachidic acid	0.5
172	Behenic acid	0.2
173	Oleic acid	19.0
174	Linoleic acid	24.1
175	Linolenic acid	47.4
176	Sterols, total	0.40%
	Mustard Oil	
177	Myristic acid	1.3% by wt
178	Behenic acid	1.1% by wt
179	Lignoceric acid	1.0% by wt
180	Oleic acid	27.2% by wt
181	Linoleic acid	16.6% by wt
182	Linolenic acid	1.8% by wt
183	Erucic acid	51.0% by wt
	Neem Oil	
184	Myristic acid	2.6% by wt
185	Palmitic acid	14.1% by wt
186	Stearic acid	24.0% by wt
187	Arachidic acid	0.8% by wt
188	Oleic acid	58.5% by wt
	Niger-Seed Oil	
189	Myristic acid	3.3% by wt
190	Palmitic acid	8.2% by wt
191	Stearic acid	4.8% by wt
192	Arachidic acid	0.5% by wt
193	Oleic acid	30.3% by wt
194	Linoleic acid	57.3% by wt
	Oiticica Oil	
195	Lauric + myristic + palmitic + stearic + arachidic + behenic acids	11.3
196	Oleic acid	6.2
197	Licanic acid	82.5
	Olive Oil	
198	Myristic acid	Trace
199	Palmitic acid	6.9
200	Stearic acid	2.3
201	Arachidic acid	0.1
202	Oleic acid	84.4
203	Linoleic acid	4.6
204	Sterols, total	0.27%
	Palm Oil	
205	Myristic acid	1.4
206	Palmitic acid	40.1
207	Stearic acid	5.5.
208	Oleic acid	42.7
209	Linoleic acid	10.3
210	Sterols, total	0.03%
	Palm-Kernel Oil	
211	Caprylic acid	2.7
212	Capric acid	7.0
213	Lauric acid	46.9
214	Myristic acid	14.1
215	Palmitic acid	8.8
216	Stearic acid	1.3
217	Oleic acid	18.5
218	Linoleic acid	0.7
219	Sterols, total	0.09%
	Peanut Oil	
220	Palmitic acid	8.3
221	Stearic acid	3.1
222	Arachidic acid	2.4
223	Behenic acid	3.1
224	Lignoceric acid	1.1
225	Oleic acid	56.0
226	Linoleic acid	26.0
227	Sterols, total	0.22%
	Perilla Oil	
228	Palmitic acid	7
229	Stearic acid	2
230	Oleic acid	13
231	Linoleic acid	14
232	Linolenic acid	64
	Poppy-Seed Oil	
233	Palmitic acid	4.8% by wt
234	Stearic acid	2.9% by wt
235	Oleic acid	30.1% by wt
236	Linoleic acid	62.2% by wt
237	Sterols, total	0.25%
	Rapeseed Oil	
238	Palmitic acid	1
239	Oleic acid	32
240	Linoleic acid	15
241	Linolenic acid	1
242	Erucic acid	50
243	Sterols, total	0.43%
	Safflower Oil	
244	Myristic acid	Trace
245	Palmitic acid	4.5
246	Stearic acid	2.5
247	Arachidic acid	Trace
248	Oleic acid	17
249	Linoleic acid	76
250	Linolenic acid	Trace
	Sesame Oil	
251	Palmitic acid	9.1
252	Stearic acid	4.3
253	Arachidic acid	0.8
254	Oleic acid	45.4
255	Linoleic acid	40.4
256	Sterols, total	0.49%
	Soybean Oil	
257	Lauric acid	0.2
258	Myristic acid	0.1
259	Palmitic acid	9.8
260	Stearic acid	2.4
261	Arachidic acid	0.9
262	Tetradecenoic acid	0.1
263	Palmitoleic acid	0.4
264	Oleic acid	28.9
265	Linoleic acid	50.7
266	Linolenic acid	6.5
267	Sterols, total	0.27%
	Sunflower-Seed Oil	
268	Palmitic acid	5.6
269	Stearic acid	2.2
270	Arachidic acid	0.9
271	Oleic acid	25.1
272	Linoleic acid	66.2
	Tung Oil	
273	Lauric + myristic + palmitic + stearic + arachidic + behenic acids	4.6
274	Oleic acid	4.1
275	Linoleic acid	0.6
276	Eleostearic acid	90.7

[1] Unless otherwise specified.

continued

43. FATS AND OILS: PROPERTIES AND COMPOSITION

Part II. Fatty Acid and Sterol Composition

	Component (Synonym)	Concentration g/100 g total fatty acids
	Wheat-Germ Oil	
277	Palmitic acid	13.5
278	Stearic acid	3.5
279	Arachidic + behenic acids	0.5
280	Oleic acid	19
281	Linoleic acid	55
282	Linolenic acid	7
283	Sterols, total	1.5%

1/ Unless otherwise specified.

Contributor: Geyer, Robert P.

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44. WAXES: PHYSICAL AND CHEMICAL PROPERTIES

Specific Gravity: Values are means calculated at 15°C and referred to water at the same temperature. Refractive Index was measured at the temperature specified at the D-line of sodium (589 nm). Data in brackets refer to the column heading in brackets.

	Wax (Synonym)	Melting Point, °C	Specific Gravity	Refractive Index [Temp, °C]	Iodine Value	Acid Value	Saponification Value	Reference
1	Bamboo-leaf wax	79-80	0.964	.....	7.8 1/	14.5	43.4	9
2	Bayberry wax (Myrtle wax)	46.7-48.8	0.989	1.436	2.2 2/-3.9 3/	3.5	20.5-21.7	7,10
3	Beeswax, crude	62-66	0.967	1.439-1.483 [40]	6.8-16.4 2/	16.8-35.8	89.3-149.0	10
4	yellow	62-65	0.968	1.447-1.465 [65]	7-11 3/	17-24	90-96	10
5	white, U.S.P.	61-69	0.967	1.443-1.449 [65]	6-11	18-24	90-97	10
6	Candelilla wax, refined	67-69	0.984	1.454-1.463 [85]	14.4-20.4	12.7-18.1	35-86	1
7	Cape-berry 4/ wax, South Africa	40.5-45	0.995	1.450 [45]	0.6-2.4	2.5-3.7	211-215	10
8	Carandá wax	79.7-84.5	0.997	.....	8.0-8.9	5.0-9.5	64.5-78.5	2,10
9	Carnauba wax	83-86	0.999	1.467-1.472 [40]	7.2-13.5	2.9-9.7	78-95	10
10	Castor oil, hydrogenated	83-88	0.993	.....	2.5-8.5	1.0-5.0	177-181	6,10
11	Chinese insect wax	81.5-84	0.951	1.457 [40]	1.4	0.2-1.5	73-93	10
12	Cotton wax	68-71	0.999	.....	24.5	32	70.6	8
13	Cranberry wax	207-218	0.975	.....	44.2-53.2 2/	42.2-59.1	131-134	10
14	Douglas-fir bark wax	59-72.8	1.020	1.468 [80]	28.5-62.5	58.6-80.7	112-200	10
15	Esparto wax	67.5-78	0.988	.....	22-23	22.7-23.9	69.8-79.3	10
16	Flax wax	61.5-69	0.974	.....	21.6-28.8	17.5-48.3	77.5-101.5	10
17	Ghedda wax (East Indian beeswax)	60.5-66.4	0.975	1.440 [50]	5.6-12.6	5.8-7.9	84.5-118.3	10
18	Indian corn wax	80-81	.....	.....	4.2 2/	1.9	120.3	5
19	Japanwax	48-53	0.975-0.993	.....	4.5-12.5	6-20	206.5-237.5	10
20	Jojoba wax	11.2-11.8	0.864-0.899	1.465 [25]	81.7-88.4 2/	0.2-0.6	92.2-95.0	10
21	Madagascar palm wax	88	.....	.....	3.2-5.3	17.7-28.0	140.0-159.6	10
22	Microcrystalline waxes, amber	64-91	0.914-0.943	1.424-1.452 [80]	0	0	0	10
23	white	71-89	0.928-0.941	1.441 [80]	0	0	0	10

1/ Wijs test. 2/ Hanus test. 3/ Hubl test. 4/ *Myrica cordifolia.*

continued

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Neutralization Value	Iodine Value	Solubility	Source	Reference	
335.53	301.74	.....	Synthetic	158,233,261,399	144
335.53	301.74	.....	*Stillingia* oils	144	145
335.53	301.74	.....	Synthetic	144	146
335.53	301.74	.....	Synthetic	529	147
222.30	201.12	sol.acet., eth., pet.eth.	Seed oils of *Acacia giraffae, Asclepias syriaca, & Macadamia ternifolia*	101,122,223	148
280.08	181.01	.....	Synthetic	18	149
280.08	181.01	.....	Synthetic	18	150
280.08	181.01	.....	Synthetic	18	151
280.08	181.01	.....	Synthetic	18	152
280.08	181.01	.....	Synthetic	138	153
280.08	181.01	.....	Synthetic	356	154
280.08	181.01	.....	Synthetic	187,287,295	155
280.08	181.01	.....	Synthetic	456	156
280.08	181.01	.....	Synthetic	377	157
280.08	181.01	.....	Synthetic	150,349,377	158
280.08	181.01	sol.acet., alc., eth., pet.eth.	Constituent of the essential fatty acids (vitamin F) and of various microorganisms; oils of cottonseed, soybean, peanut, corn, sunflower, poppy seed, linseed, & perilla	193,351,378, 409,410	159
280.08	181.01	.....	Synthetic, by (i) dehydration of ricinoleic acid (*see* entry 380), or (ii) "elaidinization" of linoleic acid (*see* entries 180 & 191)	11,266,405	160
280.08	181.01	.....	Synthetic	193	161
280.08	181.01	sol.alc., eth., me.alc., pet.eth.	Seed fat of *Chilopsis linearis*	127,193,275	162
280.08	181.01	.....	Synthetic	119,252,456	163
280.08	181.01	.....	Seed fat of *Chilopsis linearis*	118,266,378,379	164
280.08	181.01	sol.acet., cyc., eth.	Synthetic	85,117,252,266	165
280.08	181.01	.....	Synthetic	194	166
280.08	181.01	.....	Synthetic	194	167
190.56	172.39	.....	Liver oil of *Carcharodon carcharias;* Cambodia-fish oil; synthetic	274	168
181.88	164.55	sol.acet., eth., pet.eth.	Herring & menhaden oils; cattle-liver phosphatides; swine-brain lipids; shark-liver oil; synthetic	287,292	169
166.72	150.83	.....	Fish oils; synthetic	34	170
166.72	150.83	sol.acet., eth.	Synthetic	45,241	171
142.90	129.28	sol.eth., pet.eth.	Fish oils; *Spheciospongia vesparia* (a sponge); synthetic	59	172
Unsaturated (Trienoic) Fatty Acids					
224.1	304.12	sol.alc., eth.	Sardine oil	295	173
224.1	304.12	sol.me.alc., pet.eth.	.....	347	174

continued

	Fatty Acid [Synonym]	Chemical Formula	Molecular Weight	Melting Point, °C [Form]	Boiling Point, °C [Pressure mm Hg]	Specific Gravity [Temp, °C]	Refractive Index [Temp, °C]
175	6,9,12-Hexadecatrienoic	$C_{16}H_{26}O_2$	250.37	.....	.....	.....	.....
176	6,10,14-Hexadecatrienoic [Hiragonic]	$C_{16}H_{26}O_2$	250.37	.....	180-190 [15]	0.9296 [20]	1.4850 [50]
177	7,10,13-Hexadecatrienoic	$C_{16}H_{26}O_2$	250.37	.....	.....	.....	.....
178	9,12,15-Hexadecatrienoic	$C_{16}H_{26}O_2$	250.37	.....	.....	.....	.....
179	*trans*-3,*cis*-9,*cis*-12-Octadecatrienoic [Calea]	$C_{18}H_{30}O_2$	278.40	61-61.5	.....	.....	.....
180	*cis*-6,*cis*-9,*cis*-15-Octadecatrienoic[11,13] [γ-Linolenic]	$C_{18}H_{30}O_2$	278.40	−11.3 to −11	125 [0.05]	0.9164 [20]	1.4800 [20]
181	6,10,14-Octadecatrienoic	$C_{18}H_{30}O_2$	278.40	.....	.....	0.9221 [20]	1.4794 [20]
182	*cis*-8,*trans*-10,*cis*-12-Octadecatrienoic	$C_{18}H_{30}O_2$	278.40	43.5-44	.....	.....	.....
183	*trans*-8,*trans*-10,*cis*-12-Octadecatrienoic [α-Calendic]	$C_{18}H_{30}O_2$	278.40	40-40.5	.....	.....	.....
184	*trans*-8,*trans*-10,*trans*-12-Octadecatrienoic[11] [β-Calendic]	$C_{18}H_{30}O_2$	278.40	77-78	.....	.....	.....
185	*cis*-9,*trans*-11,*cis*-13-Octadecatrienoic [Punicic; trichosanic]	$C_{18}H_{30}O_2$	278.40	43.5-44	.....	0.9025 [50]	1.5113 [50]
186	*cis*-9,*trans*-11,*trans*-13-Octadecatrienoic [α-Eleostearic]	$C_{18}H_{30}O_2$	278.40	49-49.2	235 [15]	0.9028 [50]	1.5112 [50]
187	*trans*-9,*trans*-11,*trans*-13-Octadecatrienoic[15]	$C_{18}H_{30}O_2$	278.40	61.5-62.5	.....	.....	.....
188	[β-Eleostearic][15]	$C_{18}H_{30}O_2$	278.40	71-71.5	188 [1]	0.8909 [75]	1.5011 [75]
189	[Isomerized punicic][15]	$C_{18}H_{30}O_2$	278.40	70-71	.....	.....	.....
190	9,12,14-Octadecatrienoic[16]	$C_{18}H_{30}O_2$	278.40	.....	.....	.....	.....
191	*cis*-9,*cis*-12,*cis*-15-Octadecatrienoic[13] [α-Linolenic]	$C_{18}H_{30}O_2$	278.40	−11.3 to −11	125 [0.05]	0.9164 [20]	1.4678 [50]
192	*trans*-9,*trans*-12,*trans*-15-Octadecatrienoic [Elaidolinolenic]	$C_{18}H_{30}O_2$	278.40	29.5-30	.....	.....	.....
193	10,12,14-Octadecatrienoic[16]	$C_{18}H_{30}O_2$	278.40	79	.....	.....	.....
194	*trans*-10,*trans*-12,*trans*-14-Octadecatrienoic [Pseudoeleostearic]	$C_{18}H_{30}O_2$	278.40	77-77.5	.....	.....	.....
195	10,12,15-Octadecatrienoic[16]	$C_{18}H_{30}O_2$	278.40	.....	.....	.....	.....
196	5,8,11-Eicosatrienoic	$C_{20}H_{34}O_2$	306.45	.....	.....	.....	.....
197	8,11,14-Eicosatrienoic	$C_{20}H_{34}O_2$	306.45	.....	.....	.....	.....
198	11,14,17-Eicosatrienoic	$C_{20}H_{34}O_2$	306.45	.....	.....	.....	.....
199	x,y,z-Eicosatrienoic[17] [Podocarpric]	$C_{20}H_{34}O_2$	306.45	.....	.....	.....	.....
200	7,10,13-Docosatrienoic	$C_{22}H_{38}O_2$	334.52	.....	.....	.....	.....
201	8,11,14-Docosatrienoic	$C_{22}H_{38}O_2$	334.52	.....	.....	.....	.....

[11] An elaidinized α-acid. [12] Synonym: *Clupanodon melonostica*. [13] Linolenic acid exists in two isomeric forms (α- and γ-) with indistinguishable properties. [14] Oils containing <15% do not possess the enhanced drying properties

Neutralization Value	Iodine Value	Solubility	Source	Reference	
224.1	304.12	sol.alc., eth.	Herring, menhaden, & rapeseed oils	295,464,465	175
224.1	304.12	sol.acet., alc., eth., me.alc.	Oil of *Sardinops melanosticta* [12] (Japanese sardine)	488,489	176
224.1	304.12	sol.acet., alc., eth., pent.	Menhaden oil	231,295,465	177
224.1	304.12	v.sol.acet., alc., pent., pet.eth.	*Jacaranda* oils	295	178
201.51	273.51	sol.acet., alc., eth., pet.eth.	Linseed, perilla, & hemp oils	40	179
201.51	273.51	sol.acet., eth., me.alc., pet.eth.	Seed oils of *Oenothera biennis* & *O. lamarckiana* (evening primroses); drying oils [14]	352,383,415	180
201.51	273.51	sol.alc., pent., pet.eth.	*Trichosanthes* oils	495,498,499	181
201.51	273.51	v.sol.pet.eth.; sol.acet., alc., CS_2, pent.	*Catalpa ovata* seed oil; *Jacaranda* oils	125	182
201.51	273.51	sol.acet., alc., cyc., eth., pent., pet.eth.	Neou, po-yak, & tung oils	123	183
201.51	273.51	sol.alc., CS_2, eth., hept., me.alc., pet.eth.	Cattle-liver phosphatides	123,279	184
201.51	273.51	sol.alc., pent., pet.eth.	Seed oils of *Punica granatum* (pomegranate) & *Catalpa speciosa; Chilopsis* sp.; *C. linearis*	125,128-130,251	185
201.51	273.51	sol.alc., cyc., eth., pet.eth.	*Aleurites* spp. (tung), neou, & po-yak oils; oils of tropical & subtropical species	128	186
201.51	273.51	sol.acet., alc., CS_2, hept., me. alc., pent.	Cattle-liver phosphatides	131	187
201.51	273.51	sol.alc., eth., me.alc., pet.eth.	Synthetic, by isomerization of the α- acid in the presence of intense light and various catalysts, especially I_2	10,67,387	188
201.51	273.51	.....	Synthetic, by isomerization of the α- acid	131	189
201.51	273.51	.....	Synthetic, by isomerization of α-linolenic acid (*see* entry 191)	278	190
201.51	273.51	sol.acet., alc., eth., pet.eth.	Linseed, perilla, & hemp oils; drying oils [14]	400	191
201.51	273.51	sol.me.alc., pet.eth.	Synthetic, by treatment of the ester form of α-linolenic acid (*see* entry 191) with 1% Se at 205-215°C to yield this acid ester (melting point, 29-30°C)	279	192
201.51	273.51	.....	.....	276,278	193
201.51	273.51	.....	Synthetic, by alkali isomerization of α-linolenic acid (*see* entry 191)	10	194
201.51	273.51	.....	Synthetic, by alkali isomerization of α-linolenic acid (*see* entry 191)	276	195
183.06	248.48	sol.CS_2, hept., me.alc.	Fish oils; phosphatides of liver & suprarenals of land animals & of brain	292	196
183.06	248.48	sol.CS_2, hept., me.alc.	Fish oils; phosphatides of liver & suprarenals of land animals & of brain; liver oil of *Carcharodon carcharias* (a shark)	292,294	197
183.06	248.48	.....	Shark-liver & herring oil	50,51	198
183.06	248.48	.....	*Podocarpus* oil	303,304	199
167.71	227.64	.....	Reported in brain & cattle-liver phosphatides	296	200
167.71	227.64	.....	Reported in liver oil of *Carcharodon carcharias* (a shark); cattle-suprarenal phosphatides	293	201

of oils containing >25%. [15] One of three stereoisomers. [16] Isomerized linolenic acid product. [17] One isolated double bond, two conjugated double bonds.

continued

	Fatty Acid [Synonym]	Chemical Formula	Molecular Weight	Melting Point, °C [Form]	Boiling Point, °C [Pressure mm Hg]	Specific Gravity [Temp, °C]	Refractive Index [Temp, °C]
	Unsaturated (Tetraenoic) Fatty Acids						
202	4,7,10,13-Hexadecatetraenoic	$C_{16}H_{24}O_2$	248.35	.....	.....	.....	.....
203	4,8,11,14-Hexadecatetraenoic	$C_{16}H_{24}O_2$	248.35	.....	.....	.....	.....
204	4,8,12,16-Hexadecatetraenoic	$C_{16}H_{24}O_2$	248.35	.....	.....	.....	.....
205	6,9,12,15-Hexadecatetraenoic	$C_{16}H_{24}O_2$	248.35	.....	.....	.....	1.4870 [29]
206	*trans*-3,*cis*-9,*cis*-12,*cis*-15-Octadecatetraenoic	$C_{18}H_{28}O_2$	276.40	.....	.....	.....	.....
207	4,8,12,15-Octadecatetraenoic [Moroctic; morotic]	$C_{18}H_{28}O_2$	276.40	.....	208-215 [15]	0.9297 [20]	1.4911 [20]
208	6,9,12,15-Octadecatetraenoic	$C_{18}H_{28}O_2$	276.40	−57.4 to −56.6	.....	.....	1.4888 [16]
209	9,11,13,15-Octadecatetraenoic [α-Parinaric]	$C_{18}H_{28}O_2$	276.40	85-86	.....	.....	.....
210	*trans*-9,*trans*-11,*trans*-13,*trans*-15-Octadecatetraenoic [β-Parinaric]	$C_{18}H_{28}O_2$	276.40	95-96	.....	.....	.....
211	9,12,15,18-Octadecatetraenoic	$C_{18}H_{28}O_2$	276.40	.....	.....	.....	.....
212	4,8,12,16-Eicosatetraenoic	$C_{20}H_{32}O_2$	304.44	.....	217-220 [20]	0.9263 [20]	1.4915 [20]
213	5,8,11,14-Eicosatetraenoic [Arachidonic]	$C_{20}H_{32}O_2$	304.44	−49.5	163 [1]	0.9082 [20]	1.4824 [20]
214	6,10,14,18-Eicosatetraenoic	$C_{20}H_{32}O_2$	304.44	.....	.....	0.9263 [20]	1.4935 [20]
215	8,11,14,17-Eicosatetraenoic	$C_{20}H_{32}O_2$	304.44	.....	.....	.....	.....
216	4,7,10,13-Docosatetraenoic	$C_{22}H_{36}O_2$	332.48	.....	.....	.....	.....
217	7,10,13,16-Docosatetraenoic	$C_{22}H_{36}O_2$	332.48	.....	.....	.....	.....
218	8,12,16,19(20)-Docosatetraenoic [Stearidonic]	$C_{22}H_{36}O_2$	332.48	.....	.....	.....	.....
	Unsaturated (Pentaenoic) Fatty Acids						
219	4,8,12,15,18-Eicosapentaenoic [Timnodonic]	$C_{20}H_{30}O_2$	302.49	.....	.....	0.9399 [15]	1.5109 [15]
220	5,8,11,14,17-Eicosapentaenoic	$C_{20}H_{30}O_2$	302.49	−54.4 to −53.8	.....	.....	1.4977 [23]
221	4,7,10,13,16-Docosapentaenoic	$C_{22}H_{34}O_2$	302.49	.....	.....	.....	.....
222	4,8,12,15,19-Docosapentaenoic [Clupanodonic]	$C_{22}H_{34}O_2$	302.49	−78	207-212 [2]	0.9356 [20]	1.5014 [20]
223	7,10,13,16,19-Docosapentaenoic	$C_{22}H_{34}O_2$	332.49	.....	.....	.....	.....
	Unsaturated (Hexaenoic) Fatty Acids						
224	*cis*-4,*cis*-7,*cis*-10,*cis*-13,*cis*-16,*cis*-19-Docosahexaenoic	$C_{22}H_{32}O_2$	328.48	−44.5 to −44.1	.....	.....	1.5017 [26]
225	4,8,12,15,18,21-Tetracosahexaenoic [Nisinic]	$C_{24}H_{36}O_2$	356.53	.....	.....	0.9452 [20]	1.5122 [20]
	Saturated Branched-Chain Fatty Acids						
226	3-Methylbutanoic [Isovaleric]	$C_5H_{10}O_2$	102.1	−37.6	176.7	0.937 [15]	1.40178 [22.4]

18/ Also present, 3,6,9,15-hexadecatetraenoic acid. 19/ Synonym: *S. ocellata.* 20/ Synonym: *Tecoma stans.*

Neutralization Value	Iodine Value	Solubility	Source	Reference	
			Unsaturated (Tetraenoic) Fatty Acids		
225.90	407.98	sol.acet., alc., eth., pet.eth.	South African pilchard oil[18]; Japanese sardine oil	448	202
225.90	407.98	sol.acet., alc., CS_2, eth., pent., pet.eth.	Japanese sardine oil	510,511	203
225.90	407.98	sol.acet., alc., eth., pet.eth.	Japanese sardine oil	268,295	204
225.90	407.98	sol.acet., alc., CS_2, eth., pent., pet.eth.	*Sardina sagax*[19] (South African pilchard); herring oil (0.7%); menhaden body oil (1.9%)	280,281,508,510	205
202.98	367.32	.....	Seed oil of *Stenolobium stans*[20]	253	206
202.98	367.32	sol.acet., alc., eth., pet.eth.	Sardine oil; head oil of the white whale	103,472,489,500	207
202.98	367.32	sol.CS_2, me.alc.	Seed oil of *Onosmodium occidentale;* South African pilchard; Newfoundland squid; several other genera & species	269,350	208
202.98	367.32	sol.acet., alc., eth., pet.eth.	Seed oils of *Impatiens* & *Parinarium; Iris* sp.; *Nymphaea alba*	169	209
202.98	367.32	sol.eth., pet.eth.	Synthetic, by isomerization of α- acid	507	210
202.98	367.32	sol.CS_2, me.alc.	.....	347	211
184.28	333.48	sol.acet., eth., me.alc., pet.eth.	Brain phosphatides; sardine oil; whale oils; swine liver	442,489,496	212
184.28	333.48	sol.acet., eth., me.alc., pet.eth.	Confined to fats of land animals; brain, liver, glandular, & egg lipids	224,225,328,347	213
184.28	333.48	sol.acet., eth., me.alc., pet.eth.	Fish & whale oils	267	214
184.28	333.48	sol.acet., me.alc., pet.eth.	Sardine oil	464,465	215
168.76	305.35	sol.acet., me.alc., pet.eth.	Brain phosphatides	292,294	216
168.76	305.35	sol.CS_2, hept., me.alc.	Brain & cattle-liver phosphatides	292,294	217
168.76	305.35	sol.acet., me.alc., pet.eth.	Liver oil of *Carcharodon carcharias* (a shark)	50,51	218
			Unsaturated (Pentaenoic) Fatty Acids		
185.50	419.65	sol.bz., chl., eth., pet.eth.	Sardine & bonito oils	489	219
185.50	419.65	sol.hept., me.alc.	Cattle-liver lipids; various fish & seal oils	265,269	220
169.77	384.0	sol.chl., hept., me.alc.	Brain phosphatides	269	221
169.77	384.0	sol.acet., eth., pet.eth.	Marine oils; body oils of teleost fish	470,501,502,505	222
169.77	384.0	sol.bz., chl., me.alc., pet.eth.	Liver lipids of cattle; herring oil	5,6	223
			Unsaturated (Hexaenoic) Fatty Acids		
170.80	463.67	sol.bz., chl., eth., me.alc., pet. eth.	South African pilchard oil; brain phosphatides; liver lipids of cattle	292,294,526	224
157.36	427.18	sol.bz., chl., eth., me.alc., pet. eth.	Japanese sardine, fish-liver & whale oils	4	225
			Saturated Branched-Chain Fatty Acids		
549.55	.....	sol.alc., chl., eth., 23 parts w.	Tobacco; valerian root; hemp, pineapple, & lavender oils; volatile acids of sheep tallow; rumen of sheep; head & body oils of porpoise; hair grease of dog	19,20	226

continued

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continued

Neutralization Value	Iodine Value	Solubility	Source	Reference	
209.03	94.55	Crystals from light pet.; Li salt crystals from alc.	Butterfat; body fat of *Ovibos moschatus* subsp. (Canadian musk ox)	120,221,443	99
198.65	89.86	sol.bz., chl., eth.	Synthetic	371,372	100
198.65	89.86	.....	No natural source; synthetic	93,94	101
198.65	89.86	.....	No natural source; synthetic	28,164	102
198.65	89.86	.....	Only natural source, seed oil of *Thalictrum polycarpum*; synthetic in *trans*- form	164,267	103
198.65	89.86	.....	Synthetic, by the addition of HI to 5-stearolic acid followed by reduction of the addition product with Zn + acetic acid	39,398	104
198.65	89.86	sol.me.alc., aq.alc.	Various fruit & seed oils including those of various umbellates including ivy & coriander seeds, *Angelica polyclada*, & *Petroselinum crispum* [6] (parsley);	39,138,341,398	105
198.65	89.86	.....	Synthetic, by partial reduction of natural 6-octadecynoic (tariric) acid which has been synthesized by 2 different methods	41	106
198.65	89.86	.....	Synthetic, by the alkyl-acetylene method (also true for other positional isomers up to the 12-*cis*- isomer)	189	107
198.65	89.86	.....	Synthetic, by addition of HI to 7-stearolic acid, followed by reduction of the addition product with Zn + acetic acid	189	108
198.65	89.86	.....	Reported in human-hair fat & olive oil; presence in other fats suspected; synthetic, by the alkyl-acetylene method	189	109
198.65	89.86	.....	Synthetic, by isomerization of the *cis*- isomer with Se	189	110
198.65	89.86	sol.acet., alc., eth., me.alc.; insol.w.	Generally considered to be predominant fatty acid in nature—few fats known to contain <10%; purest product (98%) obtained when the oil was saponified with 3% KOH solution	14,49,189,520	111
198.65	89.86	sol.alc., chl., eth., pet.eth.	Fats of ruminants	189	112
198.65	89.86	.....	Root nodules of *Pinellia ternata* [7]; synthetic	12,13,262,478	113
198.65	89.86	.....	Root nodules of *Pinellia ternata* [7]; synthetic	12,13,262,478	114
198.65	89.86	.....	Natural fats	64,199,238,473	115
198.65	89.86	sol.acet., me.alc.	Small proportions in ox & sheep body fats; butter	62,83,209,262, 474	116
198.65	89.86	.....	Soybean oil; synthetic	44,64,199,207, 473,474	117
198.65	89.86	.....	Synthetic	44,199,473	118
198.65	89.86	.....	Synthetic, by partial hydrogenation of linolenic acid (*see* entries 180 & 191)	54,494	119
198.65	89.86	.....	Hydrolysates of butterfat and of ox & sheep fats	213,214	120
198.65	89.86	.....	Kamala-seed oil; synthetic, by reduction of ethyl undecanoate with $LiAlH_4$ to 10-undecenol, followed by further reactions to *cis*-17-octadecenoic acid	208,262	121
187.98	85.03	.....	Synthetic, by dehydrohalogenation of 2-bromononadecanoic acid	384	122
180.71	81.74	sol.acet., me.alc., pet.eth.	Natural fats; codfish; shark- & ray-liver oils; whale oil; synthetic	106,234,332,487	123

continued

	Fatty Acid [Synonym]	Chemical Formula	Molecular Weight	Melting Point, °C [Form]	Boiling Point 1/, °C [Pressure mm Hg]	Specific Gravity [Temp, °C]	Refractive Index [Temp, °C]
124	*cis*-11-Eicosenoic [*cis*-Gondoic]	$C_{20}H_{38}O_2$	310.50	23-24; 50	267 [15]	.....	.....
125	*trans*-11-Eicosenoic [*trans*-Gondoic]	$C_{20}H_{38}O_2$	310.50	52-53	.....	.....	.....
126	*cis*-14-Eicosenoic	$C_{20}H_{38}O_2$	310.50	42.5	.....	.....	.....
127	*cis*-11-Docosenoic [*cis*-Cetoleic]	$C_{22}H_{42}O_2$	338.56	33-33.7	.....	.....	.....
128	*cis*-13-Docosenoic [*cis*-Erucic]	$C_{22}H_{42}O_2$	338.56	34.7	281 [30]	0.85321 [70]	1.44438 [70]
129	*trans*-13-Docosenoic [*trans*-Brassidic]	$C_{22}H_{42}O_2$	338.56	61.9	265 [15]	0.85002 [70]	1.44349 [70]
130	22-Tricosenoic	$C_{23}H_{44}O_2$	350.57	75.1-75.2	.....	.....	.....
131	*cis*-15-Tetracosenoic [Nervonic; *cis*-Selacholeic]	$C_{24}H_{46}O_2$	366.61	42.5-43	.....	.....	.....
132	*trans*-15-Tetracosenoic [*trans*-Selacholeic]	$C_{24}H_{46}O_2$	366.61	65.5	.....	.....	.....
133	9-Hexacosenoic	$C_{26}H_{50}O_2$	394.63	.....	.....	.....	.....
134	*cis*-17-Hexacosenoic [Ximenic]	$C_{26}H_{50}O_2$	394.63	45-45.5 (?)	.....	.....	.....
135	*cis*-19-Octacosenoic	$C_{28}H_{54}O_2$	418.65	57-58	.....	.....	.....
136	*cis*-21-Triacontenoic [Lumequeic]	$C_{30}H_{58}O_2$	450.76	.....	.....	.....	.....
137	*cis*-Dotriacontenoic 10/	$C_{32}H_{62}O_2$	474.78	.....	.....	.....	.....
	Unsaturated (Dienoic) Fatty Acids						
138	2,4-Pentadienoic [β-Vinylacrylic]	$C_5H_6O_2$	98.1	80	d, 100	.....	.....
139	2,4-Hexadienoic [Sorbic]	$C_6H_8O_2$	112.1	134.5	d, 228	.....	.....
140	*cis*-2,*cis*-4-Decadienoic [*cis,cis*-Stillingic]	$C_{10}H_{16}O_2$	168.23	.....	.....	.....	1.4896 [17]
141	*cis*-2,*trans*-4-Decadienoic	$C_{10}H_{16}O_2$	168.23	.....	112-114 [0.45]	.....	1.5007 [18]
142	*trans*-2,*cis*-4-Decadienoic [*trans,cis*-Stillingic]	$C_{10}H_{16}O_2$	168.23	.....	107 [0.2]	.....	1.5078 [18]
143	*trans*-2,*trans*-4-Decadienoic	$C_{10}H_{16}O_2$	168.23	49-50	121-124 [0.3]	.....	1.5058 [31]

1/ Unless otherwise specified. 8/ Synonym: *G. callerias.* 9/ Synonym: *C. lusitanicus.* 10/ Exact structure undetermined.

Neutralization Value	Iodine Value	Solubility	Source	Reference	
180.71	81.74	sol.alc., me.alc.	Seed oils of *Cardiospermum halicacabum* (balloon vine), rape, mustard, & other Brassicaceae; reported in oils of menhaden, Atlantic cod, *Squalus acanthias* (dogfish) liver, & *Delphinapterus leucas* (beluga, or white whale) blubber	44,250,255	124
180.71	81.74	.....	Oils of pilot whale, menhaden, Atlantic cod, *Squalus acanthias* (dogfish) liver, & blubber of *Delphinapterus leucas* (beluga, or white whale); many plants, especially Buxaceae	254	125
180.71	81.74	.....	Synthetic	512	126
165.73	74.97	sol.alc.	As the ester in oils of marine animals, especially in liver oils of fish, e.g., *Gadus morhua*[8/] (Atlantic codfish)	126,482,483	127
165.73	74.97	v.sol.eth., me.alc.	Seed oils of Brassicaceae & Tropaeolaceae (40-80% of total fatty acids); seeds of rape, wallflower, mustard, & jamba; nasturtium; *Pringlea antiscorbutica* (Kerguelen cabbage); *Thlaspi arvense* (fenchweed); Argentine avocados; 3 *Ximenia* spp.; synthetic	91,151,172,235, 337	128
165.73	74.97	sp.sol.c.alc.	Synthetic, by (i) the malonic ester method, and (ii) treating erucic acid (*see* entry 128) with a variety of isomerizing agents	91,96,97	129
160.05	72.40	.....	Synthetic, by lengthening the chain of 10-undecenoic acid (*see* entry 75)	463	130
153.05	69.23	sol.acet., alc., eth.	Liver oil of *Centrophorus granulosus*[9/] (spiny dogfish shark); cerebroside of brain tissue; reported in kernel oils of several *Ximenia* spp.	92,210,290,503, 504,506	131
153.05	69.23	.....	Synthetic	92	132
143.64	64.97	.....	Reported to be a component of lipids in *Sphecispongia vesparia* (a sponge)	59	133
143.64	64.97	sol.bz., chl., eth., pet.eth.	Reported in the seed oil of *Ximenia americana* (an Indian shrub)	82,407	134
134.02	60.63	.....	Small proportion of South African *Ximenia* spp.; 4.7-12.2% of seed oils of various *Ximenia* spp.	336	135
124.48	56.31	sol.bz., chl., eth., pet.eth.	Reported to be 3-7% of fatty acids in various African *Ximenia* oils	82	136
118.18	53.47	.....	Approx. 1% in seed oils of *Ximenia americana* & *X. caffra*	336	137
			Unsaturated (Dienoic) Fatty Acids		
571.96	517.44	v.sol.alc., eth.; sol.h.w.; sl.sol. pet.eth.	Synthetic	162	138
500.53	452.82	sol.alc., eth.; sl.sol.h.w.	Berries of mountain ash; unripe sorb apples	80,229,242	139
335.53	301.74	sol.acet., eth., hex.	*Stillingia* oils	146	140
335.53	301.74	sol.acet., eth., pet.eth.	*Sebastiania fruticosa* seeds	146	141
335.53	301.74	.....	*Stillingia* oils	146	142
335.53	301.74	.....	Synthetic	146,348	143

continued

	Fatty Acid [Synonym]	Chemical Formula	Molecular Weight	Melting Point, °C [Form]	Boiling Point, °C [Pressure mm Hg]	Specific Gravity [Temp, °C]	Refractive Index [Temp, °C]
144	*cis*-2,*cis*-6-Decadienoic	$C_{10}H_{16}O_2$	168.23	[Oil]	.....	.....	1.4730 [17]
145	*trans*-2,*cis*-6-Decadienoic	$C_{10}H_{16}O_2$	168.23	.....	.....	.....	.....
146	*trans*-2,*trans*-6-Decadienoic	$C_{10}H_{16}O_2$	168.23	42-43	.....	.....	.....
147	*trans*-4,*trans*-6-Decadienoic	$C_{10}H_{16}O_2$	168.23	.....	.....	.....	.....
148	9,12-Hexadecadienoic	$C_{16}H_{28}O_2$	252.4	.....	.....	.....	.....
149	*cis*-5,*cis*-12-Octadecadienoic	$C_{18}H_{32}O_2$	280.44	.....	.....	.....	.....
150	*cis*-5,*trans*-12-Octadecadienoic	$C_{18}H_{32}O_2$	280.44	−4 to −2	.....	.....	1.4677 [20]
151	*trans*-5,*cis*-12-Octadecadienoic	$C_{18}H_{32}O_2$	280.44	−12 to −9	168-170 [0.3]	.....	1.4684 [20]
152	*trans*-5,*trans*-12-Octadecadienoic	$C_{18}H_{32}O_2$	280.44	16-19	175-176 [0.4]	.....	1.4671 [20]
153	6,8-Octadecadienoic	$C_{18}H_{32}O_2$	280.44	.....	153-155 [0.1]	.....	.....
154	*trans*-8,*trans*-10-Octadecadienoic	$C_{18}H_{32}O_2$	280.44	56-56.5	.....	0.8808 [60]	1.4682 [60]
155	*cis*-8,*cis*-11-Octadecadienoic	$C_{18}H_{32}O_2$	280.44	−12.5 to −9.5	11.8-20 [0.0001]	.....	1.4663 [25]
156	*cis*-9,*cis*-11-Octadecadienoic	$C_{18}H_{32}O_2$	280.44	42-43.2	.....	.....	.....
157	*cis*-9,*trans*-11-Octadecadienoic	$C_{18}H_{32}O_2$	280.44	−6 to −3	.....	.....	.....
158	*trans*-9,*trans*-11-Octadecadienoic	$C_{18}H_{32}O_2$	280.44	54	.....	0.8659 [77]	1.4624 [77]
159	*cis*-9,*cis*-12-Octadecadienoic [Linoleic]	$C_{18}H_{32}O_2$	280.44	−5	229-230 [16]	0.9031 [20]	1.4711 [20]
160	*cis*-9,*trans*-12-Octadecadienoic	$C_{18}H_{32}O_2$	280.44	1	.....	.....	1.4650 [27]
161	*trans*-9,*cis*-12-Octadecadienoic	$C_{18}H_{32}O_2$	280.44	1	.....	.....	1.4690 [27]
162	*trans*-9,*trans*-12-Octadecadienoic [Linolelaidic]	$C_{18}H_{32}O_2$	280.44	28-29	179-183 [0.8]	.....	1.4641 [27]
163	*cis*-10,*cis*-12-Octadecadienoic	$C_{18}H_{32}O_2$	280.44	38.2-39	.....	.....	.....
164	*trans*-10,*cis*-12-Octadecadienoic	$C_{18}H_{32}O_2$	280.44	22.23	.....	.....	.....
165	*trans*-10,*trans*-12-Octadecadienoic	$C_{18}H_{32}O_2$	280.44	56-57	.....	0.8686 [70]	1.4656 [70]
166	*cis*-10,*cis*-13-Octadecadienoic	$C_{18}H_{32}O_2$	280.44	−9 to −6.5	121-128 [0.0001]	.....	1.4670 [25]
167	*cis*-11,*cis*-14-Octadecadienoic	$C_{18}H_{32}O_2$	280.44	6-8	112-115 [0.0001]	.....	1.4664 [25]
168	*cis*-10,*cis*-13-Nonadecadienoic	$C_{19}H_{34}O_2$	294.46	.....	.....	.....	.....
169	11,14-Eicosadienoic	$C_{20}H_{36}O_2$	308.49	.....	.....	.....	.....
170	5,13-Docosadienoic	$C_{22}H_{40}O_2$	336.54	.....	.....	.....	.....
171	13,16-Docosadienoic	$C_{22}H_{40}O_2$	336.54	.....	.....	.....	.....
172	17,20-Hexacosadienoic	$C_{26}H_{48}O_2$	392.64	61	.....	.....	.....
	Unsaturated (Trienoic) Fatty Acids						
173	4,7,10-Hexadecatrienoic	$C_{16}H_{26}O_2$	250.37	.....	.....	.....	.....
174	*trans*-5,*trans*-8,*trans*-11-Hexadecatrienoic [11]	$C_{16}H_{26}O_2$	250.37	.....	.....	.....	.....

[11] An elaidinized α- acid.

	Fatty Acid [Synonym]	Chemical Formula	Molecular Weight	Melting Point, °C [Form]	Boiling Point 1/, °C [Pressure mm Hg]	Specific Gravity [Temp, °C]	Refractive Index [Temp, °C]
17	Heptadecanoic [Margaric; heptadecylic]	$C_{17}H_{34}O_2$	270.4	61.3	220 [10]	0.853 [60]	1.4324 [70]
18	Octadecanoic [Stearic]	$C_{18}H_{36}O_2$	284.5	69.6	213 [5]	0.8390 [80]	1.4337 [70]
19	Nonadecanoic [Nonadecylic]	$C_{19}H_{38}O_2$	298.5	68.6	299 [10]	0.8771 [24]	1.4512 [25]
20	Eicosanoic [Arachidic]	$C_{20}H_{40}O_2$	312.5	76.5-77.0	204 [1]	0.8240 [100]	1.4250 [100]
21	Heneicosanoic	$C_{21}H_{42}O_2$	326.5	74.3	.....	.....	.....
22	Docosanoic [Behenic]	$C_{22}H_{44}O_2$	340.6	81.5	306 [60]	0.8221 [100]	1.4270 [100]
23	Tricosanoic	$C_{23}H_{46}O_2$	354.6	79.1	203-205 [1]	.....	1.4252 [70]
24	Tetracosanoic [Lignoceric]	$C_{24}H_{48}O_2$	368.6	87.5-88.0	272 [10]	0.8207 [100]	1.4373 [70]
25	Pentacosanoic	$C_{25}H_{50}O_2$	382.6	84-85.0	.....	.....	1.4292 [70]
26	Hexacosanoic [Cerotic]	$C_{26}H_{52}O_2$	396.7	87.7-88.5	d	0.8198 [100]	1.4313 [100]
27	Heptacosanoic [Carboceric]	$C_{27}H_{54}O_2$	410.7	87.6	.....	.....	1.4324 [70]
28	Octacosanoic [Montanic]	$C_{28}H_{56}O_2$	424.7	90.0-90.9	.....	0.8191 [100]	1.4313 [100]
29	Nonacosanoic	$C_{29}H_{58}O_2$	438.7	90.3-90.5	.....	.....	.....
30	Triacontanoic [Melissic]	$C_{30}H_{60}O_2$	452.8	93.6	.....	.....	1.4323 [100]
31	Hentriacontanoic	$C_{31}H_{62}O_2$	466.8	93.1	.....	.....	.....
32	Dotriacontanoic [Laccerric]	$C_{32}H_{64}O_2$	480.8	96.2	.....	.....	.....
33	Tritriacontanoic [Psyllic; ceromelissic]	$C_{33}H_{66}O_2$	494.9	.....	.....	.....	.....
34	Tetratriacontanoic [Gheddic; geddic]	$C_{34}H_{68}O_2$	508.9	98.4	.....	.....	.....
35	Pentatriacontanoic [Ceroplastic]	$C_{35}H_{70}O_2$	522.9	98.4	.....	.....	.....
36	Hexatriacontanoic	$C_{36}H_{72}O_2$	536.9	99.9	.....	.....	.....
37	Heptatriacontanoic	$C_{37}H_{74}O_2$	550.9	.....	.....	.....	.....
38	Octatriacontanoic	$C_{38}H_{76}O_2$	564.9	101.9	.....	.....	.....
39	Hexatetracontanoic	$C_{46}H_{92}O_2$	677.2	106.8	.....	.....	.....
	Unsaturated (Monoethenoic) Fatty Acids						
40	2-Propenoic [Acrylic]	$C_3H_4O_2$	72.06	12.3; 13	141.9	1.0621 [16]	1.4224 [20]
41	*cis*-2-Butenoic [Isocrotonic]	$C_4H_6O_2$	86.09	14.4-14.6	169.3; d, 171.9	1.0312 [15.5]	1.4457 [20]
42	*trans*-2-Butenoic [Crotonic]	$C_4H_6O_2$	86.09	72.0	189	1.018 [20]	1.4228 [79.7]
43	3-Butenoic [Vinylacetic]	$C_4H_6O_2$	86.09	−39	163	1.013 [15]	1.4257 [15]
44	2-Pentenoic [β-Ethylacrylic]	$C_5H_8O_2$	100.11	8	200 [17]	0.9921 [15]	1.450 [15]
45	3-Pentenoic [β-Pentenic; β-pentenoic]	$C_5H_8O_2$	100.11	.....	193	.....	.....

1/ Unless otherwise specified.

Neutralization Value	Iodine Value	Solubility	Source	Reference	
207.5	.....	sol.acet., h.alc., eth., pet.eth.	Hair & sheep fats	182,220,329	17
197.2	.....	sol.acet., h.alc., eth., pet.eth.	Animal fats (generally)	305,368,369, 445,522,523	18
188.0	.....	sol.acet., h.alc., eth., pet.eth.	Ox fat	182,277,319, 329,360,527	19
179.5	.....	sol.bz., chl., eth., pet.eth.	Peanut oil; rambutan fat	182,220,368, 369,523,527	20
171.8	.....	inf.sol.eth., chl.; v.sol.alc.	Japanwax (?); peanut oil (?); synthetic	79,361,516	21
164.7	.....	sl.sol.alc., eth.	Brassicaceae; wool grease; waxes	181,477	22
158.2	.....	v.sol.eth.; sl.sol.alc.	Peanut oil; olive-leaf wax	79,181,230,361, 477,516	23
152.2	.....	sol.ac.a., bz., CS_2, eth.	Practically pure in beechwood tar	61,79,180,230, 361,516,521	24
146.6	.....	sol.h.acet., h.chl., h.me.alc.	*Mycobacterium tuberculosis;* synthetic wax	56,182,317,329	25
141.4	.....	sol.h.acet., h.chl., h.me.alc.	Plant & insect waxes; synthetic	102,305,445, 497,522,523	26
136.6	.....	.....	Synthetic, from 1-hexacosanol	390,420	27
132.1	.....	sol.h.ac.a., h.bz., h.me.alc.	Insect, leaf, & montan waxes	368,369,522,523	28
127.9	.....	.....	Montan wax; bitumen from peat	184,472	29
123.9	.....	sol.chl., CS_2, h.me.alc.	Plant & insect waxes (almost exclusively); mineral waxes	61,79,180,230, 331,417,521	30
120.2	.....	sol.chl., CS_2, h.me.alc.	Peat wax; montan wax (?); synthetic	390,420	31
116.7	.....	sol.h.acet., h.bz., chl.	Stick-lac wax	180,183,191, 390,420,472	32
113.4	.....	.....	*Ceroplastes ceriferous; C. rubens*	300,302,471	33
110.2	.....	sol.h.acet., h.bz., chl.	Ghedda wax, reportedly; synthetic, by oxidation of 1-tetratriacontanol by the nitrile method	184,472	34
107.3	.....	sol.h.acet., h.bz., chl.	*Ceroplastes rubens* wax	79,183,230,302, 404,417	35
104.5	.....	.....	Synthetic wax—melting point, 99.9°C	302,390,404	36
101.8	.....	.....	Synthetic wax	183,300,302, 404,471	37
99.3	.....	.....	Synthetic wax—melting point, 101.6°C	180,184,191, 390,417	38
82.8	.....	.....	Synthetic wax—melting point, 106.8°C	61,180,184,390, 472,521	39
			Unsaturated (Monoethenoic) Fatty Acids		
778.55	352.25	sol.acet., alc., tol., w.	Synthetic, by oxidation of acrolein (also by other methods)	347	40
651.71	294.86	sol.alc., CS_2, eth., pet.eth.; sl. sol.w.	Reported in the seed oil *Croton tiglium*	286,447,451	41
651.71	294.86	sol.acet., alc., pet.eth., tol., w.	Reported in the seed oil of *Croton tiglium*; synthetic	286,347,447	42
651.71	294.86	sol.alc., bz., eth.; sl.sol.w.	Synthetic	300,302,347,471	43
560.40	253.55	sol.alc., eth.	Synthetic, by reaction of 2-bromovaleric acid with quinoline	347	44
560.40	253.55	sol.bz., chl., eth.	Synthetic, by decarboxylation of the reaction product of propionaldehyde & malonic acid	347	45

continued

	Fatty Acid [Synonym]	Chemical Formula	Molecular Weight	Melting Point, °C [Form]	Boiling Point, °C [Pressure mm Hg]	Specific Gravity [Temp, °C]	Refractive Index [Temp, °C]
46	4-Pentenoic [Allylacetic]	$C_5H_8O_2$	100.11	−18	189	0.9843 [18]	1.4341 [7.5]
47	2-Hexenoic [Isohydrosorbic; β-propylacrylic]	$C_6H_{10}O_2$	114.12	32	217	0.965 [20]	1.4460 [40]
48	3-Hexenoic [Hydrosorbic]	$C_6H_{10}O_2$	114.12	12	208	0.9610 [22]	1.4355
49	4-Hexenoic [γ-Hexenoic]	$C_6H_{10}O_2$	114.12	10	206.5	0.9658 [20]	1.4367
50	5-Hexenoic [δ-Hexenoic]	$C_6H_{10}O_2$	114.2	.....	203 [121]	0.9639 [19]	1.4343 [20]
51	2-Heptenoic [α-Heptenoic]	$C_7H_{12}O_2$	128.17	.....	120-121 [11]	0.9575 [20]	1.4488 [20]
52	3-Heptenoic [β-Heptenoic]	$C_7H_{12}O_2$	128.17	−12	226-228	.....	.....
53	4-Heptenoic [γ-Heptenoic]	$C_7H_{12}O_2$	128.17	.....	98-100 [5]	0.949 [15]	1.4418 [15]
54	5-Heptenoic [δ-Heptenoic]	$C_7H_{12}O_2$	128.17	.....	117 [11]	0.9496 [20]	1.4444 [20]
55	6-Heptenoic [ε-Heptenoic]	$C_7H_{12}O_2$	128.17	−6.5	125 [15]	0.9515 [14.9]	1.4404 [14.9]
56	*cis*-2-Octenoic [3-*n*-Amylacrylic; *cis*-α-octenoic]	$C_8H_{14}O_2$	142.19	.....	154 [22]	0.9807 [20]	1.4587 [20]
57	*trans*-2-Octenoic [*trans*-α-Octenoic]	$C_8H_{14}O_2$	142.19	.....	143 [15]	0.945 [15]	1.462
58	*cis*-3-Octenoic [*cis*-β-Octenoic]	$C_8H_{14}O_2$	142.19	−25	150 [19]	0.9352 [20]	1.4433 [20]
59	*trans*-3-Octenoic [*trans*-β-Octenoic]	$C_8H_{14}O_2$	142.19	1	142 [19]	0.942 [20]	1.4456 [20]
60	*cis*-4-Octenoic [γ-Octenoic]	$C_8H_{14}O_2$	142.19	−35	96 [0.8]	0.9301 [20]	1.4417 [20]
61	*cis*-5-Octenoic [*cis*-δ-Octenoic]	$C_8H_{14}O_2$	142.19	.....	.....	.....	.....
62	*trans*-5-Octenoic [*trans*-δ-Octenoic]	$C_8H_{14}O_2$	142.19	.....	.....	.....	.....
63	*cis*-6-Octenoic [*cis*-ε-Octenoic]	$C_8H_{14}O_2$	142.19	−17	88 [0.8]	0.9378	1.4441 [20]
64	*trans*-6-Octenoic [*trans*-ε-Octenoic]	$C_8H_{14}O_2$	142.19	6	90 [1.2]	0.9422	1.4454 [20]
65	*cis*-2-Nonenoic [α-Nonylenic]	$C_9H_{16}O_2$	157.26	4	173 [20]	0.9316 [15]	1.4587 [20]
66	3-Nonenoic. [β-Nonenoic; 3-nonylenic]	$C_9H_{16}O_2$	157.26	.....	106 [1]	0.9290 [20]	1.4484 [20]
67	8-Nonenoic [8-Nonylenic]	$C_9H_{16}O_2$	157.26	5	116-118 [1]	0.9146 [16]	1.4492
68	2-Decenoic [2-Decylenic]	$C_{10}H_{18}O_2$	170.24	12	165 [15]	0.9280 [15]	1.46161 [20]
69	3-Decenoic [3-Decylenic]	$C_{10}H_{18}O_2$	170.24	18	154-163 [11]	0.914 [15]	1.4510 [18]
70	4-Decenoic [Obtusilic]	$C_{10}H_{18}O_2$	170.24	.....	148-150 [13]	0.9197 [20]	1.4497 [20]
71	8-Decenoic [Isodecenoic]	$C_{10}H_{18}O_2$	170.24	.....	155-157 [14]	0.930 [15]	.....
72	9-Decenoic [9-Decylenic; caproleic]	$C_{10}H_{18}O_2$	170.24	.....	158-163 [21]	0.9238 [15]	1.4485 [21]
73	2-Undecenoic [2-Hendecenoic]	$C_{11}H_{20}O_2$	184.27	.....	.....	.....	.....
74	9-Undecenoic [9-Hendecenoic; 9-undecylenic]	$C_{11}H_{20}O_2$	184.27	11.4	129 [1]	.....	.....
75	10-Undecenoic [10-Hendecenoic; 10-undecylenic]	$C_{11}H_{20}O_2$	184.27	24.5	275	0.9075 [25]	1.4464 [20] 2/

2/ At the α spectral line.

Neutralization Value	Iodine Value	Solubility	Source	Reference	
560.40	253.55	sol.bz., chl., eth.	Synthetic	347	46
491.54	222.39	sol.alc., bz., chl., CS_2, eth.; sl. sol.w.	Japanese peppermint oil; synthetic, from *n*-caproic acid (*see* entry 6)	347	47
491.54	222.39	sol.bz., pet.eth.	Synthetic	347	48
491.54	222.39	sol.bz., eth., pet.eth.	Synthetic, by the action of 1-bromo-2-butene on ethyl sodiomalonate	347	49
491.54	222.39	sol.bz., eth., pet.eth.	Synthetic, by (i) treatment of 6-aminocaproic acid with HNO_2, & (ii) reaction of 1-bromo-3-butene with ethyl sodiomalonate	347	50
437.74	198.06	sol.bz., eth., pet.eth.	Marine oils; milk fats; synthetic, by (i) the malonic ester method, & (ii) reaction of 7-aminoheptanoic acid & HNO_2	347	51
437.74	198.06	sol.alc., eth., pet.eth.	Synthetic, from *n*-valeraldehyde by the malonic acid method	347	52
437.74	198.06	sol.acet., eth., me.alc.	Synthetic	263	53
437.74	198.06	sol.alc., chl., eth., pet.eth.	Synthetic, by the reaction of 4-chlorobutyric acid & K or Na acetoacetate	98,398	54
437.74	198.06	sol.acet., me.alc.	Synthetic, by the reaction of 7-aminoheptanoic acid & HNO_2	192	55
394.57	178.52	.....	Synthetic, by condensation of caprylic aldehyde with malonic acid in presence of pyridine	35,157,297	56
394.57	178.52	.....	Synthetic	35,156,289,297	57
394.57	178.52	.....	Synthetic	157,260,297	58
394.57	178.52	.....	Synthetic	157,297	59
394.57	178.50	.....	Synthetic	428	60
394.57	178.50	.....	Synthetic	367	61
394.57	178.50	.....	Synthetic	260	62
394.57	178.50	.....	Synthetic	260	63
394.57	178.50	.....	Synthetic	297	64
359.7	162.47	.....	Synthetic	175,367,422,428	65
359.7	162.47	.....	Synthetic	367	66
359.7	162.47	.....	.....	192,367	67
329.55	149.09	.....	Synthetic, by condensation of caprylic aldehyde with malonic acid in presence of pyridine	13,17,69,478	68
329.55	149.09	.....	Synthetic, by distillation of hexyl paraconic acid	175	69
329.55	149.09	sol.bz., eth.	Seed oil of *Lindera obtusiloba* (Japanese spicebush)	298,484	70
329.55	149.09	.....	Butterfat & milk of man, goat, & Indian buffalo	136	71
329.55	149.09	sol.alc., eth.	Milk fats of most animals	493	72
304.47	137.74	.....	.....	13,17,69,478	73
304.47	137.74	.....	Synthetic, by debromination of 9-bromo-9-undecenoic acid with (i) Na in ethanol, or (ii) Zn & propionic acid (*see* entry 3)	13,17,69	74
304.47	137.74	sol.alc., eth.; sl.sol.chl.; insol.w.	Synthetic, by (i) pyrolysis of ricinoleic acid (*see* entry 380) & castor oil, & (ii) malonic acid method	192,381	75

continued

	Fatty Acid [Synonym]	Chemical Formula	Molecular Weight	Melting Point, °C [Form]	Boiling Point, °C [Pressure mm Hg]	Specific Gravity [Temp, °C]	Refractive Index [Temp, °C]
76	2-Dodecenoic [2-Lauroleic]	$C_{12}H_{22}O_2$	198.30	17	172-174 [8]	0.9130 [20]	1.4639 [20]
77	4-Dodecenoic [Linderic]	$C_{12}H_{22}O_2$	198.30	1-1.3	170-172 [13]	0.9106 [15]	1.4545 [15]
78	6-Dodecenoic[3/] [6-Lauroleic]	$C_{12}H_{22}O_2$	198.30	.....	.....	.....	.....
79	7-Dodecenoic [7-Lauroleic]	$C_{12}H_{22}O_2$	198.30	[Oil]	.....	.....	1.4486 [27.5]
80	9-Dodecenoic [9-Lauroleic]	$C_{12}H_{22}O_2$	198.30	.....	142 [4]	.....	.....
81	10-Dodecenoic [10-Lauroleic]	$C_{12}H_{22}O_2$	198.30	18	166-168 [9]	.....	.....
82	11-Dodecenoic [11-Lauroleic]	$C_{12}H_{22}O_2$	198.30	20	171-172 [13]	0.9030 [20]	1.4510 [20]
83	2-Tridecenoic [3-*n*-Decylacrylic]	$C_{13}H_{24}O_2$	212.32	38-39	167-171 [2]	0.8995 [30]	1.46121 [20]
84	11-Tridecenoic [Isotridecenoic]	$C_{13}H_{24}O_2$	212.32	28-29	183-185 [13.5]	.....	.....
85	12-Tridecenoic	$C_{13}H_{24}O_2$	212.32	38-39	192 [20]	.....	.....
86	2-Tetradecenoic	$C_{14}H_{26}O_2$	226.35	33; 50-53	186-188 [8]	.....	.....
87	4-Tetradecenoic [Tsuzuic]	$C_{14}H_{26}O_2$	226.35	18-18.5	185 [13]	0.9024 [20]	1.4559 [20]
88	5-Tetradecenoic [Physeteric; 5-myristoleic]	$C_{14}H_{26}O_2$	226.35	20	190-195 [15]	0.9046 [20]	1.4552 [20]
89	*cis*-8-Tetradecenoic	$C_{14}H_{26}O_2$	226.35	[Oil]	.....	.....	1.4569 [17]
90	*cis*-9-Tetradecenoic [Myristoleic]	$C_{14}H_{26}O_2$	226.35	−4	.....	0.9018	1.4519 [20]
91	2-Pentadecenoic	$C_{15}H_{28}O_2$	240.37	4.5	.....	.....	.....
92	14-Pentadecenoic	$C_{15}H_{28}O_2$	240.37	.....	198 [8]	.....	.....
93	2-Hexadecenoic [Gaidic; 2-palmitoleic]	$C_{16}H_{30}O_2$	254.40	39; 45	.....	.....	.....
94	7-Hexadecenoic [7-Palmitoleic]	$C_{16}H_{30}O_2$	254.40	21; 33; 32-33 [α-]; 40-41 [β-]	236 [15][4/]; 180-183 [1][5/]	.....	1.4583 [20][5/]
95	*cis*-9-Hexadecenoic [*cis*-9-Palmitoleic]	$C_{16}H_{30}O_2$	254.40	−0.5 to +0.5	140-141 [5]	.....	.....
96	*trans*-9-Hexadecenoic [*trans*-9-Palmitoleic]	$C_{16}H_{30}O_2$	254.40	31	.....	.....	.....
97	*cis*-10-Hexadecenoic [*cis*-10-Palmitoleic]	$C_{16}H_{30}O_2$	254.40	15-16	.....	.....	1.4593 [20]
98	2-Heptadecenoic [2-Heptadecylenic]	$C_{17}H_{32}O_2$	268.43	57.5	.....	.....	.....

3/ Occurs as α- and β- forms. 4/ Value for the α- form. 5/ Value for the β- form.

Neutralization Value	Iodine Value	Solubility	Source	Reference	
282.93	128.01	.....	Synthetic, by reaction of decanaldehyde with malonic acid in presence of pyridine	509	76
282.93	128.01	sol.bz., chl., eth.	Seed oils of *Lindera obtusiloba* & various Formosan plants	298,492	77
282.93	128.01	.....	.....	52	78
282.93	128.01	.....	.....	478	79
282.93	128.01	sol.bz., chl., eth.	Milk fats; cochineal wax; wax of *Tachardina theae*	301	80
282.93	128.01	.....	.....	134	81
282.93	128.01	.....	Synthetic, by conversion (i) from 10-undecenoic acid (*see* entry 75), & (ii) of 1-bromo-10-decene to 11-dodecenenitrile, followed by hydrolysis	478	82
264.24	119.55	.....	Synthetic, by reaction of undecanaldehyde with malonic acid in presence of pyridine	421,531	83
264.24	119.55	.....	Synthetic, by reaction of undecanaldehyde with malonic acid in presence of pyridine	106,135	84
264.24	119.55	.....	Synthetic, by (i) reaction of 1-bromo-10-undecene, ethyl malonate, & Na, & (ii) conversion of 10-undecenoic acid to 11-dodecenoic acid	13,17,69,134	85
247.45	112.13	.....	Synthetic, by reaction of lauric aldehyde with malonic acid in presence of pyridine	421,531	86
247.45	112.13	sol.bz., pet.eth.	Seed oils of *Lindera obtusiloba, Litsea* sp., *Tetradenia glauca*, & related plants	298	87
247.45	112.13	sol.bz., eth., pet.eth.	Sperm whale & dolphin oils; whale head & blubber oil; sardine oil; pilot-whale oil; sperm whale-blubber oil; human-hair fat	490-492	88
247.45	112.13	sol.bz., eth., pet.eth.	Lipids of all eubacteria investigated	23,33,429	89
247.45	112.13	sol.bz., eth., pet.eth.	Oils of whale blubber, shark liver, Antarctic whale, & turtle; eel; milk fats; Japanese coccid wax; seed fat of *Pycnanthus kombo*	23,33	90
233.43	105.59	.....	Synthetic, by conversion of myristic acid (*see* entry 14), to α-hydroxymyristic acid (*see* entry 309), then, by the action of Pb tetraacetate, to tridecanaldehyde which is then condensed with malonic acid in presence of pyridine	137	91
233.43	105.59	.....	Synthetic, by the malonic acid method from 1-bromo-12-tridecene & ethyl malonate in presence of sodium ethoxide	137	92
220.56	99.77	sol.alc., eth.	Synthetic, by the action of alcoholic KOH on 2-iodopalmitic acid	396,434	93
220.56	99.77	v.sol.alc.; sol.eth.; insol.w.	Synthetic, by the action of fused KOH on stearolic acid	81,346	94
220.56	99.77	.....	Widely distributed in animal, vegetable, & marine oils	347	95
220.56	99.77	.....	Synthetic, by treatment of the *cis*- form with Se or N oxides	53,88,437	96
220.56	99.77	.....	Synthetic	42	97
209.03	94.55	sol.eth.; crystals from acet.	Synthetic, by the malonic acid method from pentadecanaldehyde & malonic acid in presence of pyridine	310,371,372	98

continued

	Fatty Acid [Synonym]	Chemical Formula	Molecular Weight	Melting Point, °C [Form]	Boiling Point, °C [Pressure mm Hg]	Specific Gravity [Temp, °C]	Refractive Index [Temp, °C]
99	*cis*-9-Heptadecenoic [9-Heptadecylenic]	$C_{17}H_{32}O_2$	268.43	11.4-12.2; 14.5	175 [0.5]	.....	1.4598 [20]
100	*cis*-2-Octadecenoic	$C_{18}H_{34}O_2$	282.45	50.5	.....	.....	.....
101	*trans*-2-Octadecenoic [*trans*-2-Oleic]	$C_{18}H_{34}O_2$	282.45	58.5	.....	0.8484 [90]	.....
102	3-Octadecenoic [3-Octadecylenic]	$C_{18}H_{34}O_2$	282.45	56-57	.....	.....	1.4466 [65]
103	4-Octadecenoic [4-Octadecylenic]	$C_{18}H_{34}O_2$	282.45	52-53	.....	.....	.....
104	*trans*-5-Octadecenoic [5-Octadecylenic]	$C_{18}H_{34}O_2$	282.45	43-44; 47.5	.....	.....	.....
105	*cis*-6-Octadecenoic [6-Octadecylenic]	$C_{18}H_{34}O_2$	282.45	33	215-217 [2-3]	0.8824 [35]	1.4536 [65]
106	*trans*-6-Octadecenoic [Petroselaidic]	$C_{18}H_{34}O_2$	282.45	54-59	.....	.....	1.4408 [70]
107	*cis*-7-Octadecenoic	$C_{18}H_{34}O_2$	282.45	12.5-13.1	.....	.....	.....
108	*trans*-7-Octadecenoic	$C_{18}H_{34}O_2$	282.45	44.2-44.5	.....	.....	.....
109	*cis*-8-Octadecenoic [*cis*-8-Oleic]	$C_{18}H_{34}O_2$	282.45	13.4 [α-]; 16.3 [β-]	234 [15]	0.8905	1.45823
110	*trans*-8-Octadecenoic [*trans*-8-Elaidic]	$C_{18}H_{34}O_2$	282.45	52-52.6	.....	.....	.....
111	*cis*-9-Octadecenoic [9-Octadecylenic; oleic]	$C_{18}H_{34}O_2$	282.45	12 [labile]; 16 [stable]	234 [15]	0.898 [20]	1.45823 [20]
112	*trans*-9-Octadecenoic [9-Elaidic]	$C_{18}H_{34}O_2$	282.45	44.5-46.5	234 [15]	0.8568 [70]	1.4405 [70]
113	*cis*-10-Octadecenoic [*cis*-10-Oleic]	$C_{18}H_{34}O_2$	282.45	22.2-22.8	.....	.....	.....
114	*trans*-10-Octadecenoic	$C_{18}H_{34}O_2$	282.45	.....	.....	.....	.....
115	*cis*-11-Octadecenoic [*cis*-Vaccenic]	$C_{18}H_{34}O_2$	282.45	14.5-15.5	.....	.....	.....
116	*trans*-11-Octadecenoic [*trans*-Vaccenic]	$C_{18}H_{34}O_2$	282.45	44	.....	0.8563 [70]	1.4406 [70]
117	*cis*-12-Octadecenoic [*cis*-12-Oleic]	$C_{18}H_{34}O_2$	282.45	9.8-10.4; 26.5-27.5	196 [1.5]	.....	.....
118	*trans*-12-Octadecenoic [*trans*-12-Elaidic]	$C_{18}H_{34}O_2$	282.45	39.7-40.1	196 [1.5]	.....	.....
119	*trans*-15-Octadecenoic	$C_{18}H_{34}O_2$	282.45	61-61.5	.....	.....	.....
120	*trans*-16-Octadecenoic	$C_{18}H_{34}O_2$	282.45	65.6-66.2	.....	.....	.....
121	*cis*-17-Octadecenoic	$C_{18}H_{34}O_2$	282.45	55-55.5	.....	.....	.....
122	2-Nonadecenoic	$C_{19}H_{36}O_2$	298.49	66.5	297-298 [100]	.....	.....
123	*cis*-9-Eicosenoic [*cis*-Gadoleic]	$C_{20}H_{38}O_2$	310.50	23-23.5	170 [0.1]	0.882 [25]	1.4597 [25]

6/ Synonym: *P. sativum.* 7/ Synonym: *P. tuberifera.*

J. Am. Oil Chem. Soc. 48: 804-806, 1971

Monoethylenic Fatty Acids of a Partially Hydrogenated Herring Oil

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ABSTRACT

A Canadian Atlantic herring oil hydrogenated for margarine use to an iodine value of 76 and melting point of 32.5 C was found to have 30% saturated acids and 66% monounsaturated fatty acids. The monounsaturated fatty acids could be analytically determined as *cis* and *trans* isomers by open tubular gas liquid chromatography. *Trans* acids were 33% of the C_{16} and C_{18} monounsaturated acids, and 32 and 28%, respectively, of the C_{20} and C_{22} monounsaturated acids. After separation of geometric isomers by Florisil-silver nitrate chromatography the positional isomers in each class were determined by oxidative fission. The double bond positions of the original *cis* fatty acids were largely retained in both *cis* and *trans* isomers, but additional isomers were observed, especially in the *trans* fatty acids.

INTRODUCTION

The partial hydrogenation of fats and oils to produce a stable, palatable product of suitable plasticity results in the formation of new, structurally altered fatty acids which in most cases differ chemically from familiar natural products. The nutritive aspects of partially hydrogenated fats originally of vegetable origin have been the subject of a recent review (1). In Canada as much as 55 million pounds of hydrogenated marine oils have been used in margarines, shortenings or cooking fats in recent years (2). It has been presumed that the nutritional and physiological qualities of these oils would be similar to those of the partially hydrogenated vegetable oils which are primarily of C_{18} chain length except for rapeseed oil. Recent studies indicated that certain physiological responses of animals to the long chain (C_{20} and C_{22}) monoethylenic fatty acids found in most marine oils in moderate proportions (10-30%) even before hydrogenation, and especially in erucic acid-rich rapeseed oils, required further study (3). This work reports details of the analysis of the partially hydrogenated herring oil used in one such recent study (4,5).

EXPERIMENTAL PROCEDURES

. Atlantic herring oil (from reduction of whole *Clupea harengus*), which had been hydrogenated according to Canadian current commercial practice with nickel catalyst at 375-400 F and about 10 psig hydrogen pressure to a Wijs iodine value of 76 and Wiley melting point of 32.5 C, was saponified and nonsaponifiable materials removed by AOCS method C6-6b-53. The soaps were acidified and the fatty acids extracted into diethyl ether, washed and dried. The acids were converted to methyl esters by refluxing with 7% BF_3 in MeOH for 10 min.

The methyl esters were examined initially by gas liquid chromatography (GLC) and after fractionation by thin layer chromatography (TLC) on silver-nitrate impregnated silica gel (Supelcosil 12D) developed in *n*-hexane:benzene 1:1. For oxidative fission studies the monounsaturated methyl esters were separated from the saturates and polyunsaturates and into *cis* and *trans* fractions by column chromatography on a scale of 100-200 mg on acid-washe Florisil impregnated with silver nitrate (6). The solvent were essentially those reported earlier where silica gel-silv nitrate columns were employed (7,8). The esters o saturated fatty acids were almost completely eluted with ml of *n*-hexane. The *trans* and *cis* monoethylenic este were eluted in sequence with virtually no overlap b successive applications of 75 ml each of 2,4 and 8% diethy ether-*n*-hexane mixtures. The respective *cis* and *tran* fractions were further separated by preparative scale GL with recovery of four chain lengths (C_{16}, C_{18}, C_{20}, C_{22} Ozonolysis of each specific fraction was carried out methanol with oxidative work-up, the product acids bein identified by GLC after in situ esterification with 2, dimethoxypropane (9).

Basic determinations of composition were carried out o open-tubular (capillary) GLC columns (150 ft x .01 in ID coated with butanediol-succinate polyester (BDS) Apiezon-L grease (AP-L). The columns were purchase ready-coated from Perkin-Elmer and used in Perkin-Elm Model 226 or Model 900 GLC units.

Preparative gas liquid chromatography was carried o with an Aerograph A-90 (thermal conductivity detecto GLC apparatus fitted with a 6 ft x 5/15 in. OD copp column packed with 5% SE-30 silicone gum on Gas-Chro Q (100/120 mesh).

Standard *trans* monounsaturated acids were prepare from *cis* monounsaturates of known compositions (8 These were isolated from herring oil methyl esters b preparative TLC on silver nitrate-silica gel plates (Supelco 12D). After spraying with 2,7-dichlorofluorescein t monounsaturated esters visualized under UV light we recovered, treated with nitrous acid (10), and separated b TLC into *cis* and *trans* bands. The purity of the *cis* a *trans* materials prepared in this way was verified by bo infrared spectroscopy (IR) and by GLC on AP-L. Este prepared from the commercially hydrogenated sample we examined on a Varian T-60 NMR Spectrometer.

RESULTS AND DISCUSSION

The results of study of this sample of commercial prepared, partially hydrogenated herring oil would indica the use of a catalyst and conditions favoring selecti hydrogenation of the polyunsaturated acids to monou saturated acids. Only a total of 4% of a variety of artifa polyunsaturated acids remained. The proportions of 22: normally absent in herring oil, and 20:0, about 0.1-0.5% most herring oils, indicate that as little as 8% of the origi or newly formed monounsaturated acids may have be hydrogenaged to saturated acids. Total saturated acids we 30% and total monounsaturated acids 66%. Unfortunate the raw herring oil was not available for analysis and t fatty acid composition of herring oil varies with season, a of fish, and catch location. Average figures for 12 Atlan herring oils of 20% for saturated acids, range 16.4-24.4 and 60% for monounsaturated acids, range 52.1-71.5 have been reported (11).

The composition of the saturated fatty acids in t sample is not remarkable for a marine oil. Were it not f the presence of 1.64% 20:0, and 2.04% 22:0, the compo tion could well be that of the saturated acids of any

TABLE I

Fatty Acid Composition of Partially Hydrogenated Herring Oil

Principal type of fatty acid	Weight % in total sample	Monoethylenic isomers wt % of isomer in the given chain length			Monoethylenic isomers wt % of isomer in the total sample	
			Cis	*Trans*	*Cis*	*Trans*
Iso 14:0	0.10	16:1ω12	—	trace	—	—
14:0	8.11	16:1ω11	—	4.8	—	0.13
4,8,12 TMTD	0.26	16:1ω10	trace	15.9	—	0.44
14:1	0.08	16:1ω9	6.1	5.8	0.34	0.16
Iso 15:0	0.32	16:1ω8	13.6	17.3	0.76	0.48
Anteiso 15:0	0.12	16:1ω7	74.1	38.4	4.12	1.07
15:0	0.42	16:1ω6	6.1	7.9	0.34	0.22
15:1	0.05	16:1ω5	—	5.1	—	0.14
Iso 16:0	0.03	16:1ω4	—	3.7	—	0.10
Pristanic	0.31	16:1ω3	—	1.0	—	0.03
16:0	12.20					
16:1	8.35	18:1ω13	—	trace	—	—
	(*cis* 5.58)	18:1ω12	16.9	3.1	1.39	0.13
	(*trans* 2.76)	18:1ω11	8.0	6.2	0.66	0.26
16:2 + ?	0.16	18:1ω10	7.7	9.6	0.63	0.40
Iso 17:0	0.18	18:1ω9	46.6	41.0	3.83	1.69
Anteiso 17:0	0.09	18:1ω8	6.9	17.1	0.57	0.71
17:0	1.36	18:1ω7	13.8	15.7	1.13	0.65
Phytanic	0.21	18:1ω6	—	4.9	—	0.20
Iso 18:0	0.08	18:1ω5	—	2.3	—	0.09
18:0	2.13					
18:1	12.34	20:1ω17	—	trace	—	—
	(*cis* 8.20)	20:1ω16	—	trace	—	—
	(*trans* 4.13)	20:1ω15	—	trace	—	—
		20:1ω14	2.6	4.4	0.34	0.28
18:2 + ?	1.67	20:1ω13	1.3	3.8	0.17	0.24
19:0 + ?	0.38	20:1ω12	3.7	2.9	0.49	0.18
20:0	1.64	20:1ω11	4.6	11.4	0.61	0.72
20:1	19.49	20:1ω10	2.2	15.9	0.29	1.00
	(*cis* 13.20)	20:1ω9	85.6	46.0	11.30	2.89
	(*trans* 6.29)	20:1ω8	—	10.4	—	0.65
		20:1ω7	—	4.9	—	0.30
20:2 + ?	1.14					
22:0	2.04	22:1ω16	7.7 ?	1.2	1.45	0.09
22:1	26.52	22:1ω15	7.7 ?	1.1	1.45	0.09
	(*cis* 18.87)	22:1ω14	trace	2.7	trace	0.21
	(*trans* 7.63)	22:1ω13	trace	5.5	trace	0.42
		22:1ω12	trace	8.2	trace	0.63
		22:1ω11	84.6	57.0	15.96	4.35
		22:1ω10	—	8.9	—	0.68
		22:1ω9	—	14.9	—	1.14

several common marine oils.

The polyunsaturated fatty acids were estimated primarily from GLC analyses on AP-L open-tubular columns where an irregular peak preceding the *cis* monoethylenic fatty acids was shown to represent most of the materials recoverable from argentation TLC plates with mobilities less than the *cis* monoethylenic fatty acids. This figure agreed reasonably well with gravimetric recoveries from the large scale fractionation on Florisil-silver nitrate columns.

In raw fish oils *trans* fatty acids are normally undetectable by application of conventional IR, TLC or GLC technology. It is therefore reasonable to assume that all of the *trans* acids arose by geometrical conversion from pre-existing *cis* unsaturation in the same position, or accompanied formation of new positional isomers. We have drawn attention to positional isomer and chain length enrichment effects in connection with marine oil fatty acid analyses by silver nitrate chromatography (8), and subsequent publication of TLC data for a complete range of *cis* and *trans* octadecenoates (12) further indicates the potential for cross-contamination problems or inadvertent exclusion of a component or components from an otherwise homogenous group of isomeric fatty acids. Without further study we are unable to say whether the absence of a number of *cis* isomers which should have originally been present in the raw herring oil as minor components (specifically 18:1ω5, 20:1ω7, 22:1ω9) arises from deficiencies in the isolation techniques due to such undetected chromatographic mobilities, or to preferential hydrogenation effects such as those recently described for nickel catalyst by Scholfield et al. (13). However analyses reported for several commercial samples of partially hydrogenated vegetable oils of U.S. origin also show the same effect, with the *trans* monounsaturated acids having an appreciably greater proportion of acids of lesser "ω" value (14). The enrichment in *trans* isomers of low "ω" value does not lead to the retention of the vinyl isomers reported for certain hydrogenations with copper chromite catalyst (15), both nuclear magnetic resonance (NMR) and oxidative fission indicating the absence of unsaturation at or near the terminal carbons in the fatty acid chains in the partially hydrogenated fish oil. This also may be an attribute of the nickel catalyst (13). From a hydrogenation point of view, there may be some unknown significance in the accumulation of fatty acids with double bonds in the 6,7 positions (respectively ω12, ω14 and ω16 for 18:1, 20:1 and 22:1) in the various fractions.

The slight decline of percentage *trans* isomers from 33% in the C_{16} and C_{18} chain lengths through 32% in the C_{20} to 29% for the C_{22} chain length (based on GLC area comparisons) is curious as it would be expected that reduction of polyunsaturated acids, mostly of which are in the C_{20} and C_{22} chain length, would favor a higher proportion of *trans* acids. However it should not be overlooked that in herring oils the original monoethylenic acids (*cis*) are normally in proportion to $C_{22} > C_{20} > C_{18} > C_{16}$. There is therefore a greater possibility of unaffected C_{22} and C_{20} than of C_{18} and C_{16} monoethylenic *cis* fatty

acids in this particular analysis.

The reduction of the polyunsaturated acids of Canadian Atlantic herring oil, originally about 2% C_{16}, 4% C_{18}, 8% C_{20} and 6% C_{22} in the total fatty acids (11) perhaps can be detected in the higher "ω" value acids. Thus 20:1ω14 may have arisen from reduction of the 5,6 (ω15) and 8,9 (ω12) bonds of 5,8,11,14,17-eicosapentaenoic (20:5ω3) acid. In this particular case the approximate proportions (10) of 2/3 *trans* and 1/3 *cis* is indicative of new double bond formation.

Gas liquid chromatography on AP-L open-tubular GLC columns is an expeditious means of monitoring *cis-trans* separations achieved by other means (16). A virtually complete separation of the two types of acid was obtained in all chain lengths except the C_{16} on columns of 40,000 theoretical plates. Literature retention data for octadecenoates suggests that some overlap might occur (12), especially for *cis* acids of low "ω" values, but we have no evidence of this in the isomers confirmed by oxidative fission (Table I).

On open-tubular GLC columns coated with BDS the *cis* monounsaturated acids originally present could clearly be discerned as components, albeit with intermediate shoulders in the positions between the peaks regularly separable by this technique (8). The *trans* monounsaturated acids gave less recognizable peak patterns but the locations of the *trans* isomers corresponding to the original *cis* isomers were confirmed through the standards prepared with nitrous acid. For both *cis* and *trans* monounsaturated acids analyzed by GLC on AP-L and BDS semiquantitative confirmation of the individual isomer results listed in Table I was possible. A DuPont curve analyzer was not available but could usefully extend the information obtainable from the GLC envelopes.

The proportions of the various types of fatty acids in partially hydrogenated fish oils, as for vegetable oils, depend on the degree of hydrogenation and type of process as well as on the original oil composition (17,18). Comparison of isomer details in one sample is therefore of limited value. A European analysis of a partially hydrogenated herring oil indicated 41.9% saturated acids and 44.8% monounsaturated acids (19), suggesting less selective hydrogenation conditions. The details of monoethylenic isomers given are also suggestive of severe randomization of geometry and position. These results retain a residual basic relationship to the original double bond positions in the monoethylenic fatty acids of the raw oil, in agreement with our findings (Table I) and another recent study (20).

ACKNOWLEDGMENT

Canada Packers Ltd., Toronto, Canada, supplied the hydrogenated fish oil. NMR analyses were carried out by D.L. Hooper.

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[Received May 24, 1971]

EXTRACTS FROM:-

P.L. Altman and D.S. Dittmer, Editors,
Biology Data Book, vol.1, 2nd edition, 1972.
Bethesda, Maryland: Federation of American Societies for Experimental Biology (FASEB).

Table 42, pp.300-347, contributed by K.S. Markley, Rio de Janeiro, Brazil.
Table 43, pp.348-352, contributed by R.P. Geyer, Harvard University School of Public Health, Cambridge, Mass.

42. FATTY ACIDS: PHYSICAL

Fatty Acid: The first name given is the systematic name. Boiling Point: Data are for 760 mm Hg, unless otherwise specified; d = decomposes; fp = freezing point. Specific Gravity: Data are for the temperature specified and are referred to water at 4°C. Refractive Index: Data are for the temperature specified at the D-line of sodium (589 nm), unless otherwise indicated. Neutralization Value = milligrams of potassium hydroxide required to neutralize one gram of acid. Iodine Number = grams of iodine absorbed by 100 grams of acid. Solubility: sol. = soluble; insol. =

	Fatty Acid [Synonym]	Chemical Formula	Molecular Weight	Melting Point, °C [Form]	Boiling Point 1/, °C [Pressure mm Hg]	Specific Gravity [Temp, °C]	Refractive Index [Temp, °C]
	Saturated Fatty Acids						
1	Methanoic [Formic]	CH_2O_2	46.03	8.4	100.5	1.220 [20]	1.3714 [20]
2	Ethanoic [Acetic]	$C_2H_4O_2$	60.1	16.7	118.2	1.049 [20]	1.3718 [20]
3	Propanoic [Propionic]	$C_3H_6O_2$	74.1	−22.0	141.1	0.992 [20]	1.3874 [20]
4	Butanoic [Butyric]	$C_4H_8O_2$	88.1	−7.9	163.5	0.9587 [20]	1.33906 [20]
5	Pentanoic [Valeric]	$C_5H_{10}O_2$	102.1	−34.5	187.0	0.942 [20]	1.4086 [20]
6	Hexanoic [Caproic]	$C_6H_{12}O_2$	116.2	−3.4	205.8	0.929 [20]	1.41635 [20]
7	Heptanoic [Heptylic; enanthic; enanthylic; heptoic]	$C_7H_{14}O_2$	130.2	−10.5	223.0	0.92215 [20]	1.4230 [20]
8	Octanoic [Caprylic; octylic]	$C_8H_{16}O_2$	144.2	16.7	239.7	0.910 [20]	1.4285 [20]
9	Nonanoic [Pelargonic; nonylic]	$C_9H_{18}O_2$	158.2	12.5	255.6	0.907 [20]	1.4322 [20]
10	Decanoic [Capric]	$C_{10}H_{20}O_2$	172.3	31.6	270.0	0.8858 [40]	1.42855 [40]
11	Undecanoic [Undecylic; hendecanoic]	$C_{11}H_{22}O_2$	186.3	29.3	284.0	0.9905 [25]	1.4202 [70]
12	Dodecanoic [Lauric]	$C_{12}H_{24}O_2$	200.3	44.2	225 [100]	0.8690 [50]	1.4261 [60]
13	Tridecanoic [Tridecylic]	$C_{13}H_{26}O_2$	214.3	41.5	236 [100]	0.8458 [80]	1.4286 [60]
14	Tetradecanoic [Myristic]	$C_{14}H_{28}O_2$	228.4	53.9	250 [100]	0.8622 [54]	1.4273 [70]
15	Pentadecanoic [Pentadecylic]	$C_{15}H_{30}O_2$	242.2	52.3	202.5 [10]	0.8423 [80]	1.4292 [70]
16	Hexadecanoic [Palmitic]	$C_{16}H_{32}O_2$	256.4	63.1	268 [100]	0.8487 [70]	1.4309 [70]

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AND CHEMICAL PROPERTIES

insoluble; misc. = miscible; inf. = infinitely; v. = very; sp. = sparingly; sl. = slightly; c. = cold; h. = hot; a. = acid; ac. = acetic; acet. = acetone; alc. = alcohol (ethanol, unless otherwise specified); aq. = aqueous; bz. = benzene; chl. = chloroform; cyc. = cyclohexane; eth. = ether (diethyl, unless otherwise specified); gl. = glacial; hept. = heptane; hex. = hexane; me. = methyl; pent. = pentane; pet. = petroleum; pyr. = pyridine; tol. = toluene; w. = water. Data in brackets refer to the column heading in brackets. For additional information, consult General References.

Neutralization Value	Iodine Value	Solubility	Source	Reference	
			Saturated Fatty Acids		
1219	.....	sol.w.	*Formica rufa* (red ant)	347,481	1
934.2	.....	sol.w.	Vinegar	168,347	2
757.3	.....	sol.alc., chl., eth., w.	Milk & milk products	347	3
636.8	.....	sol.alc., eth., w.	Butterfat	347	4
549.3	.....	sol.alc., eth.; sl.sol.w.	Essential oils	7,201,288,334, 347	5
483.0	.....	sol.alc., eth.; sl.sol.w.	Butterfat; palm oils	347,408,449	6
431.0	.....	sol.alc., eth.; v.sl.sol.w.	Violet-leaf oil	347,408,424	7
389.1	.....	sol.alc., bz., eth.; v.sl.sol.w.	Butterfat; palm-kernel oils	347	8
354.6	.....	sol.alc., chl., eth.; v.sl.sol.w.	Butterfat; hair fat	21,22,523	9
325.7	.....	sol.alc., eth., pet.eth.; v.sl.sol.w.	Butterfat; palm-kernel oils	353,380,449	10
301.2	.....	sol.alc., chl., eth., pet.eth.	Human-hair fat	219,523	11
280.1	.....	sol.acet., alc., eth., pet.eth.	Lauraceae oils; Arecaceae	55,226,236	12
261.8	.....	sol.acet., alc., eth., pet.eth.	Human-hair fat	219,305,445,523	13
245.7	.....	sol.acet., alc., eth., pet.eth.	Myristicaceae fats	165,237,391,461	14
231.5	.....	sol.acet., alc., eth., pet.eth.	Sheep, hair, & milk fats	305,362,523	15
218.8	.....	sol.acet., h.alc., eth., pet.eth.	Palm-pulp oils	56,237,306,317, 329,388,435	16

continued

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Aetiology of Multiple Sclerosis

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Are dietary defects a cause of multiple sclerosis? There is some evidence that a lack of certain fatty acids during the maturation of the brain results in demyelination.

Nature 215: 821–823, 1967

ONE hypothesis of the aetiology of multiple sclerosis is that the disease is a result of a dietary deficiency of polyunsaturated fatty acids (PUFA). Sinclair[1] concluded that the diets of civilized countries were becoming deficient in these fatty acids and thought that one sequela of such a deficiency could be demyelination of the nervous system. Swank[2] has suggested that multiple sclerosis is the result of the ingestion of a diet rich in animal fats deficient in polyunsaturated fatty acids. These two concepts may not be incompatible, because a low saturated fat intake may cause a sparing of these fatty acids[3]. In areas of the world where there is a high incidence of multiple sclerosis (Northern United States, Central and Northern Europe), a dietary deficiency of polyunsaturated fatty acids is unlikely to occur, and this makes it unlikely that such a deficiency is a cause of the disease.

At least two of the polyunsaturated fatty acids, namely, linoleic and linolenic acid, have different biological functions[4]. In the animal organism, the biosynthesis of polyunsaturated fatty acids proceeds along two independent metabolic pathways[5,6]. Longer chain ω6 series acids are synthesized from Δ9,12 linoleic acid (18 : 2 ω6) as a precursor. (The ω-number is designated in reference to the position of the double bond starting from the methyl end of the molecule.) α-Linolenic acid (18 : 3 ω3) is required for the biosynthesis of the longer chain ω3 fatty acids. The interconversion of ω6 and ω3 series acid does not appear to occur in the animal organism[7] or in developing chick brain[8].

The two principal polyunsaturated fatty acids in mammalian[9,10] and chick brain[11] are arachidonic (20 : 4), an ω6 acid, and docosahexaenoic (22 : 6), an ω3 series acid. These acids are found primarily in the glycerophosphatides and more specifically in the phosphatidylethanolamine (PEA) and phosphatidylserine (PS) fractions, with lesser amounts in phosphatidylcholine (PC). Rathbone[12] found 8·6 per cent and 11·4 per cent of the total fatty acids of rat brain consisted of 20 : 4 ω6 and 22 : 6 ω3, respectively. In the fully developed chick embryonic brain, 20 : 4 constituted 13·5 per cent, 4·1 per cent and 3·8 per cent of the total fatty acids of the PEA, PS and PC fractions, respectively. The comparable values for 22 : 6 were 19·8 per cent, 26·2 per cent and 1·1 per cent[13]. O'Brien and Sampson[14] found that 20 : 4 ω6, 22 : 5 ω6 and 22 : 6 ω3 were present at a relatively high level in PEA and PS in human brain. It is apparent that the

brain cephalin fraction is relatively rich in ω6 and ω3 series polyunsaturated fatty acids.

The composition of brain fatty acids may be influenced by the fatty acid composition of the diet. This relationship has been demonstrated in chickens[15], young rats[16] and older rats[12]. Feeding 18 : 2 leads to an increase in longer chain ω6 acids and feeding 18 : 3 to an increase in ω3 series acid, and so Mohrhauer and Holman concluded that at least one member of each family of polyunsaturated fatty acids must be able to pass through the blood–brain barrier (this term is used in a functional sense in further discussion). Lipid-soluble substances are known to penetrate the blood–brain barrier more readily than most aqueous-soluble compounds[17], and Sperry *et al.*[18] have determined that labelled octanoic acid can penetrate the blood–brain barrier.

Usually 18 : 2 constitutes about 1 per cent of the fatty acids in the brain[19], and this fatty acid pool may be utilized for the biosynthesis of longer chain ω6 polyunsaturated fatty acids. There is a slow turnover of phospholipids in adult brain[20] and so the brain may be able to survive any 18 : 2 deficiency in the diet for extended time periods. In our work with chicks, however, as well as those already cited with mammalian species, virtually no brain phospholipid 18 : 3 could be detected. In the absence of any pool of 18 : 3, any requirements for this ω3 fatty acid would seem to depend on an adequate exogenous source. If the intake of polyunsaturated fatty acids is less than marginal during development, subsequent requirements for ω3 acids as a result of normal turnover cannot be met. Alternatively, the structure of developing nervous tissue formed during periods when an adequate exogenous source of ω3 acids is unavailable may be defective and break down after an extended time period.

The known stability of myelin and the low turnover of myelin lipids[21-23] may seem inconsistent with the foregoing concept. The same studies, however, demonstrated that the turnover of the lecithin and cephalin fractions is greater than that of spingomyelin, cerebrosides or cholesterol. It should be borne in mind that there may be a relative deficiency of ω3 acids early in life, and perhaps even *in utero*, on a diet deficient in ω3 fatty acid. The margin of safety involved here is unknown. In addition, the primary metabolic lesion may not occur in myelin, but demyelination may be a secondary effect of abnormal metabolism in other brain structures; for example, neurones, glia or neuronal processes. Thus Terry and Harkin[24] indicated in Wallerian degeneration that the periodic layers of myelin were intact at a time when the axoplasm and mitochondria are damaged. Another factor to be considered is the relatively late time of peak onset of the disease (about 30 yr) (ref. 25), a fact which may be compatible with the slow myelin lipid turnover.

Experimental data which tend to support the concept of ω3-deficiency have been reported. Weil[26] reported a decrease of phospholipids in the brain in multiple sclerosis. Gerstl *et al.*[27,28] found deficits in phospholipids and fatty acids in intact white matter from brains of patients with multiple sclerosis. They also reported that the lipid content of myelin from such brains was less than that of the control. Kishimoto *et al.*[29] analysed the fatty acids of glycerophospholipids from apparently normal white matter derived from patients with multiple sclerosis, and reported that the 22 : 6 ω3 content of the ester-linked fatty acids was less than half of that found in normal white matter. The number of cases thus far reported is too small to permit generalizations to be made. Lecithin from the white matter of patients with multiple sclerosis contains a larger percentage of saturated fatty acids than that of control subjects[30]. Analysis of the cerebrospinal fluid phospholipids in multiple sclerosis[31,32] has shown that a relative increase in the cephalin fraction occurs and the severity of the clinical picture has shown a good correlation with the increase in cerebrospinal fluid cephalin. A group of patients with multiple sclerosis studied by Tourtelotte *et al.*[33] gave results which can be recalculated in units used by McArdle and Zilkha to show a similar trend. No determinations for polyunsaturated fatty acids were attempted in these studies. Other phospholipid fractions were normal.

The two principal dietary sources of the ω3 fatty acids are a variety of seed and marine oils[34], the latter rich in polyunsaturated C_{20}- and C_{22}-acids. Differences in specie and environmental conditions influence the ω3 fatty acid content but the following generalizations may be made[34]. Edible seed oils with a significant 18 : 3 content are found in soybeans, whole wheat, millet, a number of nut and fruit oils, and some non-edible seeds yield oils which are used for cooking in many parts of the world; for example, linseed and rapeseed. In addition, a wide variety of vegetable and fruit oils indigenous to tropical and sub-tropical climates contain 18 : 3 as a minor component. Marine oils have a high content of C_{20}- and C_{22}-unsaturated fatty acids, while oils from fresh water fish contain C_{18}-unsaturates[35]. Cod-liver oil is reported[36] to contain 0·7 per cent 18 : 3 and 8·7 per cent 22 : 6. The fish oils of the sardine, herring, menhaden and mackerel are rich in C_{20}- and C_{22}-unsaturates. The principal site of lipid storage in fish is the liver[37] and the amount present in extra-liver sources depends on the nutritive condition and the specie. It appears that where the whole fish is consumed, an increased supply of ω3-type acids would be available. The ready anti-oxidizability of these highly polyunsaturated fatty acids is relevant to fish oils. Drying of the fish, lengthy exposure to air, and excessive cooking would cause peroxidation or polymerization of the essential fatty acids and make the amount of protective factor available uncertain.

The difficulties of studying the epidemiology of a low-incidence disorder like multiple sclerosis has already been detailed by McAlpine, Lumsden and Acheson[38], and the citations that follow refer to the page number in their treatise. If the known epidemiological data are correlated with the distribution of foods containing fatty acids of the linolenic acid type, the following points can be made.

(*a*) In North America, Europe, the Near East and probably Australia and Asia (except for Japan and China) there appears to exist a north–south gradient, with the incidence of the disease lower in warmer latitudes (p. 43). This low incidence occurs in areas where the consumption of animal protein is generally low and where there is an availability of a large variety of seed and fruit oils, in many cases dietary staples, which contain small to moderate amounts of 18 : 3. Thus the use of palm oil in the tropics, millet in parts of Asia, safflower, cottonseed and rapeseed oils, as well as berries and fruits containing 18 : 3 in warm latitudes, could be preventive of multiple sclerosis.

(*b*) The low incidence of multiple sclerosis in Japan (p. 5) and China (p. 27) may be attributed to the widespread use of soybeans in the diet as well as the availability of marine foods in many cases eaten uncooked, which would tend to preserve the content of polyunsaturated fatty acids.

(*c*) There are variations in the patterns of distribution of multiple sclerosis in the high incidence area of Central and Northern Europe. Where statistics are available (Scotland and Norway) they indicate that high incidence areas involve " . . . remote, circumscribed or mountainous tracts of country where there may be special problems such as . . . lack of variety in the diet" (p. 32). Thus in Norway (p. 48) the disease is uncommon in coastal fishing areas and common in interior agricultural communities. Sallstrom[39] found the incidence of the disease high in agricultural workers in Sweden.

(*d*) Whether the incidence of the disease differs in urban versus rural areas is not well documented (p. 32). Miller *et al.* (p. 38) have suggested that in England and Wales the risk of the unskilled worker developing multiple sclerosis was half that of someone in the professional or

TABLE IV
Removal of Copper From Hydrogenated Soybean Oil by Cation-Exchange Resin

Treatment	Resin used, % of oil	Copper content, ppm	Copper removed, %
Hydrogenated soybean oil	..	1.2	....
Resin (Dowex)			
50–X8, 100–200 mesh	1	0.043	96
50–X8, 100–200 mesh	2	0.027	98
50–X8, 100–200 mesh	3	0.030	97
50–X4, 50–100 mesh	1	0.027	98
50–X4, 50–100 mesh	2	0.046	96
50–X4, 50–100 mesh	3	0.034	97

Several 1 gal batches of refined, bleached soybean oil were partially hydrogenated in the presence of a copper-chromium-barium catalyst and treated with 1% cation-exchange resin and 20% water for 15 min at room temperature. After treatment, respective batches contained 0.02, 0.03, 0.04, 0.06 and 0.02 ppm copper. The original unhydrogenated oil analyzed 0.06 ppm copper but analyses were not made on the hydrogenated oils before treatment.

Analyses of our samples by the Wisconsin Alumni Research Foundation showed that hydrogenation of soybean oil with a copper-chromium-barium catalyst increased the amount of all three metals in the oil. The oil samples were ashed at the Northern Laboratory, and the ash was analyzed by emission spectroscopy at Wisconsin. For example, a soybean oil containing 0.01 ppm barium, 0.046 ppm copper and <0.017 ppm chromium before hydrogenation contained 2.46 ppm barium, >0.85 ppm copper and 0.83 ppm chromium after hydrogenation. After a sample of the hydrogenated oil was treated 15 min with 1% resin and 20% water, it contained 0.5 ppm barium, 0.059 ppm copper and 0.049 ppm chromium.

Results and Discussion

Based on the data secured and economic considerations, a preliminary comparative evaluation may be made of the five methods which we examined for removing copper from soybean oil. Alkali refining under the conditions investigated is not adequate for copper removal, although removal achieved by refining followed by bleaching may be adequate. Treatments with citric acid by the method described above, or by the ion-exchange method, have been shown to lower the copper content of a soybean oil partially hydrogenated with a copper-bearing catalyst to about 0.1 ppm or lower. Bleaching and washing with dilute acid were slightly less effective, but properly conducted countercurrent acid washing should remove a greater amount of copper than our acid-washing results show. Bleaching entails a loss of oil, but the other methods of treatment are not expected to. Consideration must be given to other factors, such as reagent cost, equipment cost, heating requirements and waste disposal in the selection of the preferred method.

Oil Stability

One gallon of commercially refined, bleached soybean oil was hydrogenated to an IV (calculated from GLC data) of 118.0 in the presence of a commercial copper-chromium catalyst. The linolenate content was 0.7% as determined by alkali isomerization. After removal of the catalyst, one-half of the oil was bleached 15 min at 110 C with 1% Super Filtrol, and the other half was treated with 1% Dowex 50–X4, 100–200 mesh, and 20% distilled water for 15 min at room temperature. The original oil contained 0.06 ppm copper and the hydrogenated oil 0.29 ppm copper. Both the bleached and cation-exchange resin-treated oils contained 0.11 ppm copper.

TABLE V
Taste Panel Evaluation of Partially Hydrogenated Soybean Oil

Samples	Hydrogenated soybean oil			
	Cation resin treated		Bleached	
	Flavor score	Peroxide value	Flavor score	Peroxide value
Storage conditions				
Initial	7.7	0.0	7.8	0.0
Stored 4 days at 60 C	6.5	0.6	6.1	0.7
Stored 8 days at 60 C	6.0	5.5	6.4	3.0
AOM peroxide value	3.9		4.9	
Copper, ppm	0.11		0.11	

Two samples of the oil, variously treated, were deodorized in the laboratory with 0.01% citric acid added at 125 C during the cooling period; these consisted of partially hydrogenated, resin-treated oil and partially hydrogenated, bleached oil. Taste panel flavor and oxidative stability results for these oils are summarized in Table V. The initial flavor scores for the partially hydrogenated, deodorized oils were typical of good quality oils. After both four and eight days' storage at 60 C, the hydrogenated oils received about the same average flavor scores and were still in the range considered acceptable by the taste panel. They also had low peroxide values after four and eight days' storage and low AOM peroxide values. Comparison of flavor scores for the bleached and the resin treated oils indicates that the resin and bleaching treatments were about comparable with respect to their effect on flavor stability.

Of the several methods available for removing copper from partially hydrogenated soybean oils, the cation-exchange resin treatment in an oil-aqueous system appears very promising from overall considerations.

ACKNOWLEDGMENT

Gary List deodorized oil samples for taste panel evaluation and Lloyd Carlson assisted with hydrogenations.

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[Received March 10, 1969]

Copy of paper submitted for pub. Feb. 5, 1973

Manuscript S-687
September 19, 1972
Draft 3

THE USE AND EFFECT OF MIXED STANDARDS ON THE QUANTITATION OF POLYCHLORINATED BIPHENYLS

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To be published in: Bulletin of Environmental Contamination and Toxicology
Publication should be in July 1973 in volume 10, No. 1.

INTRODUCTION

Polychlorinated biphenyls (PCB's) have been recognized and well documented as global pollutants. An excellent summary of the distribution of PCB's in the global ecosystem has been prepared by RISEBROUGH (1968), and a bibliography covering PCB investigations from 1881 to 1971 has been compiled by QUINBY (1972 for the Oak Ridge National Laboratory.

Despite this history of investigations, the analytical quantitation of PCB's is still largely a matter of approximation. Gas chromatography, the most common analytical procedure for these compounds, is hampered by the lack of primary standards of individual PCB components. PCB's have most often been quantitated by measuring peak heights of prominent GLC peaks, as RISEBROUGH (1969), ZITKO (1971a), and HANSEN (1971), or by measuring total area of all PCB peaks as ARMOUR and BURKE (1970) have; and relating these to a suitable standard PCB mixture, such as Aroclor 1254 or 1260. This method may work in some cases as it did for SKRENTNY (1971), but it has severe shortcomings.

If the material being analyzed for PCB's contained only one particular Aroclor, such as 1254, and if the chromatogram of the sample matched that of Aroclor 1254, then measurement of peak heights or peak areas would be satisfactory in the quantitation procedure. Unfortunately, the samples we have been analyzing (fish and fish products) often contain widely varying mixtures of PCB's, and unless the chromatograms match those of a readily available standard, such as one of the Aroclors, an accurate quantitation of the chromatograms is very difficult. Even in cases where a specific Aroclor is fed to an experimental animal, KOEMAN (1969) and MULHERN (1971) found that the profiles of the PCB's retained often do not match those of the original Aroclor.

This in itself, would not be a problem except that, as reported by ZITKO (1971b), and as substantiated by us, the GLC response is <u>not</u> constant for the variously chlorinated isomers found in a PCB mixture. As a result, the values found when analyzing for PCB's can vary by a considerable amount depending on which Aroclor, or mixture of Aroclors, is used as a standard.

The purpose of this paper is to (a) illustrate the problem created by samples containing mixtures of PCB's not readily comparable to a particular Aroclor; (b) to present some figures to illustrate, in numerical terms, the effect that the choice of standards has on the final result; and (c) to offer as a solution to the problem the use of standards of mixed Aroclors. For this reason, a detailed section on Standards has been included in the following section.

METHODS

Extraction and Cleanup

Samples were extracted into hexane and cleaned up on a Florisil (PR grade)* column, as reported by REINERT (1970). Elution from Florisil with neat hexane effected better cleanup than that achieved with the mixed solvent described by Reinert.

PCB Separation

PCB's were separated from DDT and its analogs by means of a silica gel column according to the method of REINERT (1971) with a slight modification. Reinert recommended using completely anhydrous silica gel activated at 200°C for 8 hours prior to use, but treating the silica gel this way resulted in the recovery of only 30-40% of the PCB's. Activation for 17 hours and addition of 2% H_2O by weight to the activated silica gel gave 85-100% separation of PCB's and pesticides.

Chromatography Conditions

A Wilkens 600D gas chromatograph with a tritium detector and a 5'x 1/8" silanized glass column containing equal parts of 10% DC-200 on 80/100 mesh Gas-Chrom Q** and 15% QF-1 on 80/100 mesh Gas-Chrom Q** was used for quantitation. Column temperature was maintained at 195°C by a Varian Aerograph 328A Isothermal Unit. Injector temperature was 240°C and the N_2 gas flow 50-60 ml/min.

Quantitation

The choice of appropriate mixed standard was made by visual comparison of gas chromatograms of the extract from the sample and of varying proportions of PCB standards. Quantitation was achieved by cutting out the chromatograms and weighing them on an analytical balance. Such an integrated measurement of total peak area offers the best possibility at present of compensating

*Mention of trade names and specific companies does not imply endorsement

**Prepared by Applied Science Laboratories, Inc., State College, PA

for the differences between a sample containing most of a particular Aroclor (some components having been either lost or differentially metabolized) and a standard of that particular Aroclor.

Standards

A detailed description may prove useful. Once separate standards of Aroclors 1254 and 1260 had been prepared, mixtures of the two were prepared at increments of about 10% in Aroclor 1260 content. The total (1254 + 1260) concentration for each mixture was kept approximately equal so that the chromatograms could be compared easily and an assessment could be made of the changes in total peak area with the changes in Aroclor composition. Figure 1 illustrates the change in total peak area as the composition of the standard changes.

Once all the mixed standards had been run on the GLC, the chromatograms were cut out, weighed (to give Figure 1), and placed in a reference file. This allowed the GLC operator to match rapidly the profile obtained from a sample with that of

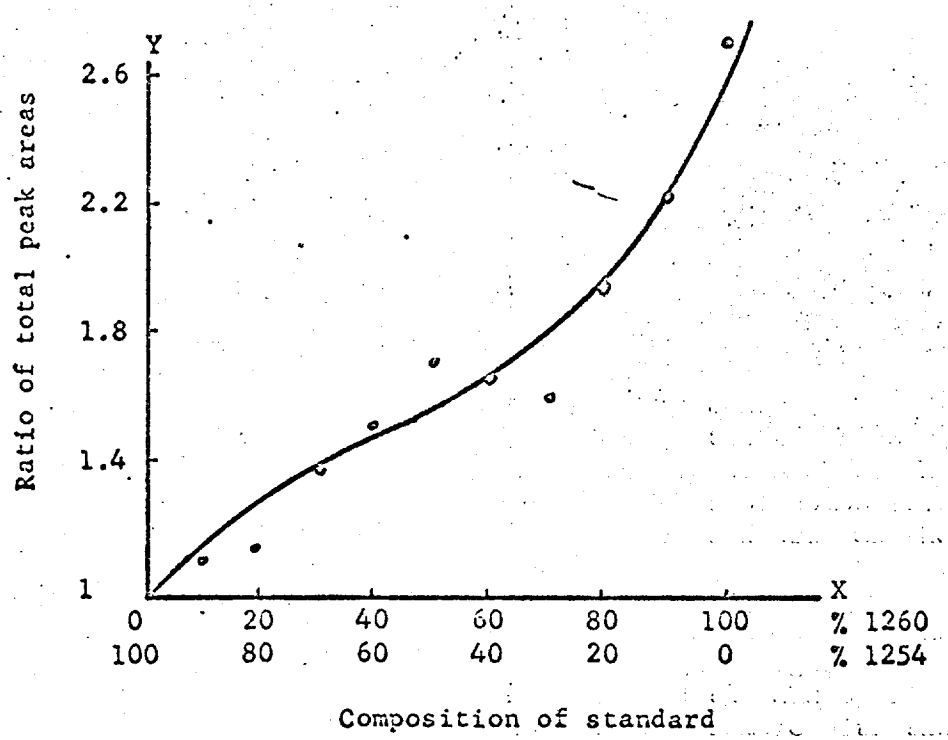

Figure 1. Dependence of total peak area on the relative amounts of Aroclors 1254 and 1260 in a mixed standard. (Total peak area = g paper/ng. PCB's injected).

the appropriate mixed standard. It was then relatively easy to make several appropriate dilutions of the mixed standard to obtain a standard curve. The same procedure can also be used for preparing mixed standards of other Aroclors, such as 1242 and 1254. Mixed standards of three or more Aroclors are feasible but obviously more complicated.

RESULTS AND DISCUSSION

Figure 2 illustrates the problem created by samples containing mixtures of PCB's not readily comparable to a specific Aroclor. Chromatograms of extracts of menhaden (Brevoortia tyrannus) meal, channel catfish (Ictalurus punctatus), and silky shark (Carcharhinus falciformis) liver*** are placed to indicate the approximate composition in terms of Aroclors present. Although the chromatograms are somewhat crowded on the page, it can be seen that neither the channel catfish nor the silky shark can be quantitated as Aroclor 1254 or 1260. The profiles more closely resemble those of the mixed Aroclors.

The table illustrates what happens when a sample is quantitated by Aroclors 1254, 1260, and various mixtures of the two. The asterisks indicate the best approximation of the true values as calculated by the standards judged most similar in profile to the samples. If one were to plot the values with the changing compositions of the standards, the curves would resemble those of Figure 1. Both Figure 1 and the table illustrate the fact that the selection of a standard can alter the analytical values by more than a factor of 2. In the case of the channel catfish, it can be seen that the standard one chooses for quantitation could determine whether or not it is over the 5.0 ppm temporary tolerance proposed by FDA for fish.

SUMMARY

A study was made of the problems encountered when the chromatogram of a sample being analyzed for PCB's via GLC cannot readily be compared to a particular Aroclor standard. The use of mixed Aroclor standards was proposed and a study made of the effect of mixed standards on the analytical results. It was found that the choice of a standard could alter the values by more than a factor of 2. In addition, the particular mixture used as a standard should be reported along with resulting values.

***From the laboratory of Dr. George Harvey, Woods Hole Oceanographic Institution

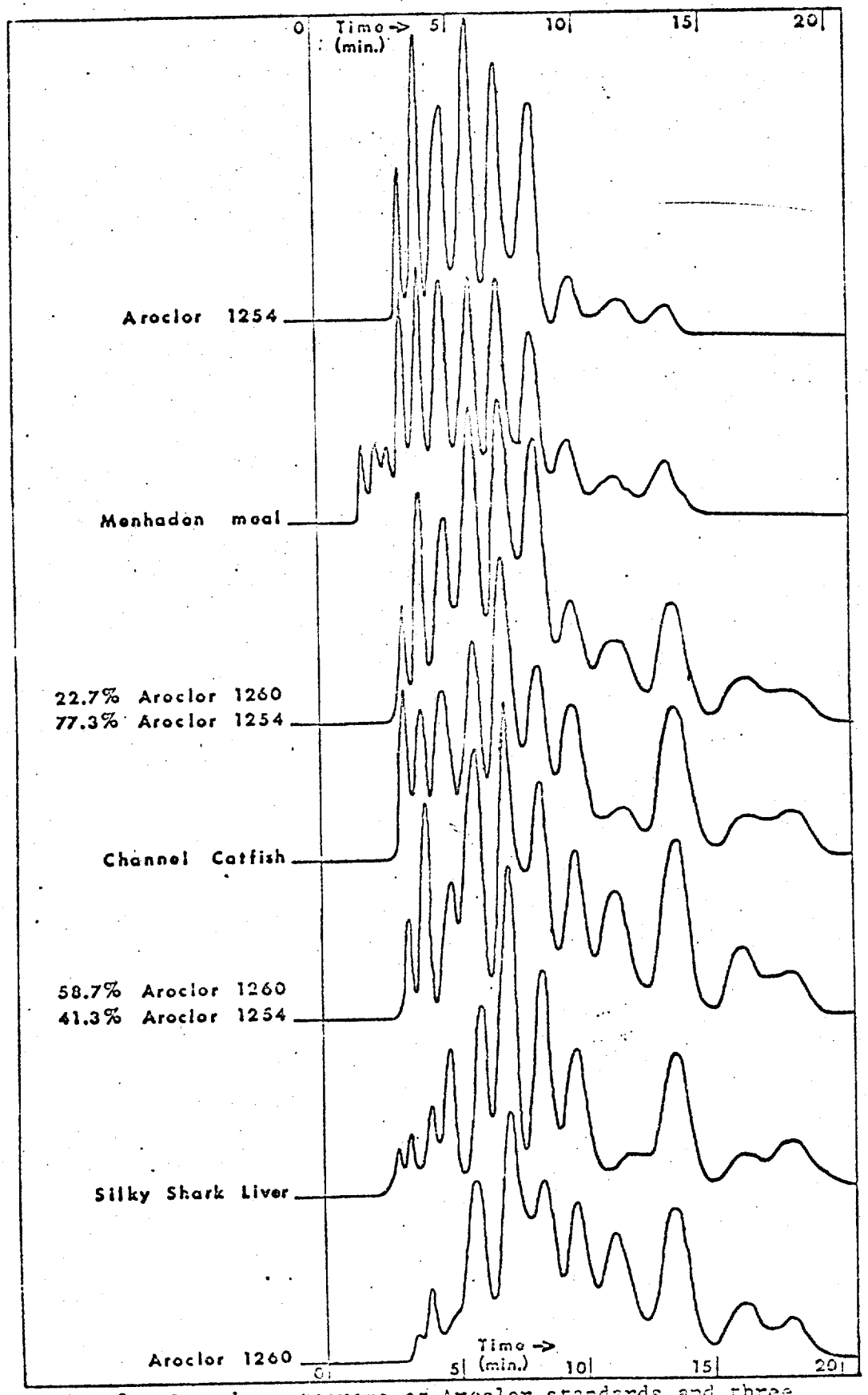

Figure 2. Gas chromatograms of Aroclor standards and three fishery products. Single vertical line represents solvent peaks.

laborious particularly if quantitative data are required. For this reason reliance is placed on tests designed to measure the concentration of certain specific oxidation products and in spite of their limitations, particularly with regard to their relationship with flavor changes, these tests have proved most useful in experimental studies.

However, where knowledge of the stability of an oil is required together with, say, the effect of treatment, e.g., with antioxidant, the simplest and most effective technique is to measure oxygen absorption by one of the standard biochemical methods (Dixon 1934; Einset *et al.* 1957). Fish oils are so unstable that studies can be completed within a reasonably short time by using slightly elevated temperatures, say, up to 40°C. The use of the standard stability tests (Pohle *et al.* 1962) employing much higher temperatures can provide results in a matter of hours, but doubts have been expressed about the applicability of these results to storage at ordinary temperatures even with the much more stable vegetable fats (Paul and Roylance 1962). Claims have been made that reliable results are obtained with marine oils by measuring the increases in weight that occur during exposure of samples of oil under standard conditions at ordinary temperatures, or slightly above (Olcott and Einset 1958B).

Often, information is required about the extent of oxidation or change in an oil of unknown history, or under conditions in which oxygen absorption cannot easily be measured. The following tests have been designed for this purpose.

Determination of Hydroperoxides.—Hydroperoxides are the first measurable components to be formed during the oxidation of fats and the present methods in use for their quantitative estimation are based on that described by Lea (1931) in which the iodine liberated from potassium iodide by a boiling solution of the peroxidized fat in a mixture of acetic acid and chloroform is titrated with 0.002 *N*-sodium thiosulfate. A popular modification of this procedure, in which the fat is allowed to react in the cold for three minutes with a small quantity of an aqueous solution of potassium iodide is that devised by Wheeler (1932). The reaction time required in the cold with a few drops of saturated potassium iodide would appear to be ten minutes and a procedure based on this has been in use in the author's laboratory for years (Banks 1937).

Considerable skill is required in carrying out peroxide determinations on fish oils, because of their high reactivity with oxygen, particularly when in solution in the solvent mixture, and there is no doubt that removal of oxygen from the reaction mixture is essential for reliable results. Heaton and Uri (1958) describe a micro-method in which the iodine liberated by the reaction carried out under oxygen-free nitrogen is measured spectroscopically and a modification of this method (Hardy and Smith 1966) has

been used for studying the relationship between oxygen absorption and the formation of measurable oxidation products.

Results can be expressed as the number of ml of 0.002 *N*-thiosulphate per gm. of fat or as m.eq. of peroxide per kg. of fat or as μ moles of hydroperoxide per gm.

Kreis Test.—This test depends on the formation of a red color when an oxidized fat is shaken with an ethereal solution of phloroglucinol. In the presence of strong hydrochloric acid it is alleged to be caused mainly by a reaction with epihydrinaldehyde. Unfortunately the colored component is thermally labile and the reaction with highly unsaturated marine oils must be carried out in the complete absence of oxygen if reliable results are to be obtained. It is considered that the reaction is too complex for practical use (Hardy and Smith 1966).

Thiobarbituric Acid Test (TBA).—This method has been developed recently and appears to be related to the Kreis test. It involves heating the fat or oil with thiobarbituric acid in the presence of strong acid, when a red coloration is produced as a result of a reaction between the thiobarbituric acid and malonaldehyde (Yu and Sinnhuber 1957). The latter may be present in the oil or it may be produced from an oxidation product during the course of the reaction. The intensity of the red color is proportional to the concentration of malonaldehyde in the mixture and it can be measured in a spectrophotometer. Results are expressed as mg. of malonaldehyde per kg. of oil (Konig and Silk 1963; Sinnhuber and Yu 1958).

More recently, it has been observed that it is necessary to carry out the reaction in the absence of oxygen in order to obtain reliable results with marine oils (Hardy and Smith 1966). This paper also compares the values of all three methods during the oxidation of herring oil at different temperatures and relates these values to the overall oxygen uptake. The results indicate that the determination of peroxides under anaerobic conditions is probably the most sensitive and reliable procedure for determining early oxidative changes.

DETERIORATION OF THE FAT OF FISH AND FISH PRODUCTS

Hydrolysis

As already indicated, the presence of free acid in marine oils does not in itself affect flavor and this applies also to the free acids that accumulate in the lipids of fish during preservation in ice or during cold storage. Only insofar as free acids will catalyze oxidation, can they be expected to have an adverse effect on such products as fish meal, and here the effect can only be marginal, in view of the other factors involved.

With frozen products, however, there seem to be indications that lipo-

lytic changes in the frozen tissue may have very important effects on overall quality, in addition to any possible influence they may have on the rate of oxidation of the neutral lipid. Frozen fish deteriorate rapidly at temperatures above —18°C. and storage at —30°C. is generally recommended for satisfactory preservation. In particular, adverse changes in appearance and texture will occur at the higher temperatures which have been attributed to denaturation of the main muscle protein, characterized by a loss of solubility of the protein in dilute solutions of sodium chloride. Dyer and Fraser (1959), working with cod, have shown that there is a remarkable similarity between the rates of protein denaturation and of the increase in acidity of the extracted lipids. It has since been shown (King *et al.* 1962) that linoleic and linolenic acids will precipitate fish muscle proteins from weak salt solutions and that there is a critical concentration at which this reaction ocurs (Anderson and Steinberg 1964). Consideration is now being given to the possibility of a reaction of this kind occurring in frozen fish, but so far a lot depends on how the results that have been accumulated over the years are interpreted (Olley and Duncan 1965). As yet, there is no direct evidence to suggest that a reaction of this kind does indeed occur *in situ* and further investigations are required to clarify the conflicting views held by different workers.

Oxidative Deterioration

All foods and food products containing fat are susceptible to rancidity. This is particularly so with fatty fish and special precautions have always been taken to prevent oxidation of fat during preservation. Traditional methods, such as salt curing and heavy salting, drying, and smoking employ procedures, which, in other forms, are used today to retard or inhibit the onset of rancidity. Thus, exclusion of air during the salting of herring, by keeping the fish completely immersed in the pickle, has long been known to be beneficial, and the good keeping quality of the heavily smoked and dried product, or red herring, can no doubt be attributed in part to impregnation of the fish with phenolic antioxidants, now known to be present in woodsmoke.

It has been known for some years now that the flesh of herring contains a catalyst of fat oxidation (Banks 1937) later shown to consist of a number of haem pigments such as hemoglobin, myoglobin and cytochrome-c and allied components (Banks 1939). These substances are not present to any great extent in the flesh of the non-fatty fish, but are to be found in the lateral band of red muscle of all fatty fish. These components catalyze the development of rancidity in frozen fatty fish. The presence of salt, arising from the freezing of the fish in cold brine activates the catalysts, probably by increasing the amount of unfrozen water in the frozen tissue and thus

providing more surfaces for catalytic action. There is no evidence that salt activates the catalysts in model systems at temperatures above the freezing point except at very high concentrations. Fatty fish are thus highly susceptible to oxidative rancidity when frozen and cold stored and special precautions involving exclusion of air and storage at —30°C. are necessary (Banks 1945). Various workers have observed that the development of rancidity in frozen herring and mackerel fillets and in salmon steaks can be retarded considerably by antioxidants (Bauernfeind *et al.* 1948; Bauernfeind *et al.* 1951; Stoloff *et al.* 1948; Tarr 1947, 1948). The antioxidants tested included ascorbic acid, various esters of gallic acid and NDGA of which ascorbic acid appears to be the most useful. They have been applied by soaking the fresh, unfrozen fish in an aqueous solution of the antioxidant or by glazing the frozen fish with such a solution or by coating the products with an extract of Irish moss containing the antioxidant. Other studies have indicated that fish treated with antioxidants at the required concentration can have an unattractive flavor (Banks 1952; Anon. 1964).

The extreme conditions required for the proper preservation of frozen fatty fish present problems during distribution. It has been shown, however, that the packaging of trout in an oxygen-impervious plastic pouch under vacuum will delay oxidation of the fat for considerable periods during cold storage of the frozen product (Bramsnaes and Sørensen 1960). A similar effect has been observed with herrings and herring products (Anon. 1964).

Foods can be preserved by the removal of water by evaporation, but the preservation of fatty fish by these means presents serious rancidity problems. Minced cooked herring dehydrated in warm air, for instance, can become rancid in a matter of days. It appears that although antioxidants can be produced during cooking, drying, and during subsequent storage, the quantities involved are too small to have any marked effect on storage life. A very stable dehydrated product has been obtained in the laboratory, however, by adding wood smoke to the air during the drying operation (Banks 1950).

Rancidity is equally a problem with products obtained with the more modern technique of accelerated freeze drying, in which frozen fish are dehydrated under a high vacuum (Goldblith *et al.* 1963; Hanson 1961; Olcott 1962). Antioxidants have not proved to be effective, and the best procedure to prevent oxidative change would appear to be to break the vacuum with nitrogen gas at the end of the process and to maintain an atmosphere of this gas over the products during subsequent storage (Olcott 1962).

Attention was drawn to the potentialities of ionizing radiations for the

long term preservation of foods by Brasch and Huber (1947) but many workers since then have observed that such treatment of foods, including fish, can lead to the development of undesirable odors and flavors as a result of chemical reactions within the products (Shewan 1962).

As already indicated, fats oxidize by a chain reaction involving free radicals and it is to be expected that high energy radiation will increase the tendency for fats to react in this way. This is in fact the case and changes in the fatty tissues of fish and in the lipid fraction of "nonfatty" fish no doubt contribute to the development of undesirable flavors in irradiated fish (Hannan 1955).

The radiation-induced changes are extremely involved, even in pure fats (Chipault 1962; Mercier 1962). They depend on the level of radiation and on the temperature at which this is carried out and on the oxygen tension during and subsequent to radiation. Other autoxidizable components of the flesh may also become involved. As a result, there is no clear picture of the fundamental aspects of the role played by fats in the changes that occur in irradiated fish.

Much work on the irradiation of fish is in progress and the trend is to submit various products to doses of radiation below the level required for complete sterilization, and so obtain a so-called pasteurized product (Proctor *et al.* 1960). Most studies are now directed towards defining the conditions required for a useful extension of the storage life of a number of products, mainly of non-fatty species, and it is now claimed that the shelf life of 13 or so varieties of fish and shellfish can be usefully extended in this way (Kaylor and Slavin 1965).

Problems associated with oxidation are encountered during the production and storage of fish meal, particularly with the products obtained from fatty fish such as menhaden, anchovy, pilchard, and herring. Meals of this type may contain up to ten per cent of oil and the general conditions employed in the more efficient processes are such that the oil is partially oxidized as it leaves the drier. Spontaneous heating of the meal can occur at this stage as a result of further oxidation, which, if not controlled, can lead to runaway heating and serious fire risks. "Curing" the meal allows this oxidation to go under controlled conditions, so that when the reactions involved come to an end, i.e., presumably, when most of the polyunsaturated components of the oil have been oxidized away, the meal can be stored without risk. Even so, however, runaway heating can sometimes occur subsequently during storage, presumably because of variations in the characteristics of meals, depending on the origin of the raw material and production methods (Brock 1959). There is some evidence of antioxidant build-up during the heating of meals (Lea *et al.* 1960) and that added antioxidants notably BHT and NDGA can have a limited effect on

oxidation and spontaneous heating (Lea *et al.* 1958; [illegible] March *et al.* 1961; Merwe and Kapp 1961; Roux 1962).

Determination of Deteriorative Changes

The assessment of deteriorative changes in the fat of fatty fish and fish products is best done by taste panel, where possible. A cooking procedure should be chosen that does not lead to a loss of flavorous components; cooking in a covered casserole on a steam bath is probably the most suitable in this respect. Results can be expressed on a numerical scale or by descriptive terms.

It is often desirable to follow changes by means of one or more of the objective methods available. This involves extraction of the fat, followed by evaporation of the solvent, or the taking of an aliquot of the extract for analysis (Banks 1938; Stansby 1935; Tarr 1947). It is essential during preparation of the fat for analysis that the conditions employed should not affect the components being estimated, in any way. Ethers, for instance, normally contain peroxides and they should not be used for extraction purposes if the solvent is removed before analysis, and if the procedure involves the taking of an aliquot, it will be necessary to run a blank on the reagents, including the solvent. Wet tissue must be dried before the fat can be extracted by most fat solvents, but high temperatures cannot be used for this purpose, since some oxidation components, e.g., hydroperoxides will decompose, and others such as short-chain carbonyls will be lost. It is usual to dry the fish tissue by mixing it with anhydrous sodium sulfate, but only material free from alkali should be employed so as to avoid destruction of hydroperoxides and other components. Finally, evaporation of solvent, if required, should be carried out under mild conditions, preferably below 40°C., for the same reasons, and air should be excluded as far as possible at all stages to prevent oxidation of unattacked glycerides.

The mainly phospholipid material of the non-fatty fish cannot be extracted completely by weak or nonpolar solvents such as ethers and hydrocarbons and a procedure such as that worked out by Bligh and Dyer (1959) should, therefore, be used for this purpose. This method depends on homogenizing the wet tissue with a mixture of methanol and chloroform followed by separation of the extract into a fatty and nonfatty layer by the addition of further chloroform and water. Olley *et al.* (1962) used a similar procedure in connection with a study of the development of free fatty acids in the phospholipids of frozen fish.

Extraction of the fat of fish meal for analytical purposes, or in preparation for assessment of oxidative change, can also present difficulties. The lipids of nonfatty fish meal, consisting as they do almost wholly of phos-

pholipids, will not be completely soluble in weak or non-polar solvents. The lipids of fatty fish meals will also tend to be insoluble in such solvents, because of extensive oxidation and polymerization during production. Nevertheless, extraction of the fats of fish meal for both purposes, using such solvents, is in common use in various countries. The formulation of an extraction procedure of the kind devised by Bligh and Dyer (1959) for wet tissue would seem to be most desirable, so as to eliminate the errors inherent in these existing methods.

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082

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J. Am. Oil Chem. Soc. 46(7): 478-500, 1969

Removal of Copper From Hydrogenated Soybean Oil[1]

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Abstract

Hydrogenation with a copper-chromite catalyst at 170 C, 30 psi, increased the copper content of a refined, bleached soybean oil from 0.02 to as much as 3.8 ppm. Removing residual copper from soybean oil is essential to the successful use of copper catalysts for selective hydrogenation. Various methods were examined to remove this copper, including alkali refining, bleaching, acid washing, citric acid treatment and cation-exchange resin treatment. Properly conducted, each of the methods except alkali refining gives 95% or higher removal of copper introduced during hydrogenation. Ion exchange appears to be the most economical, but addition of about 0.01% citric acid during deodorization may be needed to inactivate traces of unremoved copper. Soybean oil hydrogenated with a copper-chromite catalyst, bleached or treated with an ion-exchange resin and deodorized with 0.01% citric acid added had low AOM peroxide values and acceptable flavor scores after eight days at 60 C which indicate that removal of residual copper from the oil should be adequate for the production of stable oils low in linolenic acid content.

Introduction

In processing soybean oil for edible use, the practice generally accepted is to avoid contact of the oil with equipment constructed of copper or copper alloys. If not removed or deactivated, minute amounts of copper dissolved in the oil from such contact can cause the oil to have poor oxidative and flavor stabilities. As little as 0.03 ppm of copper, in active form, has been shown by Evans et al. (4) to reduce significantly the flavor stability of soybean oil. Small amounts of copper and other metals found in crude vegetable oils are largely removed during refining and bleaching (9). These metals are apparently obtained from the seed during oil extraction.

Copper catalysts, such as the Adkins type (1), promote selective linolenate reduction during partial hydrogenation of soybean oil [Moulton, Moore and Beal, Manuscript in preparation (3,5,7,8)]. These findings have focused attention on a need to remove small amounts of copper from the oil. To produce a flavor-stable soybean salad oil low in linolenate content by copper-catalyzed hydrogenation, effective removal of trace amounts of copper is necessary. Five methods of copper removal were examined to compare their effectiveness and to consider their practicality.

Materials and Methods

Commercially refined, bleached soybean oils were used for all tests. The oils were partially hydrogenated in a 1 gal autoclave with a commercial copper-chromium catalyst to iodine values (IV) of 115–120. IVs were calculated from fatty acid composition data from GLC and by using the theoretical IVs for the pure acid glycerides (9). The partially hydrogenated oils were filtered with about 2% of Hy-Flo SuperCel to remove the catalyst and obtain a clear oil.

The copper content of the hydrogenated oils varied from 0.3 to 3.8 ppm. Increase in copper appeared to be related to free fatty acid content of the oil, type and amount of catalyst used, and time of hydrogenation. The copper content of hydrogenated oils and of oils treated in various ways after hydrogenation was determined by ashing 100 to 200 g samples and analyzing for copper colorimetrically. Samples of oil were weighed into acid-washed porcelain evaporating dishes, which were placed on hot plates at low heat. As the oil samples started to decompose, the temperature was gradually raised but kept below the point where autoignition would occur. After the samples were converted to a dry, carbonaceous material, the evaporating dishes were placed in a furnace at 500 C for 16 hr and a light gray to white ash resulted. Ashing at temperatures higher than 500 C resulted in loss of copper. The ash was dissolved in dilute (1–4) nitric acid and the solution was diluted to 25 ml in a volumetric flask. Copper was then determined for a suitable aliquot of the solution by the diethyldithiocarbamate method of Andrews and Stringer (2). The method was checked by analyzing oil samples containing added copper. Samples containing 5.0, 1.0, 0.20 and 0.04 ppm copper (added as copper nitrate) analyzed 5.0, 0.75, 0.15 and 0.036 ppm by ashing at 500 C and colorimetric analysis, 4.68, 0.91, 0.16 and 0.036 ppm by low temperature dry ashing and colorimetric analysis (6), and 4.43, 0.837, 0.167 and 0.042 ppm by neutron activation analysis (6).

Copper Removal Treatments and Results

Either alkali refining plus bleaching or bleaching alone has been used to remove nickel from nickel-hydrogenated vegetable oils. Refining alone is not generally recommended because of the difficulty of washing out traces of soap left in the oil. Bleaching after refining removes substantially all traces of soap.

To study the effectiveness of refining, bleaching and other treatment methods for removing traces of copper from soybean oil, an oil containing 0.08% free fatty acid and 0.04 ppm copper was partially hydrogenated with a commercially available copper-chromium catalyst. The hydrogenated oil contained 3.4 ppm copper as shown in Table I.

To refine this oil, 200 g was vigorously mixed for 15 min at 25 C with 2 g of sodium hydroxide dissolved in 50 g of water. The resulting emulsified

TABLE I
Removal of Copper From Hydrogenated Soybean Oil by Alkali Refining or Bleaching

Treatment	Copper content, ppm	Copper removed, %
Hydrogenated soybean oil	3.4	....
Refine, 1% NaOH (on oil) in 4% solution	1.5	60
Bleach		
½% Super Filtrol	0.52	86
1% Super Filtrol	0.28	93
2% Super Filtrol	0.20	95
2% Darco G-60 activated carbon	0.13	97

[1] Presented at AOCS Meeting, Chicago, October 1967.
[2] No. Utiliz. Res. Dev. Div., ARS, USDA.

TABLE II
Removal of Copper From Hydrogenated Soybean Oil by Acid Extraction

Treatment						
Acid	Strength, %	Amount, g solution/100 g oil	Mixing time, min	No. of extractions	Copper content, ppm	Copper removed, %
Original soybean oil					0.038	....
Hydrogenated soybean oil, Cu-Cr catalyst					3.80	....
Citric	2.0	65	15	1	1.10	71
H_2SO_4	5.0	5	20	1	1.30	66
H_2SO_4	5.0	50	5	1	0.52	86
HCl	1.0	15	1	3	0.17	95
HCl	2.5	15	1	3	0.19	95
H_2SO_4	2.5	15	1	3	0.15	96
H_2SO_4	5.0	5	20	3	0.17	95
H_2SO_4	5.0	50	20	3	0.21	94

mixture was centrifuged without heating to separate the phases and the aqueous layer was removed. The refined oil contained 1.5 ppm copper. This amount corresponds to a removal of about 60% of the copper in the hydrogenated oil. Other refining conditions were not examined.

To bleach the oil, 200 g of oil was heated to about 80 C. Bleaching clay or carbon was added and the temperature was raised to 105-110 C and held 15 min after which the oil was filtered and analyzed for copper. Bleaching with ½% clay was more effective for removing traces of copper than refining with 1% sodium hydroxide. Activated carbon and activated bleaching earth were, within experimental error, about equally effective for removing copper (Table I).

Washing the oil with dilute aqueous acid has been described as an effective method of copper removal (8). Results of several tests of acid washing are shown in Table II.

For these tests 200 g samples of soybean oil partially hydrogenated with a Cu-Cr catalyst and containing 3.8 ppm of copper were vigorously mixed with aqueous acid for several minutes by means of a magnetic stirrer. The resulting mechanical emulsion was centrifuged and the oil layer was washed with 10% distilled water, centrifuged and analyzed for copper. A single wash with a 2% citric acid solution (65 g/100 g oil) with 15 min mixing removed only about 70% of the copper. One wash with a 5% sulfuric acid solution (5 g/100 g oil) for 20 min was about equally effective. A larger amount of sulfuric acid was somewhat more effective. Three consecutive treatments with either 1.0% or 2.5% solutions of hydrochloric acid or a 2.5% and 5% solution of sulfuric acid removed about 95% of the copper. These data indicate that continuous countercurrent washing with a dilute acid should remove copper effectively. However, acid washing must be followed by very thorough water washing because traces of acid left in the oil adversely affect the flavor of the oil after it is deodorized.

The complexing effect of citric acid for trace amounts of iron and copper in soybean oil is widely employed commercially in producing a stable deodorized oil. Usually the metal complex is left in the oil after this treatment because it is extremely small in amount and gelatinous in nature, but it may be separated from the oil by filtration. Some oil processors accomplish this by filtering the oil after it is deodorized.

TABLE III
Removal of Copper From Hydrogenated Soybean Oil by Citric Acid Chelation

Treatment			
Amount of citric acid, % of oil	Method of separation	Copper content, ppm	Copper removed, %
Hydrogenated soybean oil		3.4	....
0.01	Centrifugation	2.7	21
0.1	Centrifugation	1.3	62
0.1	Centrifugation + filtration	0.22	93
1.0	Centrifugation + filtration	0.04	99

In tests summarized in Table III an aqueous solution of citric acid was added to hydrogenated soybean oil, and the oil was then heated to about 125 C under high vacuum to remove the water, passing steam through the oil to promote mixing and to prevent bumping. The oil was cooled under vacuum. In the first two tests of this series, the oil was treated with 0.01% and 0.1% citric acid (1% aqueous solution) and the water then removed under vacuum. The oil was centrifuged (15 min, 900 g), decanted and analyzed. These treatments gave only partial removal of copper. In the third and fourth tests in which the oil was both centrifuged and filtered after addition of citric acid solution and dehydration, almost all copper was removed. These and other data indicate that between 0.1% and 1.0% citric acid is required to remove copper from an initial level of 3.4 ppm.

The copper removed from the oil was accounted for quantitatively in two tests. In each test, 100 g of an oil, found to contain 2.4 ppm copper by ashing the oil, was treated with 1% citric acid in 10 ml distilled water, vacuum dried and filtered as described above. The flask in which the oil was dried was thoroughly rinsed with hexane and absolute ethanol. The solution was placed in a beaker and the solvent evaporated. The filter paper was added to the beaker and its contents ashed. Analysis of the ash (two samples) showed 2.8 and 2.5 ppm copper in the oil.

Treatment of acetone or hexane solutions of vegetable oils with cation-exchange resins reported is partially effective for removing metals (10,11) but no reports have been found describing the use of such resins in a vegetable oil-aqueous system.

Results obtained by using cation-exchange resins in the hydrogen form with a water-soybean oil system are summarized in Table IV. The oil used in this series contained less copper, 1.2 ppm, than oils used in previous studies; however, the ion-exchange resin treatment was highly effective when only 1% resin (dry basis) was used. This amount of resin has several hundred times the exchange capacity required to remove the copper. Oil was mixed with one-fifth its weight of water to which a weighed quantity of resin had been added. The oil-water-resin system was stirred for 15 min at room temperature and centrifuged to recover oil for analysis. It was found that adding the resin to the oil before adding the water frequently resulted in poor copper removal, as did the use of extremely high rates of agitation. Preferential wetting of the resin by the oil appeared to be responsible.

Portions of two resins, one Dowex 50, the other Amberlite IR-120, were used in copper removal tests. Each resin was then washed with a detergent solution, rinsed on a Buchner funnel, washed with 15% HCl and then with distilled water until the effluent from the filter was neutral. These regenerated resins were used to remove copper from a partially hydrogenated soybean oil containing 0.80 ppm copper. For 15 min 200 g of oil, 40 g distilled water and 2 g resin (dry basis) were rapidly mixed. Both treated oils contained less than 0.08 ppm copper.

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PART 45—OLEOMARGARINE, MARGARINE

§ 45.1 [Amended]

7. Section 45.1 is amended by deleting the last sentence of subdivision (v) of paragraph (b)(2).

Any person who will be adversely affected by the foregoing order may at any time on or before April 13, 1973, file with the Hearing Clerk, Department of Health, Education, and Welfare, Room 6-88, 5600 Fishers Lane, Rockville, MD 20852, written objections thereto. Objections shall show wherein the person filing will be adversely affected by the order and specify with particularity the provisions of the order deemed objectionable and the grounds for the objections. If a hearing is requested, the objections must state the issues for the hearing. Such objections must be supported by grounds factually and legally sufficient to justify the relief sought and must include a detailed description and analysis of the factual information intended to be presented in support of the objections in the event that a hearing is held. Objections may be accompanied by a memorandum or brief in support thereof. All documents shall be filed in six copies. Received objections may be seen in the above office during working hours, Monday through Friday.

Effective date. All labeling ordered after December 31, 1973, and all labeling used for products shipped in interstate commerce after December 31, 1974, shall comply with these regulations.

Dated: March 7, 1973.

CHARLES C. EDWARDS,
Commissioner of Food and Drugs.

[FR Doc.73-4673 Filed 3-13-73;8:45 am]

PART 45—OLEOMARGARINE, MARGARINE

Sec.
45.1 Oleomargarine, margarine; identity; label statement of optional ingredients.
45.2 Liquid oleomargarine, liquid margarine; identity; label statement of optional ingredients.

§ 45.1 Oleomargarine, margarine; identity; label statement of optional ingredients.

(a) Oleomargarine, margarine is the plastic food prepared with one or more of the optional fat ingredients named in subparagraph (1) (i), (ii), (iii), and (iv) of this paragraph, as follows:

(1) (i) The rendered fat or oil, or stearin derived therefrom (any or all of which may be hydrogenated), of cattle, sheep, swine, or goats, or any combination of two or more of such articles.

(ii) Any vegetable food fat or oil, or oil or stearin derived therefrom (any or all of which may be hydrogenated), or any combination of two or more of such articles.

(iii) Any combination of ingredients named in subdivisions (i) and (ii) of this subparagraph, in such proportion that the weight of the ingredients named in subdivision (i) either equals the weight of the ingredients named in subdivision (ii), or exceeds such weight by a ratio of not greater than 9 to 1.

(iv) Any combination of ingredients named in subdivisions (i) and (ii) of this subparagraph, in such proportion that the weight of the ingredients named in subdivision (ii) exceeds the weight of the ingredients named in subdivision (i) by a ratio not greater than 9 to 1.

(2) One of the articles designated in subdivisions (i), (ii), (iii), (iv), (v), (vi), (vii), (viii), or (ix) of this subparagraph is intimately mixed with the fat ingredient or ingredients. The ingredients named in subdivisions (i), (ii), (iii), (iv), (v), (vi), and (vii) of this subparagraph are pasteurized and then may be subjected to the action of harmless bacterial starters. The term "milk" as used in this subparagraph means cow's milk.

(i) Cream.

(ii) Milk.

(iii) Skim milk.

(iv) Liquid sweet cream buttermilk.

(v) Any combination of dry or condensed sweet cream buttermilk and water with a total solids content of not less than 8.5 percent.

(vi) Any combination of nonfat dry milk and water, in which the weight of the nonfat dry milk is not less than 10 percent of the weight of the water.

(vii) Any mixture of two or more of the articles named in subdivisions (i), (ii), (iii), (iv), (v), and (vi) of this subparagraph.

(viii) In case only of the fat ingredient designated in subparagraph (1)(ii) of this paragraph, any combination of finely ground soybeans and water, in which the weight of the finely ground soybeans is not less than 10 percent of the weight of the water. The finely ground soybeans are subjected to a heat treatment before or after mixing with the water. The soybeans may or may not be dehulled.

(ix) Water in lieu of any of the foregoing articles of this subparagraph.

Congealing is effected, either with or without contact with water, and the congealed mixture may be worked.

(3) In the preparation of oleomargarine one or more of the following optional ingredients may also be used:

(i) Artificial coloring. For the purposes of this subdivision provitamin A shall be deemed to be artificial coloring.

(ii) Sodium benzoate or benzoic acid or a combination of these, in a quantity not to exceed 0.1 percent of the weight of the finished product.

(iii) Vitamin A (with or without any accompanying vitamin D and with or without vitamin D concentrate), in such quantity that the finished oleomargarine contains not less than 15,000 United States Pharmacopeia units of vitamin A per pound, as determined by the method prescribed in the Pharmacopeia of the United States for total biological vitamin A activity. The vitamin A potency prescribed may be furnished by fish liver oil; by concentrates of vitamin A or its fatty acid esters from animal sources; by synthetic vitamin A or its fatty acid esters; by mixtures of synthetic vitamin A or its fatty acid esters with harmless substances formed during the synthesis of the vitamin A, if the vitamin A or its fatty acid ester constitutes not less than 50 percent of the mixture; by provitamin A; or by any combination of two or more of these. For the purposes of this subdivision the term "fatty acid" may include acetic acid.

(iv) Any safe and suitable artificial flavoring substance that imparts to the food a flavor in semblance of butter. Such artificial flavoring substances are deemed to be safe if they are not food additives as defined in section 201(s) of the Federal Food, Drug, and Cosmetic Act; or, if they are food additives as so defined, they are used in conformity with regulations established pursuant to section 409 of the act.

(v) (*a*) Lecithin, in an amount not exceeding 0.5 percent of the weight of the finished oleomargarine; or

(*b*) Monoglycerides or diglycerides of fat-forming fatty acids, or a combination of these, in an amount not exceeding 0.5 percent of the weight of the finished oleomargarine; or

(*c*) Such monoglycerides and diglycerides in combination with the sodium sulfo-acetate derivatives thereof, in a total amount not exceeding 0.5 percent of the weight of the finished oleomargarine; or

(*d*) A combination of (*a*) and (*b*) of this subdivision, in which the amount of neither exceeds that above stated; or

(*e*) A combination of (*a*) and (*c*) of this subdivision, in a total amount not exceeding 0.5 percent of the weight of the finished oleomargarine.

The weight of the diglycerides in each of ingredients (*b*), (*c*), (*d*), and (*e*) of this subdivision is calculated at one-half actual weight.

(vi) Butter.

(vii) Salt.

(viii) Citric acid incorporated in the fat or oil ingredient used.

(ix) Isopropyl citrates incorporated in the fat or oil ingredient used, in an amount not to exceed 0.02 percent by weight of the finished oleomargarine.

(x) Stearyl citrate incorporated in the fat or oil ingredient used, in an amount not to exceed 0.15 percent by weight of the finished oleomargarine.

(xi) Potassium sorbate, in an amount not to exceed 0.1 percent by weight of the finished oleomargarine.

(xii) Calcium disodium EDTA (calcium disodium ethylenediaminetetraacetate), in an amount not to exceed 75 parts per million by weight of the finished oleomargarine.

(xiii) BHA (butylated hydroxyanisole) or BHT (butylated hydroxytoluene), or a combination of these, incorporated in any animal fat ingredient permitted by subparagraph (1)(i) of this paragraph in

an amount not to exceed 0.02 percent by weight of such animal fat content.

The finished oleomargarine contains not less than 80 percent fat, as determined by the method prescribed in "Official Methods of Analysis of the Association of Official Agricultural Chemists," 7th Edition, page 259 under "Indirect Method," section 15.111. [Ed. note, 10th edition. 1965, p. 242. sec. 15.132.]

(b) The name of the food for which a definition and standard of identity is prescribed by this section is "oleomargarine" or "margarine." The presence of optional ingredients, provided for in paragraph (a) of this section, in the finished margarine shall be declared as set forth in this paragraph.

(1) Fat ingredients shall be declared first in the ingredient statement by the name of the specific fat or oil or stearin used. Where combinations of fat ingredients are used, the names shall be arranged in order of decreasing predominance. If any fat ingredient is hydrogenated, the ingredient statement shall include the word "hydrogenated" or "hardened" at such place or places in the list of fats as to indicate which fats are hydrogenated; for example, "corn oil, hardened soybean oil." If any animal fat ingredient contains an ingredient provided for in paragraph (a)(3)(xiii) of this section, the statement "with _____ _____ added as (a) preservative(s)" or "with _____ added to retard rancidity" shall appear at such place or places in the list of fats as to indicate which animal fats contain these ingredients. The blank is to be filled in with "BHA" and/or "BHT" as appropriate; for example, "beef fat with BHA and BHT added as preservatives".

(2) Immediately following the listing of fat ingredients, other optional ingredients used shall be named in the order of decreasing predominance.

(i) The optional ingredients butter, salt, water, cream, milk, skim milk, sweet cream buttermilk, dried sweet cream buttermilk and water, condensed sweet cream buttermilk and water, nonfat dry milk and water, ground soybeans and water, lecithin, mono- or diglycerides, sodium sulfo-acetate derivatives of mono- or diglycerides shall each be declared by those terms.

(ii) Artificial colors shall be declared by the statement "Artificially colored" or "Artificial coloring added" or "With added artificial coloring."

(iii) Artificial flavors shall be declared by the statement "Artificially flavored" or "Artificial flavoring added" or "With added artificial flavoring."

(iv) Margarine that contains the optional ingredients citric acid, isopropyl citrate, stearyl citrate, or calcium disodium EDTA shall be labeled by the statement "_____ added as a preservative" or "_____ added to protect flavor." Margarine that contains the optional ingredients sodium benzoate or benzoic acid shall be labeled by the statement "_____ added as a preservative" or "_____ as a preservative" or "With added _____ as a preservative." Margarine that contains the optional ingredient potassium sorbate shall be labeled by the statement "_____ added as a preservative" or "_____ added to retard mold growth." The blank in each of the statements in this subdivision is filled in with the common name of the preservative ingredient used.

(v) Vitamin A shall be declared by the statement "Vitamin A added" or "With added vitamin A." Vitamin D shall be declared by the statement "Vitamin D added" or "With added vitamin D."

(vi) Where two or more optional ingredients named in paragraph (a)(3) of this section are used the words "Added" or "With added" need appear only once, either at the beginning or end of the list of such ingredients declared.

(3) Wherever the name "oleomargarine" or "margarine" appears on the label so conspicuously as to be easily seen under customary conditions of purchase, the words and statements prescribed in this section, showing the ingredients used shall immediately and conspicuously precede or follow, or in part precede and in part follow, such name, without intervening written, printed, or other graphic matter.

(Secs. 401, 701, 52 Stat. 1046, as amended, 1055, as amended; 21 U.S.C. 341, 371) [20 F.R. 9615, Dec. 20, 1955, as amended at 28 F.R. 7473, July 23, 1963; 29 F.R. 2383, Feb. 12, 1964; 31 F.R. 5433, Apr. 6, 1966; 33 F.R. 17752, Nov. 28, 1968; 36 F.R. 977, Jan. 21, 1971; 36 F.R. 2400, Feb. 4, 1971; 38 FR 6968, Mar. 14, 1973]

§ 45.2 Liquid oleomargarine, liquid margarine; identity; label statement of optional ingredients.

(a) Liquid oleomargarine, liquid margarine conforms to the definition and standard of identity and to the requirements for label declaration of optional ingredients prescribed for oleomargarine

by § 45.1, except that it is in liquid rather than plastic form.

(b) The name of the food for which a definition and standard of identity is prescribed by this section is "liquid oleomargarine" or "liquid margarine."

(Secs. 401, 701, 52 Stat. 1046, 1055 as amended, 70 Stat. 919, 72 Stat. 948; 21 U.S.C. 341, 371) [31 F.R. 5434, Apr. 6, 1966]

Fish Oil M. E. Stansby (Ed.) 1967

A. Banks

Deteriorative Changes in Fish Oils

INTRODUCTION

Two forms of deteriorative change can occur in fish oils, viz. hydrolysis of the glycerides leading to the production of free fatty acids, and union of the unsaturated compounds of the glycerides with oxygen to produce reactive molecules and rancid flavors. These changes can seriously affect the suitability of fish oils for certain purposes and thus their marketability. In addition, however, it is important not to ignore the effects that changes of this nature can have on the overall quality of fish and fish products. There are suggestions, for instance, that deteriorative changes in the lipids of fish can influence changes in other components, thus affecting overall quality, and abundant evidence that components of the flesh of fish can themselves markedly affect the progress of deterioration in the fatty part itself.

In order to cover the subject adequately, it is necessary to discuss in broad terms the nature of the changes that can occur and then to consider their practical significance in the various products—fish oils, fish, and fish products. As always, of course, it is impossible to divide a subject of this nature into a number of watertight compartments and some overlapping is unavoidable.

HYDROLYSIS AND THE FORMATION OF FREE FATTY ACIDS

General

Consisting as they do in the main of the triglycerides of long-chain fatty acids, all fats and fatty oils are susceptible to hydrolysis, involving the addition of water with the liberation of free acid and hydroxylic component. The reaction goes on in a step-wise fashion and in normal circumstances it is extremely unlikely for hydrolysis to have proceeded beyond the formation of a monoglyceride and free acid.

Hydrolysis is slow in the absence of a catalyst, particularly since fats and fatty oils are immiscible with water. The reaction can be speeded up by alkali with the production of soaps, or by a variety of "hydrolytic agents" or catalysts with the production of free acids and glycerol. In addition, however, certain enzymes or lipases present in the livers and flesh of fish will actively catalyze the liberation of free acids from fish oils. The closely related components—the phospholipids—are al[illegible]dily hydrolyzed in the presence of phospholipases.

The acidity of a fat or fatty oil can be determined by titrating the acids extracted by means of hot neutral ethanol with standard ethanolic alkali. The results may be expressed as acid values, defined as the number of mg. of potassium hydroxide required to neutralize the free acids in 1 gm. of fat, or in terms of percentages of free acid present calculated as oleic acid.

Practical Significance

The amount of free fatty acids in a sample of fat or fatty oil has for long been used for evaluation purposes; the higher the acid content of the fat, the lower the sales value. There are a number of reasons why this should be so. The presence of free acids in an oil can speed up oxidative deterioration (Olcott 1962). In addition, the free acids themselves can interfere with processing, e.g., hydrogenation, in this case by poisoning the catalyst, and all outlets other than soap making, therefore, require a neutral or near-neutral product. The free acids can be removed by treatment with alkali, but the yields of neutral fat fall off disproportionately with increasing content of free acid because of losses in the emulsions that are formed during neutralization and subsequent washing.

Finally, the factors that lead to the production of free acids in fats and fatty oils also lead to other deteriorative changes and experience indicates that an oil with a high content of free acids will also have a poor color, and flavor, and will probably be of low stability.

Fish-liver oils such as cod-liver oil are rich in vitamins A and D and although the availability of synthetic forms has reduced demand for fish-liver oils in some countries, in others these oils are used in quantity for human and animal nutrition. Such oils are not usually refined, except for the removal of the more saturated glycerides by leaving the oil at 0° to −10°C. for some time, followed by filtering (winterizing), chiefly so as to avoid loss of vitamins. In consequence, oils low in free acids are required and most have an acid value of less than 0.5 and are restricted officially in various countries to values of less than 1 to 2. Oils for veterinary purposes are only slightly inferior. Oils of this quality are obtained by processing the livers without delay and most, particularly in Europe, are therefore produced on board the fishing vessel. Oil of good quality is obtained by proper separation of the livers from other organs, steaming for sufficient time (30 min.) to destroy all lipases, followed by as efficient a procedure as possible in the circumstances on board ship to separate the oil from water and liver residue. The oil should then be "winterized" to remove some of the more saturated, solid glycerides or stearin, moisture, and liver debris on shore as soon as possible. Lower grade oils are sometimes ob-

082

tain in the residues or "foots," which, because they have been in contact with liver debris and water, will be somewhat more acid, even though the lipases have been mostly inactivated by heat. Storage of the oils should not lead to an increase in the content of free acids, provided the oils are free from moisture and liver debris and kept free from contamination with microorganisms, some of the commonest type of which can themselves contain lipases.

Because fish flesh and intestines, etc., contain lipase, the same general principles apply in the production of oils of low rancidity from offal and from whole fish. The minimum delay between capture and slaughter of the fish and the production of the oil is required. This is not always possible, and oils produced from fish offal often have high acid values. The oil produced from whole fish kept for some months in salt or calcium chloride before processing, a common practice in Europe, may also contain a high proportion of free acids.

OXIDATIVE DETERIORATION

General

Fish oils can unite readily with atmospheric oxygen to produce a variety of products. With oils that are to be used in one form or another for human or animal feeding, the reaction is a deteriorative one. The products formed have a strong, disagreeable or rancid taste and are considered by some to be toxic (Oldfield *et al.* 1963), though this belief is not general (Carpenter *et al.* 1963; Hanson 1964; Kaunitz 1962). Oxidation is the basic reaction involved in the formation of a typical fatty oil paint film. Fish oils have a limited use in paints and since in such circumstances, oxidation is required to proceed rapidly, it is encouraged by incorporating catalysts or driers with the paints. Rancidity can, therefore, be considered to be the first stage of the formation of paint films, but because it is relatively slow, the course of the reaction and the nature of the reaction products are slightly different. Nevertheless, rancid fish oils, in addition to having a strong fishy odor and flavor, are sometimes described as having a "blown-oil" or "painty" taint as distinct from rancid animal and vegetable fats, which are often described as "tallowy," "cardboardy," etc. These differences may be related to the fact that fish oils contain a high proportion of acids with 4, 5, and 6 double bonds, a factor which, of course, makes them the most labile of natural fats and fatty oils.

There is no reason to believe that the general theories covering the spontaneous and catalytic oxidation of unsaturated fatty material worked out as a result of observations on the oxidation of the esters of oleic and linoleic acids do not apply to fish oils. Nevertheless care must be taken in

applying these theories to marine oils, particularly with regard to the effects of inhibitors and antioxidants. Not enough is known about the very early stages of fat oxidation, which in the case of fish oils are probably much more important than the corresponding stages in animal and vegetable fat. The highly unsaturated hydroperoxides of fish oils are extremely unstable and without doubt break down into flavorous components at very low concentrations.

The various factors—heat, light, pro-oxidants, and antioxidants—that effect the oxidation of other fats and fatty derivatives have similar effects on fish oils. Broadly speaking, the basic requirements for the production of high quality animal fats free from oxidation products, will also apply to the production of fish oils. However, because fish oils generally are so labile, even more care is necessary during production and storage. Oxidation can occur at all stages and once it has set in it is difficult to control and the products of deterioration cannot easily be removed completely, if at all.

Practical Significance

All fats and fatty oils, including fish oils, obtained from fresh raw material contain small quantities of antioxidants. Even in the absence of antioxidants, however, exposure to oxidizing conditions for some time is required before sufficient components capable of promoting rapid oxidation are formed. In addition, depending on the conditions, the products can become contaminated by catalytic materials such as heavy metals and their complexes. The potential storage properties of fats and fatty oils will thus depend on the presence or absence of antioxidants and catalysts and it is possible to have a whole range of products some with more or less the same apparent analytical criteria but all with vastly different storage properties. Variations will be directly related to the raw material from which the product is obtained and to the process and equipment used to obtain it.

In general, the conditions required to obtain a fish oil of low acidity apply also to the production of an oil free from oxidation and of good keeping quality, if only to the extent that free acids will themselves affect keeping quality either by catalyzing oxidation or by combining with contaminating metals, thus enabling them to be more effective as pro-oxidants. In addition, keeping quality will be affected by the nature of the equipment employed in processing. Easily corroded equipment made, for example, of steel or equipment made of copper or its alloys will lead to contamination of the oil with highly reactive metallic catalysts and for this reason it is recommended that only stainless steel or tin-plated metals should be employed. In addition to contamination of this kind, both fish-

liver oils and fish-body oils are obtained from raw material rich in haem pigments, now known to be the most active catalysts of fat oxidation. The presence of these substances can affect the quality of the oil in two ways. In the first place, any contamination of the oil with debris, e.g., in the preparation of fish-liver oil, can cause oxidation if the oil is stored for some time in this condition, and in the second place, any major delay in processing can cause oxidation *in situ* so that the oil subsequently obtained will be highly oxidized. For this reason the practice employed in Europe of storing fatty fish treated with salt or calcium chloride for some months can only lead at best to an oil of low stability and at worst to a highly oxidized oil.

Medicinal oils for human and veterinary purposes need to be of high quality, free from decomposition products and of good stability and, as indicated, considerable care in production and subsequent treatment is, therefore, necessary. Bulk storage of the oils for relatively short periods is not difficult provided that storage tanks are made of noncorrodable materials and exposure to air is kept at a minimum. Usually these oils are retailed in dark glass bottles so as to reduce the adverse effect of exposure to daylight on stability, particularly of vitamin A.

In the same way, high quality fish-body oils can be obtained from fresh whole fish and these can also be stored in bulk provided that the right precautions are taken.

In addition to these high grade oils, other varieties are obtained from fish offal, fish-liver "foots," and stale fish. These oils usually have a high acidity, are dark in color, and partially oxidized. Special outlets are available for oils of this quality.

The development of antioxidants for fats and fatty oils has been in progress for a number of years and a variety of compounds are now permitted for commercial use in most countries. The most popular is propyl gallate (PG), but, in addition, various countries will allow other esters of gallic acid to be added to fats and also such compounds as butylated hydroxy anisole (BHA), butylated hydroxy toluene (BHT) and nordihydroguaiaretic acid (NDGA) together with naturally occurring antioxidants such as tocopherol and lecithin and synergists such as citric, ascorbic, tartaric, and phosphoric acids.

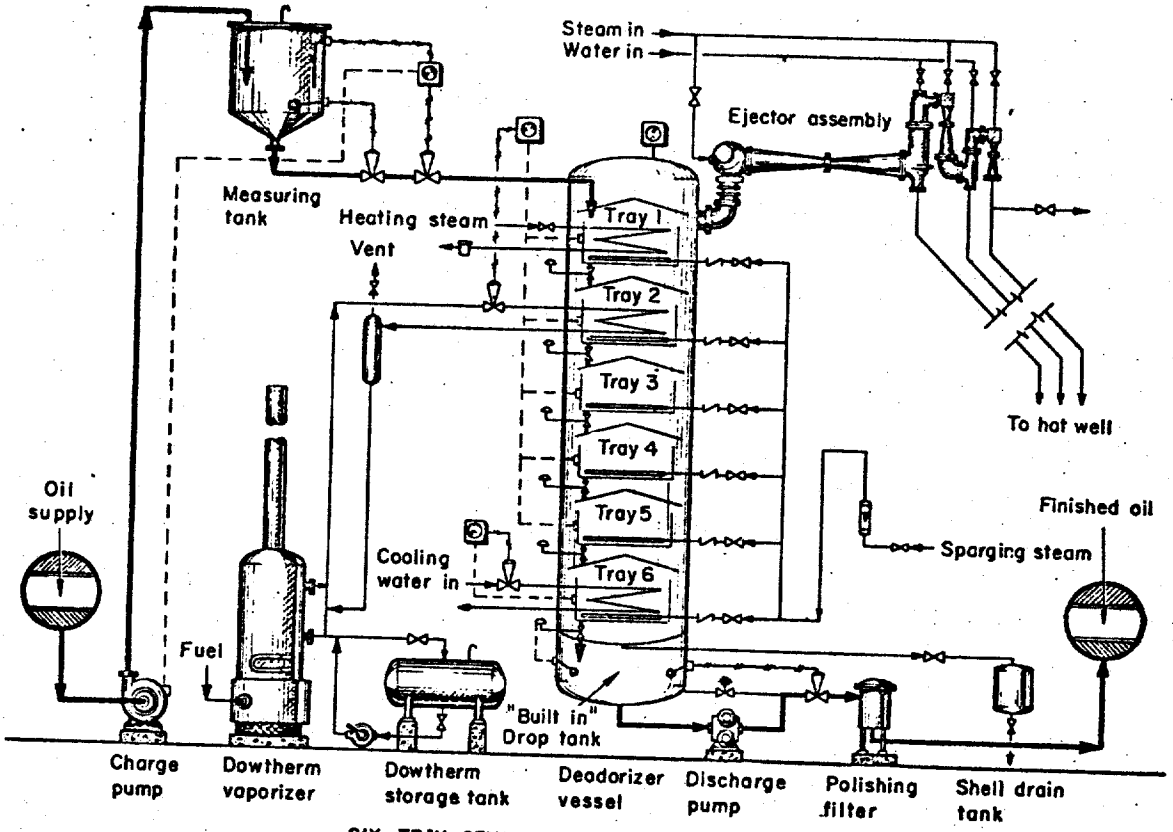
There have been relatively few reports on the effects of these antioxidants on the oxidation of fish oils and vitamin A. Bittenbender (1950) observed that gallic acid esters were useful antioxidants for menhaden oil and Otani *et al.* (1953–1954) found that BHA retarded the oxidation of sardine oil. Olcott and Einset (1958B), however, found that BHA, PG, and BHT had only minor effects on menhaden oil and that NDGA and 6-ethoxy, 2,2,4-trimethyl-1,2-dihydroquinoline (EMQ) were much more

powerful antioxidants with EMQ the stronger of the These authors (1958A) also observed that α-tocopherol at concentrations of 0.3% destroyed the effect of EMQ and depressed that of NDGA. On the other hand, Olcott and Van der Veen (1963) observed that various phospholipids including synthetic forms of lecithin and cephalin, although ineffective themselves, had very strong synergistic effects in the presence of EMQ. Toyama (1962) observed that although BHA, PG, NDGA, and BHT had little effect on the rate of oxidation of cod liver oil at 98°C., they all inhibited the oxidation of the partially hydrogenated oil, NDGA and PG being particularly effective.

As far as the oxidative destruction of vitamin A is concerned, early work by Dassow and Stansby (1949) showed that NDGA had a useful stabilizing effect on the vitamin A of halibut liver oil particularly with citric acid as a synergist. Bhattacharya *et al.* (1954) observed that α-tocopherol was ineffective on strong solutions (20,000 I.U./gm.) of the vitamin in vegetable oils, but was quite a good antioxidant when liquid paraffin (a high molecular weight hydrocarbon oil) was used as a solvent. Propyl gallate had little effect on vitamin A alcohol, but was quite effective with vitamin A esters particularly in the presence of citric and ascorbic acids. A similar picture was obtained with weaker solutions (3,000 I.U./gm.). Kinumaki and Tabei (1954–1965) observed that vitamin A alcohol is more stable than its esters and that weak antioxidants such as PG have in consequence little effect on the alcohol but can afford some protection of the esters. They found that BHA was a good antioxidant for the alcohol and that a commercial product, a mixture of BHA, PG, and citric acid was slightly weaker, with about the same strength as NDGA. Kamath and Magar (1953) on the contrary, have observed that BHA was ineffective in protecting the vitamin A of shark-liver oil, but that PG and NDGA were quite effective in this respect and also in reducing the rate of formation of peroxides. None of these antioxidants had any effect on vitamin A losses when the oils were exposed to light.

Determination of Oxidative Deterioration

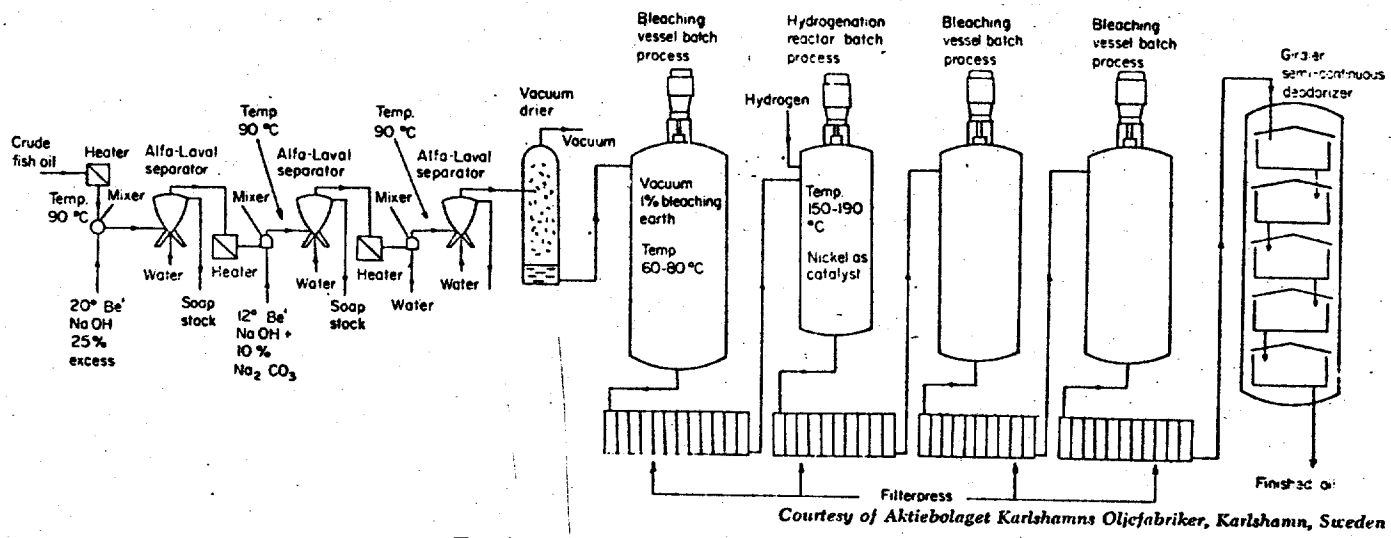
A number of methods have been proposed and used for following oxidative changes in fats and fatty oils, including fish oils, all of which depend upon the determination of the concentration of certain oxidation products in the fat or fatty oil. The number of such products that can be formed is very large, particularly in fish oils (Wyatt and Day 1963) and it is not practicable to determine the concentration of all of them. Since they all contribute in some way to the changes that occur in flavor, it follows that the assessment of flavor change is really the only strictly reliable means for detecting oxidative deterioration. Unfortunately, such techniques are



SIX TRAY SEMI-CONTINUOUS DEODORIZER FLOW DIAGRAM

Courtesy of the Girdler Process Equipment Division, Chemetron Corporation

FIG. 40. SEMICONTINUOUS DEODORIZER



Courtesy of Aktiebolaget Karlshamns Oljefabriker, Karlshamn, Sweden

FIG. 41. FLOW SHEET OF FISH-OIL PROCESSING

if deodorization were an ordinary steam distillation process of the more volatile constituents in the oil. This indicates that some of the compounds responsible for odor and flavor cannot be removed by steam distillation until they have undergone thermal decomposition.

The temperature of deodorization ranges from about 190° to 230°C. in the United States to 170° to 190°C. in Europe. The use of lower temperatures in Europe is an attempt to avoid thermal decomposition and polymerization which may yield products harmful to human nutrition. After the deodorization, an oil is usually cooled down to 38°C. and hydrogenated fat to 65°C. before it is allowed to be exposed to air.

Deodorization may be carried out industrially either by batch or by continuous processes. A typical deodorizer is shown in Fig. 39. The oil is charged directly into the deodorizer or "still" which is held under vacuum at all times. As soon as the loading starts, a small amount of sparge steam is turned on to provide agitation. The open steam for deodorization is usually distributed by means of a flat spider of perforated pipes radiating from a central steam delivery line. The charge is heated either by coils within the still itself or by circulation through an external heat exchanger. Take-off for the vapor is fitted into the top of the deodorizer. It is constructed so as to prevent reflux of the compounds responsible for odor and flavor. The distillate passes to a "catch-all" to recover any entrained oil and the vapors finally pass through a barometric condenser. After the oil has been held at the deodorization temperature for the required time, the oil is cooled and discharged.

A number of designs for continuous deodorization have been patented and several are being actively used (Mattil 1964). A semi-continuous deodorizer manufactured by the Girdler Process Equipment Division, Chemtron Corporation, has achieved considerable success both in this country and abroad (Fig. 40). In this process, successive charges of oil are pumped into a series of superheated nickel trays, with the oil remaining for one-half hour in each tray. In the top tray, the oil is de-aerated and heated with steam; in the second tray, it is heated to operating temperature with Dowtherm; in the third and fourth trays, it is subjected to steam stripping; and in the bottom tray, it is cooled before it is dumped into a drop tank from which it is continuously pumped through a pressure filter to storage tanks. Operation of the unit is made fully automatic by the use of a timing device which opens and closes the various valves.

SUMMARY

The complete processing of fish oils by the combination of the various processes is summarized by an example as shown in Fig. 41. It is through

the courtesy of the Aktlebolaget Karlshamns Öljefabriker, Karlshamn, Sweden. The flow sheet shows the processing procedure of fish oil in their plant.

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Chipault

THE ODORS OF OXIDIZED FISH OILS

Volatile compounds have been isolated from oxidized fish oils by volatilization under vacuum at 100° C., vacuum-steam distillation, and adsorption on charcoal from a stream of nitrogen swept through the oil.

The volatile fractions were separated into acidic, basic, and carbonyl fractions.

The acidic fraction was studied by paper chromatography, and only acetic and propionic acids were detected.

The carbonyl compounds were reacted with 2,4-dinitrophenylhydrazine, either directly or after removal of aldehydes by oxidation with silver oxide. Column and paper chromatography and ultraviolet spectroscopy were used to further separate and identify these components. Formaldehyde, acetaldehyde, propionaldehyde, butyraldehyde, valeraldehyde, hexaldehyde, acetone, butanone, pentanone, α,β-unsaturated monocarbonyls, glyoxal, and low-molecular weight α-keto aldehydes and diketones, such as methyl glyoxal and diacetyl, were detected in these fractions. Other 2,4-dinitrophenylhydrazones, not identified, may have been the derivatives of dicarbonyls other than the α compounds.

Small amounts of amines were detected in the basic fractions, but they have not been studied further.

NUTRITIONAL PROPERTIES OF FISH OILS

A primary objective of this project is to conduct investigations on the essential fatty acid properties of fish oils. Studies have been made on menhaden, tuna, and herring oils. These oils showed no curative or preventive potency on the dermal symptoms of EFA deficiency in white rats when fed at a level of 200 mg. per animal per day. The feeding of diets containing 10% of these oils was effective in preventing the occurrence of symptoms of EFA deficiency, with tuna oil being the most potent, followed by menhaden and herring oils in order of decreasing effectiveness. Work is in progress to define the source of the oils' EFA activity.

EFFECT OF FISH OILS ON THE LIPIDE METABOLISM OF HYPERCHOLESTEREMIC RATS

Certain dietary fats have been found to depress the plasma lipides of hypercholesteremic humans and experimental animals. Various investigators have found that this depressant action is best correlated with: (1) the fat's concentration of essential fatty acids (EFA), (2) plant sterols, (3) the net unsaturation of the dietary fat, or (4) the lack of unsaturated nonessential fatty acids. At present we are studying the changes in lipide metabolism associated with the depression of plasma lipides in rats fed bile acids and cholesterol.

Initial studies have shown that the depression of hypercholesteremia is not dependent upon the ingestion of appreciable quantities of EFA nor the avoidance of unsaturated nonessential fatty acids. Linolenate and the fish oils, tuna and menhaden, have been found significantly more effective than linoleate, the most active essential fatty acid tested in depressing plasma lipides. The observed changes are best correlated with the net unsaturation of the dietary fat. There was a significant

J. Ag. Food Chem 13: 15-17, 1965

FISH OIL ODORS

Volatile Acids from Menhaden Oil

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The acidic constituents of a highly volatile fraction collected during molecular distillation of menhaden oil have been examined by paper chromatography. Tentatively identified were formic (or acetic), acrylic, propionic, crotonic, butyric, and valeric acids, and an unknown compound with a polarity greater than formic acid but less than pyruvic or lactic acids.

Oils carefully rendered from fresh fish tissues are relatively bland or possess, at most, a mild, not unpleasant odor. The strong, undesirable odors usually associated with marine oils are caused mainly by the use of tissues in various stages of decomposition and by deterioration of the oil after it has been prepared. The first factor gives dark oils containing relatively large quantities of nitrogen and with odors of a putrid character. The second effect is associated with autoxidation of the highly unsaturated fatty acids of the oil.

The great complexity of the carbonyl compounds that can be isolated from fish oils by various techniques has been reported (*14, 19, 20*). Furthermore, fishy odors and flavors have been detected in fats of nonmarine origin, such as soybean oil, beef tallow, butter, and other dairy products. Under certain conditions, fishy flavors can be reproducibly developed in butter (*13*). According to Forss *et al.* and Stark and Forss, the fishy flavor of butterfat results from a combination of compounds responsible for an oily flavor (*n*-hexanal, *n*-heptanal, and hex-2-enal) (*7*) and a metallic flavor (oct-1-en-3-one) (*16*). However, other workers (*4, 6, 10*) have isolated carbonyl compounds identical or very closely related to those of Forss *et al.* from butterfat which was oxidized but not considered fishy.

On the other hand, Davies and Gill (*3*) concluded that fishiness developed when highly unsaturated fatty acids and tertiary nitrogen compounds were present together under autoxidizing conditions. The concept that tertiary nitrogen is necessary for fishiness to occur is supported also by Stansby (*15*) and by observations that trimethylamine, triethylamine, and even ammonia, at low concentrations, have odors akin to those encountered in products with a mild degree of fishiness. Chang *et al.* (*2*) also consider that the fishy odor of hydrogenated soybean oil is due to noncarbonyl compounds.

These observations made it desirable to examine the volatile material obtainable from fish oils for compounds other than the carbonyls which are usually associated with the odors of oxidized fats. This report describes the acidic volatile compounds isolated from menhaden oil.

Experimental

Starting Materials. The highly volatile fraction used in this study was accumulated in a cold trap maintained at dry ice–acetone temperature during fractional molecular distillation of 5 gallons of cold-pressed menhaden oil. After a degassing period at 500 microns and 60° C., six distillate fractions were collected at a pressure of 17 to 18 microns and temperatures between 190° and 212° C.

A total of 25 ml. of volatile compounds was collected during the distillation of 5 gallons of menhaden oil. When the fraction was melted, it separated into a nearly colorless lower aqueous layer with a volume of approximately 8 ml. and 17 ml. of a dark upper organic phase.

Examination of Total Volatiles. Both aqueous and organic phases had similar sharp unpleasant odors which were described by four observers as a combination of putrid, slightly fishy, and very strongly rancid. Qualitative tests indicated that both layers contained acids and carbonyls, but a Kjeldahl determination showed that the lower aqueous layer contained more than 15 times the concentration of nitrogen found in the upper layer. Spot tests for amines were positive for the lower phase, but negative for the upper organic layer. Tests for sulfhydryl groups (*5*) were negative for both layers. As a result of these observations, it was decided to examine the lower aqueous layer more thoroughly.

Separation of Acids from Aqueous Phase. A 1-ml. portion of the aqueous layer was neutralized to a pH of 10 (pH paper) with 0.1 N NaOH (0.9 ml.).

Table I. R_f Values of Volatile Acids of Menhaden Oil

Unknown Volatile Acids	No. of Chromatograms	R_f Value	Std. Dev.	Known Standard	No. of Chromatograms	R_f Value	Std. Dev.
1	6	0.13	0.010	Formic	3	0.21	0.010
2	6	0.20	0.013	Acetic	5	0.22	0.015
3	4	0.31	0.005	Acrylic	3	0.32	0.007
4	6	0.36	0.009	Propionic	5	0.35	0.010
5	6	0.45	0.016	Crotonic	3	0.44	0.010
6	6	0.51	0.016	Butyric	5	0.49	0.010
7	3	0.61	0.007	Valeric	5	0.64	0.018

The neutralized solution was transferred to flask *A* (Figure 1), which was then placed in a dry ice–acetone bath at −78° C. while the system was evacuated to a pressure of 50 microns. Stopcock *C* was closed and the dry ice bath was removed from flask *A* and placed around condensing tube *B*. Distillation was allowed to proceed overnight at room temperature. The next morning flask *A* was immersed in a bath at 50° C. for 1 hour to ensure complete removal of the neutral and basic volatile components which were condensed in tube *B* and studied separately.

Examination of Volatile Acids. The sodium salts of the volatile fatty acids remaining in flask *A* were dissolved in a few microliters of water and chromatographed on strips of Whatman No. 1 filter paper, using an ascending technique at room temperature.

The solvent used was the upper layer resulting when a mixture of 30 parts of propanol and 30 parts of butanol was thoroughly mixed and equilibrated with 20 parts of the ammonia buffer solution used by Kalbe (*8*) for separating dicarboxylic acids on paper. After 20 hours in a chromatographic jar, the papers were dried thoroughly in air at room temperature and dipped in a ninhydrin indicator solution (200 mg. of ninhydrin and 50 mg. of ascorbic acid dissolved in 100 ml. of absolute ethanol), as suggested by Burness and King (*1*). The dipped papers were allowed to dry at room temperature and the acids appeared within 1 hour as purple spots on a white background. However, the chromatograms were not stable and the background gradually darkened, so the acid spots became invisible after about 3 additional hours. Heating of the chromatograms in an oven at 100° C. hastened appearance of the acid spots but resulted, also, in a more rapid darkening of the background.

Results and Discussion

Several difficulties have been reported during paper chromatography of volatile fatty acids of low molecular weight as their nonvolatile alkali salts. The appearance of ghost spots due to impurities normally present in filter paper was eliminated by Kennedy and Barker (*9*) by thoroughly prewashing the paper. They treated the solution of the sodium salts of the fatty acids with ammonium sulfate to minimize interference, by

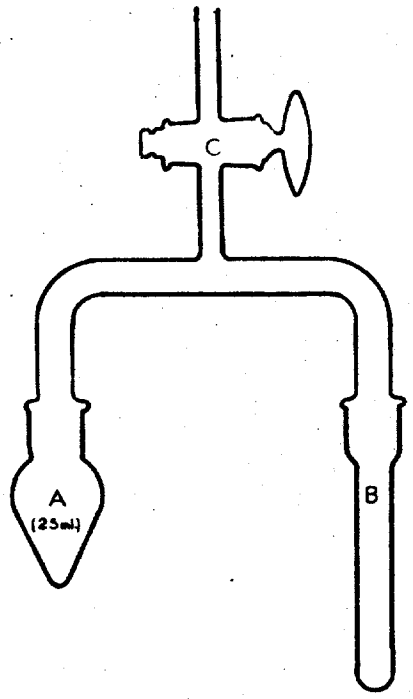

Figure 1. Apparatus for isolation of volatile acids from menhaden oil

sodium cations, with detection of the fatty acids of lower molecular weight. With the solvent system and indicator solution used here, these procedures became unnecessary, since no ghost spot was detected at any time and the sodium ions appeared as a compact yellow spot which remained very close to the base line and did not interfere with any of the fatty acids present.

Figure 2 is a tracing of a typical chromatogram of the volatile acids of menhaden oil compared with known standards. Seven spots were obtained when 1 to 10 μl. of solution were used. Larger amounts showed no additional spots but resulted in nearly complete merging of spots 1 with 2 and 3 with 4, and in considerable streaking of spot 7. With amounts less than 1 μl., spots 1 and 3 became somewhat better defined (although weaker), but spot 7 disappeared completely.

Table I shows average R_f values and standard deviations calculated from three to six chromatograms. The values

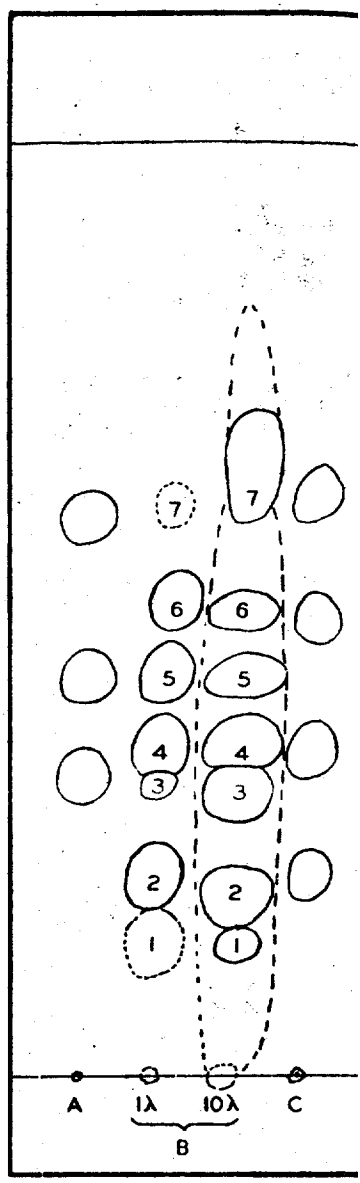

Figure 2. Paper chromatograp of volatile short-chain acids

A. Acrylic, crotonic, and isovaleric acid
B. Volatile acids from menhaden oil
C. Acetic, propionic, butyric, and valer acids

for formic and acetic acids are aln identical and, when present toget these two compounds are not separa from each other. This is in agreen with the findings of many other work who failed to separate them wit number of solvent systems. Muke (*12*), however, reported the separatio formate from acetate with methanol a mixture of methanol and acet containing 1 part per hundred of 3 ammonium hydroxide. For the p ent authors, however, these solv failed to separate these two ac Burness and King (*1*) pointed out t with the exception of ammoni formate, the ammonium salts of other fatty acids of low molecular wei require the presence of a reducing ag to react with ninhydrin, and asco

acid has been added to the ninhydrin indicator solution for this purpose. Formate, however, acts as its own reducing agent and can be detected with a ninhydrin solution free from added reducing compounds, thus providing a means of differentiating it from acetic acid.

This property of formic acid was confirmed with known mixtures containing either formate or acetate. However, when a chromatogram of the unknown volatile acids from menhaden oil was dipped in the ninhydrin solution free from ascorbic acid, all seven spots appeared, indicating that some reducing impurities were carried along all through the chromatogram.

Spot 1, with an R_f value of 0.13, is unknown. It is somewhat more polar than formic or acetic acid, but less so than keto (pyruvic) or hydroxy (lactic) acids which do not migrate from their point of application with the solvents used here. The other spots correspond, respectively, to formic (or acetic), acrylic, propionic, crotonic, butyric, and valeric acids.

The presence of these acids in menhaden oil is easily explained through autoxidation of aldehydes which, in turn, result from degradation of hydroperoxides. Loury (*11*) has suggested that aldehydes with six to nine carbon atoms readily undergo a stepwise degradation through autoxidation to the corresponding moloxide, followed by scission to yield formic acid and the next lower aldehyde. The ease with which this scission occurs decreases with decreasing chain length and is almost nil with acetaldehyde and propionaldehyde. Based on these findings, therefore, spot 2 is more likely to be formic than acetic acid. Toyama and Matsumoto (*17*) and Toyama *et al.* (*18*) isolated saturated acids from formic to caproic from oxidized methyl esters of highly unsaturated sardine oil fatty acids. The conditions of oxidation and of peroxide decomposition were considerably more severe than those used to collect the volatiles studied here, perhaps explaining why no unsaturated acids were found by these workers.

Branched-chain acids such as isobutyric and isovaleric have the same R_f values as their straight-chain isomers. Therefore, spots 6 and 7 could represent branched isomers also, but this possibility appears rather remote. Presumably, the precursors for these compounds would have to be the branched-chain fatty acids. Although these compounds are known to occur in marine lipids, they comprise only about 1.5% of the total fatty acids of menhaden oil. Furthermore, they are not highly unsaturated and they would not be expected to undergo rapid oxidative degradation. Thus, the volatile acidic compounds expected to result from autoxidation of highly unsaturated fatty acids of fish oils are the straight-chain saturated and unsaturated acids of low molecular weight, tentatively identified in this study. These acids have strong, sharp, unpleasant odors, and their presence should contribute significantly to the over-all odor of menhaden oil, although none has a characteristic fishy odor.

Undoubtedly, acids of longer chain length occur also in oxidized menhaden oil but because of their low volatility and solubility in water they are not found in the fraction studied here. Valeric acid was detected only when large amounts of the acidic material were chromatographed. The higher homologs are either completely absent or present in quantities too small to be detected.

Acknowledgment

The authors are grateful to Maurice Stansby and Eric Gauglitz of the Seattle Technological Laboratory of the Bureau of Commercial Fisheries for supplying the starting material.

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Received for review March 9, 1964. Accepted April 16, 1964. Study supported by the U. S. Department of the Interior, Fish and Wildlife Service, and by the Hormel Foundation.

Joint FAO/WHO Food Standards Programme

CODEX ALIMENTARIUS COMMISSION

CAC/RS 19-1969

RECOMMENDED INTERNATIONAL GENERAL STANDARD FOR EDIBLE FATS AND OILS

NOT COVERED BY INDIVIDUAL CODEX STANDARDS

FOOD AND AGRICULTURE ORGANIZATION
OF THE UNITED NATIONS

WORLD HEALTH ORGANIZATION

CONTENTS

	Page
Introduction	1
Recommended International General Standard for Edible Fats and Oils	5
Bibliography	13
Acceptance Procedure for Codex Commodity Standards	15
Standards for Fats and Oils Adopted by the Codex Alimentarius Commission	20

INTRODUCTION

The FAO/WHO Codex Alimentarius Commission was established to implement the Joint FAO/WHO Food Standards Programme. Membership of the Commission comprises those Member Nations and Associate Members of FAO and/or WHO which have notified the Organizations of their wish to be considered as Members. By 1 May 1970, 75 countries had become Members of the Commission. Other countries which participate in the work of the Commission or of its subsidiary bodies in an observer capacity are expected to become Members in the near future.

The purpose of the Joint FAO/WHO Food Standards Programme is to protect the health of consumers and to ensure fair practices in the food trade; to promote coordination of all food standards work undertaken by international governmental and non-governmental organizations; to determine priorities and initiate and guide the preparation of draft standards through and with the aid of appropriate organizations; to finalize standards and after acceptance by governments, publish them in a Codex Alimentarius either as regional or world-wide standards.

The Commission has held seven sessions, the most recent being in April 1970. At the sixth session, the Commission adopted as a recommended standard to be sent to all Member Nations and Associate Members of FAO and/or WHO a world-wide General Standard for Edible Fats and Oils not covered by International Codex Standards (1). Members of the Commission are requested to notify the Secretariat of the Codex Alimentarius Commission - Joint FAO/WHO Food Standards Programme, FAO, Rome - of their acceptance of the recommended standard in accordance with the acceptance procedure for Codex Commodity standards contained in paragraph 4 of the General Principles of the Codex Alimentarius. An extract from the General Principles containing this procedure is included in the Appendix to this publication. The full text of the General Principles of the Codex Alimentarius is contained in the Second Edition of the Commission's Procedural Manual (Ref. No. CX 8/7 - 1969). Member Nations and Associate Members of FAO and/or WHO which are not Members of the Commission are

(1) A complete list of standards for fats and oils adopted by the Codex Alimentarius Commission and sent to governments for acceptance is given on page 20.

also invited to notify the Secretariat if they wish to accept the recommended standard. The recommended standard will be published in the Codex Alimentarius as a world-wide Codex standard when the Commission determines that it is appropriate to do so in the light of acceptances received.

The methods of analysis and sampling referred to in this standard are not necessarily intended to replace routine methods of analysis and sampling, but are for acceptance and use as international referee methods of analysis and sampling by governments for the settlement of international disputes.

Specifications of identity and purity have been drawn up for the food additives provided for in this standard by the Joint FAO/WHO Expert Committee on Food Additives. These are being further elaborated by the Codex Alimentarius Commission into internationally recognized specifications for food additives. The statement "Not limited" should be taken to mean that the amount of food additive should be used in accordance with good manufacturing practices.

RECOMMENDED INTERNATIONAL GENERAL STANDARD FOR EDIBLE FATS AND OILS NOT COVERED BY INDIVIDUAL CODEX STANDARDS

CAC/RS 19-1969

RECOMMENDED INTERNATIONAL GENERAL STANDARD FOR EDIBLE FATS AND OILS NOT COVERED BY INDIVIDUAL CODEX STANDARDS

1. SCOPE

This standard applies to edible oils, fats and mixtures thereof, including those that have been subjected to processes of modification but not including oils and fats which must be subjected to such processes in order to render them suitable for human consumption. This standard does not apply to any oil or fat which is the subject of a specific Codex commodity standard and is designated by a specific name laid down in such standards.

2. DESCRIPTIONS

2.1 Edible Fats and Oils means foodstuffs composed of glycerides of fatty acids of vegetable, animal or marine origin. Fats of animal origin must be produced from animals in good health at the time of slaughter and be fit for human consumption as determined by a competent authority recognized in national legislation. They may contain small amounts of other lipids such as phosphatides, of unsaponifiable constituents and of free fatty acids naturally present in the fat or oil.

2.2 Virgin Fats and Oils means edible fats and oils obtained by mechanical procedures and the application of heat only. They may have been purified by washing, settling, filtering and centrifuging only.

3. ESSENTIAL COMPOSITION AND QUALITY FACTORS

3.1 Raw Materials

Edible fats and/or oils or mixtures thereof.

3.2 Colour

Characteristic of the designated product.

3.3 Odour and Taste

Characteristic of the designated product and free from foreign and rancid odour and taste.

3.4 Acid Value

Virgin fats and oils	not more than 4 mg KOH/g fat or oil
Non-virgin fats and oils	not more than 0.6 mg KOH/g fat or oil

3.5 Peroxide Value — not more than 10 milliequivalents of peroxide oxygen/kg fat or oil

4. FOOD ADDITIVES

4.1 Colours

The following colours are permitted for the purpose of restoring natural colour lost in processing or for the purpose of standardizing colour, as long as the added colour does not deceive or mislead the consumer by concealing damage or inferiority or by making the product appear to be of greater than actual value:

	Maximum level of use
4.1.1 Beta-carotene	Not limited
4.1.2 Annatto (*)	Not limited
4.1.3 Curcumin (*)	Not limited
4.1.4 Canthaxanthine	Not limited
4.1.5 Beta-apo-8'-carotenal	Not limited
4.1.6 Methyl and ethyl esters of beta-apo-8'-carotenoic acid	Not limited

(*) Temporarily endorsed.

4.2 Flavours

Natural flavours and their identical synthetic equivalents, except those which are known to represent a toxic hazard, and other synthetic flavours approved by the Codex Alimentarius Commission are permitted for the purpose of restoring natural flavour lost in processing or for the purpose of standardizing flavour, as long as the added flavour does not deceive or mislead the consumer by concealing damage or inferiority or by making the product appear to be of greater than actual value. (*)

4.3 Emulsifiers

The following are permitted but only in fats and oils not specifically designated with the name of the plant or animal from which they originate:

	Maximum level of use
4.3.1 Mono- and diglycerides of fatty acids	Not limited
4.3.2 Mono- and diglycerides of fatty acids esterified with the following acids: acetic, acetyltartaric, citric, lactic, tartaric and their sodium and calcium salts	20 g/kg of the emulsifiers specified under 4.3.2 to 4.3.11 inclusive, individually or in combination
4.3.3 Lecithins and components of commercial lecithin	
4.3.4 Polyglycerol esters of fatty acids	
4.3.5 Esters of fatty acids with polyalcohols other than glycerol: Sorbitan monopalmitate, Sorbitan monostearate, Sorbitan tristearate	

(*) Temporarily endorsed.

	Maximum level of use
4.3 Emulsifiers (Cont.)	
4.3.6 1,2 propylene glycol esters of fatty acids	)
4.3.7 Sucrose esters of fatty acids (including sucroglycerides) (*)	)
4.3.8 Stearoyl lactylic acid and its calcium salt (*)	)
4.3.9 Polyglycerol esters of interesterified ricinoleic acid (*)	)
4.3.10 Polyoxyethylene (20) sorbitan monostearate	)
4.3.11 Polyoxyethylene (20) sorbitan monooleate	)
4.4 Antioxidants	
4.4.1 Propyl, octyl, and dodecyl gallates	100 mg/kg individually or in combination
4.4.2 Butylated hydroxytoluene (BHT) Butylated hydroxyanisole (BHA)	200 mg/kg individually or in combination
4.4.3 Any combination of gallates with BHA or BHT, or both	200 mg/kg, but gallates not to exceed 100 mg/kg
4.4.4 Natural and synthetic tocopherols	Not limited
4.4.5 Ascorbyl palmitate) 4.4.6 Ascorbyl stearate)	200 mg/kg individually or in combination
4.4.7 Dilauryl thiodipropionate	200 mg/kg

(*) Temporarily endorsed.

4.5 Antioxidant Synergists	Maximum level of use
4.5.1 Citric acid and its sodium salt	Not limited
4.5.2 Isopropyl citrate mixture) 4.5.3 Phosphoric acid (*))	100 mg/kg individually or in combination
4.6 Anti-foaming Agent	
Dimethyl polysiloxane (dimethyl silicone) singly or in combination with silicone dioxide (*))	10 mg/kg
4.7 Crystallization Inhibitor	
Oxystearin (*)	1250 mg/kg

5. CONTAMINANTS		Maximum level
5.1 Matter volatile at 105°C		0.2% m/m
5.2 Insoluble impurities		0.05% m/m
5.3 Soap content		0.005% m/m
5.4 Iron (Fe)	(Virgin oil	5 mg/kg
	(Non-virgin oil	1.5 mg/kg
5.5 Copper (Cu)	(Virgin oil	0.4 mg/kg
	(Non-virgin oil	0.1 mg/kg
5.6 Lead (Pb)		0.1 mg/kg
5.7 Arsenic (As)		0.1 mg/kg

6. HYGIENE

It is recommended that the product covered by the provisions of this standard be prepared in accordance with the appropriate Sections of the General Principles of Food Hygiene recommended by the Codex Alimentarius Commission (Ref. No. CAC/RCP 1-1969).

(*) Temporarily endorsed.

7. LABELLING

In addition to Sections 1, 2, 4 and 6 of the General Standard for the Labelling of Pre-packaged Foods (Ref. CAC/RS 1-1969) the following specific provisions apply:

7.1 The Name of the Food

7.1.1 The name designated for the product conforming to the definition at 2.1 of the standard shall be such as to give a true indication of the nature of the fat or oil, and not to mislead the consumer. Names such as edible oil and salad oil which do not indicate a plant or animal source may be used without further qualification.

7.1.2 Where an oil has been subject to any process of esterification or to processing which alters its fatty acid composition or its consistency the specific name of the oil shall not be used unless qualified to indicate the nature of the process.

7.1.3 The designation virgin fat or virgin oil may only be used for individual fats or oils conforming to the definition at 2.2 of this standard.

7.2 List of Ingredients

7.2.1 A complete list of ingredients shall be declared on the label in descending order of proportion.

7.2.2 A specific name shall be used for ingredients in the list of ingredients except that class titles may be used in accordance with sub-section 3.2 (c)(ii) of the General Standard for the Labelling of Pre-packaged Foods.

7.3 Net Contents

The net contents shall be declared in accordance with sub-section 3.3 (a) of the General Standard for the Labelling of Pre-packaged Foods.

7.4 Name and Address

The name and address of the manufacturer, packer, distributor, importer, exporter or vendor of the product shall be declared.

7.5 Country of Origin

7.5.1 The country of origin of the product shall be declared if its omission would mislead or deceive the consumer.

7.5.2 When the product undergoes processing in a second country which changes its nature, the country in which the processing is performed shall be considered to be the country of origin for the purposes of labelling.

8. METHODS OF ANALYSIS AND SAMPLING

The methods of analysis and sampling referred to hereunder are international referee methods.

8.1 Determination of Acid Value (I_A)

According to the IUPAC (1964) method (IUPAC Standard Methods for the analysis of Oils, Fats and Soaps, 5th Edition, 1966, II.D.1.2. Acid Value).

Results are expressed as the number of mg KOH required to neutralize 1 g oil or fat.

8.2 Determination of Peroxide Value (I_P)

According to the IUPAC (1964) method (IUPAC Standard Methods for the analysis of Oils, Fats and Soaps, 5th Edition, 1966, II.D.13 Peroxide Value).

Results are expressed as milliequivalents active oxygen/kg fat or oil.

8.3 Determination of Matter Volatile at 105°C

According to the IUPAC (1964) method (IUPAC Standard Methods for the analysis of Oils, Fats and Soaps, 5th Edition, 1966, II.C.1.1 Moisture and Volatile Matter).

Results are expressed as % m/m.

managerial class. This may be a reflexion of economic status and the tendency to substitute animal protein for fish and vegetable proteins by the more affluent. In a study of Army inductees, Beebe *et al.*[40] concluded that the incidence of multiple sclerosis was higher in metropolitan or urban areas when compared with rural areas.

(*e*) Migration from a high risk zone to a low risk zone carries with it in part the risk of the country of origin (p. 39). Migration from a low risk to a high risk area may increase risk (p. 40). Other variables, however, such as age at the time of migration, obscure any definitive assessment. In a study of immigrants to Israel originating from Europe and Afro-Asia, Alter[41] found no change in the low incidence of the disease in the latter group. In Europeans under 15 yr old at immigration, the incidence of the disease approached that of the Afro-Asians, indicative of a protective effect of migration. In Europeans migrating at 15–29 yr the incidence was 8·9 times the Afro-Asian, and at 30–44 yr this figure was 5·4. The authors remark that their data indicate that multiple sclerosis is acquired many years before the onset of clinical symptoms, and the early life history of the patient might be examined for an aetiologic factor. Older people, however, may be habituated to dietary preferences and the statistics may reflect a resistance to changes in food selection with age.

(*f*) The tendency for the disease to be familial (p. 61) is explicable on the dietary deficiency hypothesis. Mackay and Myrianthopoulos[42], in an investigation of monozygotic and dizygotic twins, concluded that if genetic effects are involved in the aetiology of multiple sclerosis they are subjected to powerful environmental influences.

The problems that present themselves in advancing this hypothesis are numerous.

(*a*) The inability to produce demyelination of the nervous system in experimental animals on an ω3 fatty acid-deficient diet may be the result of either differences in specie sensitivity or the inability of animals to survive such a diet for the period of time required to produce lesions. Much of the experimental work reported on a linolenic acid deficiency has involved a deficiency of multi-polyunsaturated fatty acids.

(*b*) The data on the composition of polyunsaturated fatty acids in the brain in multiple sclerosis is limited. It will require more detailed analysis of various lipids and lipid fractions before any assessment of the effect of deficiency of these acids can be made.

(*c*) Some of the epidemiological data cited indicating partial protection in the young in moving from a high risk to a low risk area suggest some exogenous requirement before the brain matures.

(*d*) There may be other unknown metabolic pathways where a requirement exists in the absence of any precursor pool. Thus the aetiology of multiple sclerosis may reside in more than one dietary deficiency. Alternatively, the dietary intake of ω3 fatty acids may be adequate but the pathway from ingestion to the final deposition of 22 : 6 in the brain may be impaired. Thus absorption from the gastro-intestinal tract, transport mechanisms in the blood, passage across the blood–brain barrier, or the ω3 elongation system, may be defective in patients who develop multiple sclerosis. We have shown in chick brain[43] that the elongation of 18 : 3 ω3 to 22 : 6 ω3 may proceed with a regulatory mechanism different from that of the ω6 acids. Multiple sclerosis may thus be a result of some aberration in the control mechanism of 18 : 3 elongation to 22 : 6 resulting in an apparent deficiency in brain concentrations of the latter. Competition has been demonstrated between 18 : 2 and 18 : 3 and Rahm and Holman[44] showed in rats that an increase in the intake of 18 : 2 depressed the concentrations of 22 : 6 in liver fatty acids. We have found that the incorporation of ^{14}C-linolenic acid into 22 : 6 in brain slices was markedly inhibited by the presence of Δ6,9,12 6 isomer of linolenic acid (unpublished results of Stephanides and Bernsohn).

(*e*) It has been pointed out[45] that monomolecular surface films of fatty acids are influenced by unsaturation. Unsaturated acids produce films of the expanded type, and as the double bond moves toward the methyl end of the molecule, the film expands more readily. Thus a deficiency of ω3 fatty acids in the membrane may produce a more rigid structure which may not be consistent with normal functioning in the central nervous system.

Many basic data need to be obtained before the theoretical aspects of this problem can be fully elucidated. Such data and the development of an animal model would be a desideratum but the nature of the clinical problem and its seriousness for the patient and family has prompted this presentation despite the apparent gaps in knowledge.

We thank Dr Kevin D. Barron, head of the Neurology Service, Veterans Administration Hospital, Hines, Illinois, for his help.

This work was supported by the Veterans Administration and by a grant from the National Multiple Sclerosis Society.

Received April 10; revised July 10, 1967.

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THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 241, No. 22, Issue of November 25, pp. 5213-5219, 1966
Printed in U.S.A.

Effect of Saturated and Unsaturated Fatty Acids on the Desaturation *in Vitro* of Palmitic, Stearic, Oleic, Linoleic, and Linolenic Acids*

J. Biol. Chem. 241(22): 5213-5219, 1966

(Received for publication, March 3, 1966)

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SUMMARY

Quantitative oxidative desaturation of labeled palmitic, stearic, oleic, linoleic, and linolenic acids to palmitoleic, oleic, octadeca-6,9-dienoic, γ-linolenic, and octadeca-6,9,12,15-tetraenoic acids, respectively, by liver microsomes of rats was studied by gas-liquid radiochromatography after incubation of the acids in a medium containing adenosine triphosphate, reduced nicotinamide adenine dinucleotide, reduced nicotinamide adenine dinucleotide phosphate, $MgCl_2$, coenzyme A, glutathione, nicotinamide, NaF, and NaCN in oxygen at pH 7 and 35°.

The rates of conversion of oleic into octadeca-6,9-dienoic, linoleic into γ-linolenic, and linolenic into octadeca-6,9,12,15-tetraenoic acids were measured, and Lineweaver-Burk plots showed the existence of competitive reactions among the three acids. Saturated acids were ineffective in altering the percentage of desaturation of the acids, whereas polyunsaturated acids of 20 and 22 carbon atoms generally activated the conversion of the three acids. Docosahexaenoic acid decreased the percentage of desaturation of oleic, linoleic, and linolenic acids. The unsaturated acids of 18 carbons apparently competed for the same oxygenase, and the order of affinity was linolenic, linoleic, and oleic acids. Substrate fatty acids and products after the incubation were shown to be mainly esterified to choline and ethanolamine glycerophosphatides.

Polyunsaturated fatty acids are now known to belong to different series not interconvertible in animals. The acids of each series are individualized by the position of the first double bond proximal to the methyl group and are synthesized in animals from the first acid of the family through elongation and desaturation. Additional double bonds are added in the direction of the carboxyl group. The principal series is that derived from oleic, linoleic, and linolenic acids. Oleic acid, following a sequence of steps, is converted into octadeca-6,9-dienoic, eicosa-5,8,11-trienoic, and docosa-7,10,13-trienoic (1) acids; linoleic is transformed into γ-linolenic, arachidonic, docosa-7,10,13,16-tetraenoic (2), and docosa-4,7,10,13,16-pentaenoic acids, while linolenic acid is elongated and desaturated to eicosa-5,8,11,14,17-pentaenoic, docosa-7,10,13,16,19-pentaenoic, and docosa-4,7,10,13,16,19-hexaenoic acids (3).

Although they are not interconvertible, dietary experiments indicated that the amount of polyunsaturated acids of each series accumulated in animal tissues depends on the presence and amount of the acids of the other series. For example, eicosa-5,8,11-trienoic (4) and docosa-7,10,13-trienoic (1) acids synthesized from oleic acid in the fat-deficient rat are rapidly eliminated from the tissues by incorporation of either linoleic and arachidonic acid or linolenic, eicosa-5,8,11,14,17-pentaenoic, and docosa-4,7,10,13,16,19-hexaenoic acids (5) in the diet. The reciprocal effect was also shown (6). These interrelationships have been attributed by Dhopeshwarkar and Mead (6), Marco *et al.* (7), Machlin (8), Bozian and Coniglio (9), Edwards and Marion (10), and Mohrhauer and Holman (11) to metabolic competition among the unsaturated acids of the different families. This so-called competitive inhibition may be located at the steps involving desaturation, elongation, or esterification. However, dietary experiments are too crude to resolve this problem, and we have therefore reduced the number of variables by studying *in vitro* the relative effects of unsaturated acids of 18, 20, and 22 carbon atoms on the microsomal desaturation of labeled palmitic, stearic, oleic, linoleic, and linolenic acids.

EXPERIMENTAL PROCEDURE

Preparation of Microsomes—Livers from male white rats of the Institute strain maintained either on a full or semisynthetic fat-deficient diet (12) were used. After the rats were killed, the livers were washed in the cold with a solution of 0.15 M KCl, 5 mM $MgCl_2$, 1.5 mM glutathione, 62 mM phosphate buffer (pH 7) and 0.25 M sucrose in 0.1 mM EDTA, minced, and homogenized with the same solution (4:1, v/w). The homogenate was centrifuged at 800 × *g* for 10 min, and the supernatant solution was recentrifuged at 8,000 × *g* for 30 min, and, after decanting, at 140,000 × *g* for 1 hour and 20 min. The pellets were suspended

* This work was supported by Grant HE 08267-01-03 from the National Institutes of Health and by Grant 287 b from the Consejo Nacional de Investigaciones Científicas y Técnicas, Argentina. Part of this work has appeared in preliminary form (Brenner, R. R., De Tomas, M. E., and Peluffo, R. O., *Biochim. Biophys. Acta*, 106, 640 (1965)).

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in the homogenizing solution (2:1, v/v), and aliquots containing 5 mg of protein, estimated by the biuret method (13), were assayed for the enzymatic desaturation of labeled palmitic, stearic, oleic, linoleic, and linolenic acids.

Labeled Acids—Palmitic-1-^{14}C (29.6 mC per mmole) and linolenic-1-^{14}C (10 mC per mmole) acids were purchased from Calbiochem. All the other labeled acids were obtained from the Radiochemical Centre, Amersham, England. Stearic-1-^{14}C (37.1 mC per mmole) and oleic-1-^{14}C acids (29.4 mC per mmole) were 99% radiochemically pure, the latter having 3% *trans* isomer. Linoleic acid (24.7 mC per mmole) was 91% radiochemically pure and had 8% *cis,trans*-unconjugated acid.

Assay for Oxidative Desaturation—In animals, fatty acid desaturation has repeatedly been proven to be effected by microsomal enzymes. The first step involves conversion to the CoA derivative through activation with ATP. The fatty acyl-CoA derivatives so formed are desaturated in an ATP-independent reaction in which oxygen and NADPH or NADH are also necessary. In order to obtain a desaturating system free from elongating and oxidizing capacity, the microsomes were incubated in a medium containing suitable inhibitors. After several tests the medium of Gellhorn and Benjamin (14), without added α-glycerol phosphate, was adopted. Ten millimicromoles of each of the acids tested, dissolved in 10 μl of propylene glycol, were incubated with 5 mg of microsomal protein and 0.1 ml of supernatant in a solution containing, in micromoles: ATP, 3; CoA, 0.2; NADPH, 0.8; NADH, 1.8; magnesium chloride, 15; GSH, 4.5; nicotinamide, 1; sodium fluoride, 125; sodium cyanide, 3; phosphate buffer (pH 7) 125, in 0.15 M potassium chloride and 0.25 M sucrose. The total volume was 3 ml. After the addition of the microsomal suspension, the flasks were gassed with oxygen in the cold for 1 min and incubated in a Dubnoff metabolic shaker at 35° for 20 min. The incubation was stopped by the addition of 2 ml of 10% methanolic KOH. Myristic acid (1 mg) was added, and, after 40 min of saponification at 85°, the acidified solution was quantitatively extracted with petroleum ether (b.p. 30–40°). Fatty acids were esterified by treatment with methanolic 3 N HCl for 3 hours at 60°.

The composition of the mixture of methyl esters was determined by quantitative gas-liquid chromatography in a Pye apparatus with ionization detector. The samples were analyzed in columns packed with 10% polyethylene glycol adipate on Chromosorb W (100 to 120 mesh) at 195° or 10% diethylene glycol succinate on Chromosorb W (60 to 80 mesh) at 165° or 22% Apiezon L in Chromosorb W (100 to 120 mesh) treated with hexamethyldisilazane at 206°. The distribution of radioactivity in the fatty acids was determined by preparative gas-liquid radiochromatography with the same kinds of columns specified above. A Pye radiochromatograph with proportional counter was used. The apparatus was set with the integrator at 1000 counts per deflection. The counter efficiency detection of ^{14}C was 70%. The recovery of the radioactivity in individual incubations was 85 to 100% by means of the outlined procedure.

Structure of Fatty Acids—Labeled acids synthesized by the microsomal preparation were separated and purified by preparative gas-liquid chromatography in 10% diethylene glycol succinate on Chromosorb W (60 to 80 mesh) at 164°. The structure and position of labeling were determined by reductive ozonolysis (15) followed by gas-liquid radiochromatography with the same column conditions. Standards (5 mg) of methyl arachidonate and methyl docosa-4,7,10,13,16,19-hexaenoate were also ozonized to get aldehyde esters of 5 and 4 carbon atoms and to facilitate the identification of the expected aldehyde esters containing 6 carbon atoms.

Assay for Competitive Reactions—In order to find any competitive effect of other saturated and unsaturated acids upon the desaturation of labeled palmitic, stearic, oleic, linoleic, and linolenic acids, similar amounts of palmitic (>99% pure), stearic (>99% pure), oleic (>99% pure), linoleic (>99% pure), linolenic (>99% pure), arachidonic (>90% pure), eicosa-5,8,11,14,17-pentaenoic (>90% pure), docosa-7,10,13,16-tetraenoic (>99% pure), docosa-7,10,13,16,19-pentaenoic (>99% pure), and docosa-4,7,10,13,16,19-hexaenoic (>90% pure) acids were added to the incubation medium. Except for docosatetraenoic and docosapentaenoic acids, all of these acids were purchased from the Hormel Institute. Docosa-7,10,13,16-tetraenoic and docosa-7,10,13,16,19-pentaenoic acids were separated by preparative gas-liquid chromatography in 10% polyethylene glycol adipate in Chromosorb W (100 to 120 mesh) from bovine adrenal glands (16). Percentage of conversion was studied by gas-liquid radiochromatography as described above.

Competitive effects among oleic, linoleic, and linolenic acids were also recognized by measuring the rate of desaturation of increasing concentrations of the labeled acids during the first 4 min of incubation in the same system in the presence of the tested acids. The number of molecules converted was measured by gas-liquid radiochromatography. Approximate initial velocities were calculated and Lineweaver-Burk plots were constructed from the data (see Figs. 7 and 8).

Distribution of Radioactivity in Lipids—The distribution of labeled linoleic and stearic acids and their products of desaturation in the microsomal lipids was determined after 20 min of incubation in the same system. The incubation was stopped by pouring the contents of the flasks into 60 ml of chloroform-methanol (2:1, v/v) containing 0.5 ml of 0.25 M sulfuric acid and 1 mg of myristic acid. After shaking, 12 ml of 0.37% KCl were added and the aqueous layer was removed. The lower layer was dried, and the lipids were separated by thin layer chromatography on plates (20 × 20 cm) covered with Silica Gel G (0.5 mm thick). Chloroform-methanol-water (65:25:4, v/v) was used as the developing solvent. Standards of phosphatidylcholine and ethanolamine, fatty acids, cardiolipin, and triglycerides were run at the same time in order to identify the lipids. The surface of the plate covered by the spots of the sample was marked in 1-cm squares, and each such square was removed and directly esterified with methanolic 3 N HCl. The esters were taken up in chloroform and washed three times with water. The aqueous fraction was used for phosphorus determination by Bartlett's (17) technique, and an aliquot of the esters was counted in a Nuclear-Chicago gas flow counter while the remaining material was analyzed in the gas-liquid radiochromatograph. The distribution of label in the different lipids and the proportion of labeled acids in the main fractions were studied by this procedure.

Assay for Acyl-CoA Desaturation—Oleyl-1-^{14}C-CoA desaturation to octadeca-6,9-dienoic acid in the presence of linoleyl-CoA was also studied. Linoleyl-CoA and labeled oleyl-CoA were prepared as described by Kornberg and Pricer (18) by incubation of the free acids and CoA with guinea pig microsomes. Oleyl-CoA (10 mμmoles, 10.5 mC per mmole) was incubated at 37° for 1 hour with 5 mg of microsomal protein in a medium containing in millimicromoles: NADPH, 200; NADH, 200; EDTA, 10; and phosphate buffer (pH 7.2), 30, in a total volume of 0.5 ml.

crosomes were separated in the usual way from rats fed a complete diet.

RESULTS AND DISCUSSION

The differential gas-liquid radiochromatograms of the esterified microsomal fatty acids obtained after 20 min of incubation with labeled stearic, oleic, linoleic, and linolenic acids are shown in Fig. 1. They show the conversion of stearic into octadecenoic acid, oleic into octadecadienoic acid, linoleic into octadecatrienoic acid, and linolenic into octadecatetraenoic acid. No other labeled acid was found in our experimental conditions except for the experiments with stearic-1-^{14}C acid. When enough radioactive stearic acid was incubated, about 2% of labeling could be detected in the octadecadienoic peak. This acid would be derived from oleic acid synthesized in turn from stearic acid. Microsomal oxygenases have been shown to convert stearic into oleic acid (19), while conversion of oleic into octadeca-6,9-dienoic acid by the same subcellular fraction has been shown by Holloway, Peluffo, and Wakil (20). Linoleic desaturation to γ-linolenic acid was shown by Nugteren (21), and in our experiment the octadecatrienoic acid synthesized from linoleic-1-^{14}C acid was also proven to be γ-linolenic acid. By reductive ozonolysis and direct gas-liquid radiochromatography it was possible to localize the radioactivity in the 6-carbon aldehyde methyl ester. None was found in the other products of cleavage.

As far as we know, desaturation of linolenic to octadeca-6,9,12,15-tetraenoic acid *in vitro* has not been described in the literature. However, Klenk and Oette (3) and Klenk and Mohrhauer (22) have found that linolenic and octadeca-6,9,12,15-tetraenoic acids administered to fat-deficient rats are similarly converted into eicosa-5,8,11,14,17-pentaenoic and docosa-4,7,10,13,16,19-hexaenoic acid. Octadecatetraenoic acid synthesized by our preparation of liver microsomes from linolenic-1-^{14}C acid was shown to be the octadeca-6,9,12,15-tetraenoic acid by reductive ozonolysis and gas-liquid radiochromatography. As expected, the labeling was found only in the 6-carbon aldehyde ester that can be produced only by ozonolysis of a double bond added to the linolenic acid in positions 6 and 7.

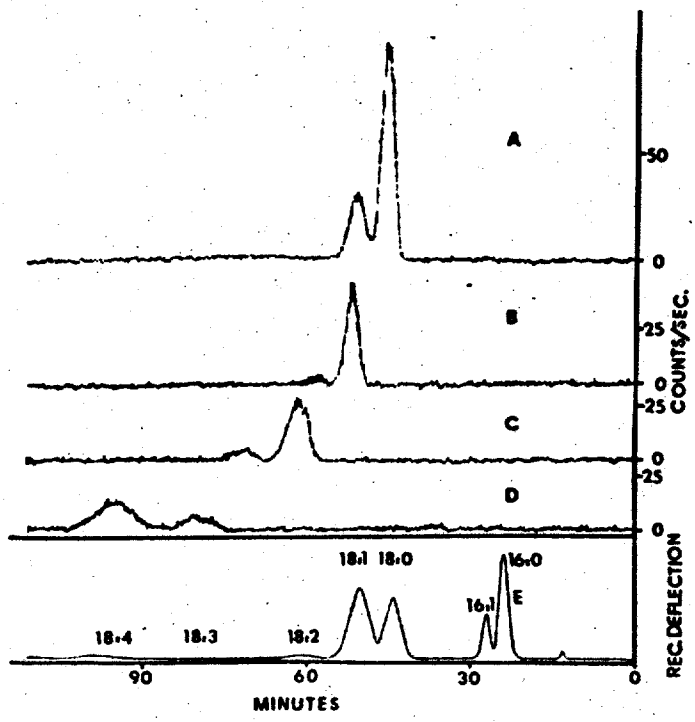

FIG. 1. Differential gas-liquid radiochromatograms after 20 min of incubation of 10 mμmoles of *A*, stearic-1-^{14}C; *B*, oleic-1-^{14}C; *C*, linoleic-1-^{14}C; and *D*, linolenic-1-^{14}C acids. Microsomal protein (5 mg) from the livers of fat-deficient rats was incubated at 35° in O_2 under the conditions described in the text. *E*, the simultaneous gas-liquid chromatogram of microsomal lipids.

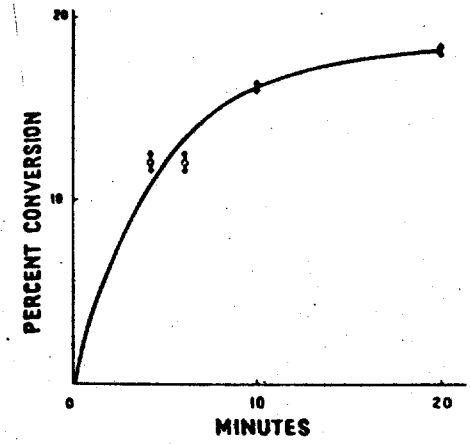

FIG. 2. Time course and percentage of linoleic-1-^{14}C acid converted into γ-linolenic-1-^{14}C acid by liver microsomes from rats fed a fat-free diet. Labeled linoleic acid (10 mμmoles) was incubated at 35° in O_2 with 5 mg of microsomal protein. The conditions of incubation are described in the text. Limits of variation in two experiments are indicated.

Desaturation of palmitic acid to palmitoleic and approximately 5% hexadecadienoic acid was also found under the same experimental conditions.

Although sufficient chain length still exists in all of the unsaturated acid molecules synthesized in our system to introduce a further double bond maintaining the divinyl rhythm, no acid with a double bond in carbon 3 and carbon 4 was found. The absence of this type of unsaturation is very probably related to polar effects due to the proximity of the carboxyl group. The other possibility that would lead to similar results is a change in the position of the double bond from carbon 3 and carbon 4 to carbon 2 and carbon 3 followed by β oxidation. Migration of double bonds from carbon 2 and carbon 3 to carbon 3 and carbon 4 has been shown by Bloch (19) to occur in the anaerobic pathway of synthesis of oleic acid by bacteria. However, this explanation must be discarded because of the lack of β-oxidative capacity of our incubation system.

The percentage of desaturation of the acids tested depended on the time of incubation. Fig. 2 shows the percentage of conversion of linoleic into γ-linolenic acid as a function of time of incubation. The conversion initially increased with time and then became approximately constant after 20 min. Similar curves were found for the other acids and are analogous to the findings of Gellhorn and Benjamin (14) for stearic desaturation. The highest percentages of desaturation found for palmitic, stearic, oleic, linoleic, and linolenic acids were 60, 41, 6, 21, and 70%, respectively, after 20 min of incubation with 5 mg of microsomal protein obtained from rats fed a fat-deficient diet for at least 4 months. However, the percentage of conversion of the acids depends on the dietary and hormonal conditions (23) of the animals. For example, 10 rats fed a fat-deficient diet for 8

TABLE I

Effect of fatty acids on oxidative desaturation of palmitic, stearic, oleic, linoleic, and linolenic acids

Labeled acids, 10 mμmoles each, were incubated with equivalent amounts of the test acids and with 5 mg of microsomal protein obtained from the livers of fat-deficient rats by using the conditions described in the text. Incubation was for 20 min at 35° in oxygen. Results are expressed as percentage of the conversion of the labeled acid without additions, and are the means of at least two experiments.

Acids added	Relative conversion				
	Palmitic	Stearic	Oleic	Linoleic	Linolenic
	%	%	%	%	%
None	100	100	100	100	100
16:0[a]		100	100	99	99
18:0	104		99	99	99
18:1	117	104		90	92
18:2 (ω 6)[b]	117	109	56		87
18:3 (ω 3)	105	109	45	72	
20:4 (ω 6)	119	111	113	102	106
20:5 (ω 3)	115	112	119	102	110
22:4 (ω 6)			100	105	
22:5 (ω 3)		112	99	102	100
22:6 (ω 3)	114	91	73	92	96

[a] In the abbreviations of fatty acids, the number preceding the colon represents the number of carbon atoms in the chain; the number following the colon represents the number of double bonds.

[b] The number following ω indicates the position of the last double bond, counting the double bonds from the terminal methyl group.

months showed 20.6 ± 1.5%[1] conversion of linoleic into γ-linolenic acid, whereas 33.4 ± 2.5% was found for 4 animals maintained on a full diet for 7 weeks and the fat-deficient diet supplemented with 5% protein for the last week.

The effect of equivalent amounts of palmitic, stearic, oleic, linoleic, linolenic, arachidonic, eicosa-5,8,11,14,17-pentaenoic, docosa-7,10,13,16-tetraenoic, docosa-7,10,13,16,19-pentaenoic, and docosa-4,7,10,13,16,19-hexaenoic acids on the oxidative desaturation of labeled palmitic, stearic, oleic, linoleic, and linolenic acids is shown in Table I. Only unsaturated fatty acids of similar chain length significantly depressed the conversion of oleic, linoleic, and linolenic acids into the corresponding, more unsaturated acids. The depressive effect increased with the unsaturation of the acid tested, higher for linolenic and lower for oleic. This order of depressive power is coincident with the sequence found by Holman (24) in the inhibition of eicosa-5,8,11-trienoic acid synthesis by fat-deficient rats after supplementation of the diet with linoleic or linolenic acid. In consequence, the very low percentage of eicosatrienoic acid found in the rat fed a complete diet may be the result of a decreased synthesis of octadeca-6,9-dienoic acid due to the presence of linoleic and linolenic acids in the diet. This result, however, does not eliminate the existence of other steps in the synthesis of eicosa-5,8,11-trienoic acid that may show regulatory effects.

Under the conditions of the experiments the polyunsaturated acids of 20 and 22 carbon atoms, belonging either to the linoleic

[1] Standard deviations of the mean.

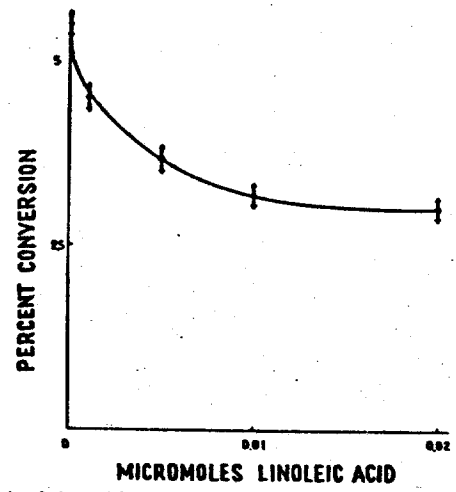

FIG. 3. Linoleic acid dependence on oleic-1-^{14}C acid conversion into octadeca-6,9-dienoic acid. Oleic-1-^{14}C acid (10 mμmoles) was incubated for 20 min at 35° in O_2 with increasing amounts of linoleic acid and 5 mg of microsomal protein from rats fed a fat-free diet under the conditions described in the text. Limits of variation in two experiments are indicated.

or the linolenic family, did not significantly depress the desaturation of oleic, linoleic, and linolenic acids, except for the case of docosahexaenoic acid. Furthermore, some of them even increased the conversion in some way. Therefore, the displacement of eicosa-5,8,11-trienoic acid from the tissues of fat-deficient rats by arachidonic acid (4) cannot be attributed to an inhibitory effect at the step of octadeca-6,9-dienoic acid synthesis but to another specific effect, possibly at the level of eicosa-8,11-dienoic acid desaturation or phospholipid synthesis.

Both palmitic and stearic acids, which were shown to be highly desaturated, when incubated as labeled acids together with linoleic-1-^{14}C and linolenic-1-^{14}C acids did not exhibit a depressing effect. The oxidative desaturation of palmitic and stearic acids was also not depressed by equivalent amounts of any of the unsaturated acids tested, whether they were of a similar or a different chain length. These results would indicate that the inhibitory effects are apparently not related either to an inadequate supply of CoA or reduced pyridine nucleotide, or to the mere presence of free fatty acids. The same or very closely related oxygenases would desaturate oleic, linoleic, or linolenic acids, but they would be different from those which desaturate palmitic acid, stearic acid, and the acids of 20 and 22 carbon atoms. Palmitic oxygenase would also be different from stearic oxygenase.

Incubation of labeled oleic and linoleic acid with increasing amounts of linoleic and linolenic acids, respectively (Figs. 3 and 4), showed a relation between the depressive effect and the concentration of the acids added. However, the effect was not linear, and became asymptotic when it reached approximately half of the maximal conversion. This kind of relationship would indicate that when higher amounts of acids are added other factors may play an important role in the desaturation of the labeled acids, in addition to a possible competitive effect. Similar results were also found (Fig. 5) when labeled linolenic acid was incubated with increasing amounts of linoleic and oleic acids.

Equivalent amounts of arachidonic and eicosa-5,8,11,14,17-pentaenoic acids incubated with labeled oleic, linoleic, or linolenic acids (Table I) seemed to increase somewhat the desaturation of any of the three acids. When increasing amounts of the

two polyunsaturated acids were incubated with 10 mμmoles of labeled linoleic acid a significant increase in the synthesis of γ-linolenic acid was found (Fig. 6). The peak corresponded approximately to 50 mμmoles of the added arachidonic or eicosapentaenoic acid and decreased with higher amounts of the acids. This unexpected result is difficult to explain and requires further research. It may be caused by a direct activating effect of arachidonic or eicosapentaenoic acids on the desaturation, or may be produced through an appropriate change in the composition of the lipids that are bound to the oxygenases. This has been shown by Holloway and Peluffo (25) to be important in determining the specific activity of the enzymes.

The mutual inhibitory effects of oleic, linoleic, and linolenic acids appear to be caused by competition reactions (Figs. 7 and 8). Lineweaver-Burk plots showed convergent lines which intersected at the ordinate when increasing amounts of linoleic-1-^{14}C acid were incubated for 4 min in the absence and presence of a constant level of oleic or linolenic acid. Similar results were also obtained when increasing amounts of either linolenic-1-^{14}C or oleic-1-^{14}C acids were incubated with constant concentrations of oleic-1-^{14}C or linolenic-1-^{14}C acids, respectively (Fig. 8).

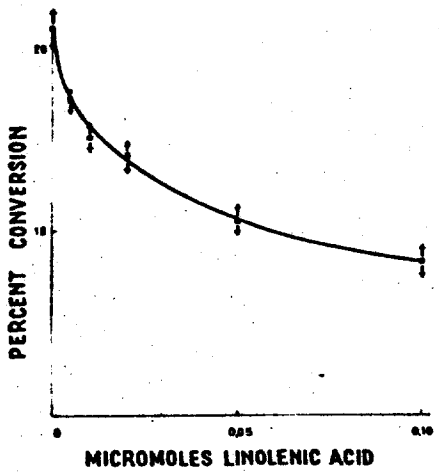

FIG. 4. Linolenic acid dependence on linoleic-1-^{14}C acid conversion into γ-linolenic acid. Linoleic-1-^{14}C acid (10 mμmoles) was incubated for 20 min at 35° in O_2 with 5 mg of microsomal protein from rats fed a fat-free diet under the conditions described in the text. Limits of variation in two experiments are indicated.

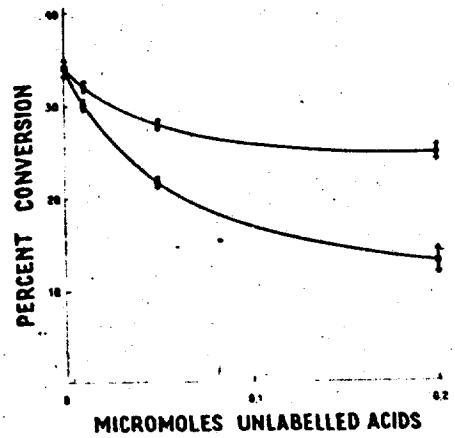

FIG. 5. Linoleic acid and oleic acid dependence on linolenic-1-^{14}C acid conversion into octadeca-6,9,12,15-tetraenoic acid. Linolenic-1-^{14}C acid (10 mμmoles) was incubated for 20 min at 35° in O_2 with linoleic (■) or oleic (●) acids and 5 mg of microsomal protein from rats fed a fat-free diet under the conditions described in the text. Limits of variation in two experiments are indicated.

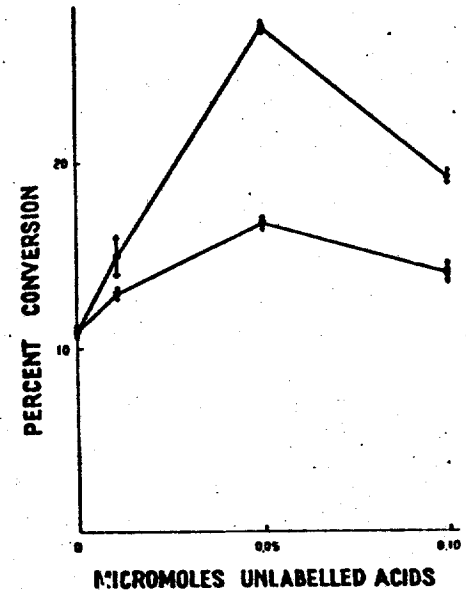

FIG. 6. Effect of arachidonic and eicosa-5,8,11,14,17-pentaenoic acids upon the conversion of linoleic-1-^{14}C acid into γ-linolenic acid. Linoleic-1-^{14}C acid (10 mμmoles) was incubated for 20 min at 35° in O_2 with increasing amounts of arachidonic (●) or eicosa-5,8,11,14,17-pentaenoic (■) acids and 5 mg of microsomal protein from rats fed a fat-free diet under the conditions described in the text. Limits of variation in two experiments are indicated.

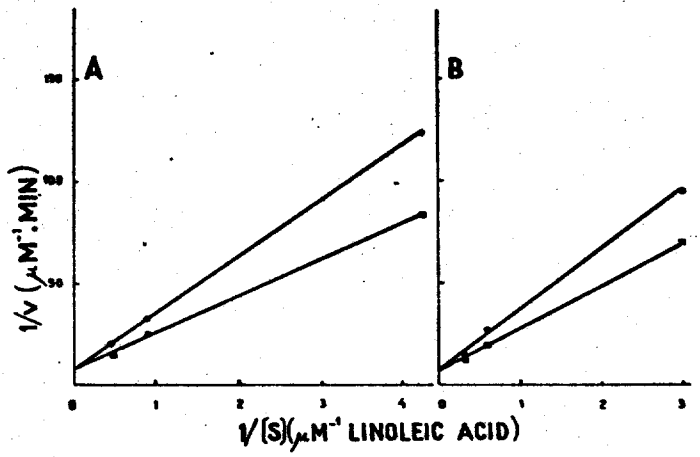

FIG. 7. Lineweaver-Burk plots for linoleic-1-^{14}C acid incubation. *A*, in the presence (●) and absence (■) of 1.58 μM linolenic acid. *B*, in the presence (●) and absence (■) of 33.3 μM oleic acid. Velocities were measured for the first 4 min of incubation at 35° in O_2 under the conditions described in the text. Each tube contained 5 mg of microsomal protein from rats fed a fat-free diet.

Approximate K_m and K_i values were calculated (Table II) from the graphs, but the enzymatic system tested was too crude to assure that they measure the affinity of the enzyme for the substrate. The K_i was used for the Michaelis constants for the unlabeled acids tested; K_i values were calculated from the graphs in the usual way from the K_m values calculated in the same experiment. They may not be real "inhibitor constants" but constants of a simultaneous reaction of the enzyme with another substrate. The maximal velocities calculated (Table II, Ex-

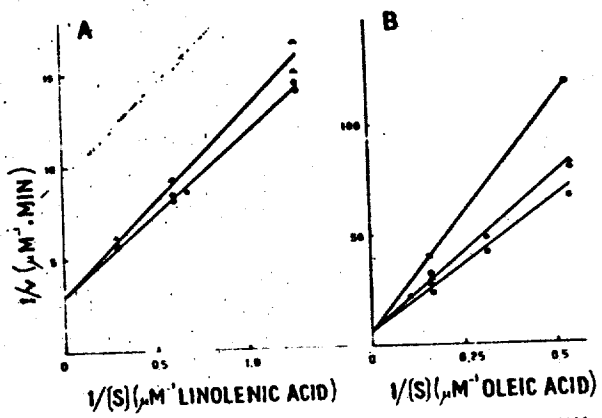

FIG. 8. Lineweaver-Burk plots for: *A*, linolenic-1-^{14}C acid incubation in the presence (▲) and absence (●) of 6.13 μM oleic-1-^{14}C acid; *B*, oleic-1-^{14}C acid incubation in the absence (●) and presence of 0.78 μM (▲) and 3.33 μM (■) linolenic-1-^{14}C acid. Velocities were measured for the first 4 min of incubation at 35° in O_2 under the conditions described in the text. Each tube contained 5 mg of microsomal protein from rats fed a fat-free diet.

TABLE II

Approximate Michaelis constants of oleic, linoleic, and linolenic oxidative dehydrogenation

The K_m values were calculated from Lineweaver-Burk plots. The velocities were measured for the first 4 min of incubation of the labeled acids with 5 mg of microsomal protein of fat-deficient rats at 35°. Conditions are as described in the text.

Labeled acids incubated	Experiment	Maximal velocities	K_m for substrate	K_i	
				Oleic	Linolenic
		mμmole/min	$M \times 10^{-6}$	$M \times 10^{-6}$	
Linoleic	6	0.16	3.2		3.3
Linoleic	7	0.37	8.6	87	
Linoleic	8	0.32	9.9		
Linolenic	12	0.32	2.9	54	
Oleic	12	0.23[a]	27		5.9[a]
		0.22[b]			4.3[b]

[a] Calculated from Fig. 8 when the inhibitory effect of 0.78 μM linolenic acid was tested.

[b] Calculated from Fig. 8 when the inhibitory effect of 3.33 μM linolenic acid was tested.

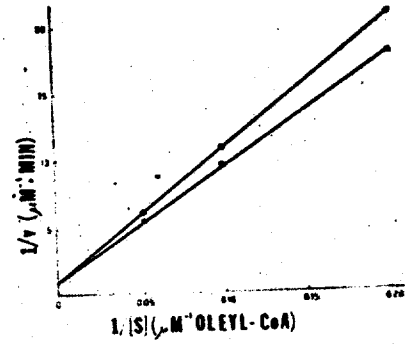

FIG. 9. Lineweaver-Burk plots for oleyl-1-^{14}C-CoA incubation in the presence (●) and absence (■) of 10 mμmoles of linoleyl-CoA. Increasing concentrations of oleyl-1-^{14}C-CoA were incubated with 5 mg of microsomal protein in a solution containing, in millimicromoles: NADPH, 200; NADH, 200; EDTA, 10; and phosphate buffer (pH 7.2), 30, in a volume of 0.5 ml. Velocities were measured from the conversion attained along the first 4 min of incubation at 37° in O_2.

periment 12) were higher for linolenic than for oleic acid, whereas K_m values were lower for linolenic than for oleic acid. Both results coincide in provoking the same effect, the displacement of oleic products of desaturation by linoleic products when both acids are added together. This result is in harmony with those obtained from experiments *in vivo* in which both acids were administered together (5).

The rates measured in our enzymatic system are the result of several reactions, including acylation of the substrate with CoA, desaturation of the acid, and esterification of substrate and newly synthesized acids. The slowest reaction undoubtedly controls the over-all rate of the reaction, and the competition found in our

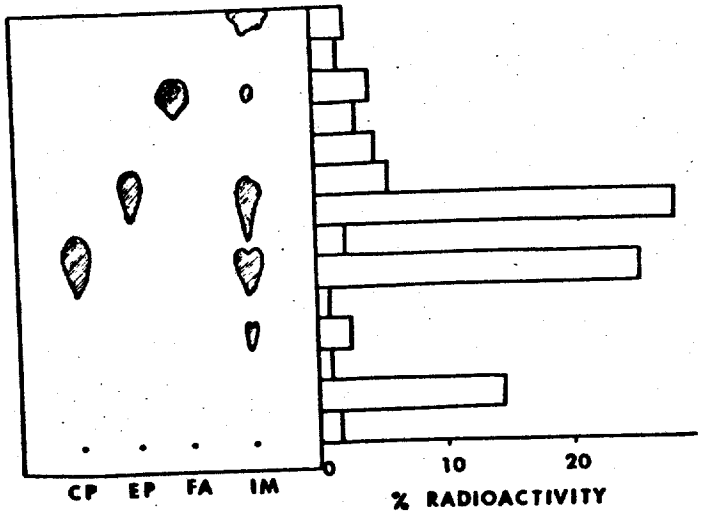

FIG. 10. Thin layer chromatogram and radioactivity distribution in the microsomal lipids after the incubation of 10 mμmoles of linoleic-1-^{14}C at 35° for 20 min in O_2 under the conditions described in the text. Each tube contained 5 mg of microsomal protein from rats fed a fat-free diet. Total lipids were chromatographed in chloroform-methanol-water (65:25:4, v/v). Chromatogram stained with iodine. *CP*, choline glycerophosphatide; *EP*, ethanolamine glycerophosphatide; *FA*, free fatty acids; *IM*, lipids of incubated microsomes.

TABLE III

Effect of α-glycerol phosphate and lysolecithin on oxidative desaturation of linoleic and stearic acids

Labeled acids, 0.01 μmole each, were incubated with 5 mg of microsomal protein for 20 min at 35° in oxygen. Conditions were as described in the text. Results are expressed as percentage of the conversion of the labeled acid without additions.

Addition	Relative conversion	
	Linoleic-1-^{14}C	Stearic-1-^{14}C
μmoles	%	%
None	100 (±3)[a]	100 (±3)
α-Glycerol phosphate		
0.01	92 (±3)	101 (±3)
0.10	96 (±2)	
1.00	82 (±7)	100 (±8)
Lysolecithin		
0.01	82 (±22)	120 (±8)
0.10	98 (±10)	
1.00	20 (±20)	97 (±18)

[a] Maximal deviation of the mean of at least two experiments.

system would correspond to this reaction. There is sufficient NADPH and CoA in our system to discard these cofactors as limiting factors. Acylation of CoA may also be discarded, since saturated acids of 16 and 18 carbons and unsaturated acids of 20 carbons do not inhibit the desaturation of oleic, linoleic, and linolenic acids. This conclusion was also definitely proved when the competition of linoleyl-CoA with labeled oleyl-CoA was tested (Fig. 9) and convergent lines which intersected at the ordinate were obtained again.

Esterification of substrate fatty acids and products by our microsomal system was shown by separation of the lipids by thin layer chromatography followed by determination of their content of radioactive fatty acids (Fig. 10). After incubation, some labeled linoleic and γ-linolenic acids were found as free acids, but they were preponderantly bound to choline and ethanolamine glycerophosphatides. Moreover, in our experimental conditions, esterification of fatty acids does not appear to be a limiting factor because α-glycerophosphate which has been proven to increase the synthesis of fatty acids (14) by regulating fatty acid esterification (26), and lysolecithin, which is a good acceptor of unsaturated fatty acids (27), do not increase the rate of desaturation (Table III).

Consequently, the experiments described above suggest that the competitive phenomena found may probably be located at the desaturating step. The K_m and K_i values calculated for oleic, linoleic, and linolenic acids would measure roughly their oxidative dehydrogenation, and it is interesting to note that K_m and K_i values for oleic acid are similar. The same similarity was also found for linolenic acid. When 3.33 μM linolenic-1-^{14}C and 6.13 μM oleic-1-^{14}C acid were added together to 5 mg of microsomal protein, the total rate of reaction for the first 4 min of incubation was 0.18 μM per min, which is less than the sum of the rates of the reactions measured separately, 0.21 μM per min. This criterion of identity of enzymes (28), together with previous results, provides some evidence that the same oxygenase and the same active center desaturate oleic, linoleic, and linolenic acids. The other oxygenases that desaturate stearic and palmitic acids, and very probably the acids of 20 and 22 carbons, would be different. However, when a more than 20-fold excess of oleic or linoleic acids was included in the system a decrease was found in the desaturation of both saturated acids, indicating that the oxygenases that desaturate saturated acids may have a similar active center able to react with oleic or linoleic acid.

The competitive reactions shown among the unsaturated acids of 18 carbons undoubtedly play an important role in the regulation of the lipidic composition of the tissues, together with similar competitions that can be predicted to exist among the unsaturated acids of 20 and 22 carbons, respectively. They may explain at least some of the effects found in dietary experiments in which different fatty acids are supplied to the animals, but many problems still remained unsolved. Other factors, such as elongation, oxidative and hydrolytical reactions, phospholipid and triglyceride synthesis, hormones, and even enzyme synthesis at the level of RNA or DNA, cannot be discarded.

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Fish Oil, M. Stansby, (Ed.), 1967

CHAPTER 15

Stephen S. Chang

Processing of Fish Oils

INTRODUCTION

The general objective of processing fats and oils is the removal of impurities which cause the original products to have an unattractive color or taste or which cause harmful metabolic effects. The impurities consist of free fatty acids, mono- and diglycerides, phosphatides, steroids, vitamins, hydrocarbons, pigments, carbohydrates, proteins and their degradation products, suspended mucilaginous and colloid-like matters, and oxidation products of fatty esters. The degree of removal of these impurities from fish oils by processing depends upon the ultimate use of the processed products. For example, a palatable oil is necessary for use in foods but not so for many industrial applications.

The processing of fish oils is similar to that of vegetable oils. However, the quality of crude fish oils is less uniform than crude vegetable oils. A representative sample of each batch of fish oil, therefore, must be carefully processed in the laboratory to determine its quality and freshness. Preferably this is done before the shipment of fish oil has arrived so that the processing conditions, refining loss, and the stability of the processed oil from that batch may be pre-determined.

A simple test for the freshness and quality of a sample of fish oil is to mix equal amounts of the oil and 50% alcoholic potash at room temperature. The FAC color of the saponified clear solution is thereby determined. Any darkening in color indicates the occurrence of autoxidation in the oil. The hydrogenation rate also serves as a measurement of the quality of fish oils. The sample of fish oil is hydrogenated under standard conditions using 0.07% of Ni as catalyst at 180°C. under 25 p.s.i.g. for 30 min. A refractive index drop of 30 or more in the third and fourth decimal place calculated on the basis of an iodine value of 100 indicates that the fish oil has good quality and can be hydrogenated in the plant with ease (Wissebach 1965). For example, a whale oil with an iodine value of 120 is considered as of good hydrogenation property if its refractive index drop is 40 or more under the standard conditions. A refractive index drop of 40 for an oil with an iodine value of 120 corresponds to a refractive index drop of 33 on the basis of an iodine value of 100. Good whale oils with iodine value of 119 have refractive index drops of 50 to 60 under standard conditions which corresponds to refractive index drop of 42 to 50 on the basis of iodine value of 100.

The processing of fish oils for edible products may consist of five steps, viz. degumming, refining, bleaching, hydrogenation, and deodorization. It is common practice in fish-oil processing to combine the degumming and refining steps. The bleaching may be done either before or after hydrogenation. To improve the quality of the oil, the hydrogenated fish oil is often mildly re-refined.

The processing of fish oil for industrial products usually consists of winterization, refining, and bleaching. However, some steps may be added or eliminated depending upon the quality of the oil required by the ultimate uses. A current trend is the application of hydrogenated fish oils and the fatty acids of hydrogenated fish oil to many industrial uses.

WINTERIZATION

Crude fish oil is usually settled and filtered before further processing. It may also be "winterized," "racked," or "cold cleared" by chilling in order to crystallize the solid fats which are then removed by filtration; the relatively saturated glycerides thus removed are called "stearines." The "pressed" or "winterized" oil has a higher iodine value and may be made to tolerate various cold tests. For fish-oil-like sperm oil which contains an appreciable percentage of esters of fatty alcohols and fatty acids, the winterization process separates not stearines but rather solid wax known as "spermaceti" which is used extensively in hand lotions, cosmetics, and in various specialized industrial applications.

According to Behr (1936), a sardine oil which has been winterized for use in paints or varnishes should have a clouding time at 0°C. of not less than 14 h. Bailey (1951) found that in order to produce a sardine oil with cold test of 20 h, it is necessary to remove approximately 30% of higher melting materials from the oil and to decrease the saturated acid content from 22 to 17%. The degree of winterization appears to depend more upon the holding time at the minimum temperature than the actual temperature employed. Brocklesby (1941) reported that a temperature of 1°C. was required to remove 21.8% of higher melting glycerides from a sample of pilchard oil, while 17–32% of such glycerides were precipitated from various pilchard oils during a 24 holding period at 9°C. Stearines, of course, can also be removed from fish oils by selective extraction with solvents and by vacuum fractional distillation.

The equipment and technique employed in winterization of oils are rather simple. However, such an operation is usually slow and the separation poor. The relatively large molecules of triglycerides are reluctant to precipitate out as well-formed crystals in a media as viscous as oil at low temperatures. The clean and dry fish oil at a temperature between 32° and 35°C. is pumped into the winterization tank which is installed in a

172

well-insulated and refrigerated room. Some winterization tanks are equipped with cooling coils. Because the cooling must be gradual, the coils are chilled by circulating cold brine instead of directly expanding a refrigerant. In order to avoid shock-chilling, the temperature differential between the oil and the brine should be about 14°C. when the oil is at 24°C. and about 6°C. when the oil is at 7°C. The cooling coils must be quite closely spaced in the tank, since the oil should not be mechanically agitated after crystals begin to form. For example, a tank of approximately 40,000 lb. of oil will drop 3° to 4°C. in about three days. As the saturated glycerides crystallize from the oil, the release of the latent heat causes the temperature of the whole mass to rise 1° to 2°C. After that point the temperature begins to fall again. The winterization may be terminated when the temperature falls below the lowest temperature which had been attained immediately preceding the rise in temperature. The mixture is then filtered in a room refrigerated to 2° to 3°C. with a plate and frame filter press.

DEGUMMING AND ALKALI REFINING

Degumming is the process by which phosphatides and certain other ill-defined mucilaginous materials are removed from the oil by treatment with 2 to 3% of water, or with an aqueous solution of boric acid or a salt such as sodium chloride or pyrophosphate at 30° to 50°C. The hydrated gum deposited by this treatment may be separated from the oil by centrifugation. Degumming may be conducted either by batch process in tank or by continuous process in mixer and centrifuge. Fish oils are usually not degummed before they are refined. The removal of phosphatides and mucilaginous materials are, therefore, accomplished together with the removal of free fatty acids by the refining process itself.

Alkali refining is the treatment of the oil with an aqueous alkaline solution. In this process, the free fatty acids combine with the alkali to form soaps; and the phosphatides and mucilaginous materials absorb alkali and are forced to coagulate through hydration or degradation. This process also removes or degrades certain chromophores. Light-colored fish oils which are currently used in certain industrial applications, particularly in the manufacture of alkyd resins, can be thus produced. It is the practice in some plants to treat fish oils with just enough alkali to remove free fatty acids. The oil is then re-refined with more alkaline solution. In such cases pigments are removed during re-refining.

Refining of an oil with caustic soda readily reduces the free fatty acid content to 0.01–0.03%. This process also practically completely removes the phosphatides. The phosphorus content in a well-refined oil should not exceed about 0.5 ppm.

Alkali refining may be done either by batch or by continuous process. A typical cylindrical kettle which can be used for batch degumming, refining, and bleaching is shown in Fig. 37. The kettle has a conical bottom equipped with outlets for oils, and for soaps and water. Swing suction-pipe is often used in order to facilitate the drawing off of the refined oil from the settled "foots." The kettle is fitted with a variable speed agitator and pipe line by which the bleaching earth can be introduced. Underneath the cover are spray connections for introducing water and aqueous alkaline solution. The closed helical steam coils can be used either for heating with steam or cooling with water; the kettle is also equipped with an open steam coil. Sampling devices are provided at various levels. The kettle is so constructed that it can be maintained under a vacuum of about 29 in. of mercury.

The crude oil or the degummed oil is pumped into the kettle, preferably at about 24°–30°C. It is usually allowed to settle in order to remove entrained air which may interfere with the refining. The amount and strength of alkali to be used is determined from the free fatty acid content and the standard cup loss of the oil, and from the experience with the particular oil stock. For fish oils, aqueous sodium hydroxide solution of 1 to 6 N is generally used. Higher concentrations are used for fish oils with higher free fatty acid content. With the oil under relatively rapid agitation, the alkaline solution is sprayed over the surface of the oil to insure uniform dispersion. When stronger solutions are used, they are usually stirred into the oil instead of spraying. At the first sign of "break," the agitation is slowed down and the oil is heated to about 54° to 60°C. The agitation is continued at slow speed until the "foots" begin to settle. After settling overnight, or at least for eight h, the clear oil is withdrawn. The desirable process is to wash this oil with water to remove any soap in the neutral oil. This is usually accomplished by spraying the oil first with about 10% its weight of hot $^1/_{10}$ N aqueous sodium hydroxide solution, followed by 5 or more times with 10% its weight of hot water.

The kettle refining process requires a long contact time between oil and alkali. It also requires long periods of settling because the process depends on gravity for the separation of the oil and water phases. With the advent of suitable centrifugal equipment for the separation of phases of different density, a continuous refining process was developed. In this process, proportioned amounts of crude or degummed oil and aqueous alkaline solution are pumped through an efficient mixer and then heated to about 54° to 85°C. by a heat exchanger. The mixture is then sent through a centrifugal separator where the refined oil and soapstock are discharged separately. The refined oil is then mixed with about 5 to 10% water, heated, and again centrifuged in order to remove residual traces of soaps. The

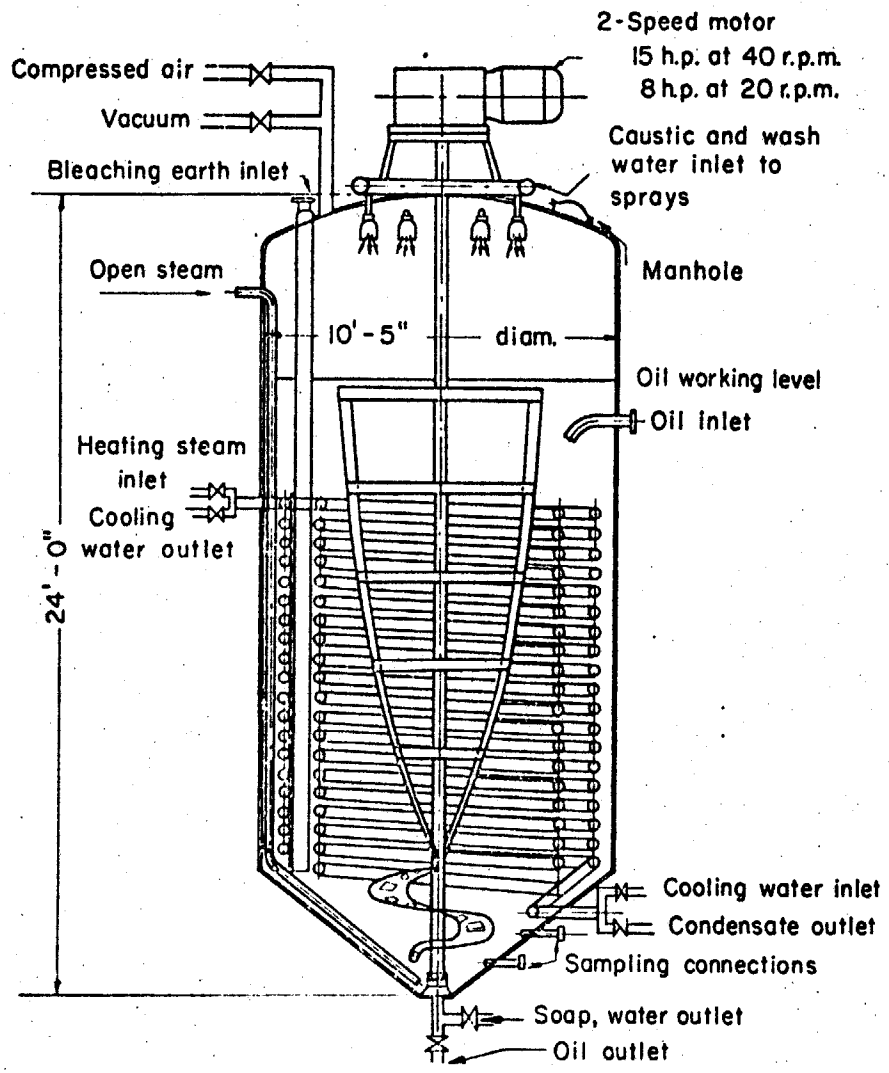

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FIG. 37. KETTLE FOR REFINING AND BLEACHING

From Devine and Williams, "Chemistry and Technology of Edible Oils and Fats" 1961.

washing may be repeated. Finally, a vacuum drier is used to remove the moisture from the refined oil.

Many modifications and new methods for the refining of oils have been developed. For example, the aqueous alkaline solution used for refining may be modified by the addition of various agents. Sodium silicate has been widely used to improve separations by "weighting" the "foots." The carbonate or soda ash process (Mattikow 1942) takes advantage of the non-saponifying character of sodium carbonate. However, the weaker alkali, sodium carbonate, can only decrease the free fatty acid content to about 0.10%. Refining may also be done with miscella in the solvent which is used for the extraction of the oil. Methods are also reported for the removal of the free fatty acids by esterification or by vacuum steam distillation.

BLEACHING

After refining, the oil is usually bleached. Although the main objective of bleaching is to reduce colored matters and natural pigments, some suspended mucilaginous and colloid-like matters are also removed. A trace of soap, if still present, is also adsorbed by the bleaching materials. In the processing of fish oil, bleaching is not particularly difficult. It should be noted that deodorization and the catalytic hydrogenation are also capable of reducing the color of oils because they destroy the carotenoids and the other red pigments.

The bleaching materials commonly used are natural clays, activated clays, and activated carbon. Natural clays are used if the oil is readily bleachable. Such clay bleaches effectively at temperatures below 100°C. and its retention of oil is the lowest, amounting to only 30% of its dry weight. Activated clay, made by acid treatment of neutral clay, is more effective in bleaching. It works best at a temperature in the neighborhood of 120°C. and retains about 50% of its dry weight of oil. The activated clay may also cause a rise in the free fatty acid content of the bleached oil. Activated carbon is normally used in combination with clays, usually in the amount of 5–10% of the clay. It is effective in removing the fluorescence sometimes found in oils, and in adsorbing certain impurities which are not affected by clays. However, both its cost and its retention of oil are the highest among the bleaching agents. Even when mixed with clays, it still retains 100 to 150% of its weight of oil. In the refining of fish oils, activated carbon is seldom used. However, Gauglitz and Gruger (1964) reported that activated carbon is effective in reducing the odors of fish oils. They succeeded in preparing a high quality menhaden oil by a combination of adsorptive bleaching with activated carbon and molecular distillation.

The optimum conditions for bleaching of fish oils are reported as 80°C. for 30 min. under a vacuum of 15 mm Hg (Gauglitz and Gruger 1964). It is reported that the best bleach is obtained when the moisture in the earth is removed in the presence of the oil (Rini 1960). The moisture in the oil is either removed under vacuum at 80° to 85°C. before the clays are added, or removed by holding the agitated mixture of the wet oil and the clay under vacuum at 85°C. for 20 to 30 min. One fish-oil processing plant uses 0.25 to 0.5% of clay at 99°C. to try to bring the color to the desired quality. If it fails, more clay (0.5 to 1.5%) is used. Another plant uses 0.5% of activated clay at 104° to 110°C. An European fish-oil processing plant uses 1.5% of activated clay at 90°C. under vacuum for 5 to 10 min. Since it takes about one hour to filter one batch of oil, a great deal of bleaching is probably taking place during filtration. It should be noted that overbleaching or prolonged agitation of clay and oil is not only not economical but also may lead to instability in flavor and even in color.

Bleaching is done industrially either in batch in open kettles or under vacuum, or continuously under vacuum. Batch bleaching in open kettle is the oldest and simplest method and is still in use in many plants. The oil is charged into a kettle fitted with a paddle agitator and coils for either heating or cooling. The clay is then added and the agitation is continued. After the bleaching is completed, the mixture is filtered through a plate and frame press.

In the batch-wise vacuum bleaching, the process is similar to the open kettle bleaching except that it is conducted in a closed vessel under a vacuum of 27 to 28 in. The clay is added either as an oil slurry or sucked in directly through a valved line to below the surface of the oil. After the bleaching is completed, the oil is cooled sufficiently before the vacuum is broken in order to avoid autoxidation. The mixture is then filtered through a plate and frame press, which may be enclosed, and kept under an inert atmosphere.

In the continuous vacuum bleaching system, the oil is first de-aerated and dried by spraying it into the lower chamber of the vessel at about 54°C. The de-aerated oil is then heated to about 99° to 116°C., filtered through a closed press containing the spent clay from a previous batch, and thence to the upper bleaching section of the vacuum vessel. A clay slurry is sprayed into the upper section where it is mixed with the main stream of oil. Sparge steam is used to aid the mixing of clay and oil. After about 10 min. of residence time, the oil is filtered through a clean plate and frame press.

To recover the oil from the bleaching material, the filter press is usually blown with air or steam, thereby reducing the oil content in the spent clay to 25 to 30% on a dry basis. Some plants are equipped to extract the spent clay with solvents such as hexane or heptane. Since fish oil contains highly unsaturated fatty acids, spontaneous combustion may take place on the large surface of clay during the blowing of the press. Some plants, therefore, bleach the fish oil only after it is hydrogenated.

HYDROGENATION

Hydrogenation of an oil consists of the direct addition of hydrogen to double bonds in the fatty acid chains of glycerides. For hydrogenation to take place, gaseous hydrogen, liquid oil, and solid catalyst must be brought into intimate contact at a suitably elevated temperature and under a suitable pressure of hydrogen. Under such conditions, isomerization may also take place, such as the migration of the position of double bonds and the formation of *trans* isomers. Primarily, hydrogen is a means of converting liquid oils to semisolid plastic fats suitable for shortening and margarine manufacture. Hydrogenation also accomplishes various other desirable results including enhancement of the stability and improvement of the color of the fat. It also destroys the objectionable color and taste of crude oils. This is particularly important in the case of fish oils (Kirschenbauer 1960). Recently, hydrogenation has also been used in the manufacture of liquid oils with an improved stability by decreasing their degree of unsaturation. In this process, the original oil is first hydrogenated and the product is winterized to remove the more saturated glycerides having higher melting points. In certain countries, it is desirable to manufacture a liquid oil of good stability through the hydrogenation of fish oils. This requires the hydrogenation of polyunsaturated fatty acids with little or no hydrogenation of the monounsaturated fatty acids. Kuwata and Ko (1958) claimed that hydrogenation does not proceed beyond the level of oleic acid when oxides of copper, chromium, and manganese are combined for use as catalyst. However, the cost of such a catalyst is high and the flavor stability of the product may be inferior because of the strong pro-oxidant effect of the copper.

The catalyst commonly used for hydrogenation is made of nickel, either by dry reduction, wet reduction, or through nickel aluminum alloy (Mattil 1964). The nickel is either deposited on kieselguhr or suspended in a highly hydrogenated oil. The latter usually contains 25% of nickel and is usually granulated or formed into flakes.

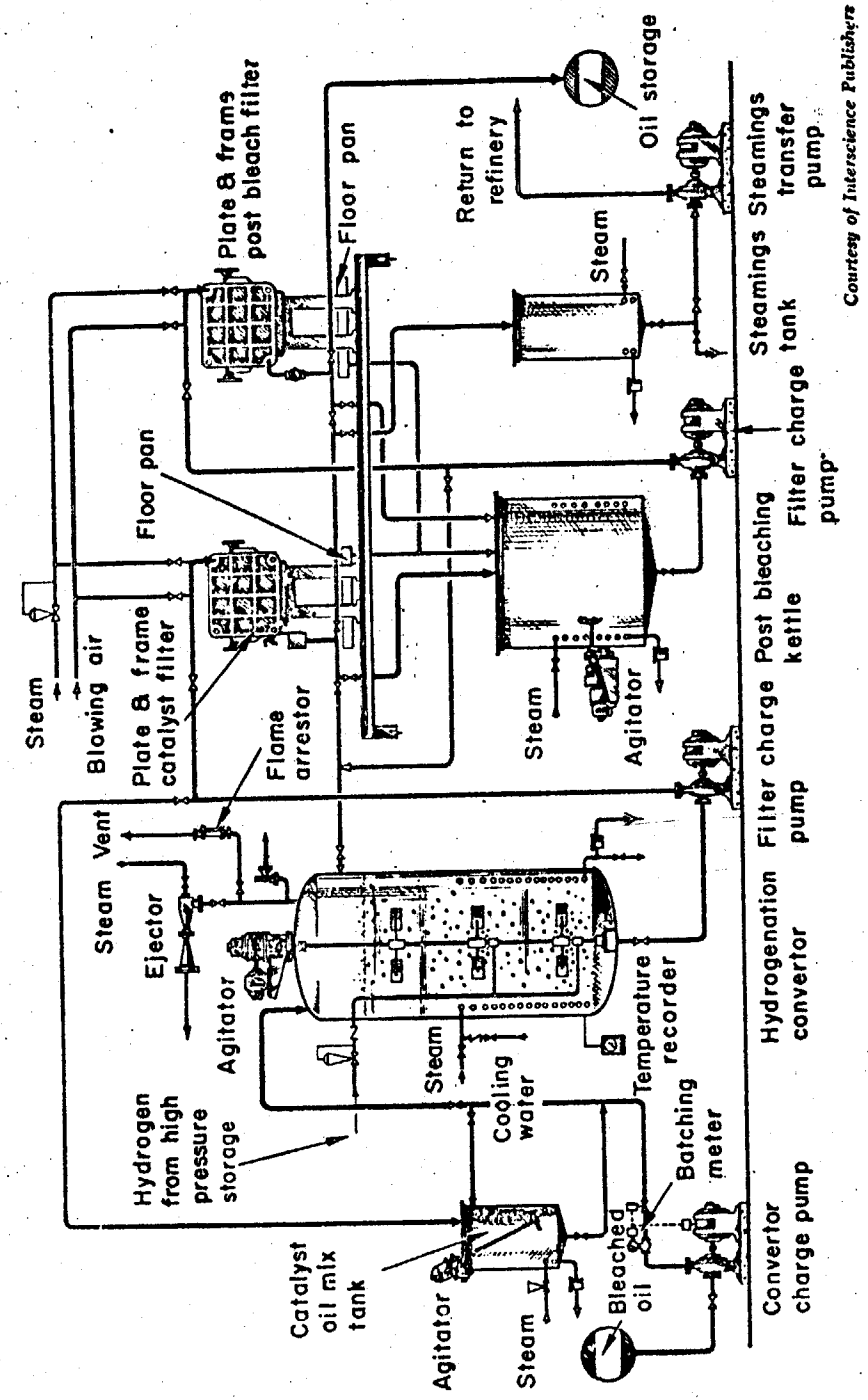
The hydrogen used for hydrogenation is usually manufactured at the hydrogenation plant by either an electrolytic process, a steam iron process, a steam hydrocarbon process, or a water gas process (Mattil 1964).

The temperature range used for commercial hydrogenation is about 107° to 204°C. under about 0 to 60 p.s.i.g. with about 0.05 to 0.10% of

nickel as catalyst. Higher temperature, lower pressure, higher catalyst concentration, and lower degree of gas dispersion in oil result in more selective hydrogenation. Selectivity here means the preferential hydrogenation of more unsaturated acid radicals with minimum formation of completely saturated ones. However, the more selective conditions also cause formation of more *trans* isomers. Too much *trans* isomers in the hydrogenated product tends to make it hard at lower temperatures and soft at higher temperatures. A typical processing plant, for example, hydrogenates fish oils at 204°C. under 10 p.s.i.g. with 0.05% of nickel catalyst in the manufacture of margarine stocks, and at 121°C. under 25 p.s.i.g. with 0.1% of nickel catalyst for shortenings. The nickel catalyst may be reused. However, the selectivity of the catalyst decreases after each use. It is a common practice, therefore, to add some fresh catalyst to each batch for hydrogenation.

Hydrogenation is done industrially either batchwise or continuously. Closed cylindrical vessels with conical bottoms of about 10,000 to 40,000 lb. capacity are used for batch hydrogenation. They are equipped with closed coils for heating with steam or cooling with water. The mixing of the oil, catalyst, and gas is accomplished by re-circulating the mixture from the bottom to the top of the vessel, by mechanically agitating with impellers, or by injecting the hydrogen gas through the mechanically agitated oil by means of a perforated distributor pipe near the bottom of the vessel. In the latter case, the hydrogen gas may be re-circulated. A typical hydrogenation plant using impellers for mixing is shown in Fig. 38. Both the oil and the catalyst are pumped into the converter and steam is admitted to the coil. The converter is then evacuated until the temperature reaches about 100°C. During the evacuation, most of the air and moisture are removed. Hydrogen is then admitted. Since hydrogenation is an exothermic reaction, the heating is discontinued and the temperature is regulated by passing water through the coils. Because there is a tendency for nitrogen and other impurities from the hydrogen gas to build up in the space above the oil, it is the practice to vent occasionally some of the gaseous contents of the converter. When hydrogenation has reached the desired point, the gas is turned off and the oil cooled down rapidly by circulating cold water in the coils. The hydrogenated oil is then filtered with a frame and plate press to remove the catalyst. Edible oils, moreover, are subjected after this filtering process to the next step of deodorization. In fish-oil processing, it is common to re-refine mildly the hydrogenated oil before the deodorization in order to improve its flavor stability.

Continuous hydrogenation has found only limited applications, particularly in fish oil processing. A number of publications and patents covering this process were reviewed by Allen (1960). In one process, a mixture



Courtesy of Interscience Publishers

FIG. 38. LAY-OUT OF A HYDROGENATION PLANT

From Bailey's Industrial Oil and Fat Products by Daniel Swern.

of oil and catalyst is pumped or sprayed into a reaction chamber which contains hydrogen under pressure. The oil-catalyst slurry may be agitated in the converter. The catalyst is filtered from the oil continuously and the catalyst may be returned to the start of the cycle for re-use. Another process involves the use of a fixed catalyst bed. The oil and hydrogen are pumped through the bed, where the reactions take place.

The progress of the hydrogenation is controlled and analyzed by refractive index, iodine number, melting point, or consumption of hydrogen. Since there is generally a linear relationship between the refractive index and the iodine number, it is common practice to use the refractive index to ascertain when the hydrogenation has reached the desired end point. However, the iodine number alone cannot represent the hardness of the oil; some other measurements are also necessary. For example, a measurement of the *trans* isomer content of the hydrogenated fat by its infrared absorption at 10.36 μ would give a somewhat better indication of its consistency. For uses in margarine and shortening, the hydrogenated fish oils are usually analyzed for their solid content index at several temperatures. This is discussed in detail in Chapter 18.

DEODORIZATION

Deodorization is the removal by vacuum steam distillation of small quantities of the more volatile components which are responsible for any odor or taste in fats and oils. The object is to yield a bland product. Before deodorization, the fats and oils may contain the volatile odor and flavor components originally present in the crude oil, the "soapy" type of odor created by refining, the "earthy" type of odor created by bleaching, and the typical hydrogenation odor created by hardening. It should be noted that compounds can impart an undesirable odor or flavor to an oil at concentrations of 1 to 10 ppm or lower. The deodorization process also serves to decrease the free fatty acid content of the oil to 0.02 to 0.04%, to decompose the hydroperoxides present, and to improve the color by decomposing some pigments.

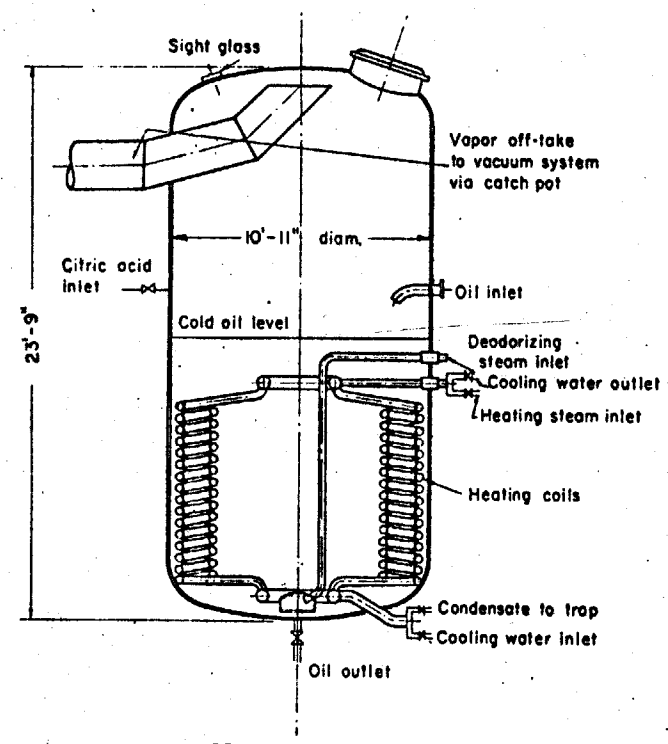
The amount of steam required or the time required at a definite steaming rate of the deodorization of fats and oils is directly proportional to the size of the batch, to the absolute pressure in the deodorizer, and to the logarithm of the ratio of initial to final concentrations of the volatile substances, and inversely proportional to the vapor pressures of volatile substances and the vaporization efficiency of the process. Within a limited range, the logarithm of the vapor pressure of the volatile constituents is approximately proportional to absolute temperature of the deodorization.

The capacity of deodorizers depend upon their designs. Batch deodorizers in the United States usually range from 10,000 to 40,000 lb. capacity,

with 25,000 lb. being the most common. In the United Kingdom, the majority of deodorizers are of 25 tons capacity. Continuous deodorizers usually have capacities of 2,000, 5,000 or 7,500 lb. per h.

A high vacuum is necessary for deodorization, not only to decrease the amount of steam required or the time required to complete the deodorization, but also to inhibit the hydrolysis of the oil during deodorization. A 6 to 8 mm absolute pressure is usually used for deodorization. This may be accomplished by wet vacuum pumps. However, steam jet ejectors with three or more stages are now being commonly used.

The open steam which is passed through the oil should be dry and free from oxygen. It is de-aerated and may be superheated, although superheating is now often omitted. The usual amount of steam used for deodorization is about 2 to 3% by weight of oil per h. Since deodorization usually requires 2 to 5 h the amount of steam used is more than necessary



25 TON DEODORIZER

Courtesy of Pergamon Press, Ltd.

FIG. 39. BATCH-TYPE DEODORIZER

From Devine and Williams, "Chemistry and Technology of Edible Oils and Fats" 1961.

TABLE

Variation of total PCB residues (Aroclor 1254 and 1260) as the composition of the quantitating standard changes

% 1260	0	10	20	30	40	60	70	80	90	100
Species	Total PCB content (ppm)									
Silky shark liver	17.0	--	--	--	--	10.2	10.9*	8.5	7.5	--
Channel catfish+	6.7	6.2	6.1	4.9	4.4	3.5*	3.6	2.9	2.6	--
Goatfish+	5.4	--	--	--	--	3.4	3.5	2.8*	2.5	2.4
Menhaden meal	0.94*	--	--	0.80	--	--	--	--	--	0.43

*Best approximation of true value
--Not calculated
+Edible portion: muscle and skin

active principle is water soluble and dialyzable as in the case of the orally effective principle in renal extracts (5).

The question arises as to whether the observed lowering of the blood pressure is a beneficent effect, as would be expected if this form of therapy is supplying a deficiency induced by injury of the kidney (9). Insofar as our observations on the human are concerned, there is no evidence of a detrimental effect as determined by the subjective feelings and objective tests of renal function when the blood pressure is markedly reduced. In the rat, also, preliminary studies (with Dr. Francis Reichsman) indicate an actual increase in survival time of animals maintained on active oils, compared to untreated controls.

From a practical therapeutic standpoint it is necessary to emphasize that excessively large amounts of material are necessary to induce a demonstrable effect in the human. Hence, although of great theoretical interest, we feel that the use of oils, as well as renal extracts, must still be considered as purely experimental. Until a more readily available source of large amounts of the active principle becomes available, it is impractical to attempt the treatment of patients except on an experimental basis.

SUMMARY

A number of refined oils derived from marine fishes when administered orally are effective in reducing the blood pressure in experimentally induced hypertension in laboratory animals (rats, dogs). Comparable effects were elicited in three human patients suffering from hypertension.

Among oils of vegetable and animal origin, only tung oil was found to have an effect in reducing the blood pressure in hypertensive animals.

Oxidation enhances the activity of certain oils; saponification prior to oxidation in some cases further increases their effectiveness. The blood pressure reducing capacity of the oils is independent of their original vitamin A content, and is retained after destruction of this vitamin by oxidation.

Fractional separation of the oil by distillation *in vacuo*, freezing, or distribution between aqueous organic solvents, partially effects a concentration of the effective blood pressure reducing agent. By adsorption on a synthetic resin and desorption with aqueous alkali, it is possible to obtain the active principle in aqueous solution.

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biodegradable detergents. Fish oil-derived alcohols, however, are mainly of research and development interest.

Nitrogen Derivatives.—An important family of products is made by reacting the carboxyl group with ammonia. Amides are the primary reaction products. These are then converted by dehydration to nitriles, the latter by hydrogenation to primary, secondary, and tertiary amines. Quaternary ammonium derivatives are obtained from the amines. Figures for the volume of fish oils consumed this way are not available. The commercial uses in general parallel those of the same products made from other raw materials—textile and fabric softeners, ore beneficiation, plastic film antiblocking agents, antistatic agents, bactericides, fungicides, corrosion inhibitors, cationic surfactants, waxes, paper coatings, petroleum additives, grease thickeners, boiler treatment, fuel additives, and asphalt antistrip agents. Textile, plastic, and petroleum applications predominate. The polarity of the amine derivatives makes them particularly useful in these fields.

Miscellaneous.—Studies have been made demonstrating that the unsaturated fish oil fatty acids are efficient, powerful, and selective collectors for the impurities in iron ores by froth flotation (Karrick 1965). The commercial possibilities for iron ores have been studied. Patent claims have been made for beryllium ore flotation (Havens 1963).

Monoglycerides, acetylated monoglycerides, and aldehydes have been investigated using the unsaturated fatty acids from menhaden, herring, tuna, and sardine oils (Karrick 1965).

The acid chlorides and the quaternary ammonium chlorides have been described.

Halogenated derivatives have been made including those from bromination of the methylene carbon separating the double-bonded carbons. Chlorinated fish oils have been examined for the fire-retarding of wood.

The sizing of hardboard with the C_{20} and C_{22} chain length fatty acids to prevent the absorption of water was tested with good results (Sinclair 1964).

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The differences most apparent from Tables 20 and 21 are in titer and iodine value.

However, when the component fatty acids are compared, as in Tables 22 and 23, the uniqueness of the fish oils becomes obvious as well as the reasons for their usefulness. The variation in values reflects the difficulties in the analyses and also different sources of oil.

TABLE 21

RANGES OF SOME NON-FISH OIL CONSTANTS

	Linseed[1]	Soya	Sunflower	Tung[2]	Rapeseed[3]	Tall[3]
Iodine value	170–200[4] 177, min.[1] 188, min.	120–141	120–135[4] 125–136[3]	160–175	97–108	127–132
Sap value	189–195	189–195	188–194	189–195	170–180	—
FFA, %	2 max.	[5]	[5]	[5]	[5]	ca. 100
Unsap, %	1 max.	1.5 max.	1.5 max.	1	1.5 max.	—
Titer, C.	19–21		16–20	36–37	11.5–15.0	—
Rosin acids	[5]	[5]	[5]		[5]	0.6

[1] ASTM, D 23 4-48 for raw oil.
[2] AOCS standards.
[3] Distilled fatty acids only—Bauer (1961).
[4] Maas *et al.* 1962.
[5] Reliable data unavailable.

TABLE 22

RANGES OF FATTY ACIDS FROM FISH OILS[1]

	Per cent Composition			
	Menhaden	Herring	Sardine (Pilchard)	Anchovy[3]
Extent of unsaturation				
Saturated acids	29.1–43.3	19.3–29.8	23–26	33–35
Monoenes	23.9–32.2	46.2–62.0		26–29
			35–48[4]	
Dienes	2.4–7.6	2.6–4.3		3.4–7.5
Trienes	0.9–1.4	0.6–4.1	[2]	0.3–3.2
Tetraenes	3.2–3.8	0.9–3.2		
Pentaenes	11.8–15.8	2.0–9.9	29–36[5]	28–30[5]
Hexaenes	5.4–17.7	7.6		
Acid chain length				
C_{14}	6.2–12.3	7.6–11.4	5.1–8.6	11
C_{15} and C_{17}	1.5	0.8–2.2	[2]	3
C_{16}	29.6–40.0	13.8–27.6	26–32	29
C_{18}	19.7–29.4	19.4–24.1	21–22	21–24
C_{20}	12.4–23.0	12.6–19.6	18–26	20–21
C_{22}	16.8–21.7	20.5–27.3	11–14	11–15
C_{24}	0.1–0.9	0.9	15.4	0.6

[1] Data from Maas *et al.* 1962; Jart 1965; Peifer *et al.* 1960; Lane *et al.* 1963; Iverson 1958 and 1965; Gruger *et al.* 1964.
[2] Reliable data unavailable.
[3] Data from Archer-Daniels-Midland: private communication.
[4] Figures are for combined monoenes and dienes.
[5] Figures are for combined tetraenes, pentaenes, and hexaenes.

TABLE 23

RANGES OF UNSATURATION OF SOME NON-MARINE OILS[1]

	Per cent Composition					
	Linseed	Soy	Sunflower	Tung	Rapeseed	Tall[2]
Extent of unsaturation						
Saturated	10–11.5	12.3–15	13.3	3.6–4.9	0.1–2	3
Monoenes	19–22	18.2–35	19.7–23.7	8.7	69.7–72	51–57.3
Dienes	14.5–18.3	44.5–57.2	60.2–69.8	14.8	14–16	35–46
Trienes	47.4–58.9	5.0–9	0.8	—	8–9.6	0–1.5
Trienes, conjugated	—	—	—	71.6	—	2.5
Acid chain length						
C_{12}	—	0.2	—	—	—	—
C_{14}	—	0–0.2	—	—	—	—
C_{16}	6.2–7.0	8–14.9	11	4	0–4	3.5
C_{18}	93.0–93.8	89–92	89–90	96	43	96.5
C_{20}	—	0–1	0–1	—	13	—
C_{22}	—	—	—	—	40	—
C_{24}	—	—	—	—	2	—

[1] Data from Mills 1964; Anon. 1959.
[2] Distilled tall oil fatty acids only.

The polyenoic acids are predominately found in the C_{20} and C_{22} chain lengths; the saturated acids in the C_{14} and C_{16} chain lengths; the monoenoics are scattered through the C_{16}, C_{18}, C_{20}, and C_{22} chain lengths.

The high iodine value (unsaturation) of menhaden oil is produced by the unique combination of mono-, tetra-, penta-, and hexaenoics as distinct from the vegetable oils that predominate in mono-, di-, or tri-enoics.

The polyenoic acids, from the most recent data (Ackman 1964), are predominately *cis* rather than *trans* in configuration, non-conjugated, and attached to the 2-position in the triglycerides. This is essentially the same as in most of the vegetable and animal oils.

Except for rapeseed oil, none of the industrial vegetable oils have carbon chain lengths over C_{18} in contrast to fish oils.

For further details on fatty acid composition of fish oils see Chapter 1.

INDUSTRIAL UTILIZATION OF FISH OILS

The major industrial uses take advantage either of the unique type and high degree of unsaturation of the fish oils to produce elastic durable polymers or of the long and diverse mixture of chain lengths to add lubricity, detergency, and plasticity functionalities. Currently fish oils are consumed mostly as the natural or crudely separated triglyceride mixtures or in hydrogenated form, neither of which fully utilizes the potential of the unique components. For drying oil purposes, some of the saturated glycerides, "stearines," and odor bodies are first removed. Alkali refining and bleaching are used to remove the minor amounts of free fatty acids,

TABLE 24

MAJOR USE AREAS FOR MENHADEN OIL

Year	Menhaden Oil Production	Domestic Use	Drying Oils	Lubricants	Feeds	Fatty Acids	Others
	MM lb.	MM lb.	MM lb.	MM lb.	MM lb.	MM lb.	MM lb.
1962	233	156	70	25	36	19	6
1963	168	104	24	27	23	12	18
1964	175[2]	82[2]	58	[4]	[4]	15.26+[3]	
1965	175[2]	75[2]	[4]	[4]	[4]	[4]	[4]

[1] MM lb. = millions of lb.
[2] Estimates.
[3] A minimum estimate for hydrogenated acids only from the Fatty Acid Producers' Council.
[4] Reliable data unavailable.

phospholipids, sterols, hydrocarbons, protein degradation products, and pigments that contribute to color and odor.

U.S. statistics (Anon. 1965C) on U.S. consumption indicate fish oils to be seventh highest among industrial fats and oils. About the same ranking occurs for world usage. More than one-half of the domestic production is exported, mainly for consumption in foods. Drying oil uses—paints, varnishes, lacquers, resins, caulks, sealants, inks, putty—are the major outlets, with lubricants, animal feeds, and fatty acids consuming the remainder about equally (see Table 24).

The classification used below is based on whether the natural unsaturated triglycerides, the natural saturated or hydrogenated glycerides, or the hydrolyzed acids are the major starting raw materials for the products described.

Natural Unsaturated Triglycerides

Most of the products below utilize the high reactivity of the carbon to carbon double bonds for polymerization, copolymerization, oxidation, isomerization, sulfation, and epoxidation.

Protective Coatings.—The amount of fish oil used varies from year to year as shown above, mainly due to price fluctuations relative to other oils. Normally fish oils are an attractive raw material for this purpose due both to the price and the high quality coatings produced. In all drying oil uses, the quantity of fish oils used ranks below that of tall oil but ahead of tung. Linseed, soy, and castor are the top-ranking oils in decreasing order. Fish oils are consumed in alkyds, varnishes, and *per se* in paints. Heat-bodied oils, mixed with raw linseed oil, go into exterior house paints. Air-blown oils are valuable in nitrocellulose plasticizers, barn and roof paints, and as rust-proofing coatings. Copolymerized with dicyclopentadiene, the oils make good primers. Bodied oils are mixed with tung oil to lower the costs of varnish manufacture.

However, synthetic resins and polymers—mainly derived from petrochemicals—have cut heavily into the market. This is true for all drying oils.

For practically all surface coatings, the high percentage of saturated fatty acids is a serious disadvantage and reduction to lower levels is necessary. "Winterizing" by cold pressing is the major process used in the U.S. Solvent fractionation—mainly propane (the Solexol process)—has been used commercially. Liquid sulfur dioxide, furfural, molecular distillation, and urea complexing have been described in the literature (Schlenk and Ebner 1959; Gruger 1960; Freeman 1940; Hixon and Miller 1953). The iodine values of the menhaden oils so treated are raised to as high as 200, which increases their value as drying oils—faster drying and harder, less tacky films are obtained. Oils such as cod-liver, having only 20% saturated acids initially have an advantage in this respect. Semi-refined anchovy with its high iodine value "dries" quite rapidly. Oils classed as drying oils usually have over 130 I.V., semi-drying oils 100–130, and non-drying oils 100. However, the desirable properties are not characterized solely by I.V. There are considerable variations in properties even among oils with the same iodine values. Anchovy versus "winterized" menhaden is an example of this. "Winterized" menhaden is reported to penetrate and adhere to rusty surfaces better than some of the other drying oils (DeSesa 1959).

The commercial "winterized" oils, known as L.C.P. (light cold pressed) or L.P. (light pressed) oils, do not give sufficiently hard films to compete with linseed oil. However, converted into alkyd resins with phthalic anhydride, isophthalic acid, or maleic anhydride, fish oils can be used to increase durability and hardness and become valuable performers. The U.S. Department of Commerce reported, for instance, that 4.7 million lb. fish oil were used in resins in 1964, and 4.0 in 1963 (Anon. 1965A). Maleic anhydride acting as a trifunctional component increases cross-linking, and is especially helpful. Pentaerythritol or other polyhydroxy alcohols are included to esterify the acids, and compatibility with nitrocellulose is increased. The "maleinized" oils, when neutralized with ammonia, become water dispersible and useful in water-based coatings. A tendency for these alkyds to yellow on exposure to ultraviolet light, however, restricts their usefulness. Most of the highly polyunsaturated oils have the same defect.

Maleinized oils for heat resistant finishes have been reported (Troupe *et al.* 1957). A broad range of industrial products from isophthalic acid alkyds have been described (DeSesa, 1959). Phthalic-based alkyds of 60% oil length show superb drying speed and hardness, exceeded only by dehydrated castor oil (DeSesa 1963).

An outstanding class of products are the copolymers made from cyclopentadiene or dicyclopentadiene. These are designed for applications in industrial enamels, varnishes, rust inhibitive paints, and primers (Anon. 1964). These copolymers have less tendency to yellow and possess less odor. Several U.S. companies produce them commercially.

Styrene is also used as a comonomer with both the oils and the oil-alkyds. Faster drying times, increased hardness, and improved water and alkali resistance are obtained. Films from methyl divinyl acetylene copolymers are claimed to give impact-stability, hardness, flexibility, and good adhesion (Marsel 1959).

Some work has been done overseas to convert the normally unconjugated carbon to carbon double bonds to conjugated systems (Reed 1965). This gives them some tung-like properties, useful in varnishes and makes copolymerization with vinyl toluene, divinyl benzene, methyl methacrylate, and acrylonitrile possible with better colors and films (Kooy 1964).

Heat-bodied fish oils are important commercial products. The stability, odor, viscosity, solubility, compatibility, and water and alkali resistance are improved. Contributions to gloss and leveling characteristics also result. Exclusion of air during the bodying process (polymerization) was an advance of modern vintage resulting in the production of a superior quality bodied fish oil.

Blown oils (air-oxidized) are also standard products with some of the same advantages as the heat-bodied ones. The surface activity is increased through the formation of oxygenated molecules, which is an advantage in some uses, such as pigment grinding. However, for the same reason, solubility in mineral spirits is decreased, and water resistance is lowered. Blown oils are also reported to be valuable in making foundry cores. In 1961, the Department of Commerce reported 1.9 million pounds were consumed in making core oils, as against 8.3 million pounds of tall oil.

The unsaturated alcohols, both conjugated and unconjugated, made by the sodium reduction route—have been reported for drying oil uses. Esterified with maleic, pyro-mellitic, and phthalic anhydride improved speed of drying to tack-free films, and high-temperature polymerization properties were obtained (Ayres 1958; Rizzo 1957).

In recent years, interest in fish oils as air entrainment agents for the protection of concrete against winter damage has been renewed.

See Chapter 17 for more details on use of fish oils in protective coatings.

Lubricants.—Most of the lubricant and grease applications use derivatives of fish oils, rather than the oils themselves. These derivatives are described later on in the chapter in the section on hydrogenated glycerides.

The Department of Commerce reported five million pounds consumed in lubricants in 1964, 5.2 million in 1963 (Anon. 1965A).

Specially oxidized heat polymers are effective lubricants in the presence of water and are used for propeller shafts and auxiliary marine steam driven engines. Sulfurized fish oils are one of the components of extreme pressure paraffin base oils (Mitacek and Greham 1965; Swensen 1942). Lead soaps of menhaden oil are used for the same purpose.

Caulks and Sealants.—Minor quantities of sealing compounds are designed around blown and heat polymerized fish oils. They are formulated to achieve prolonged elasticity, good slip, and binding properties. Oleo resinous binders for glazing putties and sealants, and building caulks are mainly prepared from soy and linseed oils. However, in many instances, formulations are interchanged between these vegetable oils and fish oils, depending on the relative costs of the oils.

Leather Treatment Agents.—Sulfated menhaden, herring, cod, and sperm oil are used extensively in fat liquoring compositions. They are usually medium SO_3 oils (4–6%) which are applied to the leather from aqueous emulsions after the tannage is completed. The oil confers internal lubricity which results in the familiar soft feel of leather.

Printing Inks.—Heat-bodied and blown fish oils mainly are used for printing ink formulations. Increased versatility for this application is often obtained from copolymerizing mixtures of these two. Menhaden, herring, and sardine oils are suitable for blending with or partial replacement of linseed oil. Printing ink manufacture has become highly complicated and the requirements quite stringent, with much of the market held by synthetic resins and polymers today. A good review of the subject was published by Dunning (1959).

Animal Feeds.—As noted in Table 24, a high percentage of the fish oils consumed in the United States ends up in animal feeds. The amount in a given feed mix is normally restricted to prevent the carryover of objectionable flavors and odors into the carcass of the animal. Sometimes hydrogenated sardine oil is added to the menhaden to raise the melting point of the mixture (Rosenberg 1963). Considerable research is being done by the U.S. Bureau of Commercial Fisheries on the nutritional effects of the oils and ways of removing the "fishy" flavors and odors. It is interesting to note that oils carefully prepared from fresh fish tissue are relatively bland (Chipault and McMeans 1965).

Insecticides.—Fish oils are excellent stickers and useful defoliant aids for emulsion application of insecticides. Potassium soaps are preferred where no interference with the leaf breathing apparatus is desired. The ammonium soaps of menhaden and salmon oil are reported to be effective against nematodes (Sokoloff 1962).

Plasticizers and Surfactants.—Expoxidation with hydrogen peroxide introduces an oxirane oxygen atom into the carbon to carbon double bond positions. Several patents have appeared recently indicating some commercial interest in these products for plasticizers, special surface coatings, surfactants, and leather fat-liquoring agents. Menhaden, herring, anchovy, and other fish oils are mentioned. The epoxidized monoglycerides and acetylated monoglycerides as well as the oils are described (Ault and Feuge 1959; Findley 1962; Dazz 1962; Plapper and Dieckelmann 1963A and 1963B; Swift & Co. 1962; Wisnick *et al.* 1964).

Saturated Natural and Hydrogenated Glycerides

The reactivity of the carbon to carbon double bonds with hydrogen is utilized to saturate the fish oils. The hydrogenated glycerides, and the corresponding hydrogenated acids that result, make up probably the second biggest industrial market for fish oils. It is difficult to assess its exact size, but it is probably growing as fast or faster than any other outlet.

The hydrogenation processes use activated metal catalysts such as nickel and in general are similar to vegetable oil hydrogenations (see Chapter 18). The "fishy" odor is destroyed by hydrogenation. The melting point and titer (hardness) are raised considerably. These are among the most important effects of hydrogenation. Hydrogenation is probably the most widely used and most studied economic development in the fat field. Some 50% of the saturated fatty acids produced commercially are of the hydrogenated type.

At times, menhaden oil has been lower priced than, or equal priced with, inedible tallows (bleachable, fancy). This, as well as the wide range of carbon chain lengths and the high percentage of chain lengths over C_{18}, gives a great range of utility. The improved stability that results from the reduction of the unsaturation is valuable in applications where only the unusual chain length composition is needed.

Lubricants—Most lubricating greases consist of lubricating oils thickened with metal soaps. The bulk of the hydrogenated fish oil soaps are actually made from the acids rather than the fat. They are discussed together in this section.

Hydrogenated menhaden, herring, and sardine oils and acids are used. The 32 to 45% content of C_{20} and C_{22} acids helps solubilize the soaps in the mineral oils. The whole oil use is limited by the effects on solubility of the soaps in the lubricating oil. Aluminum soaps are particularly sensitive to this effect. A different-type gel or thickening effect is obtained if the by-product, glycerin, is left in the grease. The metal portion of the soap is almost invariably sodium, calcium, lithium, barium, aluminum, or lead,

predominantly the first two. Lithium and barium soaps are becoming more popular because of the combination of good heat and water resistance. The composition of the fatty acids used critically affects the properties of the greases. Grease manufacture has become complicated by the ever-increasing severity of the operating conditions imposed by modern machinery.

Lithium hydrogenated fish-oil greases are notable for their chemical stability, high water resistance, and high dropping points. Block sodium greases, high temperature mixed sodium-barium greases, and hydrated calcium greases employ hydrogenated fish oils. Aluminum greases made from herring-oil acids are claimed to have good working stability, to set well at high temperatures, and to be non-grainy (Lux and Parker 1958).

The total quantity of fatty acid soaps used in grease for 1960 was approximately 32.4 million pounds. In 1962, about 23 million pounds of hydrogenated fish oils or acids were used (Anon. 1963) in the manufacture of lubricating greases. Some of this was not in soap form. Methyl and butyl esters are used, for instance, the latter as additives to improve "oiliness."

The saturated glycerides are effective in lubricating textile yarns during processing. Part of the usage includes softening, wetting, emulsifying, and water-proofing agents. Partially hydrogenated and sulfated tri- and monoglycerides are useful in these applications. Significant tonnages go into this total market in one form or another.

Leather is "stuffed" with the hydrogenated oils to contribute softening and water-proofing functions and suppleness.

Buffing Compounds.—These materials consist essentially of an abrasive held together by a waxy lubricating material. The fish oils, with their broad ranges of carbon chain lengths and high percentages of C_{20} and C_{22} acids, work well as setting agents which strengthen the buffing bar, reduce shrinkage, and provide some wetting action. The fatty acids are also used in compounding buffing materials to control the amount of shrinkage.

Miscellaneous.—There are a number of proprietary uses scattered over a wide range of end products. Hot-melt adhesives, for example, are consumers of fish oils.

Fatty Acids

The third major area for the industrial uses of fish oils is the manufacture of fatty acids. Splitting or hydrolysis of the triglycerides produces the fatty acids and glycerin. Splitting is an old art that started with batch saponification with alkali, followed by acidulation of the resulting soap with acids. The modern methods are based on continuous, automated, high-pressure splitting with steam in stainless steel equipment with

thermal efficiencies for economical operation. The fish oils are usually hydrogenated before splitting to minimize degradation. The mixtures of acids produced are either used as such or fractionally distilled, solvent separated, or crystallized into special products with segregated chain lengths. There are commercial products with 75% contents of C_{20} and C_{22} and 25% C_{18} chain lengths acids as well as 30% and 40% of C_{20} and C_{22} acids. The only other source of these chain lengths is rapeseed oil. Fish oils are the only common oils containing the C_{24} acid.

Normally only the acids in hydrogenated form are distilled commercially. Conventional fractional distillation requires such high temperatures that losses by degradation and polymerization are excessive if the unsaturated acids are the feed stock. Large-scale production of fractionated nonhardened fish oil acids is not practiced currently, although good separations of the unsaturated acids have been achieved commercially with sardine oil.

In the United States, fish oils have been an economical source of fatty acids and compete generally with the other natural fats and oils besides having their own unique applications. In 1964, there was an estimated 510.4 million pounds (Fatty Acid Producers' Council estimate) of saturated and unsaturated fatty acids produced plus approximately 266 million pounds of tall oil derived fatty acids (less than 10% rosin type only).

In addition to the above (Table 25), there are some unsaturated fish oil fatty acids included in the 10.1 million pounds of combined vegetable and marine acids reported by the council as produced in 1964. Some of the fatty acids are obtained from by-product soap stock from alkali refining.

The history of production is shown in Table 26. If these Council estimates are used, production has not shown much variation over the years. Overseas production is undoubtedly as large or larger.

TABLE 25

U.S. SATURATED FATTY ACID PRODUCTION

Year	Hydrogenated Fish & Marine	Total Saturated
	Millions of Lb.	Millions of Lb.
1961	10.3	—
1962	12.5	259.8
1963	9.6	271.2
1964	10.3[1]	297.9

[1] Fatty Acid Producers' Council estimated 15.26 million pounds in 1964, probably a more correct figure.

The major industrial applications of the acids are based on the reactivity of the carboxyl group. Neutralization, esterification, hydrogenation, and ammonolysis are the major chemical reactions used.

TABLE 26

FISH AND MARINE FATTY ACIDS PRODUCTION

Year	Millions of Lb.
1955	20.22
1956	19.40
1957	16.55
1958	10.57
1959	8.33
1960	8.73

[1] Data from Anon. 1960.

Only the clearly identified segments of the market are discussed separately below. The acids are highly flexible chemical intermediates with many uses, some of which are very proprietary. Rubber compounding agents, brake blocks, cosmetics, pharmaceuticals, slushing compounds, wood preservatives, rust inhibitors, candles, water-dispersible paint vehicles, and paper-sizing agents have been mentioned.

Metal Soaps.—The metal soaps are products that contain carboxyl groups neutralized with metals. The sodium and calcium soaps specifically are actually made to a large extent by saponification of the hardened oil itself with caustic soda or hydrated lime. Others such as the aluminum and zinc soaps are made by double decomposition with the ammonium or sodium soaps and water-soluble salts of the metals.

The use in lubricating grease was described earlier. The aluminum HFA soap is valuable also in water-proofing and as a "flatting" agent in paints. At one time the zinc and lead soaps of the unsaturated acids were used as paint driers, but this has largely been discontinued. Various soaps --aluminum and calcium—are used in leather, textile, and paper water-proofing and sizing. The unique carbon chain lengths improve the solubility of the soaps in non-aqueous media.

Esters.—Many miscellaneous commercial products are made by esterification of the acids or interesterification of the oils themselves with alcohols. Methyl, ethyl, and butyl alcohols are used. Butyl esters of saturated acids are recommended as lubricants for rolling aluminum, die-casting plastics, textile spinning, and alloy metal-working. There has been some commercial interest in the esters for conversion to the alcohols by hydogenation.

Alcohols.—The fish oil alcohols are of minor commercial interest currently. Catalytic or sodium hydrogenation of the ester group in the methyl or ethyl esters or catalytic hydrogenation of the carboxyls of the free acids yields the alcohols. The industrial applications of high molecular weight alcohols in general are many, and very large tonnages are consumed in

Committee on Specifications. 1972
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J. Am. Oil Chem. Soc., 40(5): 165-169, 1963

• TECHNICAL

The 2-Thiobarbituric Acid Reagent for Determination of Oxidative Rancidity in Fish Oils

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Abstract

A method has been devised for the quantitative determination of malonaldehyde in oxidized fish oils by means of the 2-thiobarbituric acid reagent in alcoholic solution. TBA numbers and peroxide values have been determined at intervals during hydroperoxidation of pilchard oil. The curves follow the same general pattern but the numerical relationship between them depends upon the temperature of oxidation.

In alcoholic solution, the reaction between thiobarbituric acid and malonaldehyde is carried out in the dark, because sunlight causes a decrease in optical density of the TBA-malonaldehyde complex at 532 mμ with the appearance of a second maximum at 452 mμ. The nature of the compounds responsible for absorption at these two wave-lengths is discussed.

Introduction

In recent years the Kohn and Liversedge color reaction with 2-thiobarbituric acid (TBA) has been widely used to estimate oxidative rancidity in fatty materials (1,2,4,5,12,17,18,20,22).

The TBA reagent determines the amount of malonaldehyde arising from oxidative decomposition of unsaturated fatty acids with more than one double bond (9,14). Oxidative rancidity is frequently expressed in terms of TBA number or milligrams of malonaldehyde per kilogram of material, measured by comparing the optical density of the pink TBA-malonaldehyde reaction product with that of standards prepared from 1,1,3,3-tetraethoxypropane (TEP) which gives rise to malonaldehyde on acid hydrolysis.

The TBA-reaction has been successfully applied to the measurement of rancidity in dairy (2–4,12,20), fishery (1,13,22) and meat (17,18) products. Experimentally the procedure requires dispersion of the test material in a water medium containing the TBA reagent, but Schmidt (9) has pointed out that the existence of a two-phase system can lead to errors in the case of oils and fats. We have confirmed this in measuring the oxidative rancidity of pilchard oil by the method of Yu and Sinnhuber (22), which is eminently satisfactory for materials such as fish meal. Applied to pilchard oil, the method gave results which sometimes differed by as much as 100% on the same sample. Addition of a detergent to the two-phase system of water and oil as suggested by Biggs and Bryant (2) gave turbid solutions which could not be read in the spectrophotometer.

Removal of malonaldehyde from the oil by steam distillation was tried according to the method of Sidwell et al. (12) and Tarladgis et al. (17), but was found to be unreliable in application to pilchard oil. The method requires distillation of only a certain percentage of the malonaldehyde during an arbitrarily chosen time, but in the case of fish oils with high peroxide values the amount in the distillate was not proportionate to the total amount in the sample, and duplicate estimations sometimes differed by as much as 50%.

Since previous methods did not appear to be satisfactory, a modified experimental procedure was devised to determine the TBA numbers of oxidized fish oils with a standard deviation of 10%. It was found that difficulties arising from the existence of a two-phase system during condensation between malonaldehyde and TBA could be overcome by carrying out the reaction in a monophase system of ethyl alcohol. The standard calibration curve in aqueous-alcoholic medium was identical to that obtained by Sinnhuber and Yu (13) in aqueous medium alone.[1] TBA numbers for fish meal were identical in both aqueous and alcoholic solution, but in the case of fish oils only the alcoholic medium afforded reproducible results.

In aqueous-alcoholic solution there is a marked influence of light on the absorption spectrum of the TBA-malonaldehyde reaction product. The maximum at 532 mμ disappears with the simultaneous appearance of a peak at 452 mμ. This observation has been investigated, and a study has been made of the relationship between peroxide value and TBA number during oxidation of pilchard oils at room temperature and at 75C.

Experimental

Reagents and Apparatus

TBA Reagent. This was prepared in a black-painted vessel. Sodium citrate buffer (25 ml) according to Kohn and Liversedge (5) was added to a solution of thiobarbituric acid (0.5 g) in warm ethyl alcohol (50 ml). Crystals of sodium citrate deposited on standing but this did not affect the quality of the reagent which was used within 4 hr of preparation.

Ethyl Alcohol and Methyl Alcohol. These solvents were purified according to the method of Stout and Schuette (16).

Alcoholic-HCl (0.6 N). Dry hydrogen chloride gas was passed through ethyl alcohol until the required weight increase was achieved.

1,1,3,3-Tetraethoxypropane (TEP). The TEP was supplied by K & K Laboratories, Jamaica, N. Y. and was used as an approximately 2×10^{-4} molar solution in 40% ethyl alcohol.

Epihydrin Aldehyde and Glyceraldehyde. These chemicals were supplied by Stuart Patton of the Pennsylvania State University.

All other chemicals were analytical reagent grade.

[1] The calibration curve given by Sinnhuber and Yu (13) refers to "molar concentration TEP (x 10^6)" and not "molar concentration TEP (x 10^5)" as printed.

TABLE I

Recovery of TEP Added to Commercial Sunflower Oil

% TEP added	% TEP recovered	% Recovery
0.1044	0.1039	99.5
0.1117	0.1137	101.8
0.0583	0.0545	93.5

Apparatus. The 250 ml. flat-bottom reaction flasks were painted black and all operations were carried out in subdued light. Optical densities were determined with a Unicam Model S.P. 500 spectrophotometer.

Procedure

Alcoholic-HCl (15 ml of 0.6 N) and TBA reagent (6 ml) were pipetted into a black-painted 250 ml flask in which the air had been replaced by nitrogen.

A 100–200 mg sample of the fish oil was accurately weighed in a glass capsule and added to the reagent. The flask was then connected to a reflux condenser and heated on a boiling water bath with intermittent shaking. After 30 min, 79 ml of aqueous 0.6 N HCl was added through the condenser and the refluxing continued for a further 10 min. The flask was then disconnected and cooled in running water for approximately 2 hr.

A 15 ml portion of the solution was then shaken vigorously for 30 sec with 15 ml petroleum ether (B. Pt. 40–60C) to extract residual fish oil. The upper layer was removed with a pipette and the solution shaken once more with 15 ml petroleum ether. After standing for 16 hr in the dark, the upper layer was again discarded and the optical density determined at 532 mμ against a reagent blank in a 1 cm cuvette. The TBA number in milligram malonaldehyde per kilogram of oil was obtained by multiplying the $E^{1\%}_{1cm}$ value by 46.5 [Solutions with a high optical density were diluted with 0.01 N HCl (19)].

Standardization. The calibration equation was obtained by carrying out the above procedure with appropriate quantities of standard TEP solution in place of fish oil. In each determination the volume was adjusted to 100 ml by adding the necessary amount of aqueous HCl through the condenser. The extraction with petroleum ether was omitted.

Determination of Peroxide Value. Peroxide values were determined according to the method of Wheeler (21) with the modifications suggested by Stansby (15), except that 25 ml of solvent were used instead of 10 ml as recommended. Peroxide values were expressed as milligram atoms of active oxygen per kilogram of oil.

Results

Standardization. The relationship between the concentration of malonaldehyde in terms of TEP and the optical density of the TBA-malonaldehyde complex at 532 mμ, is given by the expression

TABLE II

Reproducibility of TBA Numbers Determined on Pilchard Oils with Different Peroxide Values

	1	2	3	4
	119.8	327.5	1214	1876
	138.6	343.1	1245	1645
	122.5	350.1	1356	1351
	131.7	279.5	1508	1768
	108.4	336.1	1646	1638
	117.2	295.2	1524	1714
	115.3	312.4	1454	1638
	140.5	277.3	1443	.....
	.....	.....	1335	.....
Average TBA No.	124 ± 11	315 ± 27	1414 ± 131	1661 ± 150
Peroxide Value	21.4	92.4	464.0	600.7

$$\text{Moles TEP/litre} = 6.49 \times 10^{-6} \times OD_{532\,m\mu}$$

This equation gives a calibration curve identical that obtained by Sinnhuber and Yu (13) in aqueo medium.

Recovery of Malonaldehyde. The method show approximately 100% recovery of TEP added to a co mercial sunflower cooking oil of peroxide value 5 (Table I). Optical densities were measured against blank prepared from the cooking oil without TEP.

Reproducibility of Results. Table II shows the r sults of TBA number determinations carried out 4 different samples of oxidized pilchard oil with d ferent peroxide values. In each case the standard d viation is less than 10% of the arithmetic mean sho for 7 to 9 determinations.

TBA Number vs. Peroxide Value of a Pilchard During Oxidation at 20–25C. A fresh pilchard oil peroxide value 5.1 and TBA number 48.1 was oxidiz at 20–25C over a period of 6 months. Air was blo through the sample at the rate of 5 ml per sec, b after 640 hr the oil became too viscous to blow, a was therefore transferred to a glass dish where it w exposed to air in the form of a thin layer. Peroxi values and TBA numbers were determined at int vals with the results shown in Figure 1. Each poi on the TBA curve represents the average of 7 to determinations. Measurements were made after st ing the reaction medium for the standard time of hr in the dark. In the case of fish oil the value of t absorption maximum at 532 mμ was found to increa by about 14% after storage for an additional 80 hr the dark.

TBA Number vs. Peroxide Value of a Pilchard Oxidized at 75C. A further sample of fresh pilcha oil with initial peroxide value 5.1 and TBA numl 48.1 was oxidized by air-blowing (5 ml per sec) 75C. Figure 2 shows the changes in TBA number a peroxide value over a period of 130 hr.

Influence of Sunlight on the Nature of the TB Malonaldehyde Reaction Product. A) When the re

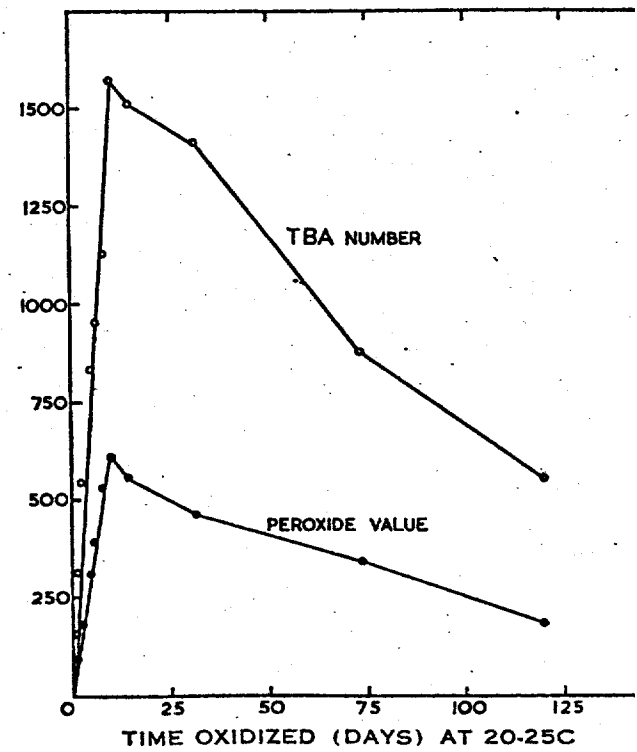


FIG. 1. Change in TBA number and peroxide value du oxidation of pilchard oil at 20–25C.

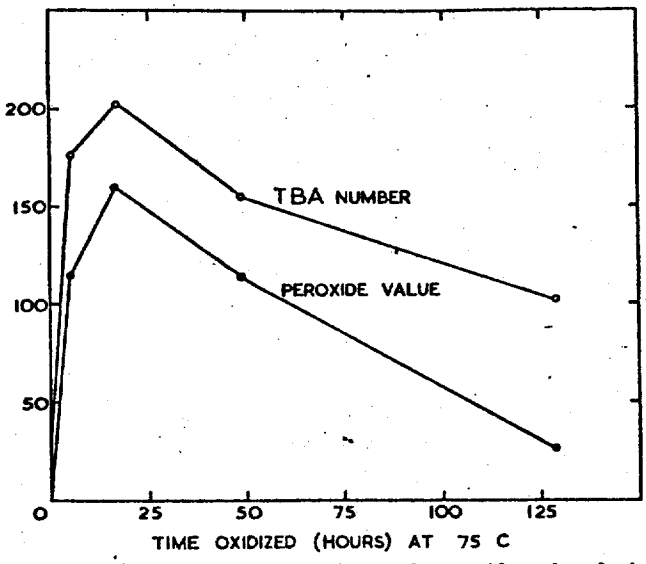


FIG. 2. Change in TBA number and peroxide value during oxidation of pilchard oil at 75C.

tion between alcoholic-TBA and malonaldehyde was carried out in the dark, the product had a pink color with an absorption maximum at 532 mμ. However, if the reaction was carried out in daylight, an orange-brown product was obtained with two absorption maxima, one at 532 mμ and the other at 452 mμ. The orange-brown solution became pink after storage in the dark for 70 hr and the 452 mμ maximum disappeared with a slight increase in absorption at 532 mμ. These effects are illustrated in Figure 3.

B) TBA undergoes a condensation to yield a colored compound without addition of malonaldehyde. Thus a solution of 0.04% TBA in a mixture of 4 parts 0.6 N HCl and 1 part 0.6 N alcoholic-HCl, turned yellow in sunlight with development of an absorption peak at 452 mμ. This maximum disappeared after 48 hr storage in the dark and a slight increase in absorption was observed at 532 mμ (Fig. 4).

The same phenomenon was observed when methyl

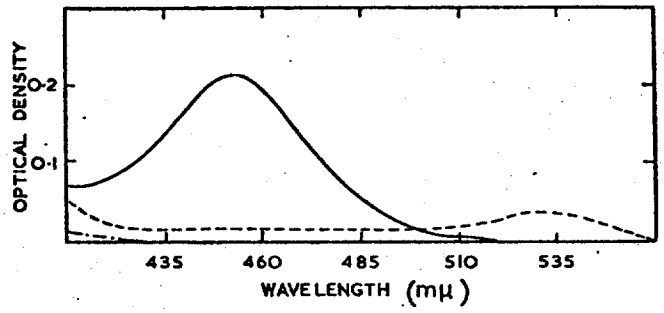


FIG. 4. A) Absorption of 0.04% TBA in a solution of 1 part 0.6 N alcoholic-HCl and 4 parts 0.6 N HCl (—·—·—). B) Absorption of solution A after exposure to sunlight (———). C) Absorption of solution B after 48 hr storage in the dark (— — —).

alcohol was used in place of ethyl alcohol, but no color was produced when TBA in aqueous solution was exposed to sunlight.

C) *Reaction of TBA with Epihydrin Aldehyde and Glyceraldehyde.* Patton (8) has shown that epihydrin aldehyde and glyceraldehyde react with TBA in aqueous solution to give a yellow product which has an absorption maximum at 456 mμ.

This reaction was compared with the development of an absorption peak at 452 mμ on exposure of an alcoholic solution of TBA to sunlight (see B above).

Epihydrin aldehyde and glyceraldehyde were condensed in the dark with TBA in aqueous-alcoholic solution to give a yellow compound with an absorption maximum at 455 mμ (Fig. 5). On continued storage in the dark the absorption maximum shifted to 485 mμ (Fig. 5).

D) *Reaction of TBA with Formic Acid.* Schmidt (10) has shown that formic acid reacts with TBA in aqueous solution to give a yellow derivative with an absorption maximum at 452 mμ.

A compound with the same absorption maximum at 452 mμ is formed by reacting TBA and formic acid in a mixture of ethyl alcohol and water (1:4) away from the influence of light (Fig. 6). A decrease in absorption at 452 mμ with a corresponding increase of

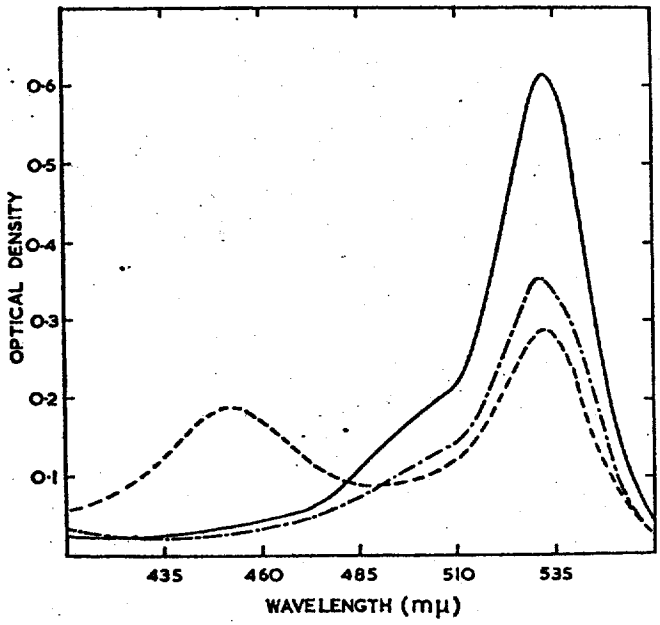


FIG. 3. A) Absorption of the derivative obtained by reaction between alcoholic-TBA and malonaldehyde in the dark (———). B) Absorption of the derivative obtained by reaction between alcoholic-TBA and malonaldehyde in sunlight (— — —). Absorption after storage of product B for 70 hr in the dark (—·—·—·). The maximum at 452 mμ disappears with an increase in absorption at 532 mμ.

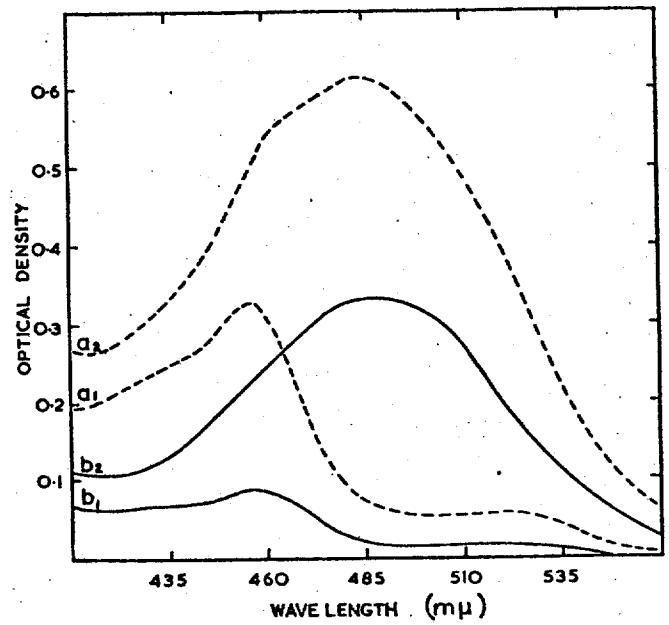


FIG. 5. a.1. Absorption of the derivative obtained by reaction between TBA and glyceraldehyde. a.2. Absorption of the TBA-glyceraldhyde derivative after 48 hr storage in the dark. b.1. Absorption of the derivative obtained by reaction between TBA and epihydrin aldehyde. b.2. Absorption of the TBA-epihydrin aldhyde derivative after 48 hr storage in the dark.

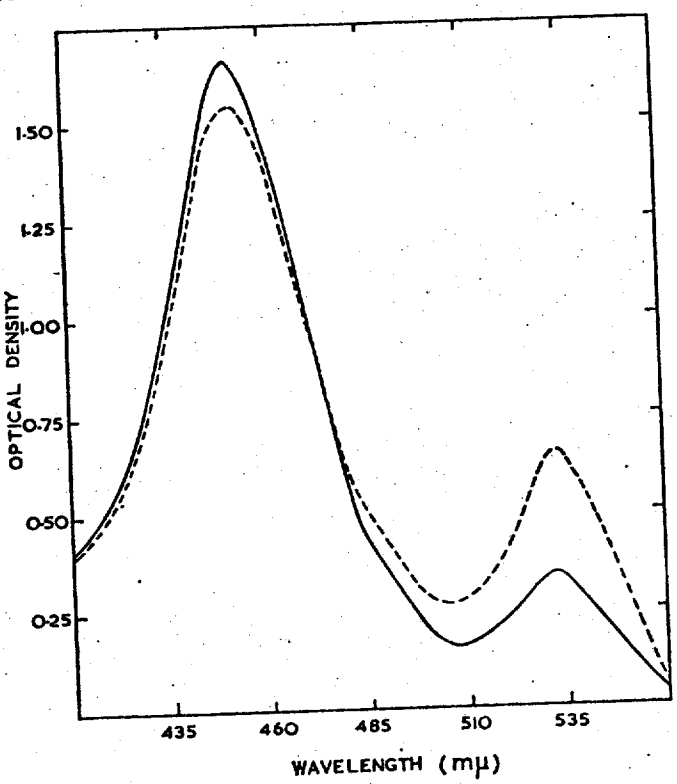


FIG. 6. A) Absorption of the TBA-formic acid derivative after 96 hr storage in the dark (———). B) Absorption of the TBA-formic acid derivative after 144 hr storage in the dark (— — —).

absorption at 532 mμ is shown after 96 hr and 144 hr storage in the dark (Fig. 6).

Discussion

The peroxide value of oils is a useful criterion of decomposition during the early stages of oxidation, but is of lesser value once the peak of the oxidation curve has been passed. The TBA number has been shown to be a useful quality index for assessment of rancidity in the early stages of oxidation (9), but no definite information could be found on the relationship between peroxide value and TBA number during continued hydroperoxidation of oils.

Several unsuccessful attempts were made to determine the TBA number of oxidized oils according to published methods. For example the procedure of Yu and Sinnhuber (22) and the distillation methods of Sidwell et al. (12) and Tarladgis et al. (17), all gave erratic results when applied to fish oil.

A new method was therefore devised to overcome errors inherent in the existence of a two-phase system between oil and water during reaction between malonaldehyde and TBA reagent. By carrying out the determination in a monophase system of ethyl alcohol it was found that the recovery of malonaldehyde added to sunflower oil was approximately 100% and that the standard deviation was less than 10% of the arithmetic mean for a series of 7 to 9 determinations on fish oils with different peroxide values (Table II).

TBA numbers determined in alcohol solution were compared with peroxide values at various intervals during oxidation of pilchard oil at 20–25C and at 75C. The curves followed the same general pattern at both temperatures (Figs. 1 and 2) but the maximum TBA number and peroxide value were less at 75C than at 20–25C. This would indicate that not only hydroperoxides, but also malonaldehyde or its precursors, are more rapidly decomposed at the higher temperature. Were it not for the fact that the malonaldehyde formed by oxidative degradation is itself labile, the TBA number might be expected to show a continued increase beyond the peak of the peroxide value curve. Since this is not the case [cf. Schmidt (9)], it would seem that the TBA number cannot be regarded as a more useful criterion of rancidity than peroxide value after the initial stages of oxidation.

Comparison of Figures 1 and 2 shows that the relationship between TBA number and peroxide value depends upon the temperature of oxidation. For example, an oil having a peroxide value of 160 may have a TBA number of 480 or 200 depending upon whether it was oxidized at 20–25C or at 75C.

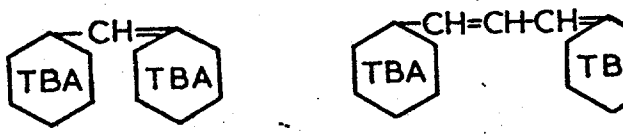
The measurement of TBA number in alcoholic medium introduces a complication which is not encountered in aqueous medium. The reaction must be carried out in the dark because daylight reduces the intensity of the TBA-malonaldehyde peak at 532 mμ and causes the appearance of a second maximum at 452 mμ (Fig. 3).

A product with an absorption maximum at 452 mμ can be obtained by the action of sunlight on an alcoholic solution of TBA alone. The yellow derivative absorbing at 452 mμ is converted to a pink compound with a peak at 532 mμ during storage in the dark (Fig. 4).

A yellow compound formed by reaction between TBA and epihydrin aldehyde or glyceraldehyde shows an absorption maximum at 455 mμ but is converted to a compound with a peak at 485 mμ after 48 hr storage in the dark (Fig. 5). The compound cannot therefore be identical with the reaction product of sunlight on TBA in alcoholic solution which is converted to a substance with a maximum at 532 mμ after storage in the dark (Fig. 4).

Formic acid, on the other hand, reacts with TBA to give a product which has an absorption maximum at 452 mμ and is converted to a substance with a peak at 532 mμ after storage in the dark (Fig. 6). Reaction between TBA and formic acid therefore yields a compound which is similar to, if not identical with that formed by the action of sunlight on TBA in alcoholic solution.

Schmidt (10) has proposed structure I (λmax 452 mμ) for the reaction product of TBA with formic acid, and structure II (λmax 532 mμ) for the condensation product of TBA with malonaldehyde.



I (λMAX 452 mμ) II (λMAX 532 m

Thus sunlight irradiation of an alcoholic solu of TBA (Figs. 4 and 6) probably gives rise to c pound I. The reaction could possibly take p through a free radical mechanism.

Compound II may be identical with the compo formed when an alcoholic solution of TBA is exp to sunlight and subsequently stored in the dark (4). If this is the case, it is possible that compoun is formed from compound I in aqueous alcoholic s tion when stored away from light (Fig. 4).

Methine dyes such as compounds I and II ap ently undergo changes in structure when in solu

This is evidenced by the TBA reaction products with epihydrin aldehyde or glyceraldehyde (Fig. 5) and with formic acid (Fig. 6), although the phenomenon has also been observed by Landucci et al. (6,7) in the case of various aldehydes present in gelatin. The mechanism of these reactions does not appear to be understood and requires further investigation.

Shepherd (11), Sinnhuber et al. (14) and Schmidt (9) have shown that certain pyrimidines are capable of forming the same pigment as malonaldehyde on reaction with TBA. The mechanism is thought to involve hydrolysis of the pyrimidine ring to produce an oxyacrolein intermediate (9) which is tautomeric with malonaldehyde and therefore undergoes condensation to produce compound II. Although TBA itself has a pyrimidine structure, it is substituted in the 4 and 6 positions and would therefore be unlikely to give rise to malonaldehyde on hydrolysis (9). No color is formed on exposure of an aqueous acid solution of TBA to sunlight, but if hydrolysis were to occur, the expected product would be malonic acid which would not give the same compound as malonaldehyde in any reaction with TBA.

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[Received March 1, 1962—Accepted December 14, 1962]

J Am oil Chem Soc 47: 126-128, 1970

Analytical Method for Determining Copper in Edible Shortenings and Oils

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Abstract

A reliable, practical method was developed for the quantitative determination of sub-ppm levels of copper in edible shortenings and oils. Copper is quantitatively extracted with a boiling solution of 0.01% EDTA in 25% HCl. The extract was analyzed with 2,9-dimethyl-4,7-diphenyl-1,10-phenanthroline (Bathocuproine), a highly specific and exceptionally sensitive reagent for the colorimetric determination of copper. An average recovery of 99.8% was obtained on 22 samples containing added oil soluble copper standards. A standard deviation of 0.002 ppm was established at the 0.05 ppm level during routine application of this method to a variety of shortenings and oils.

Introduction

Quantitative analysis of copper in edible shortenings and oils is necessary, both in quality control and experimental development. Several methods are in the literature, but are either impractical or not reliable for everyday laboratory use. The method developed and described in this report is not only precise and accurate, but applicable to high volume analyses of copper in shortenings and oils with a minimum of time and equipment. While a single sample can be analyzed in 3 hr, six samples can easily be run by an analyst in one day.

List et al. (7) adopted a low-temperature dry asher, an apparatus in which oxygen plasma is generated in a radio frequency field under high vacuum, for ashing glyceride oils. The residues were analyzed by a colorimetric procedure using zinc dibenzyldithiocarbamate as a reagent. The relative error they reported of a single determination was ±13% and that of the mean of duplicate determinations was ±9%. They used pure copper nitrate as the reagent for preparing oils containing known levels (0.04–5.0 ppm) of copper. A sample commonly employed required 60–72 hr to be ashed in their system. For lower levels of copper, 15 g of sample were needed, so three to five residues had to be combined before assay. They showed levels of copper in unhydrogenated, commercially processed soybean oils varying from 0.03–0.10 ppm. Labuza and Karel (6) described a direct solvent extraction procedure for copper and iron in fatty acids and fatty methyl esters. application of this method to shortenings and was not demonstrated. A recent symposium (8) cluded discussions on several analytical techniques copper in edible oils. Atomic absorption spectro tometry was shown to have a lower limit of 0.05- ppm when measuring copper concentration dire in fats. With several instrument modifications, li of 0.01–0.05 ppm copper in fat have been reach

Hogdahl and Melsom (5) determined copper two samples of hydrogenated fat by neutron act tion analysis. A sample of fat was irradiated for hr and the radioactive copper was extracted with HCl from a toluene solution of the irradiated The gross gamma activity was measured wit NaI(Tl) well-type scintillation crystal. A colorime measurement (as copper dibenzyldithiocarbama was also made and the absolute amount of cop in the sample calculated. The sensitivity reporte about 1–3 ppm with a reproducibility of ±7% w 2 g of fat are used.

Two papers describe determination of copper dairy products. Cranston and Thompson (2) redu the pH of milk with perchloric acid until cop protein complexes were broken. After filtering fat and precipitated protein, the copper was then c centrated by an ion exchange procedure and de mined polarographically. The amounts of cop recovered were erratic, since varying quantities sugars, salts and casein markedly influenced polarographic method. This condition was correc by using an internal standard. Willis (10) found necessary to extract butter and butteroil with ni acid and to analyze the extract for copper by ato absorption spectroscopy. Results at concentrations <0.1 ppm were quite scattered and appear to be lower limit of this method.

Van De Bogart and Beinert (9) published excellent review and technique paper for cop analysis of biological materials by wet ashing, evapo tion of excess acid, reduction, neutralization, co development and concentration of the colored comp by extraction with a small amount of organic solve

There is a wide choice of colorimetric reagents copper. Some of the more common ones, their pr erties and drawbacks are shown in Table I. T reagent of choice is Bathocuproine (2,9-dimethyl-

TABLE I
Color Reagents for Copper

Reagent	Solvent	λ Max. nm	Molar absorbance	Disadvantages
Diethyldithiocarbamate	Water	440	8,000	Unstable in acid
	Isoamyl alcohol	440	12,700	Light sensitive
	CCl_4	435	14,700	
Dibenzyldithiocarbamate	CCl_4	435	16,200	Interference from Fe^{+++} and Ni^{+}
Dithizone	CCl_4	508	24,600	Not specific for copper alone
2,2'-Biquinoline (Cuproine)	Isoamyl alcohol	546	5,220	Low sensitivity
2,9-Dimethyl-1,2-phenanthroline (Neocuproine)	Isoamyl alcohol	454	7,950	Low sensitivity
2,9-Dimethyl-4,7-diphenyl-1,10-phenanthroline (Bathocuproine)	n-hexanol	479	14,160	

diphenyl-1,10-phenanthroline), a highly specific and exceptionally sensitive reagent for the colorimetric determination of copper. Details concerning this reagent can be found in a pamphlet by Diehl and Smith (3).

Experimental Procedure

Standards

Three types of materials were used as standards. The first was 99.99% pure ACS grade copper wire. The wire surface was cleaned before each use to remove oxides. The second type was copper soaps of two fatty acids, stearic and linoleic. In theory, the two samples should contain 10.08% and 10.21% copper respectively. A colorimetric determination of the copper content after dissolving in 1:1 HNO_3 showed 10.10% and 10.40% copper, respectively. The third material employed was an oil-soluble copper standard obtained from the National Bureau of Standards [bis(1-phenyl-1,3-butanediono)copper II, 16.5% Cu]. Their directions for preparing a known concentration in lubricating oils were followed, except that a vegetable oil was used in place of the lubricating oil.

Isolation of Copper

The major problem has been to quantitatively isolate only a few μg of copper from shortenings and oils. The methods investigated for this purpose were dry ashing and acid extraction. Wet ashing was not investigated due to safety hazards associated with this method when applied to edible oils.

The dry ashing procedure has a major disadvantage in that excessive heat is required to destroy all organic material. This can cause loss of volatile metals, for example, copper soaps. Adsorbed metals on the ashing vessel can cause both low results on a sample or contamination of subsequent samples or both.

Most success was obtained with an acid extraction procedure. All glassware must be cleaned with concentrated HNO_3, rinsed with deionized distilled water, and dried prior to use. A sample, weighed to contain approximately 1–10 μg copper, is refluxed with 50 ml of a 0.01% EDTA-25% HCl solution for 30 min. Acid washed carborundum chips are used to prevent bumping. This reflux step proceeds smoothly without emulsions forming to give a clean extract. After 30 min, the sample is cooled and quantitatively transferred into a 4 in. funnel containing an 18.5 cm Whatman No. 40 filter paper which had been wetted with deionized distilled water. The aqueous phase is collected in a 400 ml beaker and the oil remaining in the filter paper is washed with several small volumes of deionized distilled water, the washings collected in the 400 ml beaker, and the oil discarded. Ten milliliters ultra pure concentrated HNO_3 (Brinkman Instruments, Inc.) are added to the beaker and the solution evaporated to 5 ml on a hot plate and finally to dryness on a steam bath. After the sample is dry, add 5–10 ml deionized distilled water, heat on a steam bath, and quantitatively transfer the contents of the beaker to a 60 ml separatory funnel for colorimetric analysis of copper.

Colorimetric Determination of Copper

Less than 1 μg copper can be quantitatively determined by using the specific colorimetric reagent 2,4-dimethyl-4,7-diphenyl-1,10-phenanthroline (Bathocuproine, the G. Frederick Smith Chemical Company). Care must be taken to eliminate and avoid traces of copper contamination as shown below. Clean all glassware with concentrated HNO_3 and rinse thoroughly with deionized distilled water. Bathocuproine (0.3600 g) is dissolved in 100 ml freshly distilled *n*-hexanol and 100 ml of a 10% solution of hydroxylammonium chloride in deionized distilled water is shaken in a separatory funnel with 10 ml of the Bathocuproine solution and 6–8 drops ultra pure NH_4OH (Brinkman Instruments, Inc.). Discard the organic phase. This removes traces of copper from the aqueous hydroxylammonium chloride solution. One hundred milliliters of a 10% solution of ammonium acetate in deionized distilled water are made copper free by shaking in a separatory funnel with 5 ml of the copper free hydroxylammonium chloride, 5 ml of the Bathocuproine solution, and 5 ml distilled *n*-hexanol. Again, the organic phase is discarded.

To the sample in the 60 ml separatory funnel pipet 2 ml of copper free hydroxylammonium chloride and the pH of the solution is adjusted to 7 ± 0.5 with ultra pure NH_4OH. Ten milliliters copper free ammonium acetate, 1 ml of the Bathocuproine solution and 5 ml *n*-hexanol are pipetted into the same and the separatory funnel shaken vigorously for 2 min. Allow the phases to separate, draw off and discard the aqueous phase and save the organic phase. These samples can be filtered through 9.0 cm Whatman No. 40 filter paper if cloudy before determining the absorbance. A Beckman "B" spectrophotometer set at 479 nm is zeroed with *n*-hexanol in a 2 cm cell. The absorbances of the samples and a blank derived from all reagents as used in the above method are determined at 479 nm in 2 cm cells. A corrected absorbance is obtained by subtracting the absorbance of the blank from that of each sample.

The amount of copper present in the sample can be obtained from a calibration curve prepared from known amounts of pure copper wire dissolved in HNO_3. The concentrations of these solutions are plotted against the color developed (absorbance at 479 nm) with Bathocuproine reagent. The copper concentration could also be approximated by calculation using a molar absorbancy (E) of 14,100.

Discussion

In the dry ashing procedure, low recovery (0–84%) of added organic copper compounds was experienced due perhaps to volatilization. These data, shown in Table II, were obtained by adding the indicated type and amount of copper to 100 g of vegetable oil in a Vycor or platinum ashing vessel. Heat was applied to the bottom of the dish with a Fischer burner flame until the oil began to smoke. The vapors from the oil were ignited with a flame.

TABLE II
Copper Analysis by Dry Ashing

Added cupric salt	Ashing vessel	Muffle temp. C	Copper added μg	Copper found[a] μg	Recovery %
Stearate	Vycor	450	2.01	1.56	77.6
Linoleate	Vycor	450	2.04	1.73	84.3
Stearate	Vycor	450	8.06	4.67	57.9
Linoleate	Vycor	450	8.16	3.93	48.2
Stearate	Vycor	500	5.04	3.24	64.1
Stearate	Platinum	500	5.04	2.25	44.6
Stearate	Platinum	550	5.04	3.50	66.5
Stearate	Platinum	550	8.06	~18.00	?
NBS Standard[b]	Platinum	550	4.97	NF	0.0
NBS Standard[b]	Platinum	550	4.97	1.60	34.2

[a] Corrected for initial copper level in samples used.
[b] NBS standard, Bis(1-phenyl-1,3-butanediono) copper (II), oil soluble material containing 16.5% Cu, from National Bureau of Standards.

TABLE III
Copper Analysis by Extraction

	Copper added to vegetable oil ppm	Copper added to vegetable oil µg	Copper found[a] µg	Recovery, %
NBS Standard[b]	0.002	1.00	0.93	93.0
	0.010	1.00	1.00	100.0
	0.010	1.00	0.95	95.0
	0.010	1.00	1.05	105.0
	0.010	5.00	4.80	96.0
	0.010	5.00	4.35	87.0
	0.014	7.00	6.48	92.6
	0.040	4.00	4.20	105.0
	0.050	4.97	4.90	98.6
	0.050	4.97	4.90	98.6
	0.050	5.00	4.99	99.8
	0.050	5.00	5.10	102.0
	0.050	5.00	5.20	104.0
	0.100	9.95	9.60	96.5
	0.22	2.00	2.10	105.0
	0.22	2.00	1.85	92.5
	0.22	2.00	2.00	100.0
Cupric stearate	0.01	1.00	1.20	120.0
	0.01	1.00	1.05	105.0
	0.02	2.00	1.95	97.5
	0.03	3.00	3.00	100.0
	0.07	7.00	7.10	101.4
			Average	99.8

[a] Corrected for initial copper levels in samples used. Copper metal, 400 to 475 µg, well over limits normally encountered can be determined by this procedure.
[b] NBS Standard, Bis(1-phenyl-1,3-butanediono) copper (II) (oil soluble material containing 16.5% Cu), from National Bureau of Standards.

After the flame went out, the residue was placed in a muffle furnace at the indicated temperature until the carbon was gone. The ash was then analyzed for copper by the colorimetric procedure described.

Extracting copper with 0.01% EDTA in 25% HCl was found to give a quantitative extraction of copper from shortenings and oils. The extracts were analyzed for copper by the colorimetric procedure described. These data are presented in Table III. An average recovery of 99.8% added oil soluble copper standards was obtained with a standard deviation of 0.002 ppm established at the 0.05 ppm level during routine application of this method to a variety of shortenings and oils.

Using 25% HCl alone without added EDTA resulted in low recovery (88–90%) of added copper. Two somewhat similar methods are available for determining trace metals in petroleum distillates. Barney II (1) extracted an oil with sulfuric acid and then with a mixture of hydrochloric acid, acetone and water. The extract was analyzed by spectrographic analysis. Sulfuric acid chars edible oil so it canno be used. Hackett (4) extracted mineral oils wit alcoholic hydrochloric acid and colorimetrically de termined the copper by extraction of the 2,2-diquinoly complex with chloroform. The method operates sati factorily when more than 10 µg copper are prese EDTA was not needed for a quantitative extractio of copper in mineral oils but was needed for edibl shortenings and oils.

Less than 90% of added copper was extracted fro shortenings and oils with 25% HCl alone. The add tion of EDTA leads to a quantitative extraction copper from shortenings and oils. Postulating the ro of EDTA in assuring successful extractions of copp from shortenings and oils is beyond the scope of th report. Little, if any, information is available co cerning the role of EDTA in highly acid media n at the elevated temperature used in the heated syste One is that the EDTA becomes increasingly protonated, the extent of which is not known, maki it a better chelating agent. The other is that a copp EDTA complex would be less stable or more likely dissociate at high temperatures.

The method as written is particularly applica to the determination of copper in shortenings a oils at low levels (0.002–0.10 ppm), but could also applied for analysis of copper in other media. seems quite apparent that the isolation method co be extended to other metals (e.g., nickel in hydrogenation catalyst).

ACKNOWLEDGMENTS

Initial work on the colorimetric analysis of copper, P. F. Vornh constructive advice, H. W. Wharton.

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[Received September 10, 1969]

Proc. Soc. Exp. Biol. Med., 21: 139-141, 1923

70 (2302)

Studies on the chemistry of cod liver oil.*

I. The effect of hydrogenation upon the vitamine content.

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A survey of the literature reveals comparatively little on the hydrogenation of cod liver oil. Drummond,[1] working with whale oil, hydrogenated at 250° C. for 4 to 6 hours, found that the hardened oil was entirely deficient in "fat-soluble A," which at that time was generally also regarded as the "antirachitic vitamine". Since then the bulk of evidence has tended to show that

*This investigation was started in January, 1922, and is still being continued.

1 Drummond, J. C., *Biochem. J.*, 1919, xiii, 81.

"fat-soluble A"—the antiophthalmic, growth-promoting vitamine—is not identical with the antirachitic vitamine.

Paal and Roth,[2] studying the catalytic action of colloidal metals of the platinum group, hydrogenated cod liver oil and butter. The process used by these investigators is a very mild one. As modified to suit our needs, it is carried out at about 55° C. for a period of about 36 hours. This is in sharp contrast to the drastic hydrogenation reported by Drummond.

Employing both the curative and preventive type of experiment the various fractions obtained by us, and about to be described, were tested on more than 150 rats. The diets used included a synthetic diet practically free from antirachitic vitamine, the McCollum diet No. 3143 and the Pappenheimer-Zucker diet D.

Fraction I.—This fraction, which possesses no vitamine activity, is the concentrated water reduction mother-liquor.

Fraction II.—The hydrogenated cod liver oil, obtained as a snow-white product, is odorless and tasteless, and melts at about 55° C. The yield is about 95 gm. from 100 gm. cod liver oil. When used in amounts corresponding to 0.100 gm. cod liver oil daily, it prevents and cures rickets. Smaller dosage was not tried.

Fraction III.—The residue left after extracting fraction II with alcohol. The yield is about 90 gm. from 95 gm. of fraction II. Fraction III is inactive in amounts corresponding to 0.250 gm. cod liver oil daily. Larger doses were not tried.

Fraction IV.—A creamy-white substance, almost odorless and tasteless, melting at about 30° C., extracted from fraction II by means of alcohol. The yield is about 4.5 gm. from 95 gm. fraction II. When given in amounts equivalent to 0.100 gm. cod liver oil daily, it prevents and cures rickets. Smaller dosage was not tried.

Fraction IVa.—This is the same as fraction IV, resembling it in appearance, but with the cholesterol removed. Yield is about 0.045 gm. from 4.5 gm. fraction IV. Administered in amounts corresponding to 0.250 gm. cod liver oil daily, it prevents and cures rickets. Smaller dosage was not tried.

Fraction C.—The cholesterol fraction proved to be inactive.

In the few cases in which the above active fractions were tested in attempts to cure ophthalmia, unsatisfactory results were

2 Paal, C., and Roth, K., *Ber. chem. Ges.*, 1908, xli, 2282; 1909, xlii, 1541.

obtained. However, we do not conclude that hydrogenation destroys the antiophthalmic vitamine, inasmuch as growth is a better index than ophthalmia, and our animals grew fairly well.

In this connection, it is of interest to note that Goldblatt and Zilva,[3] using the preventive type of experiment with animals fed on the McCollum diet No. 3143, found that 33.7 mg. daily of a hardened cod liver oil protected against rickets, and that 1.8 mg. promoted growth. The original oil, before hardening, also promoted growth in daily doses of 1.8 mg. The antirachitic value of the original oil was not determined.

From the above, it appears that the vitamine potency of cod liver oil is not appreciably destroyed by a suitable hydrogenation process.

It is thus possible to prepare a potent concentrate which may be used as a starting-point for further chemical fractionation, and as a source of vitamine possessing obvious advantages over cod liver oil. Further work along this line is in progress and detailed data will appear in an early publication.

Fish Oils, Stansby (Ed.), 1967

CHAPTER 16

H. Fineberg
and A. G. Johanson

Industrial Use of Fish Oils

INTRODUCTION

The world's waters annually produce a tremendous harvest of fish, being in 1962 40.4 million tons (Chapman 1965), close to 90% of which came from the oceans. The catch has doubled in the past 24 years and is increasing at the rate of 8% each year. The herring-like fishes, including anchoveta and sardines, made up 41% of the total in 1962 and an estimated 50% + in 1964. About one-third of the total catch is used for industrial purposes, mainly as fish meal for animal feeding, and the rest as fresh, frozen, dried, or canned for food.

The bulk of the fish oils of commerce are obtained as by-products from fish-meal production or from food fish wastes. World fish-oil production was an estimated 865,000 tons in 1966. Production has increased 25.5% since 1961. All other oils have increased enormously also so that fish still is only 2.5% of the total edible fats and oils produced and 13.4% of the inedible ones.

The energy needs of the approximately three billion world population and the normally lower price of fish oils versus the major edible oils and fats such as soybean, peanut, sunflower, cottonseed, palm, butter, and lard have steered the flow into food uses. They are used overseas for shortening, margarine, and compound cooking-fat manufacture. Probably over 75% is used for edible purposes, except in the United States. Outside the United States, the fish oils are refined, selectively hydrogenated, and deodorized for these applications. Fish oils in food products were discontinued in the United States very shortly after the new Food, Drug, and Cosmetic Act was enacted in 1938 (Culbertson 1963). They were considered adulterated products because the fish raw materials used in their manufacture were not all edible. Among the non-edible oils and fats, fish oils rank about 8th in volume, below tallow and grease, coconut, soybean, linseed, castor, sperm, and tall oil.

Supplies of fish oil could increase several fold if edible fish protein concentrates now being developed by the U.S. Bureau of Commercial Fisheries, Peru, and others become commercial on a big scale. An isopropanol extraction process is being developed at Beltsville, Maryland, by the Bureau (Anon. 1966B). There is currently also a great waste, through spoilage and scrapping, of fish that could be prevented and converted into still larger oil and meal supplies.

TABLE 18

WORLD OCEAN FISH CATCH 1962

Species	Volume of Catch Millions of Metric Tons
Herring & anchovy	14.6[1]
Cod, haddock, & hake	5.51
Horse mackerel & sea perch	4.27
Tuna and mackerel	2.38
Flat fish (halibut, sole, flounder)	1.2
Salmon & smelt	0.55
Sharks & rays	0.37
Others	6.77
	35.65

[1] Includes 1.1 MM tons of U.S. menhaden.

The composition of the world fish catch in 1962 is illustrated in Table 18.

Industrial uses (Anon. 1965B) today are mainly for protective coatings on wood, metal, fiber, and concrete; lubricants, medicinals; and some soap. The last use has declined so greatly that it is only a minor one. In the United Kingdom, for example, fish oils were only 0.3% of the total fatty raw materials used in soap. In protective coatings, fish oils compete mainly with linseed, soybean, castor, safflower, tung, and tall oils. It is the relatively low fish-oil cost contrasted to that of all other drying oils (except tall oil) as well as the high degree of unsaturation that make fish oils so useful in this field. The unique mixture of fatty acid chain lengths makes fish oils valuable also in lubricants, greases, paper sizes, buffing agents and textile auxiliaries.

TABLE 19

WORLD OIL PRICES IN 1964

Oil	Price Cents per Lb.
Tung	22.0
Peanut	14.3
Safflower, non-break	14.0[1]
Castor	12.4
Sunflower	11.5
Lard, prime	11.4
Linseed	10.8
Soybean	10.4
Peru fish oil	9.2
Menhaden	9.0[2]
Tallow, bleachable, fancy	7.6
Tall oil	7.5

[1] FOB San Francisco, Tanks, Dec. 20, 1965. Safflower has become an important high-quality oil for protective coatings in the U.S.
[2] The highest price in 14 years. The low in recent years was 4.6¢ in 1962.

World oil prices as of January-December 1964, CIF European ports, illustrate the relative market positions of fish oils and their major competitors (Table 19).

Food demand, including salad and cooking oils, margarine and shortening, controls prices for most of the oils, including fish. Peruvian fish oil, tallow, lard, and tall oil are obtained as by-products, complicating the pricing structure and supply still further. There exists considerable interplay and substitution of one oil for another, depending on relative prices and availability in the world markets.

Menhaden is protected in the United States at present by tariffs which prevent encroachment by the tremendous amount of Peruvian anchovy oil exported into the world market.

SOURCES

The major fish-oil-producing countries are Peru, Chile, Iceland, Norway, South Africa, Canada, and the United States. China and Japan are also big producers but do not engage in world trade. Peru and Chile are late comers, but have experienced a meteoric increase in production. Peru has become the largest exporter in the world—295 million pounds in 1964, mostly anchovy, and an estimated 298 million pounds in 1965. Norway and Iceland are the chief sources of herring on the world scene. The South African countries have an annual production of approximately 132 million pounds of sardine (pilchard) oil. The fish catch is increasingly of the "oily" type—the Peruvian anchovy, American menhaden, South African pilchard, and North Atlantic herring.

The United States in 1964 produced 172 million pounds of menhaden oil, an estimated 185.3 million pounds total of all fish oils. In the last ten years, the total fish oils produced ranged from 165 million in 1957 to 266 million in 1961. Menhaden was 90% of the total, the remainder being mainly herring, Pacific sardine, and a little tuna and mackerel (Anon. 1966 A). The menhaden catch of the United States is used entirely for industrial oil and meal production, none for edible fish use. Menhaden are indigenous to the United States. Approximately two-thirds of the catch is from the Gulf Coast, one-third from the North Atlantic. Alaska and western Canada are the chief sources of domestic herring, with Maine being the least prolific. In addition to the domestic sources, the United States imported 3.9 million pounds of herring oil in 1964, mainly from Peru,[1] 7.7 million pounds of cod, and 7.2 of cod liver oils. However, the actual consumption of all fish oils is lower than the production figures indicate since the U.S. exports much of its oil—151.5 million pounds in 1964, mainly

[1] The Peruvian herring oil may actually have been anchovy.

menhaden. U.S. consumption ranged from a low of 83 million pounds in 1954 to a high of 168 million pounds in 1961.

It is interesting to note that preliminary gross results from a study by the Bureau of Commercial Fisheries (Chapman 1965) indicate roughly a standing crop of 4–6 million tons of anchovy off southern California and northern Mexico, about 3 million tons of hake, and about 11 million tons for all types (maximum sustainable yield) off the U.S. coasts. Thus, a tremendous potential for oil exists but has still to be confirmed or proven economically feasible. The total actual catch was 2.26 million tons in 1964. The size of the catch hasn't changed much in the last thirty years, but the composition has greatly. This is a complicated study and not too much is yet known reliably.

COMPOSITION

There is still much to learn about fish oils even though progress has been considerable. Research interest in these oils remains high since they are the best known natural sources of long-chain, polyunsaturated fatty acids, although from a food point of view they are very low in the normal "essential fatty acids." There is much work being done to determine the functionality of the fish acids in human nutrition.

Commercially, the composition of the oils sold is not as clearly standardized by trading, association, ASTM, AOCS, and Federal rules and specifications as are other industrial oils such as soybean, cottonseed, linseed, dehydrated castor, and tung. Table 20 lists representative values.

Corresponding values for industrial vegetable oils with which the fish oils are competitive are shown in Table 21.

TABLE 20

RANGES OF FISH-OIL CONSTANTS

Constant	Menhaden Gulf	Menhaden Atlantic	Herring	Sardine (Pilchard)	Anchovy[1]
Iodine value	145–170	150–185 175–190[2]	120–150	160–194	180 minimum 196–198.5[3]
Saponification value	[4]	185–190	185–195	185–195	191–193.5[3]
Free fatty acid, %	[4]	3 maximum	0.2–5.0	0.1–13	1 maximum 0.2[3]
Moisture and insolubles, %	[4]	1 maximum	1 maximum	[4]	0.5 maximum
Gardner color, #	[4]	[4]	[4]	[4]	9 maximum
Unsaponifiables, %	[4]	0.6–1.6	2–3	0.5–2	3 maximum
Titer, °C	[4]	30–34	23–27	28–32	3.9 10[1]

[1] Peruvian semi-refined, industry practice.
[2] NPVL Association raw material index, October 1965.
[3] Representative samples entering U.S in 1962 and 1964—courtesy of Archer-Daniels-Midland Company.
[4] Reliable data unavailable.

8.4 Determination of Insoluble Impurities

According to the IUPAC (1964) method (IUPAC Standard Methods for the Analysis of Oils, Fats and Soaps, 5th Edition, 1966, II. C. 2 Impurities).

Results are expressed as % m/m.

8.5 Determination of Soap Content

According to the FAO/WHO Codex Alimentarius Method (FAO/WHO Codex Alimentarius Methods of Analysis for Edible Fats and Oils, CAC/RM 13-1969, Determination of Soap Content).

Results are expressed as % m/m sodium oleate.

8.6 Determination of Iron (*)

According to the FAO/WHO Codex Alimentarius Method (FAO/WHO Codex Alimentarius Methods of Analysis for Edible Fats and Oils, CAC/RM 14-1969, Determination of Iron Content).

Results are expressed as mg iron/kg.

8.7 Determination of Copper (*)

According to the AOAC (1965) method (Official Methods of Analysis of the AOAC, International Union of Pure and Applied Chemistry Carbamate Method, 24.023 - 24.028).

Results are expressed as mg copper/kg.

8.8. Determination of Lead (*)

According to the AOAC (1965) method, after complete digestion, by the colorimetric dithizone determination procedure (Official Methods of Analysis of the AOAC, 1965, 24.053 (and 24.008, 24.009, 24.043 j, 24.046, 24.047 and 24.048)).

Results are expressed as mg lead/kg.

8.9 Determination of Arsenic

According to the colorimetric silver diethyldithiocarbamate method of the AOAC (Official Methods of Analysis of the AOAC, 1965, 24.011 - 24.014, 24.016 - 24.017, 24.006 - 24.008).

Results are expressed as mg arsenic/kg.

(*) Might be replaced by Atomic Absorption Spectrophotometry in the future.

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ACCEPTANCE PROCEDURE
FOR
CODEX COMMODITY STANDARDS

EXTRACT OF THE GENERAL PRINCIPLES OF THE CODEX ALIMENTARIUS

ACCEPTANCE OF CODEX COMMODITY STANDARDS

"4.A. A Codex standard may be accepted by a country in accordance with its established legal and administrative procedures in respect of distribution of the product concerned, whether imported or home-produced, within its territorial jurisdiction in the following ways:

i) Full acceptance

a) Full acceptance means that the country concerned will ensure that a product to which the standard applies will be permitted to be distributed freely, in accordance with (c) below, within its territorial jurisdiction under the name and description laid down in the standard, provided that it complies with all the relevant requirements of the standard.

b) The country will also ensure that products not complying with the standard will not be permitted to be distributed under the name and description laid down in the standard.

c) The distribution of any sound products conforming with the standard will not be hindered by any legal or administrative provisions in the country concerned relating to the health of the consumer or to other food standard matters except for considerations of human, plant or animal health which are not specifically dealt with in the standard.

ii) Target acceptance

Target acceptance means that the country concerned indicates its intention to accept the standard after a stated number of years and will meanwhile not hinder within its territorial jurisdiction the distribution of any sound products conforming with the standard by any legal or administrative provisions relating to the health of the consumer or to other food standard matters except for considerations of human, plant or animal health which are not specifically dealt with in the standard.

iii) Acceptance with minor deviations

Acceptance with minor deviations means that the country concerned gives full acceptance as defined in paragraph 4. A(i) to the standard with the exception of minor deviations which are recognized as such by the Codex Alimentarius Commission; it being understood that a product complying with the standard as qualified by such minor deviations will be permitted to be distributed freely within the territorial jurisdiction of the country concerned. The country concerned will further include in its declaration of acceptance a statement of such deviations, the reasons for them, and also indicate:

a) whether products fully conforming to the standard may be distributed freely within its territorial jurisdiction in accordance with paragraph 4. A(i);

b) whether it expects to be able to give full acceptance to the standard and, if so, when.

B. A country which considers that it cannot accept the standard in any of the ways mentioned above should indicate:

i) whether products conforming to the standard may be distributed freely within its territorial jurisdiction;

ii) in what ways its present or proposed requirements differ from the standard, and, if possible, the reasons for these differences.

C. i) A country which accepts a Codex standard according to one of the provisions of 4. A is responsible for the uniform and impartial application of the provisions of the standard as they apply to all home-produced and imported products distributed within its territorial jurisdiction. In addition, the country should be prepared to offer advice and guidance to exporters and processors of products for export to promote understanding of and compliance with the requirements of importing countries which have accepted a Codex standard according to one of the provisions of 4. A.

ii) Where, in an importing country, a product claimed to be in compliance with a Codex standard is found not to be in compliance with that standard, whether in respect of the label accompanying the product or otherwise, the importing country should inform the

competent authorities in the exporting country of all the relevant facts and in particular the details of the origin of the product in question (name and address of the exporter), if it is thought that a person in the exporting country is responsible for such non-compliance."

RECOMMENDED INTERNATIONAL STANDARDS FOR FATS AND OILS

CAC/RS 19-1969 Recommended International Standard for Edible Fats and Oils not covered by individual Codex Standards

CAC/RS 20-1969 Recommended International Standard for Edible Soya Bean Oil

CAC/RS 21-1969 Recommended International Standard for Edible Arachis Oil

CAC/RS 22-1969 Recommended International Standard for Edible Cottonseed Oil

CAC/RS 23-1969 Recommended International Standard for Edible Sunflowerseed Oil

CAC/RS 24-1969 Recommended International Standard for Edible Rapeseed Oil

CAC/RS 25-1969 Recommended International Standard for Edible Maize Oil

CAC/RS 26-1969 Recommended International Standard for Edible Sesameseed Oil

CAC/RS 27-1969 Recommended International Standard for Edible Safflowerseed Oil

CAC/RS 28-1969 Recommended International Standard for Lard

CAC/RS 29-1969 Recommended International Standard for Rendered Pork Fat

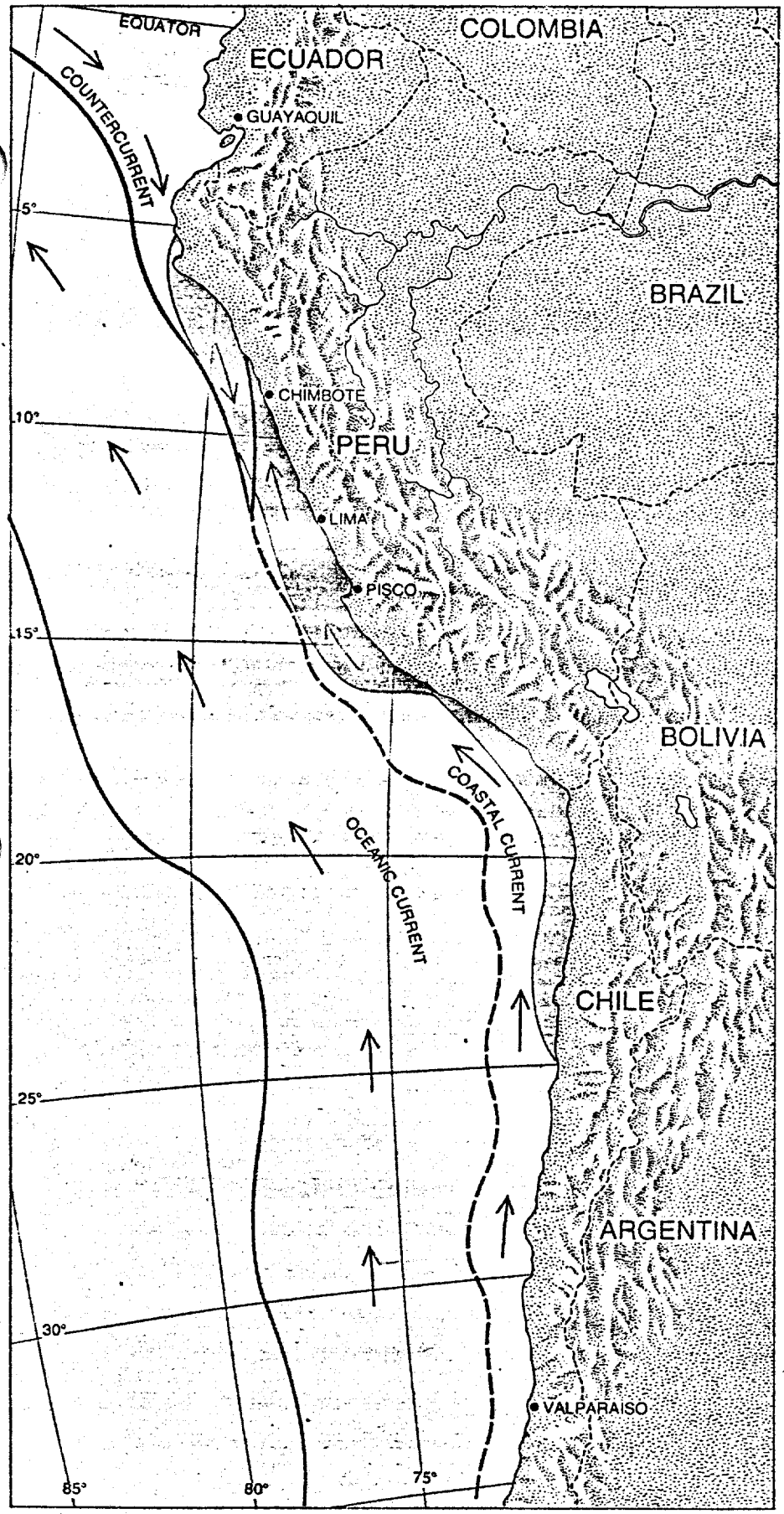
CAC/RS 30-1969 Recommended International Standard for Premier Jus

CAC/RS 31-1969 Recommended International Standard for Edible Tallow

CAC/RS 32-1969 Recommended International Standard for Margarine

CAC/RS 33-1970 Recommended International Standard for Olive Oil, Virgin and Refined, and for Refined Olive Residue Oils

CAC/RS 34-1970 Recommended International Standard for Edible Mustardseed Oil



TWO NORTH-FLOWING COMPONENTS of the Peru Current are the deep, narrow Coastal Current that hugs the land from Valparaiso in Chile to north of Chimbote in Peru and the deeper and wider Oceanic Current that reaches the latitude of the Gulf of Guayaquil. The anchovies (*dark color*) are normally found in the Coastal Current between 25 and five degrees south latitude; they may consist of a northern and a southern population.

the amount of carbon fixed photosynthetically per cubic meter of water per day, the range in the upwelling region is from 45 to 200 milligrams, compared with less than 15 milligrams in the waters immediately adjacent. Perhaps only one other part of the world ocean is richer: the Benguela Current off the southwestern coast of Africa.

The Peru upwelling sustains an enormous flow of living matter. The food chain begins with the microscopic diatoms and other members of the phytoplankton that comprise the pasturage of the sea. The plants absorb the nutrients and grow in rich profusion, providing fodder for billions of grazing animals, principally minute crustaceans such as copepods but including arrowworms and a wide variety of other small marine herbivores. The food chain can then go on to several more links, progressing from the small fishes that eat the herbivores to the larger fishes and squids that prey on the small fishes and perhaps continuing to include one or more further advanced levels of marine predation. The food chain in the Peru Current does go on in this fashion to some degree, but most of its energy flow stops with the anchovies. This single fish species has succeeded in capturing an extraordinarily high proportion of the total energy available in the ecosystem and in converting it into enormous quantities of living matter. At the height of the anchovies' annual cycle the total bulk of the species is probably of the order of 15 to 20 million metric tons.

The Peruvian anchovy belongs to the same genus (*Engraulis*) as the common anchovy of the eastern Atlantic and the Mediterranean (*E. encrasicolus*), but it comprises a separate species (*E. ringens*). Its life begins in the form of an egg, a tiny oval spot of nearly transparent protoplasm adrift in the sea. Eggs can be spawned at almost any time of the year but there are two periods when the anchovies' reproductive activity is highest. The major spawning occurs in August and September, during the southern winter, and it is repeated on a lesser scale in January and February. Anchovies are precocious: most females are capable of spawning when they are a year old. By then each female, a little over four inches long, may produce 10,000 eggs. If she survives to the age of two and reaches a length of six inches, her output increases to some 20,000 eggs.

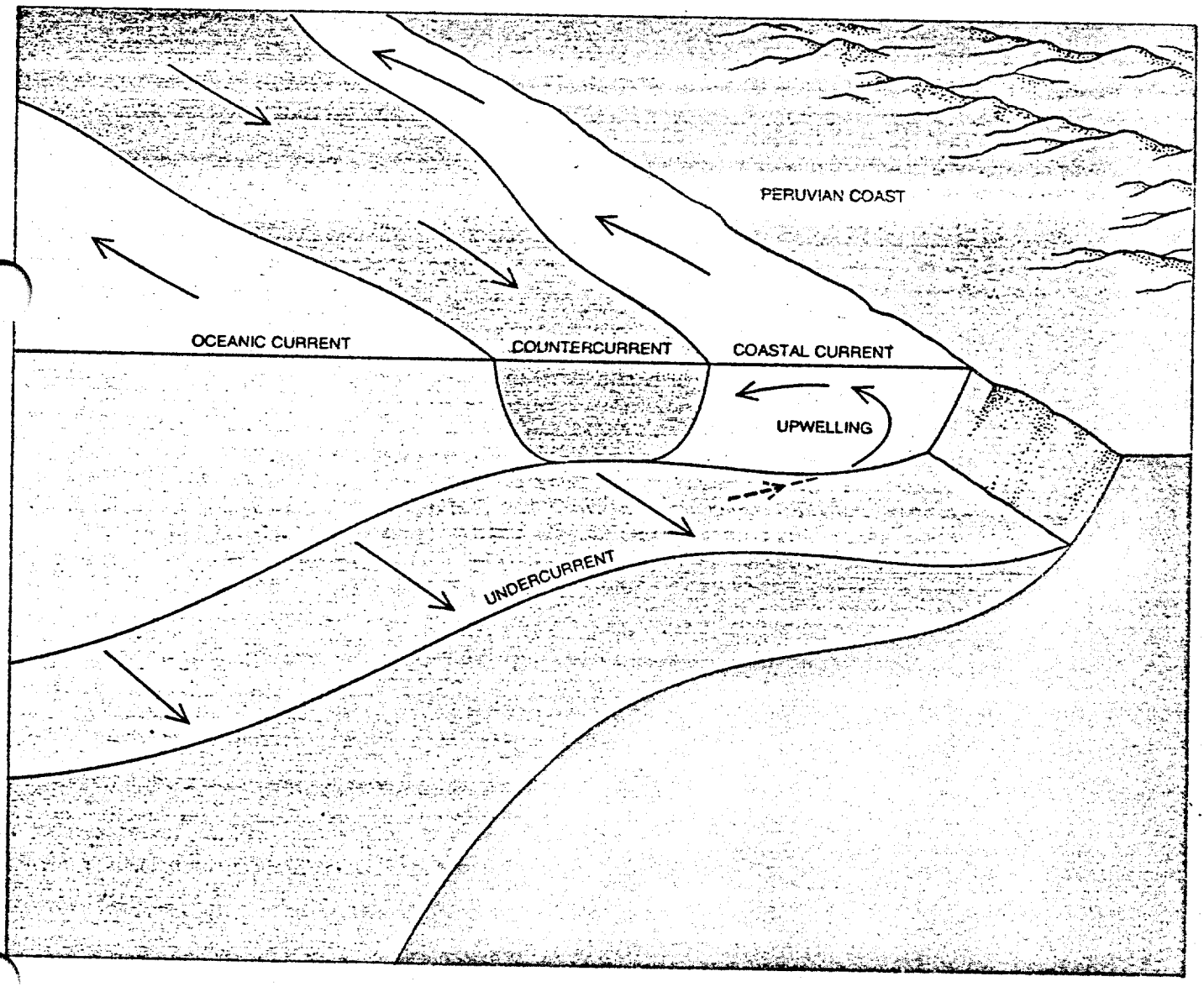
The delicate larvae that hatch from the eggs lead a perilous existence. Many species of fish produce eggs that contain a considerable store of yolk; the reserve

of nutrient helps to sustain the newly hatched young until they adjust to finding their own food. The anchovy egg has a negligible yolk store, and so the larva must locate food quickly or starve. To make matters worse, the larva has limited swimming powers and a high rate of metabolism. If more than a few wiggles are required to obtain the food it needs, it will not survive. Because every larva consumes plankton in substantial amounts and because the peak hatches produce larvae numbering in multiples of billions, only enormous swarms of microscopic plants and larval crustaceans can sustain the anchovy stock. Nor is starvation the only peril: the larval anchovies feed swarms of predators. They are eaten by the same copepods that will, if the little fish survive, be the anchovies' own main sustenance. Arrow-worms also devour them, and so do their own parents.

One month after the time the anchovy larvae are hatched more than 99 percent of them have perished. Even with such a high mortality rate, a process that begins with billions of spawning fish, each casting 10,000 to 20,000 eggs, produces enormous quantities of anchovy larvae. The little fish grow rapidly; in the course of the first year they attain a length of 4.2 to 4.3 inches. The year-old fish are so slender, however, that they weigh a scant third of an ounce.

The anchovy schools do not move at random; apparently because of a strong preference for the cold water of the Coastal Current they remain within a comparatively restricted zone. The Coastal Current is at its narrowest during the southern summer, running close to shore and seldom exceeding 200 meters in depth. Within this shrunken world the anchovies press together in enormous concentrations near the shore and close to the surface. It is now that predators fare best. The larger fishes and the squids feed well; the several species of guano birds have to fly only short distances from their island nesting grounds and need not dive deep to reach their prey. The greatest of the predators, the fisherman, finds summer work the easiest. He can often set his purse seine within sight of port and gather in anchovies 100 tons at a time.

El Niño occurs at irregular intervals. There is said to be a seven-year cycle, but in actuality the phenomenon is far



TWO SOUTH-FLOWING COMPONENTS of the Peru Current are seen schematically in relation to the two north-flowing components in this diagram. The Countercurrent (*dark color*), a surface or near-surface stream of water, intrudes between the north-flowing cold Coastal and Oceanic currents. Normally the Countercurrent does not extend much south of the Equator, but when the wind that moves the north-flowing currents along falters or changes direction, its warm water pushes far to the south with disastrous biological consequences. Deep below all three currents is the second, far larger south-flowing component, the Undercurrent (*light color*).

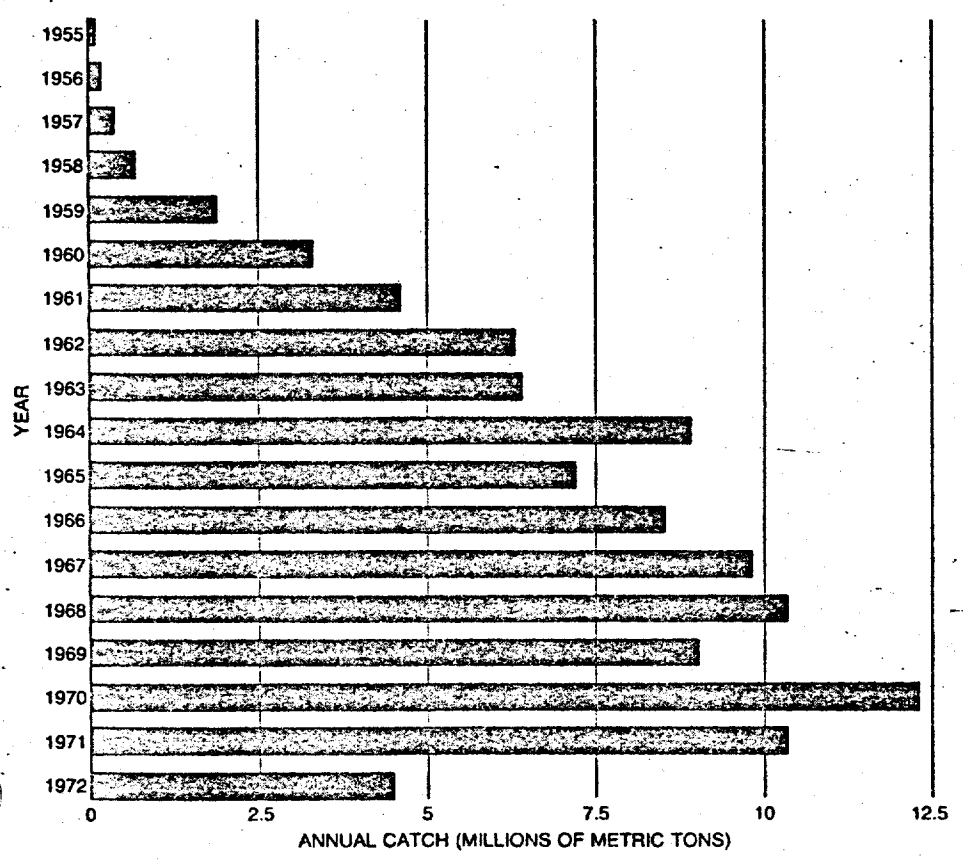
less precise in its appearance. The severe environmental dislocations may be repeated for two or more years in a row or may not recur for a decade or longer. Another kind of regularity, however, has given El Niño its name. The change usually begins around Christmastime and so is given the Spanish name for the Christ Child. The complicated chain of events in a year of El Niño disturbs the anchovies, sometimes profoundly. The wind now comes from the west rather than from the southeast, and it is laden with moisture from the Pacific. With no mountains to rob the air of its burden of water the arid coast is often subjected to torrential rains and severe windstorms. In a desert region where even a heavy mist can cause problems the floods of El Niño are often devastating. Oddly enough, however, in some Niño years no rain falls.

A warning that the sea change may be on its way is given when the temperature of the coastal water begins to rise. If the increase in temperature persists and spreads, the delicately adjusted world of the anchovy tilts. With the warm water come unfamiliar inhabitants of the northern Tropics: the yellowfin tuna, the dolphinfish, the manta ray and the hammerhead shark. Some of them feed on the anchovies. A greater threat to the anchovies' survival, however, is a slowing of the northbound Coastal Current and a decline or even a halt in the usual upwelling of subsurface waters. As the supply of nutrients diminishes, the planktonic plant life that provides the base of the ocean food chain becomes less abundant. As a result herbivorous planktonic animals become scarcer, and so it goes link by link up the chain. Furthermore, the water temperature is now too high to suit the anchovies themselves. Even if the shortage of food has not yet greatly reduced their numbers, the fish scatter, no longer forming the enormous schools that normally afford the guano birds and fishermen such rewarding targets.

The effect on the guano birds and marine animals is among the most serious of the changes wrought by El Niño. The birds starve or fly away, deserting their nestlings. Fishes, squids and even turtles and small sea mammals die. Their decaying bodies release evil-smelling hydrogen sulfide that bubbles up through the water and blackens the paint on the boats in the harbors. This unpleasant phenomenon is called El Pintor (The Painter). Patches of reddish, brownish or yellow water similar to the "red tides" that upset the Florida tourist industry become relatively common. They are caused by prodigious blooms of dinoflagellates: microscopic planktonic plants that are toxic in high concentrations. The greater frequency of the blooms during Niño years may be because the nutrient composition of the seawater suits the organisms better then or because the less vigorous currents fail to disperse accumulating clusters of dinoflagellates as quickly as usual.

The causes of El Niño are wind changes and sea changes on a very large scale. When the steady southeast trade winds weaken or when the wind blows from the west, the ocean currents that run to the northwest are no longer pushed along with the same vigor. Under normal circumstances the south-flowing Peru Countercurrent is weak, but when the prevailing winds fail or are reversed, the Countercurrent thrusts a tongue of warm water into the cleft between the now less vigorous Coastal and Oceanic currents. As it meets less resistance the Countercurrent penetrates farther south, pushing the weak north-flowing currents aside and covering their cold waters with a 30-meter layer of warm tropical water. The water may have come from as far away as the Panama Bight, north of the Equator, and part of it may even have originated as land runoff in Central America. It can be as much as seven degrees C. warmer than the north-flowing Coastal Current and is lower in salinity, deficient in oxygen and poor in nutrients. In some Niño years the Countercurrent pushes the tropical water as far as 600 miles south of the Equator.

The organisms most obviously affected by a Niño year are the guano birds, various species that are colloquially lumped together under the same name that is applied to the droppings that accumulate in large quantities on the rocky islands where they nest. There are three principal species: the guanay, or cormorant (*Phalacrocorax bougainvillii*); the piquero, or booby (*Sula variegata*), and the alcatras, or pelican (*Pelicanus thagus*). Over the millenniums the bird droppings have accumulated in piles as high as 150 feet on some islands. Because guano is perhaps the finest natural fertilizer known, the guano islands have provided the foundation for a valuable industry. Of the guano birds' diet between 80 and 95 percent is made up of anchovies, and the coastal waters of Peru support what is probably the largest population of oceanic birds anywhere in the world. In recent years estimates of the birds' total number have gone as



ANCHOVY CATCH remained below two million metric tons until the 1960's. Exploitation of the fishery skyrocketed thereafter; the annual rate approached or exceeded nine million tons for six of eight consecutive years. The peak year, 1970, saw a catch of 12.3 million tons.

SACKS OF FISH MEAL awaiting shipment abroad line a wharf at a Peruvian port. The meal is used to enrich feeds for poultry and other livestock. In 1970 the export of fish meal and fish oil earned some $340 million, a third of Peru's total export revenue.

high as 30 million. The five million individuals that inhabited one particular guano island are believed to have consumed 1,000 tons of anchovies a day. The guano birds' annual anchovy catch in recent years is calculated to average 2.5 million metric tons, or between a fourth and a fifth of the commercial-fishery catch.

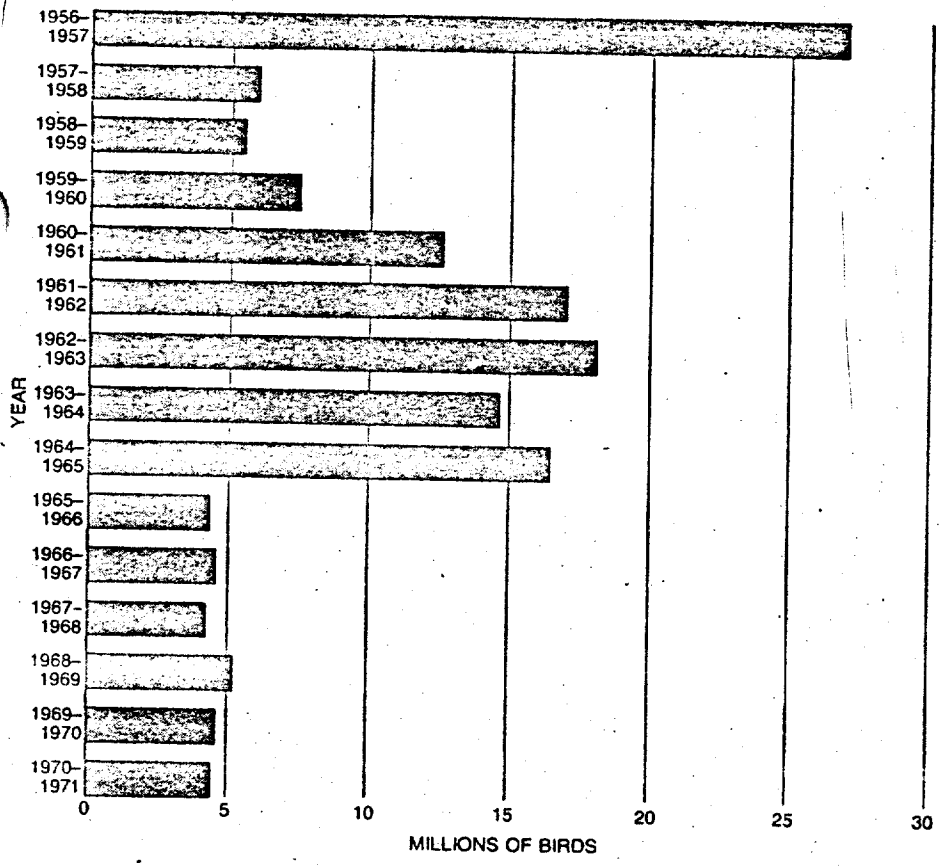
Following every Niño year of any consequence the bird population declines just as the anchovy population does. When the warmer water scatters the dense surface schools of fish, the birds find it harder to feed themselves, let alone their nestlings. Adult birds fly to other areas. Juvenile birds, less efficient fishers than their parents, perish in large numbers. The deserted nestlings are doomed to starvation. After the severe Niño year of 1957 the guano-bird population, then estimated to be 27 million, plummeted to six million and dropped to a low of 5.5 million the following year. Numbers slowly increased thereafter, so that there were 17 million birds when the Niño year of 1965 arrived. That year the population fell to 4.3 million.

Since then the guano birds have failed to recover at the normal rate. There is concern that the commercial anchovy fishery, which has expanded greatly in the same period, is depriving the birds of so much food that their numbers may fall below the level that is critical to their survival as social species. The late Robert Cushman Murphy of the American Museum of Natural History devoted some years to the study of these populations, and it was his opinion that the birds and the fishermen were essentially incompatible. It seems likely, however, that in spite of Murphy's contrary view the two competitors will be able to coexist at some suitable level of commercial fishing. At the same time it may well be that the size of the commercial catch in recent years has prevented the bird population from regaining its former numbers.

It is not commonly known that in the past few years the anchovy fishery has made Peru the world's leading fish-producing nation. Until recently Peru was harvesting anchovies at a rate of 10 million metric tons or more per year. This is a greater weight than that of all the species of fish being caught by any one nation in the Old World, and is twice the tonnage of the combined all-species catch of all the nations of North and Central America. The fish meal made from the Peruvian catch is sold around the world to enrich feeds for poultry and other livestock; the fish oil goes into margarine, paint, lipstick and a score of other products. In 1970 the export of fishery products brought Peru some $340 million, nearly a third of the nation's foreign-exchange earnings. In addition to this the tax revenues from the industry and the domestic employment it provides have become major elements in Peru's economy.

The anchovy industry began in earnest in 1957. Within 10 years the profits that could be made from catching and processing the fish attracted hundreds of fishing boats and led to the construction of dozens of fish-meal factories. No fish stock, however, can stand unchecked exploitation. Government authorities and fishery biologists became concerned about the future of the resource. Peru was a newcomer to large-scale commercial fishing and had neither fishery scientists nor administrators with experience in the complexities of fishery research and management. The Peruvian government turned to the United Nations for help.

In 1960, with a grant from the UN Development Programme and a matching amount in Peruvian funds, the Insti-



GUANO-BIRD POPULATION, hard hit by El Niño in 1957, had regained more than half its former numbers when El Niño reappeared in 1965. The population did not recover at the normal rate thereafter, possibly because the large commercial anchovy catches cut down the birds' food supply. Only a million or so guano birds may have survived the 1972 Niño.

tuto del Mar del Peru was set up to conduct research on the anchovy stocks and to advise the government on management of the fishery. The Food and Agriculture Organization of the UN (FAO) recruited experienced fishery scientists from around the world to work at the institute, conduct research on the anchovy stocks and train a Peruvian staff. Located in Lima, the institute is now a firmly established fishery-research center where more than 50 young Peruvian scientists are conducting the studies needed to establish conservation regulations for the anchovy fishery. The Peruvian staff is advised by a few resident FAO scientists and by a panel of distinguished experts from around the world, organized by the FAO, that meets twice a year.

It has taken biologists nearly a century to unravel the intricacies of fish populations and the complexities of their response to the dual stresses of environmental change and human exploitation. Until last year fishery biologists could point to the management of the Peruvian fishery as an exemplary application of this hard-won knowledge. A major stock had been put under rational control before exploitation had depleted it, and conservation measures seemed to be ensuring an enormously high yield at the limit of the biological capacity of the Peru Current ecosystem. Then in 1972 such pride was chastened, if not utterly humbled.

After the Niño year of 1965 the fishery had enjoyed several very successful seasons, culminating in 1970 with an anchovy catch of 12.3 million metric tons. Then, toward the end of April, 1972, and only a few weeks after the start of the season, fishing suddenly faltered. By the end of June catches had dwindled to almost nothing, and at the close of the 1972 season only 4.5 million tons of anchovies had been harvested. The catch this year threatens to be even poorer. Indeed, there is some reason to fear that the world's greatest stock of fish may have been irreversibly damaged, in which case the Peru fishery would be destined to collapse altogether.

That is the gloomiest outlook. It is based on two disturbing circumstances. First, the size of the "standing stock" (the total anchovy population) now appears to be far smaller than normal. It may be as low as one or two million metric tons, compared with an average of 15 million tons in recent years and an estimated 20 million tons in 1971. Second, "recruitment" (the numbers of fi grown big enough to enter the commercial fishery in any year) has been by far the smallest ever observed. It is scarcely 13 percent of the recruitment in a normal year.

What is causing the trouble? Very possibly El Niño has been a major factor, but some puzzling circumstances have made the scientists closest to the subject uncertain about the extent of the relation between the sea change and the reduction in the anchovy stock. It is clear, however, that the Niño year of 1972 was one of the most severe ever observed. Instead of remaining at the normal level of 22 degrees C. the surface temperature of the Coastal Current rose to 30.3 degrees in February. Although the temperature fluctuated thereafter, it remained high for the rest of the year and was still above normal in January, 1973. It did not fall to near-normal temperature until March of this year. At the same time that the temperature rose the salinity of the water declined from a normal 35 or 36 parts per 1,000 to 32.7 parts per 1,000.

Tropical and subtropical marine plants and animals began to appear far south of their usual limits: dolphinfish, skipjack tuna and the tropical crab *Euphilax*. The guano birds fled from their nesting islands, abandoning their young; their population may now number no more than a million. The warm Countercurrent forced the cold-seeking anchovies so close to the shore that the fishermen often found the water too shallow for their nets. Moreover, the crowded fish did not spawn as abundantly as usual and the eggs and larvae, already reduced in numbers, did not survive at the usual rate because of greater predation by their own close-packed parents.

Biologists are nonetheless unwilling to blame El Niño for all these occurrences. For example, with respect to the anchovies they note that recruitment of young fish to the adult population was observed to fall below normal levels before it became obvious that the surface temperature of the Coastal Current had risen. In seeming contradiction to this, however, the tropical crabs too made their appearance before the surface temperature rose, apparently indicating that a body of tropical water had by then already invaded the world of the anchovies. In the light of such oddities the experts frankly admit that they do not know how much influence El Niño exerted on the anchovies in 1972.

Fish Oils, M. E. Stansby (Ed.), 1967

CHAPTER 4

Clifford R. Houle

Gas-Liquid Chromatography

INTRODUCTION

This chapter deals with the applications of gas-liquid partition chromatography as it is applied to the analysis of the components of fish oils. Because of limited scope and space, the reader interested in a more thorough discussion of gas chromatography is referred to the many excellent texts on this subject. Among these, the books by Pecsok (1959) and Keulemans (1959) are particularly recommended.

CHROMATOGRAPHY: GENERAL ASPECTS

The analytical method known today as chromatography is generally acknowledged to have been first employed by Tswett in 1906. Because he separated plant pigments into discrete colored bands by his technique, he used the term "chromatography" to describe the method. Chromatography has long since evolved into the separation of colorless compounds, necessitating varied physical and chemical methods of detection. Gas-liquid partition chromatography, since its introduction by James and Martin in 1952, has rapidly become a powerful tool for the separation and identification of the numerous components of lipid mixtures (e.g., fish oils). Its usefulness, however, can be fully exploited only when it is used in conjunction with complementary methods of separation and identification.

INSTRUMENTATION

The commercial equipment presently available for gas-liquid chromatography are numerous and varied; the manufacturers should be consulted for the features of a specific system. However, there are certain characteristics essential to all instruments that will be used for the analysis of the components of fish oils. These features are as follows:

(1) A detector sensitive to the substances derived or isolated from fish oils; (2) a column containing a suitable packing enclosed in an oven which may be thermostatically regulated from room temperature to about 350°C.; (3) an apparatus to provide a regulated flow of the carrier gas through the column; and (4) a method for introducing the sample with a minimal disruption of the system.

There are numerous refinements provided by commercial manufacturers, such as flash heaters at the sample injection point to vaporize the sample, which are common to most instruments. In addition, the basic

equipment requires the use of a stable, sensitive recorder to graphically record the electrical impulses transmitted by the detector.

The method of detection of the effluent of the gas chromatograph is of primary importance: the detector must rapidly both sense and measure the compounds separated by the column in the presence of the carrier gas. For the analysis of lipid classes from fish oils, there are three types of detectors in wide use because of their effectiveness in this regard. They are the thermal conductivity detector, the argon ionization detector, and the hydrogen flame ionization detector.

Each of these methods of detecting lipids in the column effluent have various advantages and disadvantages in relation to each other. The thermal conductivity (katharometer) detector is probably the most widely used system, although data obtained by this method requires the application of correction factors for accurate quantitative analysis. The argon and the hydrogen flame ionization detectors are the most sensitive, and thus are particularly advantageous when the analysis of a limited amount of sample is required. Since the sample is destroyed in the hydrogen flame detector, if the system is not equipped with an effluent splitter, it cannot be used where the investigator wishes to conduct further analyses upon the separated sample components. A stream-splitting device must be used with caution, however, to avoid invalidating quantitative data (Karmen *et al.* 1963).

The sensitivity of the argon ionization detector may be reduced if the sample or solvents have not been carefully dried (Evans and Scott 1961). This detector has an additional disadvantage of requiring careful calibration, since its response is linear only over a narrow sample range at a given voltage (Fowlis *et al.* 1964).

FACTORS AFFECTING THE SEPARATION

The column used in gas-liquid partition chromatography contains a packing composed of a liquid stationary phase supported in the column by an inert, size-graded solid. Separation of a single class of lipids (e.g., fatty acid methyl esters) obtained from a fish oil is effected by applying a small amount of the mixture on to the column through which an inert gas is flowing. The individual compounds of the mixture are partitioned between the inert gas and the liquid phase of the column. Because each compound of the mixture varies in its physical properties (e.g., solubility, vapor pressure, polarity) a separation into individual compounds is obtained as the mixture passes through the column.

The type of column is dictated to a large extent by the physical properties of the class of compounds to be separated. Fatty acid methyl esters, for example, are most commonly separated on polyester phases such as

380

ethylene glycol succinate (EGS) or on diethylene glycol succinate (DEGS). β-cyclodextrin acetate has been shown by Schlenk *et al.* (1962) to be a very useful liquid phase for both analytical and preparative gas chromatography of numerous lipids because of its stability at high temperatures. These and other liquid phases which may be used for the separation of the numerous lipid classes obtained by chemical isolation techniques from fish lipids are listed in Table 8. The primary requisite of a good liquid phase is that it be virtually nonvolatile at the temperature of operation.

TABLE 8

LIQUID PHASES RECOMMENDED FOR THE ANALYSIS OF FISH LIPIDS

Lipid Class	Liquid Phase	Maximum Column Temperatures (°C.)
Fatty acid Methyl esters	Butanediol succinate (BDS)	225
	Diethyleneglycol adipate (DEGA)	225
	Diethyleneglycol succinate (DEGS)	225
	Ethyleneglycol succinate (EGS)	225
	β-Cyclodextrin acetate (β-CDXA)	300
	SE-30 Silicone gum rubber	375
	Apiezon L	300
	Apiezon M	275
Hydrocarbons	Dioctylphthalate	150
	Squalene	30
Alcohols	Carbowax 20M	250
	β-Cyclodextrin acetate	300
Aldehydes	Carbowax 20M	250
	Apiezon L	300
	β-Cyclodextrin acetate	300
Steroids	SE-30 Silicone gum rubber	375
	Fluoro silicone (QF-1)	250

The inertness and size of the solid support is of importance for quantitative separations. Commonly used solid supports are diatomaceous earth (Celite and Celatom) or crushed firebrick (Sil-O-Cel C 22) and flux calcined derivatives. These solid supports may be obtained commercially: size-graded, acid and base washed in ready-to-use form. Some may be obtained specially treated with dimethyldichlorosilane (Vandenheuvel and Vatcher 1956) or other deactivating reagents to prevent surface adsorption of the compounds being analyzed. These treated support materials are especially advantageous when low per cent liquid phase loadings are used and where surface adsorption is most likely to become a problem. Column packings are usually made up to provide from 3 to 20% by weight of liquid phase on the solid support. To prepare a column packing, the liquid phase is dissolved in a suitable solvent, poured over the solid support

in a round-bottom flask, and the solvent is then removed by evaporation at reduced pressure with agitation.

For most analytical work, column tubing is from one-eighth to one-quarter inches outside diameter and from 4 to 10 feet in length. The tubing material may be of copper or aluminum, although glass and stainless steel are often used for labile substances. Optimal flow rates of the inert gas through a one-quarter inch outside diameter column are from 30 to 100 ml per minute. The optimum flow rate of the carrier gas is best determined by an actual test of each individual column with appropriate standards.

The column temperature must be held constant over very narrow limits. In addition, operating temperatures should be below the maximum column temperatures for each phase listed in Table 8 in order to extend the useful life of the column.

Polyester columns are commonly conditioned for 24 hours at 20°C. above their optimum operating temperature to decrease the effects of liquid phase bleed upon detector sensitivity.

PREPARATION OF THE SAMPLE

The most essential consideration when analyzing fish oils for their constituents is to protect the fish oil under study at all times from atmospheric oxygen. This is easily accomplished by maintaining a blanket of an inert gas such as nitrogen or carbon dioxide over the sample. Heat and light are also detrimental. However, their effects are minimal for short exposure times when the air above the sample has been replaced by nitrogen.

Although fish oils are quite diverse in the nature of their components, they usually contain substantial amounts of fatty acids in the form of esters of glycerol. Gas-liquid partition chromatography techniques have been most often applied to the analysis of the fatty acid composition of these triglycerides. The most convenient form in which fatty acids have been analyzed via gas-liquid chromatography is as their methyl esters. While there are many techniques by which one may achieve this conversion, the methods given below are particularly suitable for micro amounts of sample. Radin and co-workers (1960), for example, have described a method of esterification of fatty acids utilizing 2, 2-dimethoxypropane, methanol, and aqueous hydrochloric acid. Another esterification method using boron trifluoride-methanol reagent has been outlined by Metcalfe and Schmitz (1961). Esterification of small amounts of fatty acids with diazomethane generated from N-nitrosomethylurea or N-methyl-N-nitroso-p-toluene-sulfonamide has been described by Roper and Ma (1957) and by Schlenk and Gellerman (1960), respectively.

Any of these micro methods are suitable for fish oils. For larger sam-

ples, conventional methods are quite adequate. The complete conversion of the fatty acids to methyl esters and the absence of other classes of compounds should be confirmed, however, by thin-layer chromatography (Mangold 1961) prior to GLC analysis.

SAMPLE INTRODUCTION

While a few instruments may make use of a glass capillary pipette for sample introduction, most systems utilize a micro-syringe for this purpose. The syringe, from one-half to ten microliters in volume, is inserted through a rubber septum into the sample vaporization chamber of the gas chromatograph and a dilute solution of the sample is injected. Depending upon the number of components in the sample, from 0.05 to 3 microliters of a $^1/_{10}$ to 10% solution of the sample in acetone, hexane, or chloroform is injected into argon or flame ionization detection instruments.

The less sensitive thermal conductivity detectors permit samples ranging from 100 to 500 μg. Samples of this size may be readily injected without prior dilution in a solvent.

QUALITATIVE IDENTIFICATION

The identification of the components of an unknown mixture must be correlated with standard reference compounds and reference mixtures of known composition. Some of the fatty acid methyl esters common to fish oils are listed in Table 9. All of these are available from the Hormel Institute, Austin, Minnesota. If the unknown mixture has been previously characterized to contain only one class of compounds (e.g., fatty acid methyl esters) via thin-layer chromatography, then the reference standards will provide a basis for the assignment of specific structures to the individual peaks separated by gas-liquid partition chromatography. The analysis will be even more certain if the mixture and standards are analyzed both on a polar and on a non-polar gas-liquid chromatographic column (Farquhar *et al.* 1959). Since the acquisition of pure reference standards for all of the possible fatty acid methyl ester components of a sample is impractical, the application of graphically determined identification constants is required to establish the identity of many of the peaks observed during gas chromatographic analysis. Woodford and Van Gent (1960) and Miwa *et al.* (1960) proposed basically similar methods of plotting on a semi-log graph the retention times (log-scale) of two or more known, normal saturated monocarboxylic methyl esters against their chain length (number of carbon atoms of the parent acid) to obtain what was called the "carbon number" and "equivalent chain length," respectively. By the use of this method, Hofstetter *et al.* (1965) presented excellent data on the equivalent chain lengths of numerous standard saturated, un-

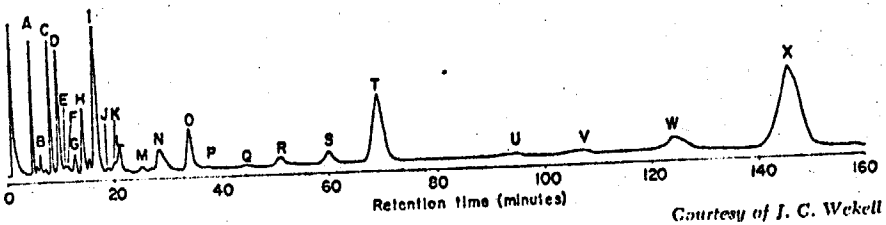
TABLE 9

FATTY ACID METHYL ESTERS AVAILABLE AS STANDARDS FOR GAS CHROMATOGRAPHY

Systematic Name	Common Name
Saturated Fatty Acid Methyl Esters	
Methyl dodecanoate	Methyl laurate
Methyl tridecanoate	—
Methyl tetradecanoate	Methyl myristate
Methyl pentadecanoate	—
Methyl hexadecanoate	Methyl palmitate
Methyl heptadecanoate	—
Methyl octadecanoate	Methyl stearate
Methyl nonadecanoate	—
Methyl eicosanoate	Methyl arachidate
Methyl henicosanoate	—
Methyl docosanoate	Methyl behenate
Methyl tetracosanoate	Methyl lignocerate
Unsaturated Fatty Acid Methyl Esters	
Methyl *cis*-9-hexadecenoate	Methyl palmitoleate
Methyl *cis*-9-octadecenoate	Methyl oleate
Methyl *cis*-9,*cis*-11-octadecadienoate	Methyl linoleate
Methyl *cis*-9,*cis*-12,*cis*-15-octadecatrienoate	Methyl linolenate
Methyl *cis*-11-eicosenoate	—
Methyl 5,8,11,14-eicosatetraenoate	Methyl arachidonate
Methyl eicosapentenoate	—
Methyl *cis*-13-docosenoate	Methyl erucate
Methyl docosahexaenoate	—

saturated, and branched-chain fatty acid methyl esters analyzed on both polar and non-polar liquid phases. Because the equivalent chain length is independent of operating parameters other than the type of liquid phase used, the data of Hofstetter and co-workers should be of great assistance in the identification of individual fatty acids.

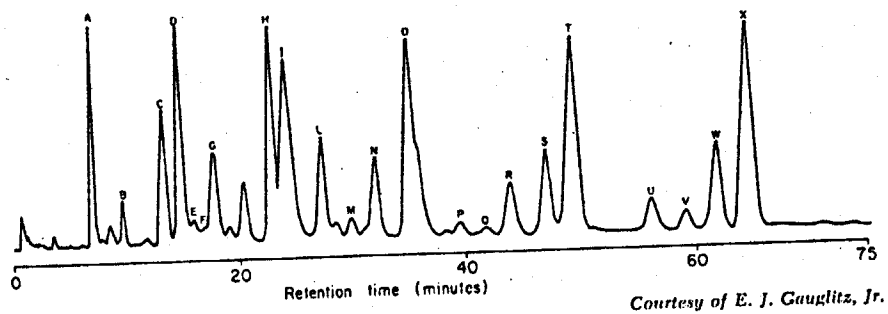
There are several techniques for verifying the structural determinations other than those based upon retention time of peaks on liquid phases of differing polarity and the use of equivalent chain length data as described above. For example, the sample may be hydrogenated so that only saturated compounds are observed. The sum of the saturated and unsaturated esters of a single chain length from the original sample compared to the amount of that chain length present in the hydrogenated sample should agree if the proper structures have been assigned to the unsaturated components. In addition, hydrogenation may permit the detection of small amounts of saturated branched-chain compounds previously obscured by unsaturated peaks. The preparation of acetoxymercuri-methoxy derivatives may also aid in the determination of questionable structures (Mangold and Kamereck 1961). This technique may be used to separate methyl esters upon the basis of unsaturation via thin-layer chromatogra-



Courtesy of J. C. Wekell

FIG. 2. ISOTHERMAL GLC ANALYSIS OF THE FATTY ACID METHYL ESTERS OF MENHADEN OIL

Instrument: Aerograph 6000, hydrogen flame ionization detector. Column: Ten per cent diethyleneglycol succinate on 100/120 mesh Chromosorb W. Ten feet times one-eighth inch outside diameter pyrex glass column. Conditions of analysis: Temperature, 178°C.; Nitrogen carrier gas flow rate, 30 ml/min.; Hydrogen flow rate, 24 ml/min.; and Air flow rate, 400 ml/min. Identification of individual peaks on the chromatogram. (Fatty acid chain length; number of double bonds). A, 14:0; B, 15:0; C, 16:0; D, 16:1; E, 17:0; F, 16:2; G, 17:1; H, 18:0; I, 18:1; J, 19:0; K, 17:3 or 18:2; L, 18:2; M, 20:0; N, 18:3; O, 20:1; P, 20:2; Q, 22:0; R, 22:1 or 20:4; S, 20:4; T, 20:5; U, 24:1 and 22:4; V, 22:4; W, 22:5; and X, 22:6.



Courtesy of E. J. Gauglitz, Jr.

FIG. 3. PROGRAMMED TEMPERATURE GLC ANALYSIS OF THE FATTY ACID METHYL ESTERS OF MENHADEN OIL

Instrument: Barber-Coleman Model 5000, dual hydrogen flame ionization detectors. Column: Six feet times one-quarter inch outside diameter, packed with five per cent stabilized diethyleneglycol succinate on 80/90 mesh Anakrom ABS. Conditions of analysis: Column temperature at start of run, 130°C. Temperature program rate of 1°C./min. Nitrogen carrier gas flow rate of 60 ml/min.; Hydrogen flow rate of 39 ml/min.; Air flow rate of 400 ml/min. Identification of individual peaks on the chromatogram. (Fatty acid chain length; number of double bonds). A, 14: 0; B, 15: 0; C, 16: 0; D, 16: 1; E, F, G, identity uncertain; H, 18: 0; I, 18: 1; L, 18: 2; M, 20: 0; N, 18: 3; O, 20: 1; P, Q, identity uncertain; R, 22: 1 or 20: 4; S, 20: 4; T, 20: 5; U, 24: 1 and 22: 4; V, 22: 4; W, 22: 5; and X, 22: 6.

phy. The separation of methyl esters into groups of equal degree of unsaturation prior to gas chromatographic analysis reduces the complexity of the analysis.

Another method for improving resolution when analyzing a mixture containing compounds of widely varying boiling points is programmed temperature gas chromatography. Figures 2 and 3 present a comparison of gas chromatographic analysis by means of isothermal and programmed temperature methods, respectively, of menhaden oil methyl esters. The programmed temperature method of analysis is particularly advantageous for fish oils because minor peaks near the end of the run are more clearly indicated (Fig. 3).

QUANTITATIVE DETERMINATION OF COMPONENTS

Of equal concern with the identification of the individual compounds of an oil is the need to know the relative amounts of each component. For fatty acid methyl esters, it has been established that the measurement of peak areas gives the weight per cent of saturated and unsaturated (up to six methylene-interrupted double bonds) components when an appropriately designed argon ionization or hydrogen flame ionization detector is used (Horning *et al.* 1964). The validity of this statement for a particular instrument, however, must be established through the analysis of standard mixtures of known concentration. Preferably, these mixtures should contain at least some components identical to the components of the unknown mixture. Standard quantitative mixtures containing equal weight concentrations of numerous fatty acid methyl esters are available from the Hormel Institute, Austin, Minnesota. In addition, standard mixtures comparable to the NIH standards (Horning *et al.* 1964) are offered by Applied Science Laboratories, State College, Pennsylvania. Such standards should be used prior to the analysis of the unknown sample to determine the overload characteristics and the response with respect to a variation in chain length and unsaturation of the particular instrument in use.

The determination of the composition of a mixture that has been analyzed by gas chromatographic means requires the measurement of the area under each peak. Numerous methods of measurement of these curves have been developed.

The area under a peak may be approximated by cutting out the paper beneath a curve and weighing it, by planimetry, by triangulation, by automatic integration, or by application of a formula of either the product of the peak height and the base at one-half height or the product of the retention time and the peak height. Application of some of these methods is shown in Fig. 4. Since the retention time of each peak must be measured for qualitative analysis, the method of measuring peak areas by the

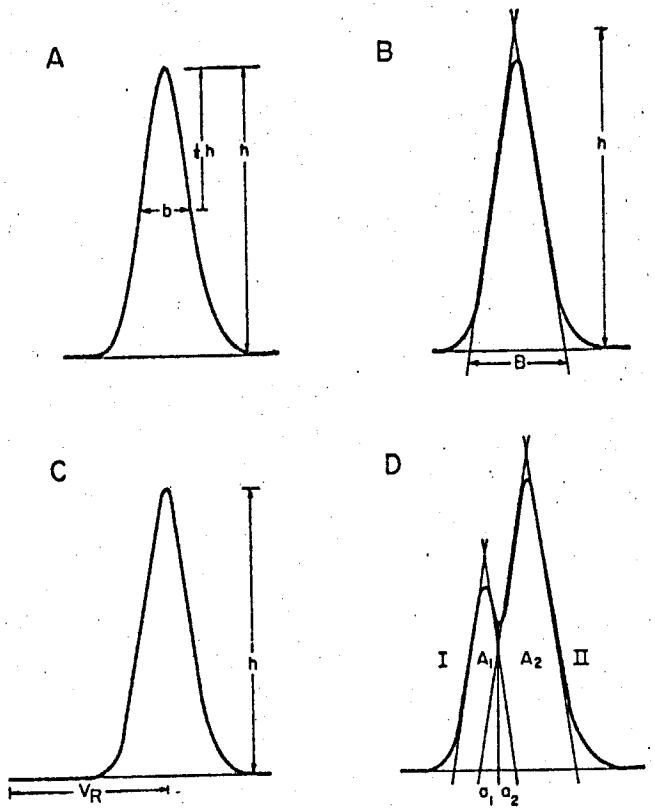


FIG. 4. COMMON METHODS OF MEASUREMENT OF PEAK AREAS

A, Peak height times base at one-half height. B, Triangulation; Peak height times one-half the base. C, Retention time times peak height. D, Measurement of incompletely resolved peaks by triangulation.

product of retention time times the peak height is the most convenient method (Carroll 1961). However, the more direct methods of measurement, such as triangulation or the product of the peak height times the base at one-half height, are probably more accurate where overlapping peaks occur. Figure 4-D shows two overlapping peaks and the method of correcting the areas by triangulation.

COMPLEMENTARY METHODS TO GLC

Gas-liquid chromatographic analysis of fatty acid methyl esters or of other lipid components of fish oils should not be used to the exclusion of other methods of identification. Thin-layer chromatography, for example,

is a valuable aid to the separation and identification of individual fatty acid as well as other lipid components of fish oil (Malins and Mangold 1960). Furthermore, characterization of individual acids after isolation may be accomplished by determination of their critical solution temperature (Schmid *et al.* 1965). Structural analysis of the unsaturated fatty acids may be accomplished by trapping the individual unsaturated peaks from the effluent of the gas chromatograph and subjecting these compounds to ozonolysis followed by gas chromatography of the products of the degradation (Privett *et al.* 1963). Gas chromatography should be considered to be a useful tool that in conjunction with other techniques, it aids in the more complete analysis of fish oils.

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Sci. Am. 228(6): 22-29, 1973

THE ANCHOVY CRISIS

Over the past decade the world's largest fishery has been in the Peru Current. A periodic ecological disturbance, combined with the heavy fishing, now threatens to destroy the industry

by C. P. Idyll

The world of the Peruvian anchovy is the sweep of a great cold ocean current. In a slow northward drift the current carries the little fishes along in company with countless tiny plants and animals that the anchovies avidly devour and with larger fishes, squids and a host of other marine animals. The anchovies form thronging legions that wheel and dart in the current. Their world is often entered by aliens: birds that plunge from above, snatching up the anchovies by the hundreds of thousands, and men who cast great net enclosures around the fishes, carrying them off to shore by the millions.

In the brief life-span of the anchovy, rarely longer than three years, its cold-current environment usually changes only within narrow limits. During the lifetime of some generations, however, their world may be put out of joint. The slow northward drift of the current, only two-tenths to three-tenths of a knot (compared with the six knots of the Gulf Stream off Florida), becomes still slower and may even reverse itself. The water grows warmer and less salty; the makeup of its populations changes and many of the usually abundant microscopic plants and animals dwindle in number. Finding their world poorer, the little anchovies scatter; many may die prematurely and many in the successor generation may not be born at all. The sea change known as El Niño has arrived.

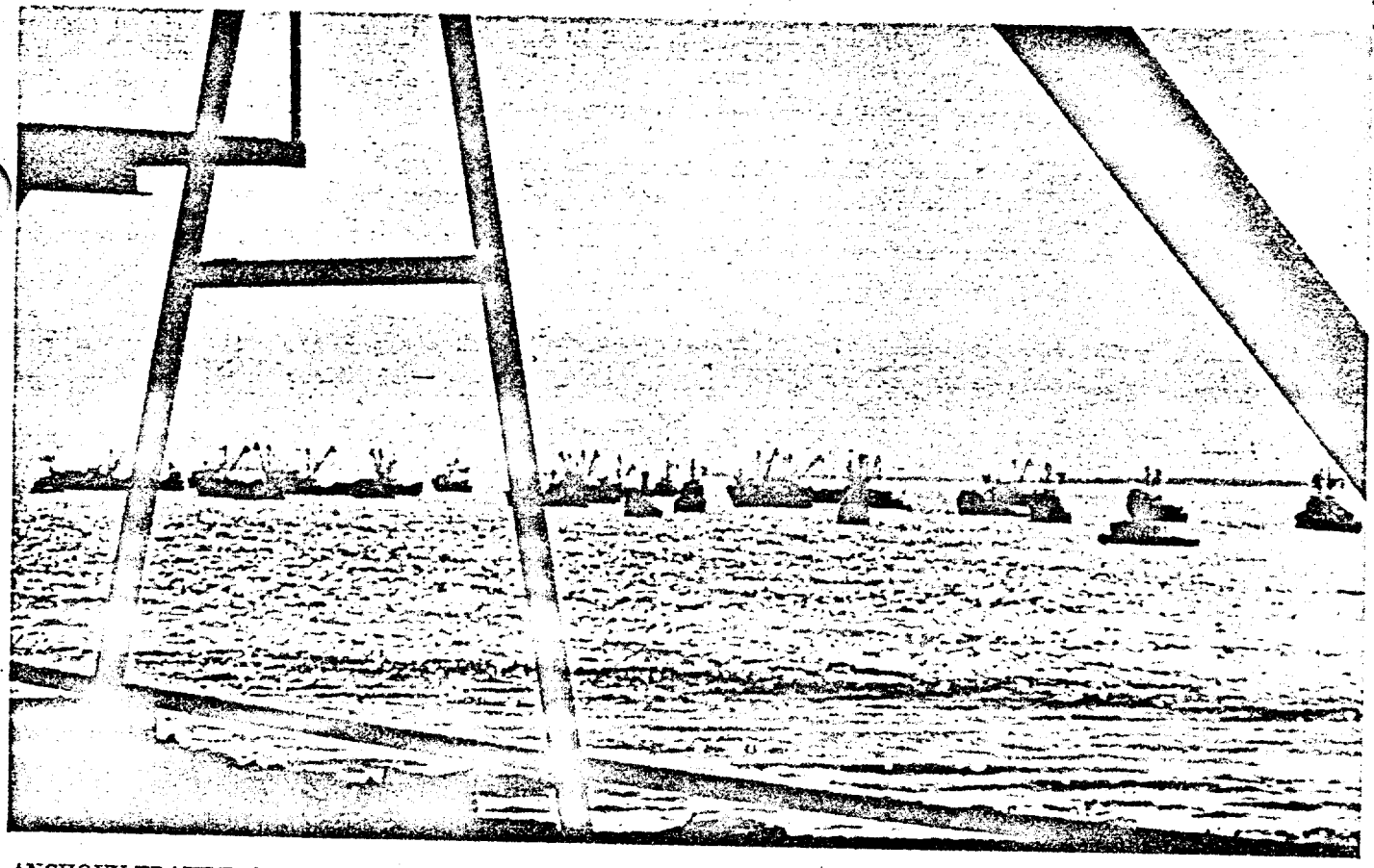
Coastal Peru is normally a cool and misty land, quite unlike the steamy Tropics that occupy the same latitudes on the eastern coast of South America. The Peru coast is kept that way by the temperature of the ocean current, which in the south can be as cool as 10 degrees Celsius (50 degrees Fahrenheit) and only reaches about 22 degrees C. (71.6 degrees F.) in the north. Although the northernmost part of Peru is a mere three degrees of latitude below the Equator, the average air temperature is a moderate 18 to 22 degrees C. (64.4 to 71.6 degrees F.). Along the 1,475 miles of Peruvian coastline, a distance 100 miles longer than the Pacific coast of the U.S., there are no marshes, mud flats and estuaries—only arid desert, most of it a treeless, monotonous, barren brown. This bleak strip of sand extends a short distance inland, rarely more than 40 miles or so, to the upthrust Andes, one of the most awesome mountain ranges in the world. The high rain shadow of the Andes robs the prevailing southeast winds of their moisture and keeps the coastal region arid. A few streams that rise in the mountains cross the desert; their narrow valleys support what little agriculture exists along the coast.

The Peru Current consists of four components, the interaction of which creates, molds and changes the world of the anchovy. Two of the components travel in a northerly direction: the Coastal Current, flowing next to the shore, and the Oceanic Current, located farther out to sea. Between the two, on and near the surface, runs the Peru Countercurrent. Beneath all three runs the Peru Undercurrent. Both the Countercurrent and the Undercurrent flow southward.

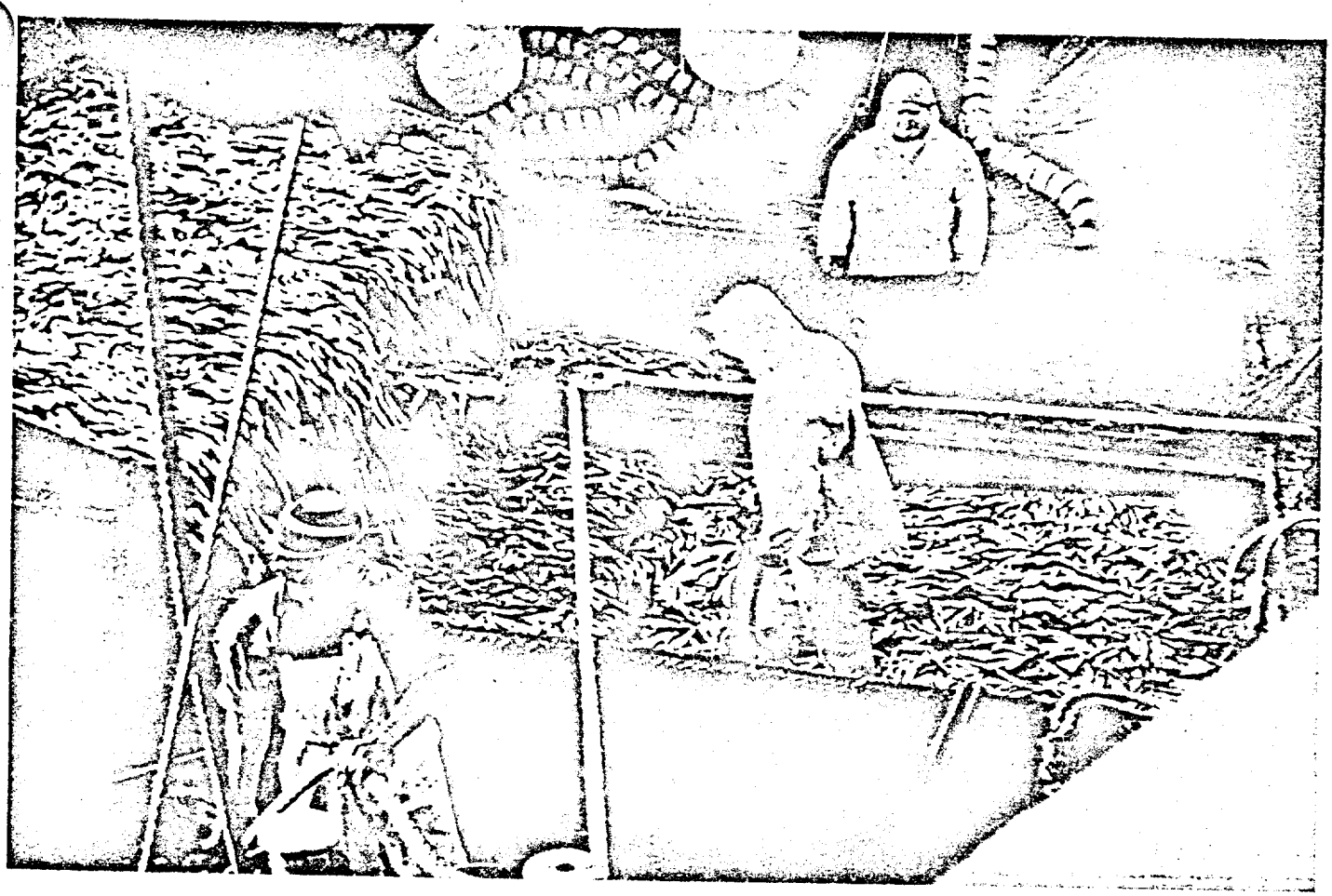
The Coastal Current runs deep and hugs the land from about Valparaiso in Chile in the south to north of Chimbote in Peru, a stretch of some 2,000 miles. The anchovies live mostly in the northern part of this great band of water, which constantly changes shape and size, becoming wider or narrower, deeper or shallower, altering and twisting like an elongated amoeba. The Oceanic Current is longer than the Coastal Current. It is often several hundred miles wide, and it runs as deep as 700 meters. It flows north to a point about opposite the Gulf of Guayaquil before bending west.

The great northward sweep of water is often called the Humboldt Current, after the German naturalist who described the phenomenon following a visit to South America in 1803. Humboldt thought that the cold of the water was the chill of the Antarctic. His conjecture was partly right: the current does include subantarctic water. Much of the cold, however, is the cold of subsurface water. As the water on the ocean surface is swept away by the prevailing winds, deeper low-temperature water wells up slowly to replace it. The trade winds in this part of the world, channeled and bent by the Andes, blow from the south and southeast, mostly parallel to the shore. This prevailing wind urges the surface water northward at the same time that another influence, the Coriolis force, deflects it to the west. As the resulting steady offshore drift skims off the surface layer the cold subsurface water rises with stately slowness to replace it, traveling vertically at a rate ranging from 20 to 100 meters per month, depending on the location and the season.

The biological effect of the upwelling is enormous. That stretch of water, only a tiny fraction of the ocean surface, produces fully 22 percent of all the fish caught throughout the world. Its richness springs from a constantly renewed supply of the chemical nutrients—principally phosphates and nitrates—that stimulate plant growth. Accumulated gradually in the deep layers of the ocean as the debris of dead marine plants and animals sinks to the bottom, the nutrients travel with the upwelling water to the top levels. There the light is sufficient to drive photosynthesis, and the nutrients help the marine plants to flourish. The concentration of nutrients in the Peru upwelling is many times greater than that in the open ocean. In terms of



ANCHOVY TRAWLERS by the score lie at anchor in the fishing port of Pisco in Peru. Structures in the foreground are part of a fish-meal factory. Tonnage of anchovies alone landed in Peru in a normal year outweighs the combined fish catch of any other nation.



CASCADE OF ANCHOVIES spills from a draining grate into the cargo hold of a trawler as crewmen keep watch. The fish gather by the billions near shore and close to the surface, particularly in summer months; trawlers can then net anchovies 100 tons at a time.

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Biochem. J., 32: 462-466, 1938

LX. A NOTE ON THE DIGESTION OF FATS BY PANCREATIC LIPASE

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(*Received 19 January 1938*)

Fats have for some time been recognized as basal dietary constituents, though even now it is not definitely established whether or not these constituents should be regarded as essential food factors. That animals eat a variety of fats, both of animal and vegetable origin, is widely recognized and there is evidence that man can digest a number of fats other than those which are normally included in his diet.

Special consideration was given to this type of food when a Committee of the Royal Society [1918–19] considered the possibility of utilizing fatty acids as food. Burns & Sharpe [1918–19] concluded that the fatty acids of whale oil were as thoroughly digested and absorbed as was the hardened oil from which they were prepared. Gardner & Fox [1919] found that cocoabutter could be satisfactorily utilized by man. Barbour [1934], working with rats, showed that peanut oil or cottonseed oil could be effectively metabolized when it constituted 20% of the diet. Langworthy & Holmes [1917, 1], from experiments on man, found that olive oil, cottonseed oil, peanut oil, coconut oil, sesame oil and cocoabutter could safely be included in his diet, but concluded that the limit of tolerance was less for cocoabutter than for the other fats studied. With animal fats these observers [1915] found the coefficient of digestibility of butter to be 97·1%, of lard 97·3%, of beef fat 93·1% and of mutton fat slightly less, 87·6%: for cream the figure was 96·9% [1917, 2].

In the experiments just quoted the subjects were left entirely free as to the amount of food they consumed and wide variations were noted. For instance, with beef fat the average amount taken per day was 100 g. and with mutton fat only 53 g. This work demonstrated that various fats could be adequately digested and assimilated by man, but afforded no information as to the respective rates of digestion of the fats. Weber & King [1935] found that the α- and β-monoglycerides of lauric, myristic, palmitic and stearic acids were hydrolysed with equal facility by pancreatic lipase, but on the whole there appears to be little experimental evidence as to the comparative rates of digestion of different fats.

Experimental

Various methods for emulsifying fats have been used by different workers. A method in general use involves the use of alcohol, but other methods have been suggested such as the use of gum acacia or neutral sodium oleate as emulsifying agents [Weinskin & Wynne, 1935–36]. Such methods have no direct application to digestion in man and it was desirable to use the fat in a form in which it could be taken as food. At first an emulsion in water [Plimmer, 1933] was tried, but this method could not be used because it was applicable only to some and not to all fats. Reconstituted creams in which various fats were substituted for butter were next tried and this method proved satisfactory.

Preparation of creams

The creams used in these experiments were made with a well-known type of machine and it was found that once the machine was "run in" a standard product could always be obtained. It had been shown previously [Hartwell, 1937] that the best emulsification was obtained with the proportion of 57 g. butter to 105 ml. milk: the cream so made contains approximately 33 % of fat. With other fats which contained little or no water, 47 g. fat were added to 105 ml. milk and 10 ml. water. When creams were kept overnight they were placed in a refrigerator, previous experiments [Hartwell, 1937] having shown that keeping them in this way made no difference to their rate of digestion. Such creams were allowed to stand for half an hour after being made, so that the layer of air bubbles which came to the top could be removed. If this precaution were not taken, a thick crust formed which rendered an even mixing of the cream impossible.

Beef, mutton and bacon fats were prepared in the following way. Any lean was removed and the fat was cut into small pieces and heated in a gas oven for ½–¾ hr., after which the liquid fat was poured off, allowed to cool and used for the experiments.

In making the creams the fat was melted and the milk was heated to boiling point; the machine was also heated.

Butter cream was made by pumping the mixture once through the machine. With all the other fats the mixture was pumped through three times to ensure complete emulsification, because it was found that some fats (especially olive oil and lard) were apt to oil out on top if only put through once. Some preliminary experiments were made with creams which had been put through the machine six and nine times. These creams, however, digested at the same rate as those which had been put through only three times, therefore it seemed unnecessary to take this extra precaution. Microscopic examination showed that all the creams consisted of very small fat globules, and no differences in the sizes of the fat droplets could be detected.

Method of experiment

The commercial form of pancreatic substance used as a source of lipase was that found by Clifford [1934] to be suitable for this type of work. In a typical experiment 50 ml. cream, 175 ml. distilled water and 25 ml. of 2–4 % pancreatic substance (the exact strength depending on the activity of the sample) were used.

The cream was measured in a 50 ml. graduated cylinder, and washed into a flask with the distilled water, the pancreatic substance being added last. The reagents were mixed cold, not warmed separately as is usual in digestion experiments.

The sample of lipase used was active at room temperature (19–19·5°), and in some cases digestion started so rapidly that the initial stages would not have been recorded had the reagents been at 38° when mixed.

Immediately after mixing 20 ml. were taken for titration, further 20 ml. samples being removed every 15 min. To the 20 ml. digestion mixture were added approximately 150 ml. boiling distilled water and 2 ml. phenolphthalein and the whole was titrated hot with 0·1 *N* NaOH. The flasks were placed in a large water bath and the temperature was maintained at 38–39°.

Butter cream was used as a standard for comparison and control experiments were made with milk and with boiled pancreas substance and cream. The fats

used were almond oil, arachis oil, bacon fat, beef fat, beef oleo, beef stearin, castor oil, cocoabutter, coconut oil, cod liver oil, cottonseed oil, lard, mutton fat, olive oil, palm oil, palm kernel oil, premier jus, soya bean oil, hardened whale oil and hardened arachis oil.

Blank titrations of the creams alone gave 0·25–0·3 ml. of 0·1 N NaOH for butter. The other creams ranged from 0·2 to 0·3 ml. except beef oleo, premier jus and castor oil which gave slightly higher values, 0·4–0·45 ml. The slightly higher figure for the first titrations of some creams is due to the rapid start of digestion. Palm oil had an appreciably higher value, 1·8 ml., and owing to its yellow colour, it was difficult to judge the end point of the titration.

Results and comments

Butter cream was taken as a standard for comparison and each experiment included three or four of the other creams. These were arranged in different combinations and at least three experiments were made with each.

A comparison of lard, fat from smoked bacon and fat from green bacon showed that there was no difference in the rates of digestion of these three fats. Fat prepared from beef suet and from scrap beef fat also gave similar digestion curves, as did mutton suet fat and scrap mutton fat, so it was concluded that it was unnecessary to use more than one type of fat from any animal.

When it was found that beef and mutton fats were digested so slowly when compared with butter fat, it was thought possible that heating the fats might have had some detrimental effect. In order to see if this supposition were true two samples of butter were heated in a gas oven, one sample at the same time as some beef suet, and the other sample with some mutton suet. The digestion of the fat in creams made from these heated butters showed no alteration in rate, therefore it was unlikely that heating the beef and mutton fats was the cause of the slower digestion.

The majority of the fats used were digested by lipase much more slowly than was butter fat, three only showing a more rapid rate of digestion. Coconut oil was digested more rapidly than any other fat: three separate samples were used and all gave similar digestion curves. Palm kernel oil also showed a rapid rate of digestion, but not so rapid as the coconut oil. Castor oil was digested more rapidly than butter, but the curve obtained with this fat was of rather a different type from the other curves.

Almond oil, arachis oil, bacon fat, beef fat, beef oleo, beef stearin, cocoabutter, cod liver oil, cottonseed oil, lard, mutton fat, olive oil, premier jus, soya bean oil, hardened whale oil and hardened arachis oil were digested at a much slower rate than butter and all at approximately the same rate. This is in agreement with the experiments of Gardner & Fox [1919] who found that cocoabutter was rather less digestible than butter. Palm oil digested slowly, but could hardly be compared with the others, owing to the difficulty in judging the end point because of the orange colour of the fat itself. The experiments, however, served to demonstrate that palm kernel oil belongs to the group of fats which are digested slowly by pancreatic lipase.

That coconut oil is digested so much more quickly than butter may be of dietetic interest in that this oil is said to be used for the making of cream buns. It seems possible that if margarines contain this fat they may be more rapidly digested than butter. Except in the case of castor oil, the rate of digestion runs parallel with the saponification value of the fats. Thus of the fats used, coconut oil has the highest saponification value [Bolton, 1928] and is the most rapidly

digested. According to Lewkowitsch [1923] palm kernel oil is very nearly related in its chemical composition to coconut oil and its digestion curve (Fig. 1) is nearer to that of coconut oil than that of any other fat. Wright & Mitchell [1921] state that in butter and coconut oil fatty acids of low molecular weight

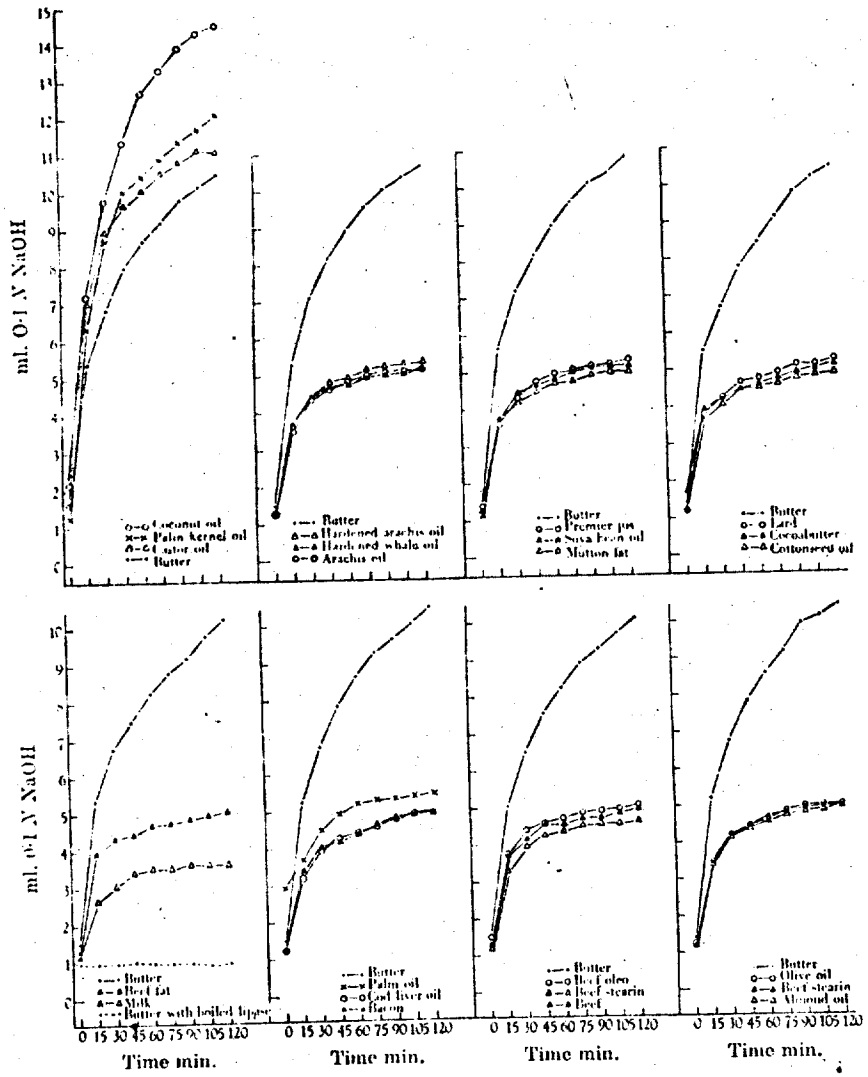

Fig. 1.

are present "to some notable extent", while as a general rule natural oils and fats rarely yield fatty acids of that description. It is possible that this may be correlated with the more rapid rate of digestion of coconut oil and butter fat by lipase. Evans *et al.* [1934] put coconut oil first in order of fats able to spare vitamin B. If, as suggested by Burr *et al.* [1932] linoleic is an essential fatty

acid, then coconut oil which contains 1% [Chemists' Year Book 1934] and is rapidly digested, may prove a more valuable food than has hitherto been supposed.

Summary

1. Coconut oil was digested by pancreatic lipase more rapidly than any other fat.

2. Palm kernel oil and castor oil were digested more rapidly than butter fat, while almond oil, arachis oil, bacon fat, beef fat, beef oleo, beef stearin, cocoabutter, cod liver oil, cottonseed oil, lard, mutton fat, olive oil, palm oil, premier jus, soya bean oil, hardened whale oil and hardened arachis oil were digested appreciably more slowly, and at approximately equal rates.

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Fed. Proc., 23: 1062-1067, 1964

Nutritional and metabolic interrelationships between fatty acids[1]

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THE INFLUENCE of dietary components upon the composition of depot fat of animals has been known for generations. It is common knowledge that swine fed starchy cereals develop a hard fat, whereas peanut-fed swine develop a liquid lard. The interrelationships between composition of dietary fat and the composition of animal fats have been studied repeatedly by empirical feeding trials involving different fats and, more recently, by more precisely controlled nutritional experiments, and this information already comprises a large literature. The transition from practical trial to detailed knowledge has encompassed several decades and has moved in proportion to the precision of the analytical methods available. In recent years the availability of gas chromatographic methods has made possible the detailed study of the composition of complex fats, and simultaneously the advent of methods of preparation of pure fatty acids on a large scale has made possible nutritional study involving single fatty acids and combinations of pure fatty acids. The result of these developments is that at present one can resolve the effects of dietary fat into several effects of dietary fatty acids. It has been possible to study the metabolism of single fatty acids and to study the influences of each fatty acid upon the metabolism of others. This field of study is still in infancy and the results available thus far encompass only a few of the numerous fatty acids found in foodstuffs. An attempt will be made here to treat the interrelationships thus far known to exist between polyunsaturated acids (PUFA) and between these, monoenoic acids, and the saturated acids.

INTERRELATIONSHIPS BETWEEN POLYUNSATURATED FATTY ACIDS

Shortly after Burr and Burr (3) described the essential fatty acid (EFA) syndrome, Nunn and Smedley-Maclean (25) investigated the fatty acids of the liver of EFA-deficient rats. They discovered that although the highly unsaturated acids of livers of normal rats reacted with bromine to produce octabromarachidic acid, the liver unsaturated fatty acids from deficient rats yielded polybromides with a lower bromine content. This they deduced to be due to the presence in the deficient animal of what they called dihydroarachidonic acid. This trienoic acid was found to increase sharply in several tissues of EFA-deficient rats by Rieckehoff et al. (31), using the alkaline isomerization procedure developed in the 1940's for analysis of polyunsaturates. Later, Fulco and Mead (10) found this acid to be 5,8,11-eicosatrienoic acid and showed it to be a metabolite derived from oleic acid. The appearance of this trienoic acid in deficient animals and its suppression by the administration of linoleate probably constitutes the first observations of an interrelationship between specific fatty acids.

The development of alkaline isomerization permitted study of the fate of dietary PUFA in the animal. Rieckehoff et al. (31) showed that the PUFA composition of the lipids of several organs of the rat were related to the dietary fat. For example, the feeding of corn oil to rats increased markedly the tetraenoic acid content of tissue lipids, and feeding cod liver oil altered the composition of tissue lipids in quite a different way. The deposition of pentaenoic and hexaenoic acids was so marked that the lipids of the heart approached cod liver oil in respect to its content of those acids. This study was extended by Widmer and Holman (36) to a study of the changes occurring when single unsaturated fatty acids were fed to rats receiving no other fat. Their results indicated that the tetraenoic acid, arachidonic acid, was formed from dietary linoleate and that pentaenoic and hexaenoic acids appeared in the animal when linolenate was fed. Thus, the suspected relationship of linoleate and arachidonate was confirmed and the relationship of linolenate to pentaenoic and hexaenoic acids was indicated. The study also revealed that linoleate and linolenate were not interconverted, for the metabolites from one did not appear when the other was the only dietary fatty acid.

The metabolic relationships suggested by nutritional and compositional studies have been confirmed by elegant studies in which radioactive fatty acids were used. By this means the metabolic conversion of linoleic to arachidonic acids has been proven by Steinberg et al. (32). The comparable conversions of linolenate to

[1] This work was supported in part by Research Grant AM-04524 from the National Institutes of Health.

eicosapentaenoic and docosahexaenoic acids have been shown as well (16, 33, 36). Some steps in the metabolic sequences remain to be confirmed, but the general outlines of the interconversions are now well known.

Much of the nutritional experimentation which led to the knowledge of the essentiality or nonessentiality of individual acids and their isomers and to the metabolic conversions of polyunsaturated acids in animals was by means of comparisons of nonsupplemented animals with animals supplemented with single dose levels. This approach does not allow description of how tissue lipid composition varied with changes in the composition of diet. The usefulness of the latter approach was inadvertently revealed through an experiment in our laboratory designed to be a multiple comparison of supplements fed at single dose levels. The interpretation of the results was very confusing until it was realized that the several supplements could be considered as multiple dose levels of linoleate (12). This realization has led to a program of study of dose-response curves for several single pure unsaturated fatty acids and for combinations of them. These studies shall be the major portion of the discussion to be presented here.

Despite the use of three levels of total dietary fat and three quite different dietary fats, the influence of dietary linoleate upon the content of dienoic, trienoic, and tetraenoic acids in the lipids of supplemented rats was found to follow exponential curves (12). In order to combine the responses of trienoic acid and tetraenoic acids in a single expression related to deficiency and in order to remove variation due to differences in extraction and handling of lipids, the ratio of triene to tetraene in the tissue lipids was plotted against dietary linoleate. Triene and tetraene have the inherent advantage that neither is a significant component of common dietary fats, and both are metabolic products, one from oleate, the other from linoleate. When this was done, the curve described a hyperbolic function, one leg of which represented high values in deficiency and the other low values in well-supplemented animals. The curve was used to set the minimum requirement for linoleate, which lies between 1 and 2% of calories for rats (12), swine (11), and infants (13).

The dose-response curves have also been obtained for linoleate, linolenate, and arachidonate fed as single pure supplements, and the curves have been described for liver (20), heart (21), brain (22), and erythrocytes and adipose tissue (23). The family of curves for heart polyunsaturates in linoleate-supplemented rats is shown in Fig. 1. In this study the conversion of linoleate to 20:4ω6 and 22:5ω6 was clearly demonstrated. Likewise, linolenate is converted primarily to 20:5ω3, 22:5ω3, and 22:6ω3.[2] The nonconvertibility of members of the linoleate family to members of the linolenate family and vice versa was also confirmed conclusively.

[2] The notation used here is required to distinguish the various isomers of PUFA and indicate relationships. The first number indicates chain length, the second indicates the number of double bonds, and the number following the omega indicates the position of the last double bond, counting from the terminal methyl groups.

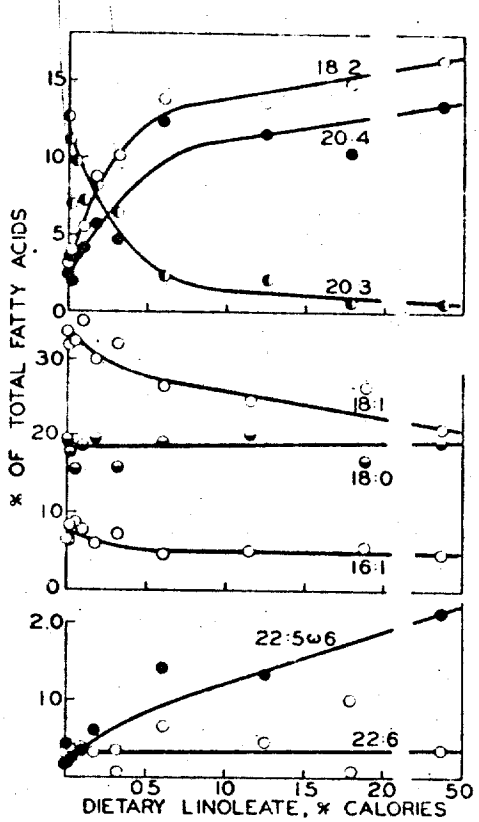

FIG. 1. Relationship of fatty acid composition of heart lipids to level of dietary linoleate in the rat (21).

The study also indicated that the PUFA of rat tissues belongs principally to the linoleate, linolenate, and oleate families although other minor groups are present.

In these several studies a depression of metabolites of linoleate by high levels of dietary linolenate is also indicated. Depression of PUFA of one family by feeding members of another family has been observed several times previously (5, 9, 14, 17, 28, 29, 31, 36), but the significance of the observations was not then apparent. Dhopeshwarkar and Mead (7) postulated an inhibition of the metabolism of linoleate by oleate, and Marco et al. (19) and Machlin (18) observed a depression of arachidonate by dietary linolenate and postulated a competitive inhibition between linolenate and linoleate in their metabolisms. Bozian and Coniglio (1, 2), Edwards and Marion (8), and Mohrhauer and Holman (20) have postulated that linoleate, linolenate, and arachidonate competed with oleate metabolism to depress the synthesis of eicosatrienoic acid.

Once the competitive inhibition in the metabolism of PUFA was recognized, experiments planned for the study of the phenomenon were necessary. A repetition of the observations of Marco et al. and Machlin, in which linolenate was the antagonist of linoleate metabolism, were studied first, by using purified linoleate and linolenate in appropriate ranges of dietary intake (24). At three levels of dietary linoleate (0.1, 0.3, and 0.6% of calories) the metabolism of linoleate to arachidonate

was significantly and consistently depressed by increasing levels of dietary linolenate. Figure 2 shows the effect of level of linolenate intake upon the metabolites of linoleate measurable in liver total fatty acids. In addition to the depression of arachidonate content of liver lipids, an equally significant depression of the content of 22:5ω6 was also observed. The effect upon 20:3ω6 was minimal, but the accumulation of 18:2ω6 itself, as reported by Machlin, is clearly seen. These results indicate that the higher metabolites of linoleate are inhibited most and that linoleate itself accumulates under conditions of inhibition.

In a short-term study of the inhibition of arachidonate metabolism by linolenate, similar results have been observed (Rahm and Holman, unpublished data). In EFA-deficient rats which were fed a constant level of

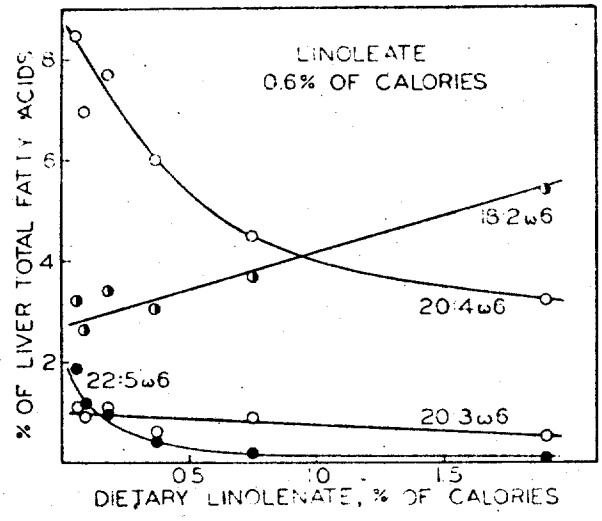

FIG. 2. Effect of dietary linolenate upon metabolism of linoleate (24).

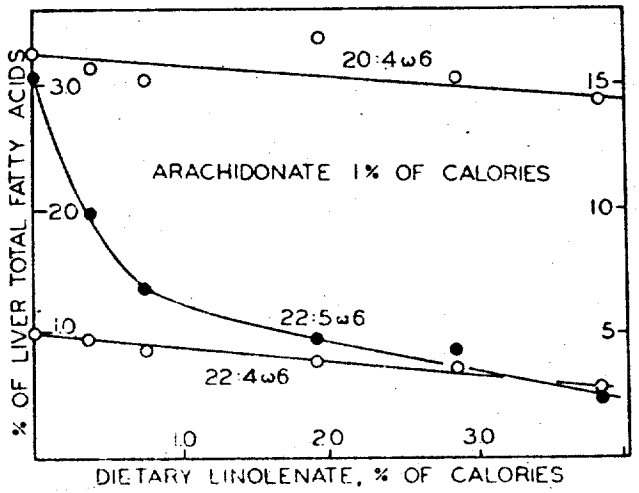

FIG. 3. Effect of dietary linolenate upon metabolism of arachidonate as demonstrated in a short-term experiment. (Rahm and Holman, unpublished data).

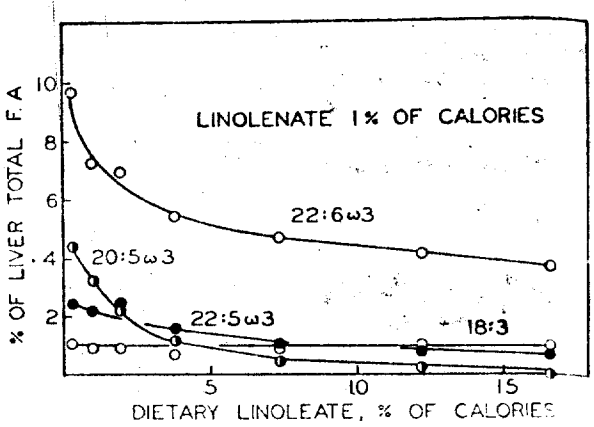

FIG. 4. Effect of linoleate upon metabolism of linolenate. (Rahm and Holman, unpublished data).

arachidonate for 5 days, a second supplement of linolenate caused inhibition of its conversion to 22:4ω6 and 22:5ω6. These results are shown in Fig. 3. In this study arachidonate did not accumulate as a consequence of inhibition of its metabolism. The study revealed that inhibition at the later steps of metabolism is not due only to inhibition at earlier steps. Linolenate apparently competes at more than one step in the metabolic chain.

If this phenomenon is indeed competitive inhibition, then inhibition of linolenate metabolism by linoleate should be demonstrable. This had been indicated fortuitously in one of the earlier studies (30). Therefore, an experiment was planned to test this possibility. Linolenate was fed at a constant level of 1% of calories to all groups of weanling male rats, and the groups were given different levels of dietary linoleate. The effect upon the metabolites of linolenate is shown in Fig. 4. The concentration of linolenate itself seems unaffected by levels of linoleate which cause striking depressions in the concentrations of the metabolites of linolenate. All three of the measurable linolenate metabolites, 20:5ω3, 22:5ω3, and 22:6ω3, were inhibited by dietary linoleate. Thus, the inhibition of linoleate metabolism by linolenate is paralleled by the inhibition of linolenate metabolism by linoleate. The inhibition is competitive.

The competitive phenomena observed between the linoleate and linolenate families of polyunsaturates are not mere dilution effects. If that were so, the appearance of, for example, linolenate and its metabolic products in the lipids of a tissue should diminish the concentrations of the metabolites of linoleate in a proportional manner. This is not the case, for as dietary linolenate is increased, linoleate itself increases, whereas 20:4ω6 and 22:5ω6 decrease exponentially in a parallel fashion, and 22:3ω6 decreases only slightly in a linear fashion (Fig. 2). The same conclusion can be drawn from an examination of the successive precursor:product ratios for the metabolite sequences (15).

A comparison of the curves in Figs. 2, 3, and 4 suggests that perhaps the chain-lengthening steps in the metabolism are inhibited to a different degree than are

the dehydrogenation steps. The information is not complete on this point, and the relative effects of inhibition of individual steps in the chain cannot be deduced from steady state concentrations. Nevertheless, the steady state concentrations of those substances formed by dehydrogenation steps respond to dietary inhibition in a sharply exponential manner, whereas those formed by chain lengthening respond weakly in a seemingly linear fashion. This point should be investigated with labeled fatty acids.

MONOENE-POLYENE RELATIONSHIPS

Similar attempts have been made to describe the competition between oleate and linoleate (Rahm, Mohrhauer, and Holman, unpublished results). Groups of rats were fed either 0, 0.2, or 1.0% of calories of methyl linoleate, and subgroups were fed levels of methyl oleate varying from 1 to 7.6% of calories. The effects of 100 days of such dietary treatment upon the content of 20:4ω6 and 22:5ω6 in heart and liver lipids was minimal. However, when the effects upon 20:3ω9 and 20:4ω6 were combined in the 20:3–20:4 ratio, the inhibition by oleate is apparent (Fig. 5). At the zero level of dietary linoleate, the residual 18:2 in the deficient animals is variable and sufficiently low to permit a heightening of the triene:tetraene ratio by supplements of oleate. At 0.2% of calories of linoleate, the effect is still perceptible, but at 1% of linoleate calories the effect is not exerted.

Dhopeshwarkar and Mead reported that dietary

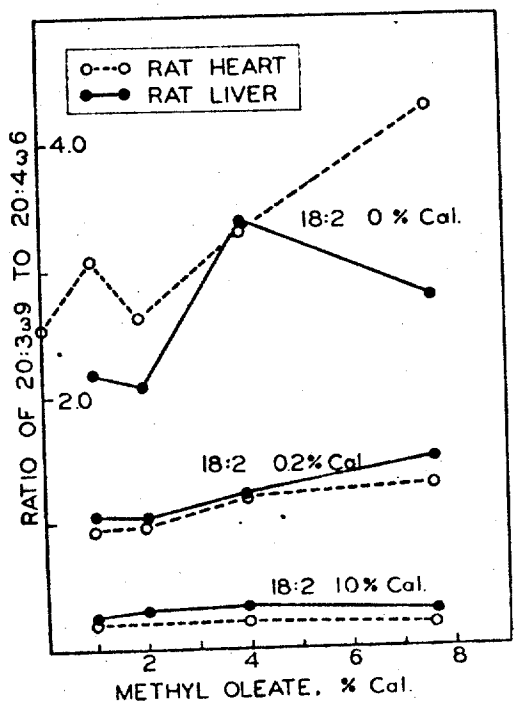

FIG. 5. Inhibition of linoleate metabolism by oleate as revealed by an examination of the triene:tetraene ratio in heart and liver lipids of the rat. (Rahm and Holman, unpublished data.)

TABLE 1. *Dietary levels of fatty acids required to induce 50% inhibition of metabolic reactions*

Inhibited Reaction	Inhibitor	Calories %	Reference
18:2ω6 → 22:5ω6	18:1ω9	ca. 70	
18:3ω3 → 22:6ω3	18:2ω6	3.2	
18:2ω6 → 22:5ω6	18:3ω3	0.1	(23)
20:4ω6 → 22:5ω6	18:3ω3	0.6	
18:1ω9 → 20:3ω9	18:2ω6	0.6	(19)
18:1ω9 → 20:3ω9	20:4ω6	0.25	(19)
18:1ω9 → 20:3ω9	18:3ω3	0.2	(19)

oleic acid induced an EFA deficiency (7). A similar study from this laboratory has shown that 10% of olive oil as sole dietary fat raises the triene:tetraene ratio in liver lipids and that more stainable fat was observed in the livers and the kidneys of the animals (6). These effects are consistent with marginal EFA deficiency. Tinsley (35) has observed that in rats fed pork diets which varied in oleate content, the fatty acid composition of the rat liver lipids varied in relation to the oleate intake. Moreover, there was a strong correlation between the oleate level in the liver lipids of the rats and the ratio of arachidonate to linoleate (product:precursor ratio). This suggests that the level of dietary oleate has an influence on the conversion of linoleate to arachidonate. Tinsley recently has reported (34) that as the proportion of dietary oleate increased to about 7% of the diet (about 15% of calories), the triene:tretaene ratio was observed to increase fourfold in heart phospholipids and liver mitochondria without any observable gross changes in the animals' performance.

The antagonistic effects of oleate upon linoleate metabolism appear to be minimal effects, produced only by high dietary levels of oleate or high oleate:linoleate ratios. Attempts in this laboratory to induce suppressions of linoleate metabolites of the order observed in inhibitions between linoleate and linolenate have thus far failed, although triolein has been fed at levels as high as 22% of calories. The only observable effects of oleate at dietary levels less than this limit are changes in the triene:tetraene ratio. The development of changes in composition indicative of EFA deficiency was stimulated only by very high ratios of dietary oleate to linoleate at comparatively low absolute levels of linoleate. This is demonstrated in Fig. 5.

The relative competitive abilities of oleate, linoleate, arachidonate, and linolenate are shown in Table 1. From the appropriate dose response curves for final metabolite shown in Figs. 2–4, and from other data from this laboratory, the levels of dietary inhibitor were chosen at which 50% inhibition of the over-all reaction occurred. In the instance of oleate, the value was found by extrapolation from a 15% inhibition at 21.6% of calories (Rahm and Holman, unpublished data). The data clearly indicate that oleate is the weakest inhibitor; that is, very high dietary levels are required to exert its effect. Linolenate is the most active inhibitor. Only 0.1% of calories of linolenate are required to inhibit the metabolism of linoleate to 50%, whereas in the converse 3.2% of calories of linoleate are required.

When a series of natural polyunsaturated acids was

fed at 0.8% of calories as sole supplements to a fat-free diet, the suppression of the conversion of oleate to 20:3ω9 was somewhat in proportion to the degree of unsaturation (29). This is shown in Table 2. The inhibition exhibited by the natural ω6 and ω3 acids and the relatively low activities of 19:2ω6 suggests that the terminal structure may not be the definitive feature in the metabolic competitive inhibition. There is no strict correlation between EFA activity and 20:3ω9 suggestion.

In the metabolism of oleate, linoleate, linolenate, and also other minor families of fatty acids, the fatty acids can undergo either chain lengthening or dehydrogenation, and either reaction can proceed first. This is illustrated in Fig. 6 for the metabolism of linoleate. In the metabolic mechanism depicted, all of the products of linoleate shown have been found in nature. Moreover, all have been found by gas chromatography in one tissue, in beef testis tissue, and all except 18:3ω6 in sufficient quantity for isolation and proof of structure by ozonolysis (Hofstetter and Holman, unpublished

TABLE 2. *Suppressing of metabolism of oleate and relieving of dermal signs of EFA deficiency by 0.8% of calories of several polyunsaturated acids**

Fatty Acid	Suppression of 20:3ω9, %	Relief of Dermatitis, %
18:2ω6	63	100
18:3ω3	81	77
20:4ω6	91	100
20:5ω3	92	69
22:6ω3	88	35
19:2ω6	28	65

* Data from (29).

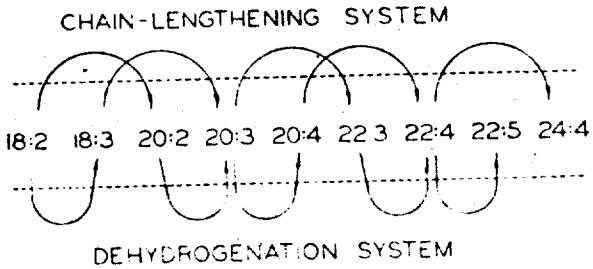

FIG. 6. Metabolism of linoleate, showing major possible pathways of chain elongation and dehydrogenation. All metabolites depicted have been isolated from testis tissue.

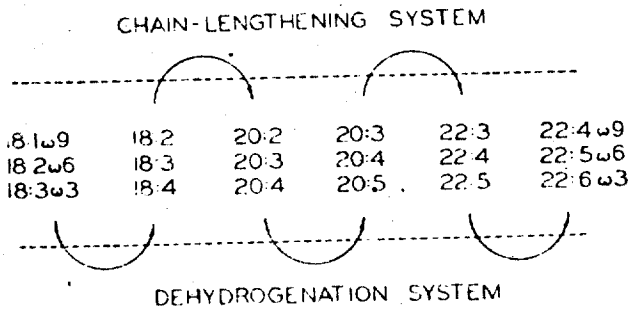

FIG. 7. Competitive metabolism of oleate, linoleate, and linolenate.

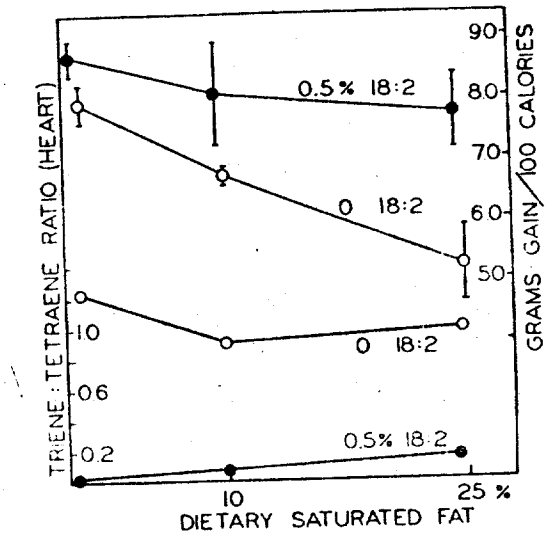

FIG. 8. Influence of dietary saturated fat upon caloric efficiency and triene:tetraene ratio of heart in rats fed diets containing zero or 0.5% linoleate (27).

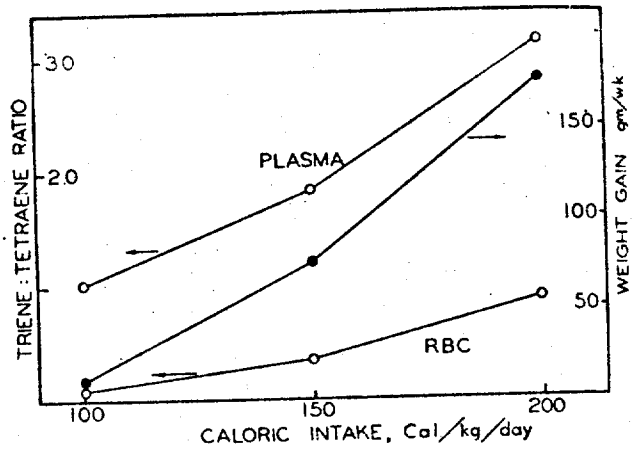

FIG. 9. Relationships between total calories, growth, and plasma triene:tetraene ratio in dogs. From data of Patil and Hansen (26) and Wiese et al. (37).

data). The competitive inhibition which exists between oleate, linoleate, and linolenate is depicted for alternate dehydrogenation and chain lengthening along the principal pathway in Fig. 7. Members of all three families exist in the metabolic pool at each step in the sequence, and competition may be exerted between these and between these and other members of the sequences. In the absence of linoleate and linolenate (EFA deficiency), the conversion of endogenous 18:1ω9 to 20:3ω9 and 22:3ω9 becomes dominant. When linoleate is present, its metabolic products are formed and suppress those of oleate. When linolenate is also present, its conversion takes precedence, and the metabolism of linoleate is damped. Thus, the composition of the tissue PUFA is dependent upon the dietary supplies of their precursors and is regulated by competitive inhibitions of their metabolisms.

RELATIONSHIPS BETWEEN SATURATED AND UNSATURATED ACIDS

In addition to the competitions between unsaturated fatty acids, dietary saturated fatty acids likewise exert influences upon the polyunsaturated acids of tissues. When the content of saturated fat in the diet is increased and linoleate is held constant, the weight gain of animals is decreased. Figure 8 shows this relationship for rats fed a diet containing either zero or 0.5% linoleate and varying amounts of hydrogenated coconut oil (27). In the linoleate-free diet, caloric efficiency decreased sharply as saturated fat was increased. The same was true for the diet containing the marginal level of linoleate, but the decrease was less. The triene:tetraene ratio of the groups fed linoleate-free diets was consistently high and did not respond to additional nutritional insult. However, at the minimal linoleate level, increased saturated fat produced a small but consistent increase in the triene:tetraene ratio.

The response quoted above for saturated fat may well be merely a response to caloric intake for, the diets fed were not isocaloric nor pair fed. An examination of data of Patil and Hansen (26) and of Wiese et al. (37), gathered in a study on dogs, reveals that the triene:tetraene ratio of the plasma lipids increases as caloric intake and consequent growth are increased (see Fig. 9). A correlation between the dermatitis of fat-deficient rats and their growth has been noted previously (4). The probable explanation for this effect is that when stores of essential fatty acid are limited, the growing organism depletes its supplies for the formation of new cells more rapidly than the organism whose growth is retarded. That is, on diets in which EFA content is marginal, a low caloric intake and low growth induces less severe deficiency than high intake and rapid growth.

From the various evidences reviewed here it is clear that the metabolism of unsaturated acids is influenced by the concentrations and kinds of other fatty acids present in the diet and in the metabolic pool. The metabolic conversions of polyunsaturated acids are dependent not only upon the concentrations of the various precursors, but also upon the concentrations of other families of polyunsaturated acids which act as competitive inhibitors. Thus, the concept of balanced diet must include a consideration of balanced concentrations of the several polyunsaturated, monounsaturated, and saturated fatty acids.

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Table 2

Fatty Acid Composition of Shellfish Oils

Name of Species: Common	Name of Species: Scientific	Fatty Acid Percentage Composition[1]: 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	Reference
Abalone	*Haliotis discus*	3.9	2.9	20.9	3.3	NK	5.1	16.4	1.2	—[2]	NK	4.8[2]	—[3]	8.8	10.7[3]	7.3	NK	Shimma 1964B
Abalone, Kid	*Haliotis japonica*	4.9	3.2	19.8	4.4	+	3.9	17.1	1.6	—[2]	0.9	5.9[2]	—[3]	10.0	12.3[3]	8.4	NK	Shimma 1964B
Barnacle, Goose	*Mitella polymerus*	1.9	0.5	24.7	2.4	1.8	8.1	10.5	1.2	NK	NK	3.6	1.8	20.4	1.0	0.5	17.2	Rodegker 1964
Clam	*Meretrix meretrix lusoria*	2.1	2.3	18.2	4.9	4.5	4.9	6.7	1.0	—[2]	4.4	6.8[2]	—[3]	10.0	5.1[3]	3.2	16.5	Shimma 1964B
Clam, Hen	*Mactra sulcataria*	4.0	3.2	29.8	10.5	NK	9.7	8.2	+	—[2]	2.0	6.7[2]	—[3]	9.5	1.7[3]	NK	4.6	Shimma 1964B
Clam, Littleneck	*Protothaca stimirea*	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 1964
Clam, Shortneck	*Tapes japonica*	2.7	2.6	18.8	9.4	+	8.9	8.9	+	—[2]	3.1	7.7[2]	—[3]	10.9	3.0[3]	+	14.3	Shimma 1964B
Corb shell	*Corbicula japonica*	4.7	3.1	20.3	10.7	+	5.1	8.3	1.9	—[2]	3.4	9.0[2]	—[3]	8.6	4.8[3]	3.9	10.3	Shimma 1964B
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 1964
Mussel	*Mytilus californianus*	3.5	0.2	24.5	1.1	NK	1.7	3.2	3.2	NK	1.6	2.6	1.0	14.0	NK	1.1	27.7	Rodegker 1964
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 1964
Oyster, Pacific	*Crassostrea gigas*	5.7	2.2	14.5	7.0	NK	3.2	10.9	2.1	—[2]	4.2	5.1[2]	—[3]	22.8	3.9[3]	NK	10.7	Shimma 1964B
Scallop, Sea	*Placepecten 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 1964
Starfish, Pacific	*Pisaster ochraceus*	14.0	1.2	20.3	6.2	2.1	7.0	5.4	1.2	NK	NK	23.0	NK	NK	2.0	NK	NK	Rodegker 1964
Top shell	*Turbo cornutus*	1.4	4.9	13.6	NK	NK	4.9	6.6	3.5	—[2]	+	3.3[2]	—[3]	6.7	16.4[3]	8.7	NK	Shimma 1964B
"Tori-gai"	*Fulcia mutica*	3.4	4.0	18.9	10.3	NK	10.0	5.5	0.8	—[2]	NK	5.1[2]	—[3]	11.6	2.9[3]	+	16.7	Shimma 1964B

[1] Fatty acid not known or not mentioned in reference indicated by NK. Trace amounts indicated by + sign, i.e. less than 0.1%.
[2] Combined 18:3 and 20:1 acids.
[3] Combined 20:4 and 22:1 acids.

Table 3

Fatty Acid Composition of Oils from Fish Livers[1]

Name of Species: Common	Name of Species: Scientific	Fatty Acid Percentage Composition: 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	Reference
Cod	*Gadus callarias*	1	+	12	5	1	3	24	1	1	1	9	1	8	5	1	19	Klenk 1962
Cod	*Gadus callarias* (?)	2.8	0.3	11.6	8.6	0.3	2.7	25.2	2.5	0.7	2.2	13.1	NK	9.3	6.3	1.0	8.6	DeWitt 1963
Cod	NK	2.9	NK	14.6	6.2	NK	3.5	39.0	1.7	0.3	+	9.1	2.1	2.6	4.6	1.5	9.7	Ito 1963B
Cod, Atlantic	*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 1964
Cod, Atlantic	*Gadus morhua*	3.5	0.5	10.4	12.2	0.1	1.2	19.6	0.8	0.2	0.7	14.6	1.7	5.0	13.3	2.0	10.5	Ackman 1964A
Dogfish, Piked	*Squalus acanthias*	3.0	0.6	15.0	4.9	1.0	3.4	19.0	1.4	NK	1.4	9.5	1.1	5.4	14.0	2.1	12.2	Olley 1962
Dogfish, Spiny	*Squalus acanthias*	1.6	0.3	13.2	5.7	1.0	4.3	28.5	0.7	0.6	0.8	10.5	0.8	3.7	10.3	3.1	6.5	Gruger 1964
Dogfish, Spiny	*Squalus acanthias*	2.7	0.3	23.2	6.8	0.8	4.0	35.7	0.7	0.5	NK	7.0	0.6	3.7	5.5	1.5	5.1	Malins 1965
Haddock	*Gadus aeglefinus*	2	+	16	6	1	3	30	1	+	1	4	1	15	1	1	14	Klenk 1962
Ling	*Molva molva*	2	+	15	4	1	3	37	1	1	1	6	1	6	3	1	15	Klenk 1962
Plaice	*Pleuronectes limanda*	3	1	14	8	2	1	22	1	1	+	2	3	15	+	6	12	Klenk 1962
Puffer	*Sphoeroides vermicularis*	5.7	NK	19.0	11.5	NK	7.3	24.0	NK	—[2]	NK	10.6[2]	—[3]	4.6	7.4[3]	4.2	5.7	Shimma 1964A
Sea devil	*Lophius piscatorius*	4	1	15	6	1	3	21	2	1	2	7	1	6	5	1	16	Klenk 1962
Shark	NK	3.3	NK	13.3	10.8	NK	2.9	25.2	2.3	0.4	1.4	9.2	3.1	9.2	6.6	3.4	7.3	Ito 1963B
Shark, Herring	*Isurus cornubicus*	3	+	13	3	1	2	9	1	1	2	22	+	3	31	1	5	Klenk 1962
Squid	*Illex illecebrosus*	4.4	0.2	13.7	9.0	0.4	1.8	16.3	0.8	0.7	0.8	12.4	0.8	13.9	8.2	1.4	16.9	Jangaard 1965
Turbot	*Rhombus maximus*	4	+	16	12	1	2	35	1	1	+	5	1	5	2	3	9	Klenk 1962
Whitefish	*Gadus merlangus*	4	+	16	4	1	3	25	2	1	2	7	1	8	6	1	16	Klenk 1962
Cod (eggs)	*Gadus morhua*	0.6	0.1	23.4	6.1	+	1.3	23.4	0.2	0.2	0.1	1.0	2.5	16.5	0.2	0.8	22.1	Ackman 1964C
Salmon, Pink (eggs)	*Oncorhynchus gorbuscha*	2.9	0.5	9.5	7.0	0.8	2.9	20.5	1.5	1.2	1.8	1.1	1.5	20.6	0.4	4.6	16.0	Gruger 1964

[1] Not known or not mentioned in reference is designated as NK. Trace amounts designated by a + sign.
[2] Combined 18:3 & 20:1 acids.
[3] Combined 20:4 & 22:1 acids.

respectively. The fatty acids listed in the tables are those generally common to all species. There are fatty acids that occur in trace amounts, which have been left out of the tables only to simplify the presentation. Where the latter acids seem important to a species, particular attention is drawn to them in the discussion.

Fish-Body Oil Fatty Acids

The data from many laboratories throughout the world illustrate markedly the wide distribution of fatty acids that can be found in fish body oils. Not only are there wide distributions in the types of fatty acids, i.e., those of different chain lengths and different degrees of unsaturation, as previously discussed, but more important there are wide differences in the relative amounts of individual fatty acids. The distribution of oleic acid, for example, which is generally thought to be the most common monoenoic acid in fish oils varies (in Table 1) from about 5 to 38% of the total fatty acids. Docosahexaenoic acid (22:6), as another example, can vary from about 3 to 38% according to the compiled data. As seen from the numerical values in Table 1, these fatty acid distributions are associated with the species differences and the sources of oil samples with respect to particular parts of fish. We emphasize that this latter point is quite important to interpretations that one may make because, for instance, the phospholipids, as a lipid fraction, contain proportionately more unsaturated fatty acids than do the triglycerides. As indicated earlier, the anatomical source of the sample has a bearing on whether or not an analysis is based more on one class of lipids than on another class. Thus, in some cases, it is noted in parentheses under the common name of the species in Table 1 the words dorsal and ventral, which cite the particular oil sample for those anatomical parts of the fish. Geographical and seasonal influences upon composition are not obvious from the data in Table 1, but these factors can be assumed for the cases of multiple data from the same species, e.g., Atlantic cod, spiny dogfish, menhaden, and mullet.

The data given in Table 1 represent only those of recent GLC analyses and do not represent all of the data available from other techniques. One might assume that wider distributions in fatty acid compositions than is presented in Table 1 are possible as additional data become available.

Oils from certain species of fish appear to exhibit above-average amounts of particular fatty acids. Nearly 10% myristic acid (14:0) is observed for capelin, menhaden, Peruvian sardine, skipjack tuna, some whales, and ventral tissues of shad. Palmitic acid (16:0) is usually about 15 to 20% of the total fatty acids, but some species, such as Atlantic cod, flying fish, mullet, and puffer, can have about 30% 16:0 acid. Rather large amounts of palmitoleic acid (16:1) have been found in capelin, mullet, lemon sole, and harbor seal. Similarly, stearic acid (18:0), which is generally about 4% of fish oil acids, can apparently range as high as 12 to 16% of the total.

The mullet appears to be a unique source of C_{15} and C_{17} acids. Up to 25% straight-chain odd-carbon fatty acids have been found in mullet (Gruger *et al.* 1960; Sen and Schlenk 1964). Sen and Schlenk (1964) report these acids to range from 15:0 (11%) to C_{21} (0.9% total) with unsaturated components having up to four ethylenic bonds, e.g., 17:4 and 19:4. The 17:1 acid was found as 4.6% of the total mixed fatty acids.

Based on the compiled data, the species of capelin, dogfish, goby, marine herring, and whale have among the highest percentages of monoenoic acids. However, this statement is made cautiously because we know that the sample source can be an influencing factor in the types of lipids of the oil analyzed. As an illustration, Pacific herring oil from the ventral area of the fish is found to contain more than twice the amount of 20:1 and 22:1 acids (38 vs 14%) compared to oil from the dorsal area of the fish (Shimma and Taguchi 1964A). By comparison, blubber oils of seal and whale appear very high (>65%) in monoenoic acids.

In regard to the pentaenoic and hexaenoic acids of the oils listed in Table 1, only a few species appear to have exceptional proportions of these fatty acids. These species, with percentages of pentaenes and hexaenes in parentheses, include the following: Atlantic cod (56%), Pacific cod (49%), halibut - *Hippoglossus hippoglossus* - (53%), pond smelt (45%), porbeagle shark (45%), and squid (53%). Most other species contain these component fatty acids in about 10 to 20% of the total fatty acids.

Recalling the previous discussion, one should recognize that the values just mentioned may be quite different due to several environmental factors. The values reported for halibut and lemon sole by Olley and Duncan (1965) illustrate this point. Their values show comparisons between summer and winter catches, whereby the amount of 22:6 acid was nearly doubled from one time of year to the other. Olley and Duncan (1965) imply that the phospholipid levels are relatively stable in comparison to the neutral lipid levels. For this reason, one could anticipate that their finding seasonal differences is reflected principally in the fatty acids of the triglycerides.

Seasonal effects are also shown for mullet-body oil (Gruger *et al.* 1964). Based on the work of Reiser *et al.* (1963) and others, one could say that the seasonal differences observed by Gruger *et al.* (1964) on mullet fatty acids could be complicated by maturity or size of fish and by availability and nature of food supply for these fish. The same comment can be made of seasonal observations of others as well.

Fatty acid analysis of some freshwater species, other than those found in

Table 1, have been reported by Brenner *et al.* (1961). Analyses of depot fats of *Prochilodus lineatus, Pimelodus maculatus, Salminus maxillosus, Parapimelodus valenenciennesi,* and *Pseudoagenius brivifilis* showed fatty acids from C_{11} to C_{24}.

Shimma and Taguchi (1964A, 1964C) reported extensive evaluations of fish and marine-animal oils by GLC. Their investigations are particularly interesting from the view that related parts of several species were examined, and their results show differences in composition related to the differences in anatomical sample source.

Whale oils are especially interesting oils because some contain fatty acids that are largely in the form of wax esters. Jangaard *et al.* (1963B) report GLC data of the Atlantic bottlenose whale (*Hyperoodon ampullatus*) and compare its fatty acids with the fatty acids of commercial winterized sperm whale oil. They found 1.2% 10:0 acid as well as the usual monoenoic acids in the sperm whale oil; however, the bottlenose whale oil contained nearly four times the amount of 20:1 and 22:1 acids compared to the sperm whale oil.

Sano and Murase (1965) report the GLC analysis of minor component fatty acids of Sei whale blubber oil. They found a wider range of fatty acids than has been usually found from recent fish oil analyses. Noteworthy is their report of fatty acids such as 13:1, 14:2, 19:5, 21:1, 21:5, and 24:6.

Fatty acids of the blubber of North Atlantic finback whale (*Balaenoptera physalus*) were reported by Ackman *et al.* (1965). Sections of blubber consisting of lateral slices of equal thickness were the source of fatty acids, which were shown to differ in total composition. The greatest differences in composition were between that of the outermost and of the innermost section, the data of which are summarized in Table 1. Ackman *et al.* (1965) point out that the total C_{20} and C_{22} monoenoic and polyenoic acids in each layer of blubber is nearly constant, but the ratios of the monoenoic to the polyenoic acids change very significantly. One can consider these findings in light of a physiological function of blubber as insulation. It would be interesting to know if this kind of distribution is present in depot fat layers of other species.

Relatively little is known about fish-oil fatty acids of chain lengths greater than C_{22}, because these acids occur in quite minor amounts. Also, these acids have been difficult to measure with the same degree of confidence as other fatty acids are measured. Ito and Fukuzumi (1963B) observed from 1.0% to 2.5% 24:1 acid in some species. Gruger *et al.* (1964) implied that 24:1 accounted for nearly all of the C_{24} acids found by the GLC analyses of numerous species of fish. Ackman and Sipos (1964) report that the normal range of C_{24} acids in marine teleost fish does not exceed 2% as determined by the classical methods (Hilditch and Williams 1964). Particular attention was given by Ackman and Sipos (1964) to pilchard-oil fatty acids, which were believed to contain abnormal amounts of C_{24} acids. Their results showed 0.5% 24:1, 0.2% 24:5, and 0.1% 24:6 acids in the pilchard oil. Such results are not at all unreasonable for most other fish oils.

Work by Adachi (1960) on cuttlefish oil was directed towards the C_{24}, C_{26}, and C_{28} acids. Results showed that these chain-length acids were more monoenoic, trienoic, and tetraenoic in character than they were pentaenoic and hexaenoic. Investigations of triglycerides of sablefish (*Anaplopoma fimbria*) by Dolev and Olcott (1965B) have shown that 24:6 and 26:4 acids are present. These findings were the result of very tedious chromatographic separations of triglyceride fractions.

Shellfish-Oil Fatty Acids

Relatively little is known about the fatty acid composition of shellfish oils compared to fatty acids of marine and freshwater fish oils. Only in the last few years has GLC data become available for shellfish oils. A comparison of the numerical values in Table 2 with those in Table 1 will show very few differences between the fatty acids of shellfish and the fatty acids of freshwater and marine fishes.

Gruger *et al.* (1964) reported about four per cent 20:4 acid in extracted oils of blue crab and sea scallop. This amount of the 20:4 acid may be considered rather high compared to the usual range of values for 20:4 in marine oils.

Fatty acids of hen clam differ from those of littleneck clam and shortneck clam in the relative amounts of the 22:6 acid. Not as great a difference in 22:6 is seen from the data on Pacific oysters from obviously wide geographic origins. Of the listed shellfish, the common mussel fatty acids contained the highest amount of 22:6.

It appears strange that the 22:6 acid is not observed in abalone and top shell (Shimma and Taguchi 1964B) and in starfish (Rodegker and Nevenzel 1964). Also, the amounts of 22:5 in abalone and top shell appear rather high for shellfish fatty acids, indicating a possible unusual biosynthetic pattern in these species or a selection of tissue samples which are high in phospholipid content.

Pacific starfish oil, analyzed by Rodegker and Nevenzel (1964), contained 14% 14:0, which appears high for shellfish but which may be normal for starfish. They also found 18 to 29% 20:1 and 30 to 45% C_{20}-C_{22} polyunsaturated fatty acids in starfish lipids.

Fatty acids from abalone and top shell show between 10 and 16% 22:1, while fatty acids from the remainder of shellfish oils indicate less tha

about 5% 22:1. Sea scallop oil had as little as 0.2% 22:1, but contained 23% 20:1. The other shellfish oils ranged from about 2 to 9% of the 20:1 acid.

Fatty acid composition of *Chlamys nipponesis* and *Pecten yessoensis* are reported by Igarashi *et al.* (1961) using the classical distillation method of analysis. These oils had iodine values of 203 and 226, respectively. Other data similar to these are summarized by Hilditch and Williams (1964).

Fatty Acids Composition of Fish-Liver and Egg Oils

The livers of fish are known generally to be high in oil content. In many cases, research has demonstrated a considerable difference in fatty acid composition of fish liver oils compared to body oils. Fish-egg fatty acids, such as those from salmon, are usually higher in unsaturation than those acids from oils of other fish tissues (Kyte 1956).

Table 3 lists principally the fatty acid compositions for liver oils. Only the egg-oil fatty acids of two species are included for comparison.

Fatty acid mixtures from oils of dogfish livers and cod livers can be quite high in the amounts of 20:1 and 22:1 acids. With such oils, it is not surprising that the amounts of 20:5 and 22:6 acids may be correspondingly low. Oil of herring shark livers has the highest combined amounts (53%) of 20:1 and 22:1 (Klenk and Eberhagen 1962) of the liver oils listed in Table 3.

These data and others might indicate that high monoenoic acid content could be a general characteristic of shark-liver oil. However, it can be argued from such data as by Pathak *et al.* (1955, 1957) and by Kamath and Magar (1955, 1956A, B) that 20:1 and 22:1 in several other elasmobranch liver oils cannot be very high in proportion to the total amounts of C_{20} and C_{22} acids. For instance, data from the classical distillation technique show that if unsaturated C_{20} and C_{22} acids amount to 19 and 7%, respectively, and taking into account the presence of 20:4, 20:5, 22:5, and 22:6 acids, then there may not necessarily be high proportions of 20:1 and 22:1 present. As is the case with body oils, we have here the situation where extensive analyses of many liver samples are necessary to establish general patterns.

Dogfish-liver oils have provided interesting materials for the investigation of fatty acids associated with alkoxydiglycerides in the presence of triglycerides (Malins 1960). GLC analysis of fatty acids of alkoxydiglycerides of *Squalus acanthias* livers has shown that 20:1 comprised 90% of the C_{20} acids and that 22:1 comprised 68% of the C_{22} acids (Malins *et al.* 1965). This distinction was not as striking, however, among the fatty acids of the triglycerides of the same oil.

Fatty acid analyses of yellowtail (*Seriola quinqueradiata*) liver oil

showed over 50% C_{20} and C_{22} acids and over 8% polyunsaturated C_{24} acids (Tsuyuki *et al.* 1958). Analysis of liver-oil fatty acids of *Octopus dofleini* resulted in the isolation of 24:5 acid, among others (Hatano 1958A). Additional work on this oil showed the presence of 10:1ω7, 14:1ω9, and 12:1ω8, the latter of which is said to occur only in this octopus and not in other aquatic life (Hatano 1958B).

Concerning fatty acids of rainbow trout-egg oil, Ando (1962) observed changes during egg development. It was found that, after fertilization, the percentage of C_{16} acids decreased once and then increased gradually after which a marked increase occurred in the degree of unsaturation.

It should be pointed out for salmon-egg fatty acids that about 40% of the total fatty acids are 20:5, 22:5, and 22:6 acids. Only about 1% are 20:1 and 22:1 acids, as seen in Table 3 (Gruger *et al.* 1964).

Ackman and Burgher (1964C) reported GLC analysis of cod-roe fatty acids, (cf. Table 3), which contained larger amounts of 16:1 and 18:1 acids than the flesh lipids of a male cod. They also found that the amount of 22:6 was not as high among the roe fatty acids as in the male flesh lipids. This was partly attributed to a significant triglyceride content in the roe in contrast to the large phospholipid content of the flesh. There were marked differences in fatty acid composition between that of the roe oil and liver oil of the female cod and that of the liver oil of the male cod.

Other Sources of Marine Fatty Acids

There are a few other areas of research where the fatty acid composition of fish oils are of interest. Fatty acids of three species of sea anemone were analyzed by Toyama *et al.* (1955). Based on classical data, the fatty acids of sea anemone show little difference compared to fatty acids of body oils of other marine fish. It is desirable to have GLC analyses of such oils, however, to be conclusive.

The component fatty acids of the milk of Atlantic grey seal were analyzed by Ackman and Burgher (1963). Their GLC data confirmed the belief that the milk fat of marine mammals resembles the composition of the depot fats of these animals. The fatty acid composition of grey seal milk fat is compared to that of the blubber of lactating grey seal and the blubber of an adult harbor seal in a recent report by Ackman and Jangaard (1965). Different proportions were noted for 16:0 and 22:6ω3, as well as other acids. The milk fat had lower proportions (1:3) of 20:1 and 22:1 acids than the blubber.

Fatty acids of salmon offal oil were compared between two production years by Swain (1954). Such comparisons are important, since salmon offal is a large by-product of the salmon canning industry.

Some fatty acids from marine life mitochondria were investigated by E

chardson *et al.* (1961, 1962). The mitochondrial samples were from the livers and hearts of marine birds, seals, and several fish. The results of GLC analyses showed very low levels of linoleic, linolenic, and arachidonic acids and high levels of 20:5 and 22:6 acids from these sources. The results of the study were biochemically implicated to the need of marine life for certain fatty acids.

Lipoproteins as a source of fatty acids in fish have gone virtually unnoticed in research. Olley (1961) has investigated phospholipids associated with fish lipoproteins, but did not include the constituent fatty acids. It would be interesting to speculate on the fatty acid composition of these and other conjugated proteins, such as those related to lipids complexed with ribonucleoproteins.

SUMMARY AND CONCLUSIONS

The fatty acid distribution patterns of fish and marine animal oils are very complex from the standpoint of molecular structure and also the overall composition at any given time for each species. Environmental factors can effect the makeup of body-oil fatty acids, and maturity and sex of fish have influences upon composition as well.

Fatty acid composition varies not only from species to species, but often to an even greater extent from one fish to another of the same species. From the compiled data, it should be apparent that an average value for a component fatty acid has little meaning. It is most important to establish ranges of composition values (Stansby 1954). This can only be done by numerous analyses of fish oils over periods of years, taking into account the many factors that influence fatty acid composition.

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Fatty Acid Composition

INTRODUCTION

Knowledge of the chemical nature of fish-oil fatty acids and their distribution in marine life is important for the development of fishery products and for the evaluation of the nutritional significance of fatty acids in fish oils. Knowledge of the distribution of fatty acids is also important in order to understand the physical and chemical properties of fish oils and the biochemical role of fatty acids in fish and marine animals.

The history and early developments of the fatty acid composition of fish oils are well documented by Hilditch and Williams (1964) and by Bailey *et al.* (1952). Also, Lovern (1942, 1964) has reported extensive investigations during the early era. Recent investigations continue to add to current understanding of fish oils, and one now finds renewed interest in the many types and classes of compounds associated with fatty acids of marine life.

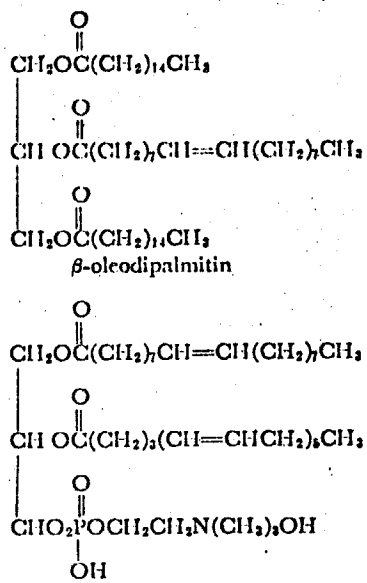
In this chapter, some background information is given about the nature of fatty acids and their chemical distributions in fish oils, the origin of fatty acids in fish, and the effects of environment on fish-oil fatty acids. Following the background information, a discussion is given about fatty acid mixtures found in fish oils common to North America. Fish oils from other areas of the world are also included for comparison.

NATURE OF FATTY ACIDS AND CHEMICAL DISTRIBUTIONS

Fish oils and marine-animal oils are generally characterized by a rather large group of saturated and unsaturated fatty acids, which are commonly associated with mixed triglycerides. In addition to triglycerides, body oils from fish and marine animals usually include minor amounts of fatty acids as substituents of phospholipids and other lipids. In comparison to body oils, on the other hand, liver oils and oils from particular parts of fish and marine animals can often contain large amounts of fatty acids associated with phospholipids, glyceryl ethers (alkoxydiglycerides), and wax esters, depending on the source of oils and lipids (Lovern 1962). For a detailed discussion of classes of fish lipids see Chapter 2.

Nature of Fatty Acids

The fatty acids derived from fish oils are of three principal types: saturated, monounsaturated, and polyunsaturated. The formula,



β-oleodipalmitin

α′-oleyl-β-cicosapentaenyl-α-lecithin

Additional information about the kinds of lipids associated with fatty acids may be found in such texts as those by Deuel (1951), Gunstone (1958), and Hanahan (1960).

Fatty acids of the triglycerides, lecithins, and phosphatidyl ethanolamines (cephalins) from livers of cod and lobster and from muscles of cod and scallop were analyzed by Brockerhoff *et al.* (1963). They demonstrated that the polyunsaturated fatty acids were preferentially located in the β position of both the triglycerides and the lecithins. The phosphatidyl ethanolamines were also found to be highly unsaturated.

Brockerhoff and Hoyle (1963) analyzed the fatty acid distribution in the α and β positions of triglycerides from fish body and liver oils, and found that the polyenoic acids were preferentially distributed in the β position. They also analyzed seal blubber and whale oils, and found that the polyenoic acids occurred in the α position rather than in the β position of the blubber triglycerides.

Lecithin fractions from tuna, salmon, and menhaden muscle were shown by Menzel and Olcott (1964) to have fatty acids distributed similar to the lecithins isolated from muscle tissues by Brockerhoff *et al.* (1963). For the three species, 91–99% of the fatty acids in the β position were unsaturated and 36–86% of the fatty acids in the α position were saturated. Tuna lecithin, for example, had 8.4% 20:5[1] acid and 39% 22:6 acid in the α position and 15% 20:5 acid and 48% 22:6 acid in the β position. In another investigation, for example, Schuster *et al.* (1964) showed that white muscle of five species of tuna contained cephalins and lecithins having 47% and 50% of the 22:6 acid, respectively Other instances of fatty acid distributions in fish phospholipids are cited by Schuster *et al.* (1964).

An interesting investigation of the triglycerides of sablefish (*Anaplopoma fimbria*) was begun by Dolev and Olcott (1965A), in which they found much less polyunsaturated fatty acids in the total triglyceride fraction than is customarily found in most marine oils. The most recent results of this work demonstrated that some of the α- and β-substituent fatty acids of sablefish triglycerides do not follow the usual distributional patterns (Dolev and Olcott 1965B). In this species, they observed for example that 18:3, 20:3 and 20:4 occur mainly in the α, α′ positions compared to 14:0, 16:0 and 16:1 which occur mainly in the β position. The 18:0 and 18:1 acids appeared to be distributed evenly. Notably important was the finding that 22:6, which was present in the glycerides, was not detected as free fatty acid from hydrolysis by lipase.

ORIGIN OF FATTY ACIDS IN FISH

It has been known for many years that the nature of fat in fish diets can influence the proportionate distribution of fatty acids in fish oils (Lovern 1942). Natural oils in marine plant life, planktonic crustacea, and other plankton are consumed by fish in varying degrees depending on feeding habits. Feeding habits of fish can vary according to such factors as availability of food, which may be a geographic factor, periods of fasting, and spawning cycles. The food fat metabolism in fish is being investigated continuously in order to understand better the origin of the unique fatty acids in fish oils. To illustrate how the fatty acids of fish can change according to dietary fats, some recent findings are discussed.

[1] It has become common practice to use abbreviations when referring to particular fatty acids (Farquhar *et al.* 1959). Generally, the abbreviation is written as two numbers separated by a colon. The first number designates the number of carbon atoms in the fatty acid molecule and the second number (after the colon) designates the number of methylene-interrupted *cis* ethylenic bonds. In addition, polyunsaturated fatty acids may be regarded according to families of which the terminal structures are the same. For this reason, the position of the ethylenic bonds along the carbon atom chains may be designated by the Greek letter omega or ω and a number that refers to the number of carbon atoms separating the terminal methyl group from the ethylenic bond nearest to the terminal methyl (Mohrhauer and Holman 1963). For example, 18:3ω3 stands for 9,12,15-octadecatrienoic acid (linolenic acid), and fish-oil fatty acids designated with an ω 3 are said to belong to the linolenic acid family. Similarly, endings of ω 6 and ω 9 refer to the linoleic and oleic acid families, respectively.

$$CH_3(CH_2)_x(CH{=}CHCH_2)_n(CH_2)_yCOOH$$

where $n = 0$ to 6, illustrates the type of fatty acid structures common to fish oils.

The saturated fatty acids have carbon chain lengths that generally range from C_{12} (lauric acid) to C_{24} (lignoceric acid). Also, traces of C_8 and C_{10} acids may be found in some fish oils. A C_5 acid (isovaleric), however, occurs in jaw-bone oil of dolphin and porpoise.

The monounsaturated type is comprised of monoethenoic acids, and the polyunsaturated type is comprised of polyethenoic acids which contain from 2 to 6 ethylenic bonds per acid. The carbon chain lengths of the unsaturated acids range generally from C_{14} (9-tetradecenoic acid) to C_{22} (4,7,10,13,16,19-docosahexaenoic acid). Small amounts of C_{10} and C_{12} monoenoic acids have been found in some fish oils. There are no naturally occurring acetylenic acids and hydroxy carboxylic acids presently known in fish oils.

Even-numbered carbon fatty acids make up about 97% of the total fatty acids, with a few notable exceptions. It was relatively recent that odd-numbered carbon fatty acids were generally acknowledged to be part of all fish oils. Branched-chain odd-carbon acids were isolated by Morice and Shorland (1956) from shark-liver oil. They isolated 13-methyltetradecanoic acid, (+)-12-methyltetradecanoic acid, and (+)-14-methylhexadecanoic acid, and found that together these acids comprised 0.1–0.2% of the liver-oil fatty acids. Earlier work by Morice and Shorland (1952) demonstrated the presence of other branched-chain acids in shark-liver oil that resembled 2,3-dimethyloctadecanoic acid and 2,3,4-trimethylhexadecanoic acid. The application of gas-liquid chromatography by Farquhar *et al.* (1959) to the analysis of menhaden-oil fatty acids has also demonstrated the existence of straight-chain and branched-chain, odd-carbon acids of fish oils. Saturated and unsaturated odd-carbon fatty acids were isolated from menhaden oil by Gellerman and Schlenk (1959) and from mullet oil by Sen and Schlenk (1964), respectively. Normal and branched-chain odd-numbered fatty acids in fish depot fats, seal blubber, and whale blubber were examined by Ackman and Sipos (1965). These workers noted the ratios of *iso* and *anteiso* fatty acid to be comparable in the fish and seal samples, but differed in a whale sample. (Cf. discussion under heading of Fish Diets, p. 8).

The nature of the ethylenic bonds in the unsaturated acids from fish oils has been known for many years to be of the *cis* geometric configuration. In addition, however, evidence has been sufficient only recently to prove as far as is known that the carbon-carbon atom separations of the ethylenic bonds of polyunsaturated acids are of a methylene-interrupted type (Klenk 1958; Farquhar *et al.* 1959; Kayama *et al.* 1963B). This type of

separation is also referred to as a divinylmethane structure. Numerous investigators have shown that the divinylmethane structure is common as far as we know for individually isolated polyunsaturated acids of fish oil. Among these investigators, Silk and Hahn (1954) positively identified a 6,9,12,15-hexadecatetraenoic acid (C_{16} acid) in South African pilchard oil. The work of Klenk and Brockerhoff (1957) and Matic (1958) revealed the presence of a 6,9,12,15-octadecatetraenoic acid (C_{18}) in herring oil and South African pilchard oil, respectively. The structures of 5,8,11,14,17-eicosapentaenoic acid (C_{20}), 7,10,13,16,19-docosapentaenoic acid (C_{22}), and 4,7,10,13,16,19-docosahexaenoic acid (C_{22}) were determined by such workers as Whitcutt and Sutton (1956); Whitcutt (1957); Klenk and Brockerhoff (1958); Toyama *et al.* (1959); Farquhar *et al.* (1959); Ackman *et al.* (1963); and Ackman (1964). Ahrens *et al.* (1959) were the first to point out that menhaden (*Brevoortia tyrannus*) oil is composed of at least 44 different fatty acids.

Other workers have approached the problem of proving the general divinylmethane structure by analyzing mixtures of fish-oil fatty acids. Privett (1956) investigated the effects of lipoxidase catalyzed oxidations on concentrates of fatty acids, and demonstrated the 1,4-diene nature of the double bond structures. More conclusively, however, Hashimoto *et al.* (1963) proved the gross divinylmethane structure in fish-oil polyunsaturated acids by analysis of nuclear magnetic resonance spectra.

Chemical Distribution of Fatty Acids

There are differences in the natural distribution of fatty acids associated with lipids such as triglycerides and phospholipids. For example, it is generally believed that phospholipids, such as lecithins and cephalins, contain more polyunsaturated fatty acids than do the triglycerides when isolated from the same oil or tissues. Also, it is believed generally that depot fats consist largely of triglycerides, while the total lipids of various body organs and muscle tissues can by comparison contain large proportions of phospholipids. The reasons for such distributions are the subject of much research, which will not be discussed here. It is only important to point out some examples of these differences in order to understand better the composition of fish-oil fatty acids.

To aid the following discussion, examples of the molecular structures of triglycerides and phospholipids may be represented by the formulas for β-oleodipalmitin and α'-oleyl-β-eicosapentaenyl-α-lecithin, respectively, as follows:

Fish Diets

Experiments by Kelly *et al.* (1958A) with mullet (*Mugil cephalus*) gave indications that fish, like land animals, probably convert fatty dienoic acid into tetraenoic, pentaenoic, and hexaenoic acids. Unlike land animals, however, the mullet seemed capable of converting 18:2ω6 acid to a fatty trienoic acid. Other experiments have shown that young freshwater fish on low fat or cottonseed oil diets had no significant change in fatty acids, but when fed a diet containing other fish oil the fatty acids of the freshwater species changed to resemble the dietary fish oil (Kelly *et al.* 1958B). Similar results have been observed by Brenner *et al.* (1960, 1963), Mead *et al.* (1960), and Brockerhoff *et al.* (1964).

In another instance, rainbow trout were fed for 103 days on a diet containing soybean oil. Results showed that no 20:4, 20:5, and 22:6 acids could be detected in the trout oil after such treatment (Toyomizu *et al.* 1963).

Work on goldfish (*Carrassius auratus*) by Reiser *et al.* (1963) demonstrated that controlled diets depleted of polyunsaturated fatty acids, but containing principally 18:0, 18:1, 18:2 and 18:3, produced changes after 76 to 120 days in fatty acids of both triglycerides and phospholipids. It appears from their data that the triglycerides in these fish showed significantly less 20:5 and 22:6 acids when compared to the phospholipids.

Marine plankton, as a major food source for fish, have commanded the attention of scientists in recent years. Crabs and shrimps, which when immature are included in the crustacean class of plankton, are known to exhibit fatty acid changes in body fats analogous to fish, depending on their diets (Kelly *et al.* 1959). Klenk and Eberhagen (1962A, B) isolated from marine plankton a group of polyunsaturated fatty acids, which are like those found in fish and marine animal oils. Others have also shown the similarities between the fatty acids of fish oils and plankton-oil fatty acids (Farkas and Herodek 1961, 1964; Lewis 1962; Kayama *et al.* 1963A).

Ackman and Sipos (1965) examined a number of samples of fish oil fatty acids and made a detailed study of the saturated fractions. They noted that the normal odd-numbered fatty acids found in marine lipids originate in phytoplankton, but that there is little evidence to indicate that branched-chain fatty acids are prominent in these plankton. It is suggested that branched-chain fatty acids are formed to some degree in zooplankton.

Biogenesis of Fatty Acids

In order to understand how fatty acids are transformed or converted from the food chain to the fat and oil depots in the fish, it has been necessary to investigate what happens to the individual acids after ingestion.

Klenk and Kremer (1960) showed that when radioactive carbon-14 labelled acetate was incubated with liver slices of frog, carp, trout, flounder, smelt, and dab, the C_{20} and C_{22} polyenoic acids were synthesized from linoleic and linolenic acid precursor.

Kayama *et al.* (1963B) tested the supposition that dietary linolenic acid is incorporated in toto into the carbon chains of 20:5 and 22:6 acids of fish. Using mature kelp bass (*Paralabrax clathratus*) for intraperitoneal injections of methyl linolenate-1-C^{14} diluted with pure methyl linolenate, the investigators found that the acid of the latter ester was converted to 20:5 acid and that the 20:5 acid was incorporated into 22:6 acid. The probable conversion pathway for linolenic acid is as follows: 18:3ω3→18:4ω3→20:4ω3→20:5ω3→22:5ω3→22:6ω3. This indicates a retention of the linolenic-type structure throughout the conversions. However, it is possible, as Kayama *et al.* (1963B) pointed out, that the linolenic acid, unlike linoleic, undergoes extensive partial degradation and re-synthesis before chain elongation to the final products.

ENVIRONMENTAL INFLUENCE ON FATTY ACID COMPOSITION

It has been observed in the past that environmental factors such as geographic locations of catch and seasons of the year, which may be related to water temperatures, are related to the proportions of fatty acids of fish oils (Lovern 1942; Swain 1953). Re-evaluations of these factors have been important in light of modern instrumentation like gas-liquid chromatography (GLC) (Farquhar *et al.* 1959). Seasonal variations in fatty acids of commercial cod-liver oils were investigated by DeWitt (1963). He observed a range in iodine values, 146 to 168, for GLC analyzed fatty acids of the liver oils sampled from May to November.

The influence of season, as well as water temperatures and pressure effects, was observed by Lewis (1962) on fatty acids of Arctic plankton. When comparing plankton fatty acids from Arctic plankton and California coastal plankton, Lewis (1962) found two changes in the fatty acids, namely, an increase in the degree of unsaturation and a reduction in chain length with a reduction in environmental temperature.

It is unfortunate that Lewis (1962) reported for some two dozen species only those fatty acids with GLC retention times up to that of 20:1. Rodegker and Nevenzel (1964) point out that this indicates only about 45 to 90% of the total component fatty acids to be anticipated. Lewis (1962) proposed the ratio of 16:0 to 16:1 acids as a valuable index of the temperature of the habitat of marine ectotherms. However, in the work by Rodegker and Nevenzel (1964) on fatty acids of mussel, barnacle, and starfish, only one of their samples agreed with the 16:0 to 16:1 ratio reported by Lewis. They suggested that problems may be due to attempts to com-

pare fatty acids of different tissue types and, hence, different lipids which may have different biological functions. Rodegker and Nevenzel (1964) suggest that a clear picture of temperature effects, etc., would be anticipated from analyses of the same type of lipid fatty acid source, say for example the muscle phospholipids.

The effects of temperature on growing mullet (*Mugil cephalus*) and goldfish (*Carrassius auratus*) were investigated by Reiser *et al.* (1963). They concluded from analyses of fish raised in water at 13°C. and at 23°C. that these temperatures had little or no influence on the deposition or interconversion of polyunsaturated acids in the mullet. These findings were surprising. Based on controlled diets, they reasoned that possibly saturated fatty acids (12:0 and 14:0) are not absorbed at the lower temperature, thereby causing a lack of significant influence on the unsaturated fatty acids. Contrary to these findings, however, Kayama *et al.* (1963A) have demonstrated that guppies (*Lebistes reticulatus*) raised in water at 17°C. possessed more 22:6 acid in the oil than did guppies raised in water at 24°C.

In other work, Farkas and Herodek (1964) showed that the amount of C_{20} - C_{22} polyunsaturated acids in freshwater planktonic crustacea increased with decreasing temperatures, and in some species these acids exceeded the values common to marine animals. The fatty acids of freshwater fish which fed on these crustacea resembled fatty acids of marine fish. One might say then that temperature had an indirect influence on the fatty acids via the food chain.

NATURAL FATTY ACID MIXTURES

Now that we have discussed somewhat the chemical structures associated with fatty acids of fish oils, the origin of fatty acids, and the influence of some environmental factors upon the natural patterns of fatty acid mixtures, it will be easier to evaluate the fatty acid compositions of fish oils. The mixtures of fatty acids to be discussed below are related to fish oils and marine animal oils that are common principally to North America, but oils from elsewhere in the world are included for comparison.

Fatty acid compositions of fish oils have been known for many years to be complex. Only after the development and widespread application of gas-liquid chromatography (GLC) to the analysis of fish-oil fatty acids has this complexity become more vivid (Ahrens *et al.* 1959). The results of GLC have provided the data given in the subsequent tables. The technique of GLC is discussed in another chapter.

The fatty acid composition of some body oils from marine and freshwater fishes and marine animals are listed in Table 1. Tables 2 and 3 show similar compositions for oils of shellfish, and fish-liver and egg oils,

TABLE 1

FATTY ACID COMPOSITION OF BODY OILS OF MARINE AND FRESHWATER FISHES[1]

Name of Species		Fatty Acid Percentage Composition																
Common	Scientific	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	Reference
Angler	*Lophiomus setigerus*	1.6	NK	28.2	6.1	NK	15.6	20.8	3.5	—[2]	NK	1.7[2]	—[2]	5.5	5.2[3]	NK	11.8	Shimma 1964A
Barracuda	*Sphyraena schlgeli*	2.9	NK	17.2	7.2	NK	4.0	20.3	1.1	—[2]	NK	1.8[2]	—[2]	17.2	1.4[3]	1.2	25.7	Shimma 1964A
Bonito	*Sarda chilensis*	7	+	17	12	1	2	21	1	+	2	1	NK	22	NK	2	5	Klenk 1962
Butterfish	*Centrolophus japonicus*	1.3	+	18.6	4.2	+	8.4	16.1	1.2	—[2]	NK	4.6[2]	—[2]	3.7	6.1[3]	4.4	31.4	Shimma 1964A
Capelin (male)	*Mallotus villosus*	8.8	NK	12.0	14.9	NK	1.8	9.0	NK	NK	NK	21.5	NK	5.8	18.5	0.5	3.6	Ackman 1963
Capelin (female)	*Mallotus villosus*	8.6	NK	13.3	16.5	NK	1.6	10.7	NK	NK	NK	17.2	NK	8.6	14.2	0.9	4.8	Ackman 1963
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 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	—[2]	NK	2.0[2]	—[2]	17.9	5.6[3]	1.9	29.2	Shimma 1964A
Conger	*Astroconger myriaster*	5.5	0.8	17.8	9.7	NK	3.3	30.0	2.5	—[2]	1.6	4.9[2]	—[2]	8.0	3.4[3]	3.1	7.8	Shimma 1964A
Cuttlefish	*Ommastrephes sloani pacificus*	3.3	NK	15.0	7.2	NK	3.0	22.0	1.7	1.1	1.9	11.2	2.1	5.9	10.8	2.9	10.5	Ito 1963B
Dogfish	*Squalus sucklii*	1.9	NK	15.7	6.5	NK	3.7	24.7	2.3	—[2]	NK	12.6[2]	—[2]	8.7	4.4[3]	3.5	16.0	Shimma 1964A
Dogfish, Piked	*Squalus acanthias*	1.8	0.7	17.0	4.6	1.7	2.9	16.1	2.2	NK	1.3	5.2	2.7	8.3	4.0	2.7	21.8	Olley 1965
Dogfish, Spiny	*Squalus acanthias*	2.0	0.5	21.2	6.0	1.2	2.7	27.5	1.3	0.6	0.7	5.8	2.5	7.9	4.1	2.3	10.4	Gruger 1964
Dogfish, Spiny	*Squalus acanthias*	1.6	0.2	23.5	5.7	0.9	3.0	33.6	0.6	0.5	NK	6.4	2.8	6.5	3.5	1.7	7.0	Malins 1965
Flatfish	NK	4.0	NK	15.7	5.9	NK	2.3	16.5	1.5	0.8	0.7	11.9	1.9	11.9	7.7	1.3	15.4	Ito 1963B
Flying fish (dorsal)	*Prognichthys agoo*	1.1	NK	34.4	2.7	NK	11.7	10.9	1.1	—[2]	NK	1.8[2]	—[2]	4.8	4.8[3]	NK	25.6	Shimma 1964A
Flying fish (ventral)	*Prognichthys agoo*	3.5	1.2	20.5	6.7	3.1	6.9	20.3	1.4	—[2]	NK	7.5[2]	—[2]	4.7	3.4[3]	2.9	16.6	Shimma 1964A
Goby	NK	5.4	NK	12.8	9.5	NK	2.3	26.2	1.4	0.3	1.2	18.6	0.9	3.5	12.5	1.4	3.3	Ito 1963B
Goby	*Acanthogobius flavimanus*	2.2	1.4	20.2	5.8	2.0	10.4	11.8	3.4	—[2]	NK	3.2[2]	—[2]	15.6	4.4[3]	3.9	11.8	Shimma 1964A
Gurnard	*Chelidonichths kumu*	0.7	NK	13.9	2.8	1.2	8.9	10.9	0.7	—[2]	NK	1.3[2]	—[2]	7.3	4.1[3]	2.4	38.8	Shimma 1964A
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
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 1964
Halibut	*Hippoglossus hippoglossus*	0.8	0.4	9.6	2.5	0.6	9.0	12.3	+	+	NK	4.0	14.4		2.8	2?	37.6	Olley 1965
Halibut	*Hippoglossus hippoglossus*	2.3	0.6	12.9	5.3	1.2	4.2	17.1	NK	NK	1.4	7.3	0.9	12.6	5.0	2.3	19.2	Olley 1965
Hake, Pacific	*Merluccius productus*	1.6	0.2	23.2	8.5	0.9	2.4	32.4	0.9	0.6	1.4	1.3	0.7	12.8	0.7	0.7	7.8	Stansby 1966
Herring	*Clupea harengus*	6	+	13	6	+	1	22	1	+	2	15	+	6	16	1	6	Klenk 1962
Herring, Lake	*Coregonus artedii*	5.5	0.4	17.7	7.1	0.6	3.0	18.1	4.3	3.4	1.8	1.2	3.4	5.9	2.8	3.3	13.3	Gruger 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 1964
Herring, Pacific (dorsal)	*Clupea harengus pallasi*	3.2	NK	24.9	4.9	NK	3.6	13.5	+	—[2]	NK	7.4[2]	—[2]	11.4	8.6[3]	NK	22.5	Shimma 1964A
Herring, Pacific (ventral)	*Clupea harengus pallasi*	6.1	NK	14.1	7.1	NK	1.8	21.7	1.7	—[2]	NK	19.2[2]	—	4.6	19.9[3]	NK	3.8	Shimma 1964A
Herring, Round (dorsal)	*Etrumeus micropus*	3.7	0.9	27.2	6.2	1.7	7.3	12.1	1.4	—[2]	NK	1.4[2]	—[2]	10.2	3.2[3]	NK	24.1	Shimma 1964A
Herring, Round (ventral)	*Etrumeus micropus*	5.7	1.3	16.3	7.8	3.0	7.4	14.5	1.9	—[2]	NK	3.0[2]	—[2]	10.1	3.5[3]	3.7	20.3	Shimma 1964A

305

TABLE 1 (*continued*)

Name of Species: Common	Name of Species: Scientific	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	Reference
		Fatty Acid Percentage Composition																
Loach	*Misgurnus fossilus*	2.2	NK	18.4	16.7	NK	5.9	14.7	6.2	7.6[2]	NK	—[2]	—[3]	4.2	8.6[3]	2.7	2.5	Shimma 1964A
Mackerel	*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 1964
Mackerel, Horse	*Decapterus lajang*	2.4	0.6	33.0	4.4	+	13.7	12.3	2.7	—[2]	NK	2.0[2]	—[3]	4.8	3.6[3]	NK	20.5	Shimma 1964A
Mackerel, Jack	*Caranx mertensi*	NK	NK	18.8	1.2	NK	10.7	19.6	2.2	—[2]	NK	3.2[2]	—[3]	3.7	3.7[3]	3.0	34.2	Shimma 1964A
Menhaden	*Brevoortia tyrannus*	7.2	0.5	17.0	9.8	0.4	3.1	14.5	2.7	1.3	3.2	2.1	0.6	12.5	NK	2.1	8.9	Ahrens 1959
Menhaden	*Brevoortia tyrannus*	7.7	NK	25.3	6.7	NK	3.1	15.4	NK	1.6	4.0	NK	2.0	12.9	NK	2.3	14.0	Peifer 1962
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 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 1964
Mullet, Striped	*Mugil cephalus*	7.5	4.5	13.9	15.5	1.0	5.1	9.1	2.2	1.0	3.1	0.6	3.6	11.8	NK	3.2	3.2	Gruger 1964
Mullet	*Mugil cephalus*	3.9	7.4	29.6	6.3	5.5	4.8	5.8	2.6	1.5	NK	NK	3.6	4.7	NK	3.1	10.3	Peifer 1962
Mullet	*Mugil cephalus*	5.3	11.2	32.0	6.0	1.2	2.0	4.6	1.5	0.8	0.6	1.1	2.5	5.0	NK	NK	5.7?	Sen 1964
Perch, Ocean	*Sebastes marinus*	5.5	NK	23.0	3.8	NK	3.9	18.5	1.6	NK	NK	7.2	1.2	7.9	NK	+	13.6	Peifer 1962
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 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
Puffer	*Sphoeroides vermicularis*	1.9	NK	29.7	3.0	NK	12.4	18.3	3.0	—[2]	NK	4.7[2]	—[3]	5.1	4.8[3]	NK	17.1	Shimma 1964A
Redfish	*Sebastes* sp.	NK	NK	23.1	5.5	NK	10.7	14.4	4.8	—[2]	NK	5.1[2]	—[3]	6.1	3.7[3]	NK	26.6	Shimma 1964A
Rockfish	*Sebastodes pinniger*	4.1	0.6	14.9	6.6	2.6	6.0	20.8	1.6	0.8	1.3	1.4	1.5	11.7	0.8	1.6	17.4	Gruger 1964
Sablefish	*Anoplopoma fimbria*	4.6	0.4	18.1	8.0	0.8	3.1	20.4	0.8	0.5	1.3	5.6	0.8	8.5	8.9	1.8	12.1	Gruger 1964
Sablefish	*Anoplopoma fimbria*	4.1	0.5	14.8	11.6	1.0	3.8	38.0	2.1	1.3	NK	6.6?	1.0	4.6	4.1?	NK	2.2	Dolev 1965
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 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 1964
Salmon, Chum (dorsal)	*Oncorhynchus keta*	6.2	NK	16.3	8.3	NK	2.7	24.8	1.4	—[2]	NK	14.4[2]	—[3]	6.5	12.8[3]	NK	6.6	Shimma 1964A
Salmon, Chum (ventral)	*Oncorhynchus keta*	6.6	NK	13.5	9.0	NK	2.8	25.3	1.9	—[2]	NK	15.7[2]	—[3]	6.8	14.8	NK	3.6	Shimma 1964A
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 1964
Salmon, Silver	*Oncorhynchus kisutch*	3.7	NK	22.5	5.0	NK	3.9	23.6	0.9	1.0	NK	5.3	1.4	11.0	NK	3.3	12.9	Peifer 1962
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 1964
Sardine	NK	6.6	NK	15.5	9.5	NK	3.7	17.3	2.5	1.3	2.9	8.1	2.5	9.6	7.8	2.8	8.5	Ito 1963B
Sardine, Peruvian	*Clupea pilchardus*	8	+	19	10	+	3	14	1	+	3	2	1	22	+	2	4	Klenk 1962
Saury, Pacific	*Coloabis saira*	6.9	0.7	18.4	5.4	NK	3.5	7.3	1.7	—[2]	4.7	13.8[2]	—[3]	6.6	14.7[3]	1.2	13.5	Shimma 1964A
Sea bass	*Lateolabrax japonicus*	2.5	NK	20.1	8.8	2.3	5.9	16.9	1.7	—[2]	2.6	1.9[2]	—[3]	10.6	3.1[3]	1.8	21.8	Shimma 1964A
Sea-bream, "Ki"	*Taius tumifrons*	1.3	NK	30.4	5.0	NK	8.1	15.8	1.9	—[2]	NK	3.1[2]	—[3]	3.9	4.2[3]	2.9	23.4	Shimma 1964A
Sea-bream, "Ma"	*Chrysophyrs major*	2.8	NK	26.7	5.8	1.2	10.3	15.4	1.5	—[2]	NK	2.7[2]	—[3]	12.2	7.3[3]	2.8	11.3	Shimma 1964A
Seal, Harbor	*Phoca vitulina concolor*	3.3	0.1	6.5	17.6	0.1	0.4	30.2	0.8	0.2	0.7	13.6	0.5	5.5	6.0	4.3	7.5	Jangaard 1963A
Shad (dorsal)	*Dorosoma thrissa*	4.5	NK	24.5	5.1	NK	5.8	16.8	1.6	—[2]	1.2	1.5[2]	—[3]	8.4	2.1[3]	NK	28.5	Shimma 1964A
Shad (ventral)	*Dorosoma thrissa*	9.7	0.8	22.0	9.5	NK	5.1	22.9	2.7	—[2]	2.9	2.1[2]	—[3]	8.0	0.9[3]	1.4	10.9	Shimma 1964A
Shark, Porbeagle	*Lamna cornubica*	2.5	NK	12.2	8.1	NK	2.4	16.8	1.0	—[2]	NK	4.1[2]	1.2	2.8	1.5?	13.7	29.0	Shimma 1964A
Skipper	NK	6.9	0.7	13.4	5.1	NK	2.4	10.1	2.3	2.3	5.9	13.4	0.9	8.4	13.8	1.7	12.0	Ito 1963A
Smelt, Pond	*Hypomesus olidus*	1.9	NK	22.5	6.4	NK	3.8	11.3	1.1	—[2]	NK	2.7[2]	—[3]	13.4	2.8[3]	1.5	28.9	Shimma 1964A
Smelt, Sweet	*Pleocoglossus altivelis*	7.3	1.1	29.1	15.0	NK	2.6	11.5	4.8	17.2[2]	NK	—[2]	—[3]	2.4	1.2[3]	1.3	1.3	Shimma 1964A
Snapper	*Etelis evurus*	1.0	NK	24.9	3.4	+	9.0	12.8	1.3	—[2]	NK	1.4[2]	—[3]	3.7	2.9[3]	1.4	33.8	Shimma 1964A
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 1965
Sole, Lemon	*Microstomus kitt*	3.3	1.1	20.0	4.7	2.9	2.2	18.1	1.0	NK	0.5	6.6	1.6	17.5	0.5	6.4	6.6	Olley 1965
Squid	*Illex illecebrosus*	2.2	0.3	27.6	0.4	0.3	4.4	4.9	0.2	0.1	0.1	4.9	0.8	15.8	0.5	0.3	37.1	Jangaard 1965
Swordfish	NK	2.9	NK	17.5	6.3	NK	4.9	27.9	1.9	0.4	0.7	4.6	3.0	4.4	2.0	3.1	17.8	Ito 1963B
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 1964
Trout, Rainbow	*Salmo gairdneri irideus*	1.6	NK	13.2	6.5	NK	4.3	19.2	9.1	9.3[2]	2.2	—[2]	—[3]	14.0	2.9[3]	1.7	16.0	Shimma 1964A
Tunny	NK	5.9	NK	19.2	11.5	NK	4.6	12.8	2.2	1.2	4.4	1.2	2.7	13.9	1.3	2.2	17.0	Ito 1963B
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 1963B
Tuna, Bluefin	*Thunnus maccoii*	1.9	NK	15.4	4.8	1.2	8.2	21.2	1.7	—[2]	NK	7.3[2]	—[3]	6.8	2.0[3]	4.5	24.4	Shimma 1964A
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 1963B
Tuna, Bluefin (dark meat)	*Thunnus thynnus*	4.0	0.7	25.7	3.5	0.7	12.5	14.5	0.6	NK	1.0	3.2	1.4	5.5	2.1	1.8	21.0	Roubal 1963A
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 1963B
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 1963B
Whale	NK	10	+	14	13	1	1	38	3	1	1	2	+	6	NK	2	3	Klenk 1962
Whale, Beaked (blubber)	*Hyperdon rostratus*	4.2	0.2	4.3	11.9	NK	0.9	28.1	2.0	NK	NK	30.1	NK	NK	11.3	NK	NK	Mori 1964
Whale, Finback (outer blubber)	*Balaenoptera physalus*	5.7	0.2	7.1	8.4	0.4	1.4	28.9	2.0	0.8	0.5	19.6	0.6	0.9	17.9	0.7	1.1	Ackman 1965
Whale, Finback (inner blubber)	*Balaenoptera physalus*	5.1	0.3	12.1	7.1	0.8	3.0	27.9	1.6	0.4	0.7	14.8	0.8	4.1	11.0	2.5	5.2	Ackman 1965
Whale, Sperm, Antarctic	*Physeter macrocephalus*	8.3	0.4	11.9	18.0	NK	1.1	32.8	0.1	NK	NK	11.9	NK	NK	3.8	NK	NK	Mori 1964
Whale, Sperm, Arctic	*Physeter macrocephalus*	7.4	0.6	10.4	15.9	NK	1.0	27.7	0.7	NK	NK	16.6	NK	NK	8.6	NK	NK	Mori 1964
Whale, Sperm, Japan	*Physeter macrocephalus*	7.5	1.0	10.0	15.4	NK	1.2	26.2	0.5	NK	NK	16.7	NK	NK	9.9	NK	NK	Mori 1964
Whitebait	*Salangichthys microdon*	3.5	0.9	15.8	9.3	2.1	4.5	15.7	4.5	—[2]	2.3	5.1[2]	—[3]	10.8	6.3[3]	2.9	14.9	Shimma 1964A
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 1964

[1] Not known or not mentioned in reference is designated as NK. Trace amounts indicated by a + sign.
[2] Combined 18:3 and 20:1 acids. More 18:3 than 20:1 acid in freshwater species.
[3] Combined 20:4 and 22:1 acids.

3. Dried fruits
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27. Edible safflowerseed oil
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J. Pharmacol. Exp. Therap., 84: 128-135, 1945

THE PREPARATION OF EXTRACTS FROM OXIDIZED MARINE AND OTHER OILS FOR REDUCING THE BLOOD PRESSURE IN EXPERIMENTAL AND HUMAN CHRONIC HYPERTENSION

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Received for publication March 19, 1945

In earlier reports (1, 2) it has been shown that various oxidized marine oils, when administered orally to hypertensive rats, reduces their blood pressure in a way similar to that of renal extracts (3). In view of the expense and technical difficulty involved in preparing active extracts from kidney tissue, compared to the less laborious procedure of obtaining equally effective agents from readily available marine oils, the latter appeared to be a better source of the desired product. We have, therefore, investigated a number of marine, vegetable and animal oils, as sources of this material as well as methods for the preparation and concentration of extracts suitable for administration to animals or to human patients. Our results have consistently shown the effectiveness of these products in the experimental animal (rats and dogs) and show promise in preliminary studies on man. The present paper reports details of the source, preparation and methods of concentration of the effective agent.

METHODS OF ASSAY. The effectiveness of the various preparations described later was determined on rats rendered hypertensive by compression of the renal parenchyma (4). Oral administration of the extracts by incorporating them in the animal's food has been used exclusively. This avoids the effects of a non-specific depression of the blood pressure which may follow the parenteral administration of various noxious agents and which are responsible for some of the effects observed following the administration of certain renal extracts (5, 6).

Groups of six hypertensive rats were used in each assay. The animals had been operated upon at least three months previously. In this way the spontaneous fluctuations which are observed in the months immediately following operation are avoided. After a preliminary daily training period for several weeks, the blood pressure remains essentially constant except for a slowly progressive rise which continues until the death of the animal (7). The mean blood pressures of the animals ranged from 150 to 200 mm. with an average pressure of the group of six used in each assay varying from 165 to 175. In this way one compensates for the fact that the blood pressure reducing capacity of extracts varies with the blood pressure of the animal and renders the results of separate experiments comparable.

The blood pressures were determined at the same time of the day by the procedure previously described (8). Experiments were discarded in which, due to distastefulness of the oil-mixtures, the animals refused to eat and lost a significant amount of weight. Daily

[1] Aided by grants from the John and Mary Markle Foundation, The National Life Assurance Co., and Mr. Alfred Starr.

[2] This work was begun at the Bowman Gray School of Medicine, Winston-Salem, N. C. The clinical data presented are based on observations of patients at The North Carolina Baptist Hospital.

weighings permitted one to detect this and thus avoid the error introduced by the drop in blood pressure which accompanies starvation.

We have also administered orally the active marine oils, described later, to three hypertensive dogs, with drops in blood pressure comparable to that observed in the rat. The blood pressure of these dogs was determined by puncture of the femoral artery with direct readings on a mercury manometer. It is impracticable to use the dog for systematic assays, but the results indicate that the observed effects are not limited to the rat.

MARINE OILS. We have investigated the common commercially available marine oils for their blood pressure reducing activity. This varied considerably in oils of various sources and sometimes in different samples of oils derived from the same species. Active preparations have been obtained from residues of cod, dogfish, hake, basking shark and skate liver oils. Sardine body oil was also found to be slightly active as was also crude fish oil obtained by distillation, principally of menhaden. One sample of a fresh water fish (Burbot liver) was found to be entirely inactive.[3]

The commercially available samples of cod liver oil were found to be entirely inactive as were also some samples of the concentrates obtained from this source. On the other hand, some samples of concentrate (containing 50,000 to 100,000 units of vitamin A) per gram were found to be active and first prompted the present investigation (1).

In table 1 are reproduced the effects of thirty-one fractions of oils derived from the four species of fish, designated in the first column. In the second column are recorded the distillation temperature at which the oils were derived when distilled at pressures between 6 to 11 micromillimeters.[4] The vitamin A content of the various fractions is given in the third column; their blood pressure reducing properties in the last three columns. Each fraction was tested without further treatment (column 4), following oxidation with thirty percent hydrogen peroxide at room temperature in slightly alkaline solution (column 5), and following saponification with aqueous alcoholic potash, acidification and oxidation (column 6). Fractions which in doses of 5 grams per rat reduced the blood pressure 15 mms. or more, for a period of at least two days are designated in the table as active (A). Those in which the blood pressure at this dosage level was reduced between 10 and 15 mms. as slightly active (SA); between 5 and 10 mms. as minimally active (MA). Obviously, the last named must be considered as manifesting only questionable activity.

It is evident from table 1 that there is no correlation between the vitamin A content of the various oils investigated and their effectiveness in reducing the blood pressure. Moreover, fractional distillation, although effecting to some extent the separation of the active material is not a satisfactory procedure for concentrating this material which apparently is carried over at a considerable

[3] Most of the oils used in the present study were supplied by Eli Lilly and Co. The others were furnished by Distillation Products, Inc., Parke, Davis and Co., The Winthrop Co., The Abbott Laboratories, The National Oil Products Co., Industrial Chemical Sales, Emery Industries, Procter and Gamble and Bogalusa Tung Oil, Inc.

[4] We are indebted to Mr. A. Lee Caldwell of The Lilly Research Laboratories for Supplying these fractionated oils and the data on their vitamin content.

range of temperatures. In general, however, the active material distills over at a temperature of 215° with a second active fraction appearing in the distillate at about 250°.

TABLE 1

The pressure reducing activity of various fractions of four marine oils

SOURCE OF OIL	DISTILLATION TEMPERATURE	VITAMIN A CONTENT	EFFECTIVENESS IN REDUCING THE BLOOD PRESSURE		
			Untreated	Oxidized	Hydrolyzed and oxidized
	°C.	*Units per gram*			
Dogfish oil	200	240,000	A	A	A
	225	44,000	I	MA	A
	230	4,000	I	SA	A
	235	900	I	I	MA
	240	200	I	I	MA
	250	100	I	I	SA
	255	100	MA	MA	A
	Residue	100	I	I	I
Hake liver oil	210	215,000	I	MA	A
	215	40,000	MA	MA	A
	220	6,750	I	MA	A
	225	2,300	I	SA	MA
	230	500	I	SA	MA
	235	100	I	I	I
	240	100	SA	MA	A
	Residue	100	I	MA	A
Basking shark liver oil	100	Trace	I	I	I
	120	Trace	I	SA	SA
	130	Trace	I	MA	MA
	150	2,200	I	I	MA
	230	1,200	I	I	I
	250	100	I	I	MA
	255	None	I	I	SA
	Residue				I
Skate liver oil	165	635	A	A	A
	215	3,900	SA	MA	A
	230	180	I	SA	MA
	242	None	I	SA	SA
	245	None	I	I	I
	248	None	I	I	I
	Residue	None	MA	MA	SA

A comparison of the last three columns in the table also shows that oxidation or hydrolysis prior to oxidation in many cases fails to impart or increase the activity of certain oil fractions. In some cases oxidation alone increases the activity. In others, prior saponification is necessary in order to obtain the maxi-

tis. This patient was under observation continuously in the hospital for a period of sixteen months. Because of the more complete and convincing nature of the results obtained on this patient, the details of the experiment are confined to this one case. After a control period of observation from June 1 to July 7, 1943, the

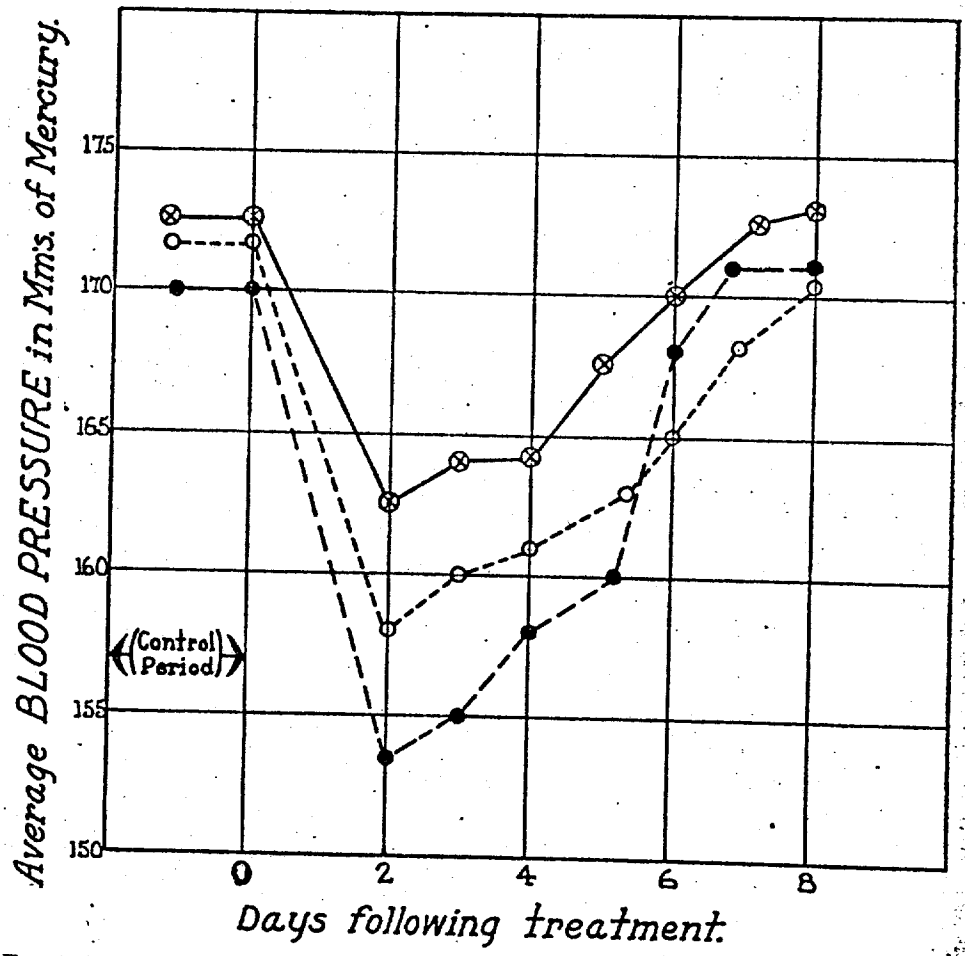

FIG. 1. THE EFFECT OF TUNG OIL ON THE BLOOD PRESSURE OF THE HYPERTENSIVE RAT. Curves showing the effect of administering 12 grams of refined tung oil (⊗———⊗), the same amount of oxidized oil (●———●), and the aqueous solution obtained by adsorption and elution of the active principle (○— — — —○), to groups of six animals. Each reading is the average of the observed mean blood pressures. After a control period, the animals were fed the material under investigation mixed with their food, at the time designated on the abscissa as 0.

patient was treated with an active oxidized marine oil in doses of 15 cc every two hours from July 7 to September 7, 1943. He was then given an equal dose of an inactive oil as a placebo during the period from September 7, 1943 to February 28, 1944, at which time the administration of active oil was resumed and continued until July 1, 1944, when a placebo was again administered until the

discharge of the patient from the hospital on October 1, 1944. Blood pressure readings were made by three different members of the nursing, interne and resident staff of the hospital.[5] The six daily readings obtained in this way were averaged and the resulting readings are recorded in the chart. In this way the inevitable fluctuations are eliminated in the final value which is composed of at least forty-two separate readings.

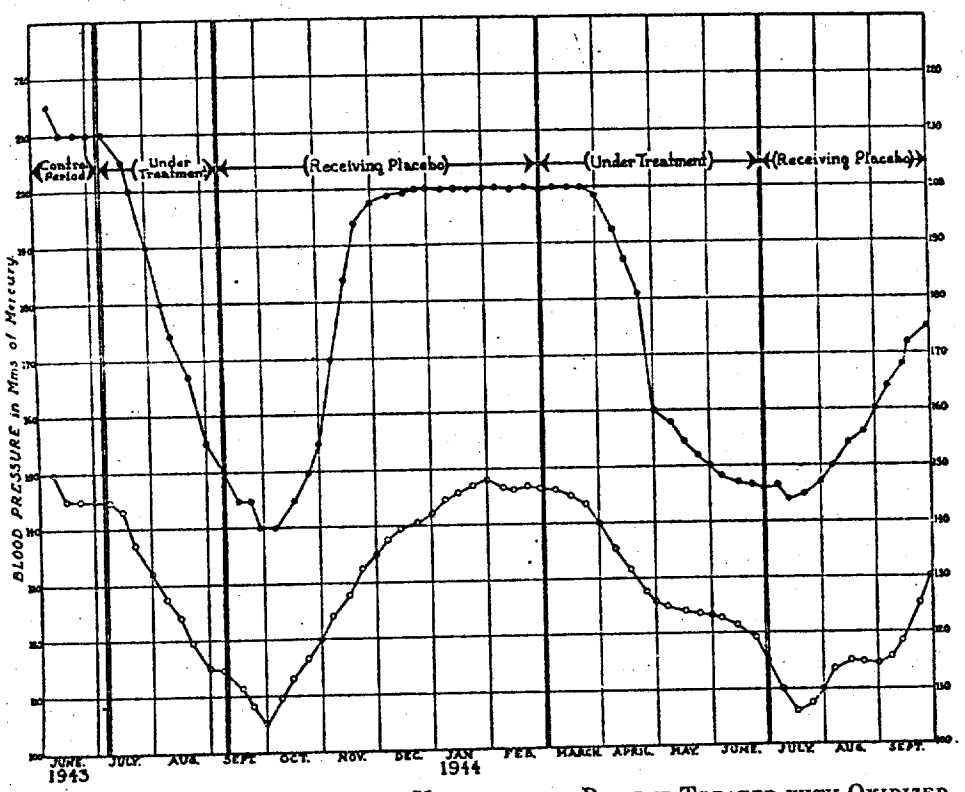

FIG. 2. THE BLOOD PRESSURE OF A HYPERTENSIVE PATIENT TREATED WITH OXIDIZED MARINE OIL

Each point on the curve is the average of at least forty-two readings obtained during the period of observation. The patient was alternately receiving an active oil (as tested on hypertensive rats) during the periods of July 7 to September 7, 1943, and February 28 to June 30, 1944, and an inactive oil as a placebo during the periods of September 7, 1943, to February 28, 1944, and July 1 to October 1, 1944.

●———● systolic blood pressures.
○———○ diastolic blood pressures.

It is evident from the curve that, as in the experimental animal, the administration of an active oil to this patient resulted in a definite decline of the blood pressure. It should be noted that there is a latent period of several weeks following the beginning of therapy before any decline in pressure is noted and that the

[5] We are indebted to the members of the house staff of The North Carolina Baptist Hospital, and particularly to Dr. William L. Venning, for their cooperation in following the blood pressure of these patients.

minimum values are found several weeks after cessation of therapy. The return of the blood pressure to the pretreatment level while the patient was receiving a placebo is also delayed. In general, therefore, the response is similar to that observed in the smaller experimental animal (rat, dog) except for the more gradual change with time, as is to be anticipated.

CONCENTRATION OF THE ACTIVE PRINCIPLE IN AN AQUEOUS MEDIUM. Although the oxidized marine oils are sufficiently palatable to permit their direct use in the hypertensive patient and experimental animal, it was desirable to concentrate the active principle and obviate the necessity of feeding large amounts of inert oil.

Distillation, as we have already seen, is not uniform in its effects, nor does it effect the desired concentration. Steam distillation also fails to remove the active principle. Fractional freezing was equally unsatisfactory, although in some oils it was possible to concentrate the active principle two or three fold by this method. The extraction of the oils, dissolved in ten volumes of petroleum (skellysolve) or ethyl ether, by eighty percent aqueous ethyl alcohol was laborious and, although effecting a considerable degree of concentration, was discarded for the following procedure:

Trial of a number of adsorption agents (charcoal, kieselguhr, alumina, diatomaceous earth, etc.) led to the adoption of a commercially available synthetic resinous ion-exchanger (amberlite I R 100) as the most convenient and effective. When this adsorbant was agitated with the oil in a ratio of about one part (by weight) of the adsorbant to twenty parts of oil, the active principle was removed from the oil. After filtering the oil through cloth, the adsorbant was placed in a continuous extraction apparatus arranged as in the ordinary Soxhlet extractor and treated with low-boiling skellysolve until free of adherent oil. The adsorbant was then dried in the air with the aid of a fan and stirred with two percent aqueous sodium or potassium hydroxide solution. After filtration, this process was repeated three or four times. The combined filtrates are found to contain the active material in aqueous solution, and may be further concentrated by evaporation and fractional solution in aqueous ethyl alcohol or other water-soluble organic solvents.

DISCUSSION. The results of the present study demonstrate the capacity of certain marine and vegetable oils to reduce the blood pressure of experimental animals and human patients with chronic hypertension. The same materials do not affect the pressure of normal animals. The question arises as to the relation of the active principle responsible for this action to that present in renal extracts. Insofar as the available facts are concerned, the results obtained by extracts of these two sources are comparable and it is possible that we are dealing with the same substance or substances of related chemical structure. The active principle present in oils is apparently a derivative obtained by oxidation of a fatty acid or other esterifiable substance, since saponification prior to oxidation in many cases increases the yield of the active material. The fact that auto-oxidation and the use of relatively feeble oxidation agents results in the formation of active extracts suggest that the precursor concerned is unsaturated. The resulting

Thin layer chromatography on Silica Gel G (Merck) in light petroleum, b.p. 30–60°, revealed at least six components, and charring with sulfuric acid yielded brown to black coloration, suggesting the absence of conjugation. After complete hydrogenation, thin-layer chromatography yielded only one spot, suggesting that the six or more original components were unsaturated.

To achieve partial identification of some of the hydrocarbons, the mixture was completely hydrogenated and the straight-chain and branched components were separated by urea fractionation in isopropanol. The non-complex fraction had a near-infrared spectrum similar to pristane[1] and the complexed fraction had a spectrum similar to eicosane. Gas chromatographic analysis was performed on each fraction using a Barber Coleman Model 20 instrument, with a 1.25 meter × 6 mm column containing 15 % Apiezon L on celite. Chromatograms were run at 180° and 230° for short and long chain components, respectively. The results of these analyses are given in Table 1.

All straight-chain paraffins from C_{14} through C_{33} were observed in the urea-complexed fraction. These were identified by semilog plots using a few normal paraffins as standards. No other components were observed except one unknown substance having a carbon number of 17.6. Odd numbered paraffins were dominant. The 15, 17, 19, and 21 carbon hydrocarbons, probably originally unsaturated, might correspond to decarboxylation products of the principal fatty acids of herring oil. The 25, 27, and 29 carbon members of the series correspond in chain length to the principal wax hydrocarbons of plants. It should be noted that the most prominent plant wax hydrocarbons are saturated and have 29 and 31 carbons[4], whereas the hydrocarbons of these chain lengths in herring oil are probably unsaturated.

The branched chain hydrocarbons were dominated by pristane, and squalane (carbon number 26.3) was of next importance. The structures of the minor constituents remain unknown.

Acknowledgements. We are indebted to the J. C. Martens Co., Bergen, for the herring oil, to Prof. N. A. Sørensen of Trondheim for authentic pristane, and to the American Petroleum Institute for a series of normal paraffin standards. The work was done at the Hormel Institute (1958–1959) under a stipend to G.L. by the *Royal Norwegian Government*. The work was partially supported by a grant from the *National Institutes of Health* (H3559).

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Received December 7, 1962.

J. Am. Oil Chem. Soc. 48: 389-391, 1971

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

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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

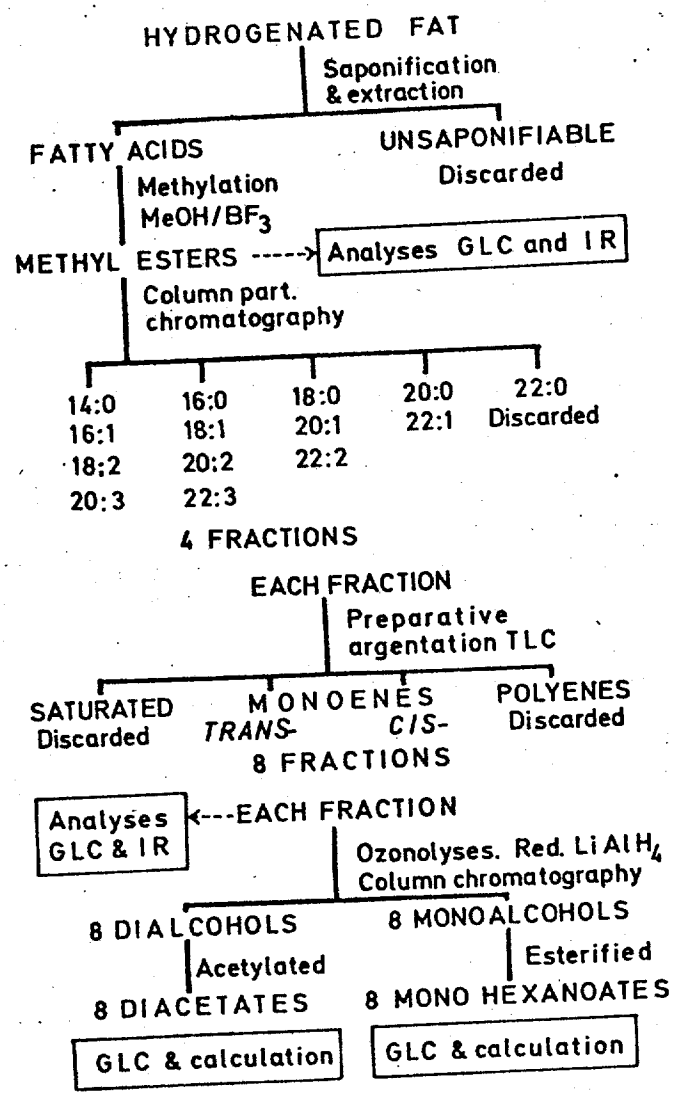

FIG. 1. Analytical procedure.

[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 TLC, 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 of 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

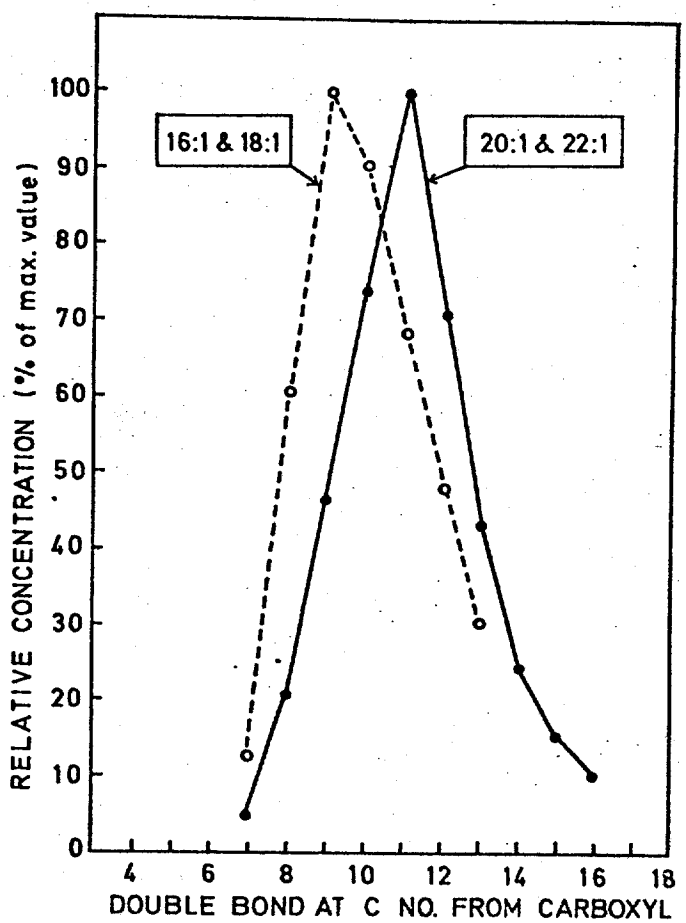

FIG. 2. Double bond positions in monoene acids of hydrogenated fish oils from dialcohol GLC. Mean concentrations for *cis* and *trans*-isomers.

complexes hydrolyzed by careful addition of dilute sulfuri acid. This hydrolysis was carried out at a temperatur around 0 C. The mixture was diluted with water and th mono- and dialcohols extracted with ethyl ether. Hexan was added to this extract to give a final mixture of ethy ether-hexane 20:80, followed by chromatography throug a small column of alumina which retained the dialcohol giving a solution of the monoalcohols. The dialcohols wer subsequently eluted with pure ethyl ether.

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

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

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

RESULTS AND DISCUSSION

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

The agentation chromatographic step is mostly used t separate according to unsaturation. Our problem was t 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

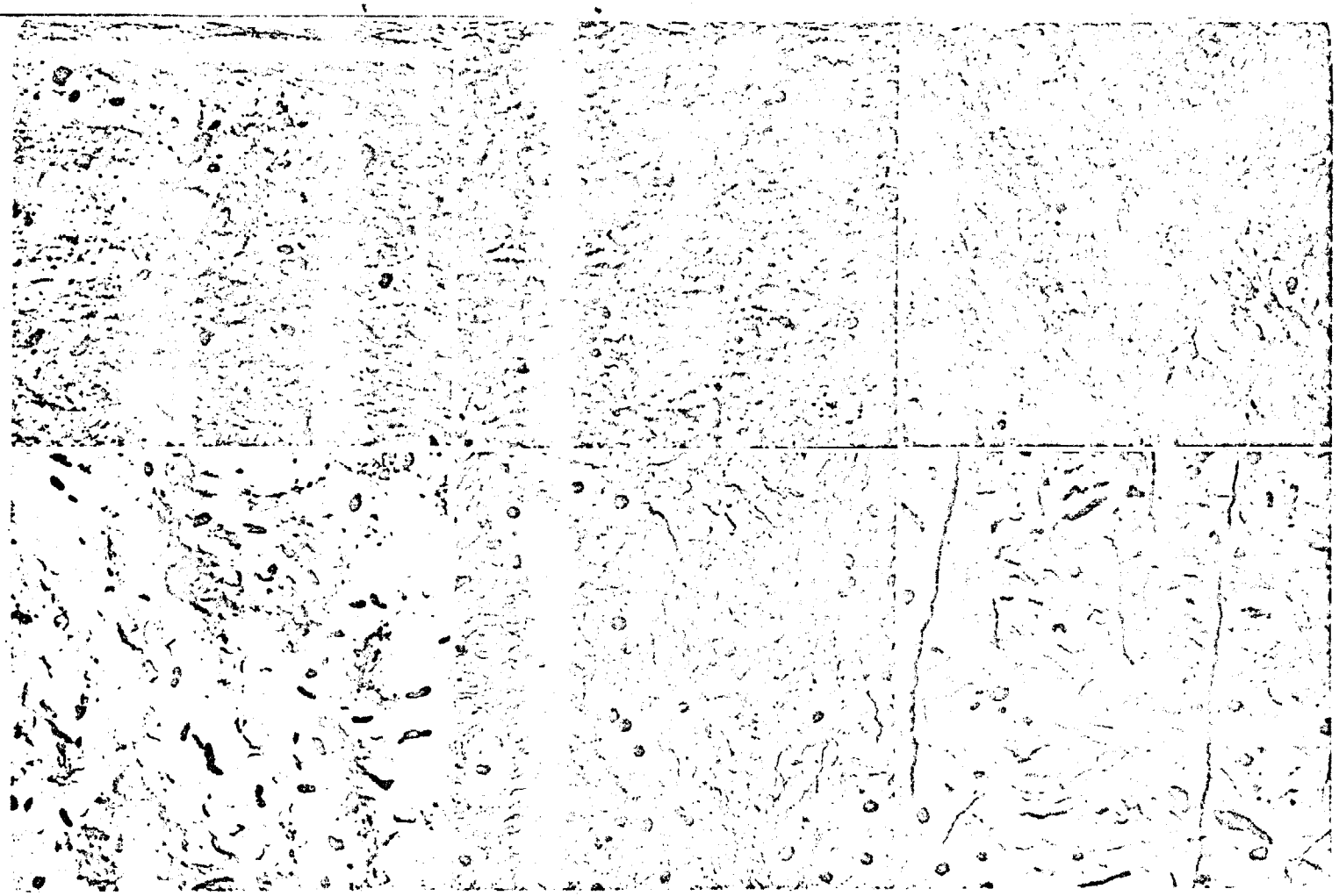

Fig 1.—Photomicrographs from occipital lobe white matter. Upper panels stained for myelin, lower stained for axons. *Right column*, control 1, measured axonal density index=54. *Middle column*, MS 1 NAWM, index=86. *Left column*, MS 1 plaque, index=14. The plaque shows total demyelination. The increased 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.[14] 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.[19] 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 (10 ml total) was followed by elution with 15 ml of chloroform-methanol 1:4, which yielded the gangliosides.[20] (A 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.[21] Bromthymol blue, followed by 2-naphthol/sulfuric acid,[22] 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 demylination (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 concentration of gangliosides in fresh tissue is 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[illegible]. This retention of ganglioside is emphasized by the comparison with the weight of total lipid. Where control and NAWM values range between 610 and 1,670 mμmoles/gm lipids, the demyelinated areas range between 2,540 and 6,890 mμmoles/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, 22:5ω3, which is not measurable in normal white matter, is seen as a distant 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 116 (standard error of the mean = 17), while the two NAWM samples examined yielded a lower value, 73 (58 and 87). The density index for seven

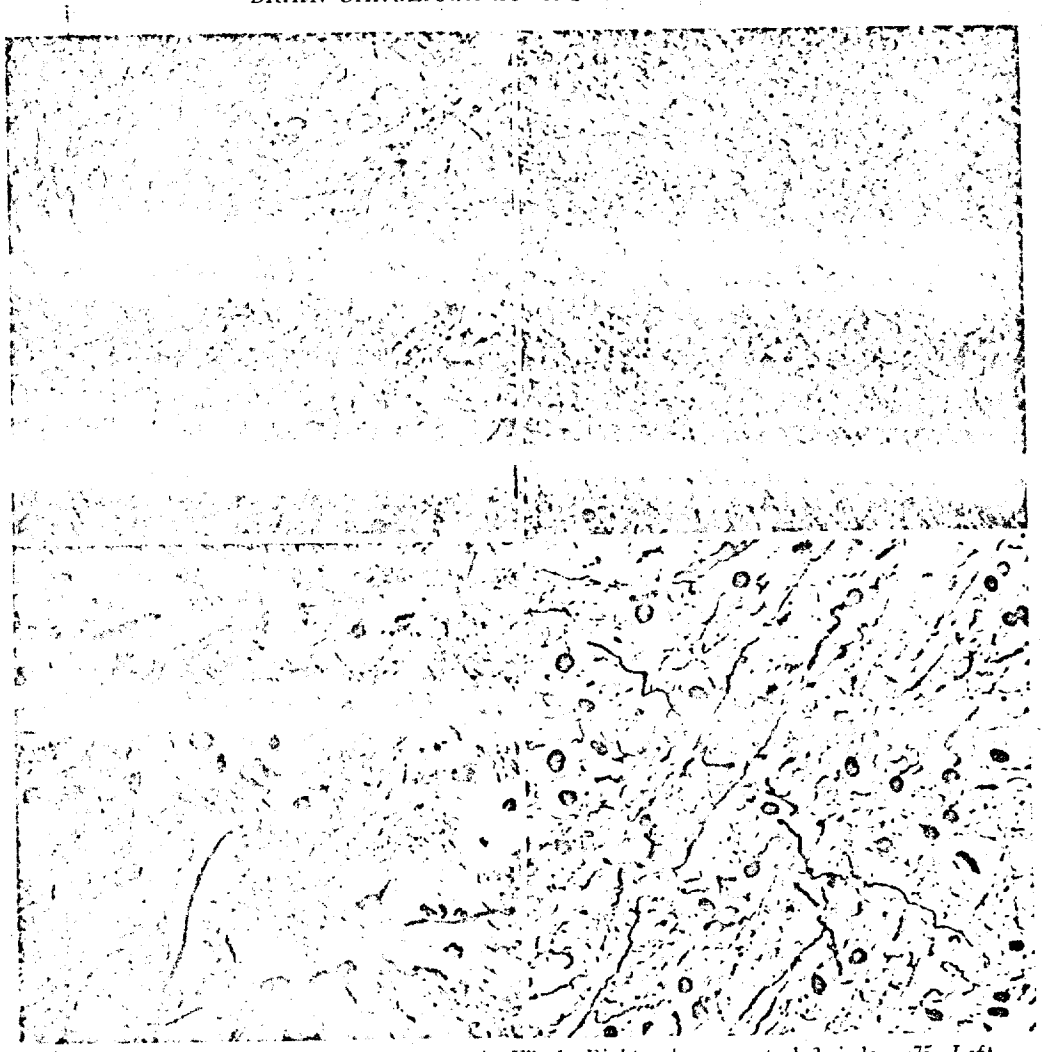

Fig 3.—Frontal lobe white matter, as in Fig 1. *Right column,* control 3, index=75. *Left column,* MS 3, index=19.

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±0.46 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	890		161	24
		740	875		159	25
MS 3			833			13
			883			19
			884			17
			910			30
			918			22
Control 1	670			180		
	728			152		
Control 2	683			216		
	694			202		
	698			216		
	710			187		
Control 3	724			125		
	734			141		
	742			147		
	743			152		
	752			129		
Average	716	712	884	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
		306	235		1170	6890
MS 3			48			3640
			115			6000
			92			5370
			137			4530
			109			4980
Control 1	118			660		
	93			610		
Control 2	184			850		
	269			1330		
	190			870		
	233			1240		
Control 3	209			1670		
	156			1100		
	212			1430		
	247			1610		
	171			1320		
Average	189	223	126	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*

Patient	mg/gm Fresh Tissue			mg/gm Total Lipids		
	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			165
			4.1			187
Control 1	23.2			129		
	21.2			149		
Control 2	36.6			169		
	34.3			170		
	35.9			166		
	32.9			176		
Control 3	21.6			173		
	22.5			169		
	22.7			154		
	26.4			164		
	22.5			174		
Average	27.3	25.0	4.8	162	152	221

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*

Fatty Acid *	NAWM and Normal Tissue					Demyelinated Tissue		
	MS 1	MS 2	C 1	C 2	C 3	MS 1	MS 2	MS 3
16:0	15.8	15.0	15.3	14.0	15.3	27.3	23.5	26.2
16:1	2.2	2.1	1.6	1.8	2.0	3.7	4.0	3.1
18:0	21.2	20.6	20.7	20.4	21.4	10.0	14.8	13.4
18:1	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	5.3	1.5	1.5	1.8
20:4ω6	4.1	5.9	3.7	5.2	4.1	12.0	10.1	10.5
22:5ω3	...	...	...	...	...	1.7	2.0	1.5
22:5ω6	5.9	7.0	5.6	7.4	5.8	4.4	2.8	2.3
22:6ω3	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 the α is shown, by the position of the first double bond, counting from the methyl end of the molecule. Where the double bond position is not shown, the fatty acid is a mixture of isomers differing in the location of the bond.[10]

The numbers in the table refer to percentages of the total fatty acids in the glycerophospholipid samples. The averages of replicate analyses 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

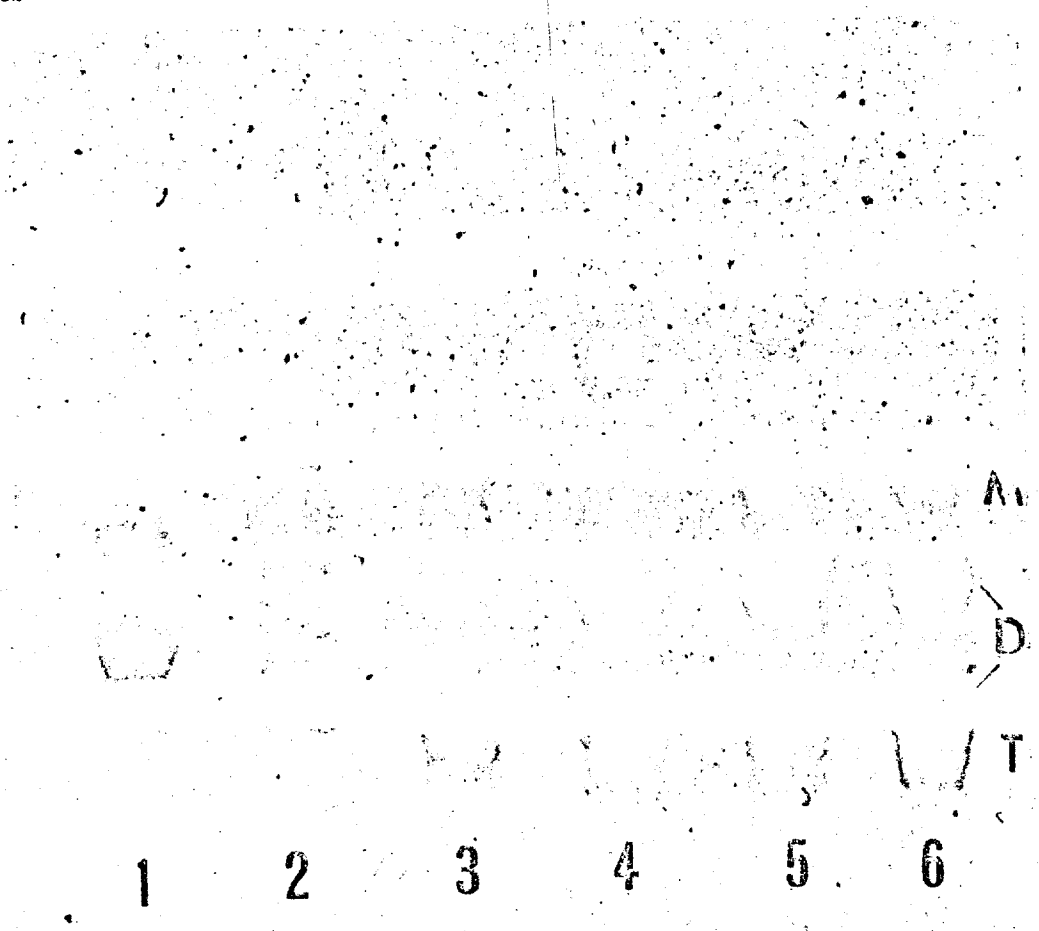

Fig 4.—Thin-layer chromatogram of gangliosides from different brain areas. *Lane 1*, mixture of standards; *2*, MS 3; *3*, MS 2 plaque; *4*, MS 2 NAWM; *5*, control 1 white matter; *6*, control 1 gray matter. *M* indicates monosialo; *D*, disialo, *T*, trisialogangliosides.

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 higher 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	20.6
18:1	34.7	23.8	43.7	44.0
20:1	1.6	1.1	5.1	4.8
20:4	10.9	8.8	5.0	4.6
22:5	4.9	4.6	6.5	7.7
22:6	5.9	16.7	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.[24] 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.

This study was supported in part by grants from the National Multiple Sclerosis Society (275-5) and the National Institute of Neurological Diseases and Blindness, US Public Health Services (NB 03192). A brief account of this work has been reported previously.[29]

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 Mendelssolm and Samuel Morley. But nobody thought of babies. Everybody was thinking of battles.

J. Am. Oil Chem. Soc., 45: 197-209, 1968

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

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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_{Le}/K_{Lo}) 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_{Le}/K_{Lo})
1	Cu:3A–K_2CO_3	37	15.0	2.1	9.8	5.0
2	Cu:4A–Na_2CO_3	19	10.3	1.4	7.2	10.9
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	2.6	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%)	24	14.8	1.0	9.9	9.1
9	Cu:SK40–Na_2CO_3	95	10.5	1.7	7.8	9.8

TABLE II

Hydrogenation of Soybean Oil with Copper-Exchanged Molecular Sieve Catalysts (300 ml oil at 170C and 30 psi)

Expt. No.	Catalyst	Reaction time, min	IV Drop (GLC)	% Linolenate (alkali isomerization)	*trans*, %	Selectivity, (K_{Le}/K_{Lo})
10	Cu:4A–Na_2CO_3	60	12.8	0.9	8.8	11.1
11	Cu:13X–Na_2CO_3 (0.26%)[a]	40	13.0	1.4	9.1	8.6
12	4A–Na_2CO_3–$Cu(NO_3)_2$ (0.2%)	22	11.8	1.1	7.2	12.1
13	Cu:SK10–Na_2CO_3	37	12.2	1.0	9.1	10.7
14	Cu in sieve $\frac{SiO_2}{Al_2O_3} = 1.2$ (0.2%)	12	12.7	0.7	9.7	12.4
15	Cu in sieve $\frac{SiO_2}{Al_2O_3} = 1.2$ (0.1%)	45	12.8	0.8	9.3	12.2
16[b]	Cu in sieve $\frac{SiO_2}{Al_2O_3} = 1.2$ (0.1%)	28	10.7	1.3	6.6	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_{Le}/K_{Lo}) 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 30 psi; catalyst, 0.1% as copper oxide)

Expt. No.	Support	Precipitating agent	Reaction time, min	IV Drop (GLC)	% Linolenate (alkali isomerization)	*trans*, %	Selectivity (K_{Le}/K_{Lo})
18	Celite	NaOH	165	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	7.9	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	66	11.1	1.3	8.0	12.3
24	Washed Silica 923[a]	NaOH	54	14.9	0.9	7.7	11.6
25	MS Gel	NaOH	45	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	25	12.0	0.9	10.0	12.0
29	Syloid 63	NaOH	28	13.7	0.6	9.9	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	Harshaw alumina[b]	Na_2CO_3	57	11.4	1.7	8.5	9.0
33	Al_2O_3	Na_2CO_3	54	15.4	0.6	7.6	14.2
34	SiO_2	NaOH	37	13.6	0.9	9.0	12.1
35	None	$NaAlO_2$[b]	64	13.0	1.3	10.1	9.2
36	None	Na_2SiO_3[b]	40	12.5	1.2	8.9	10.0
37	Raney copper[c]		21	11.9	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)	% Linolenate (alkali isomerization)	*trans*, %	Selectivity (K_{L_3}/K_{L_2})
38	Cab-o-Sil M5	Na_2CO_3	13	12.1	0.8	9.8	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	11.5	12.8	0.5	8.6	15.3
42[b]	Cab-o-Sil M5	Na_2CO_3	12.5	12.6	0.5	10.1	16.0
43[a]	Cab-o-Sil M5[c]	Na_2CO_3	16	13.0	0.6	9.1	13.9
44[a]	Cab-o-Sil M5	NaOH	55	11.4	1.4	7.2	11.4
45[a]	Santocel 62	Na_2CO_3	13	13.4	0.6	9.6	14.4
46	Cab-o-Sil EH5	Na_2CO_3	22	15.9	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[a]	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	7.2	13.2
50[a]	Aerosil[d]	Na_2CO_3	31	10.0	1.2	7.7	14.9
51[a]	Nickel catalyst[e]		2	15.1	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] G-15 commercial nickel catalyst (Girdler 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

Infrared analyses were performed by Miss Janina Nowakowska and Richard L. Hoffmann. The author is grateful to H. J. Dutton and J. C. Cowan for their counsel in the investigation.

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[Received October 13, 1967]

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The Sterols of Seafood

SUMMARY--The sterol composition of several varieties of seafood was assayed by gas-liquid chromatography. Cholesterol is the major sterol (over 90%) in haddock, pollock, salmon, shrimp, and lobster. The predominant sterols in oyster are cholesterol (41%) and 24-methylenecholesterol (26%), and in crab the major sterols are cholesterol (57%) and brassicasterol (37%). Scallop and clam contain a large number of sterols, the predominant one being cholesterol (26 and 37%, respectively). The sterols other than cholesterol give different chromogens in the colorimetric analysis for sterols, which may account in part for the variability in the "cholesterol" values found in the literature.

INTRODUCTION

The question of the cholesterol content of shellfish and other seafood often arises in the design of special low-cholesterol diets. The values reported in available literature for the cholesterol content of shellfish vary, making difficult an accurate assessment of dietary cholesterol content. The work presented in this report was undertaken to ascertain what percentage of the nonsaponifiable fraction of certain shellfish and other fish was indeed cholesterol.

METHODS

Finely-minced samples of the meat of the seafood under study were extracted by stirring continuously with chloroform-methanol (2:1) for 6 hr. The chloroform layer was dried, and aliquots were saponified with 15% alc. KOH. The nonsaponifiable material was extracted with petroleum ether, and the sterol digitonides were prepared and analyzed colorimetrically by the method of Sperry and Webb (1950). The values obtained were compared with a cholesterol standard. Other samples of fish were saponified directly with 15% alc. KOH, the nonsaponifiable content assayed gravimetrically, and the sterol content determined colorimetrically.

Gas-liquid chromatography was carried out with a Barber-Colman gas chromatograph. The liquid phase 4.1% SE-52 adsorbed on Gas-Chrom P (80-100-m The column (4 ft) was operated at 231°C. Thin chromatography was carried out on silver-nitrate-im nated silica gel H (DiFullio *et al.*, 1965). The s was 10% acetone in chloroform; the compounds detected by spraying with concentrated H_2SO_4.

Table 1. Gas-chromatographic retention times of various non-saponifiable compounds (compared with cholesterol = 1.00).

Compound	This study	Idler *et al.* (1964)
Squalene	0.49	.....
C_{26} compounds	.....	0.64
22-Dehydrocholesterol	.....	0.90
Desmosterol	1.13	.....
Brassicasterol	1.13	1.13
Zymosterol	1.24	.....
Dehydrobrassicasterol	1.29	.....
24-Methylene cholesterol	1.31	.....
C_{29} sterols	.....	1.58 and 1.73
Lanosterol	1.69	.....

RESULTS AND DISCUSSION

The identity of the peaks obtained from gas-chron graphic analysis of the various nonsaponifiable frac was based on the relationship of their retention tim those of cholesterol. Table 1 presents the values obt in the present study and compares them with those ob by Idler *et al.* (1964) in a study of the sterols of se muscle. Fig. 1 presents a typical gas chromatogram.

The average sterol content of a number of diff shellfish and fish is given in Table 2. It is evident

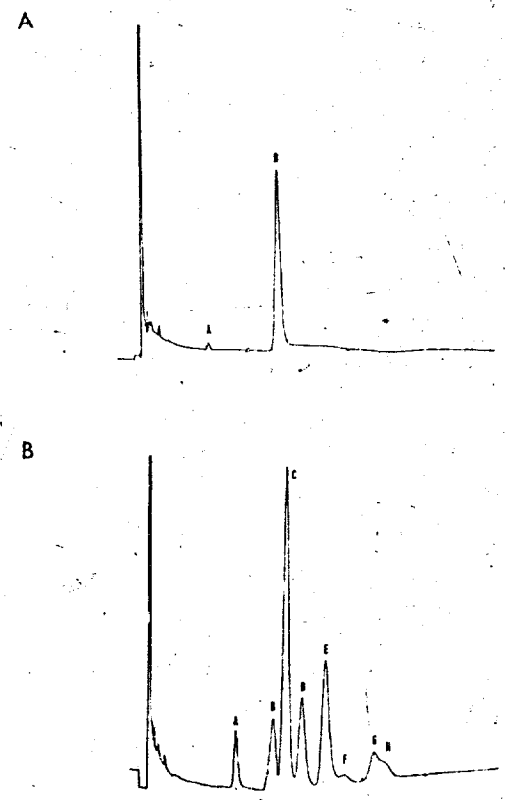


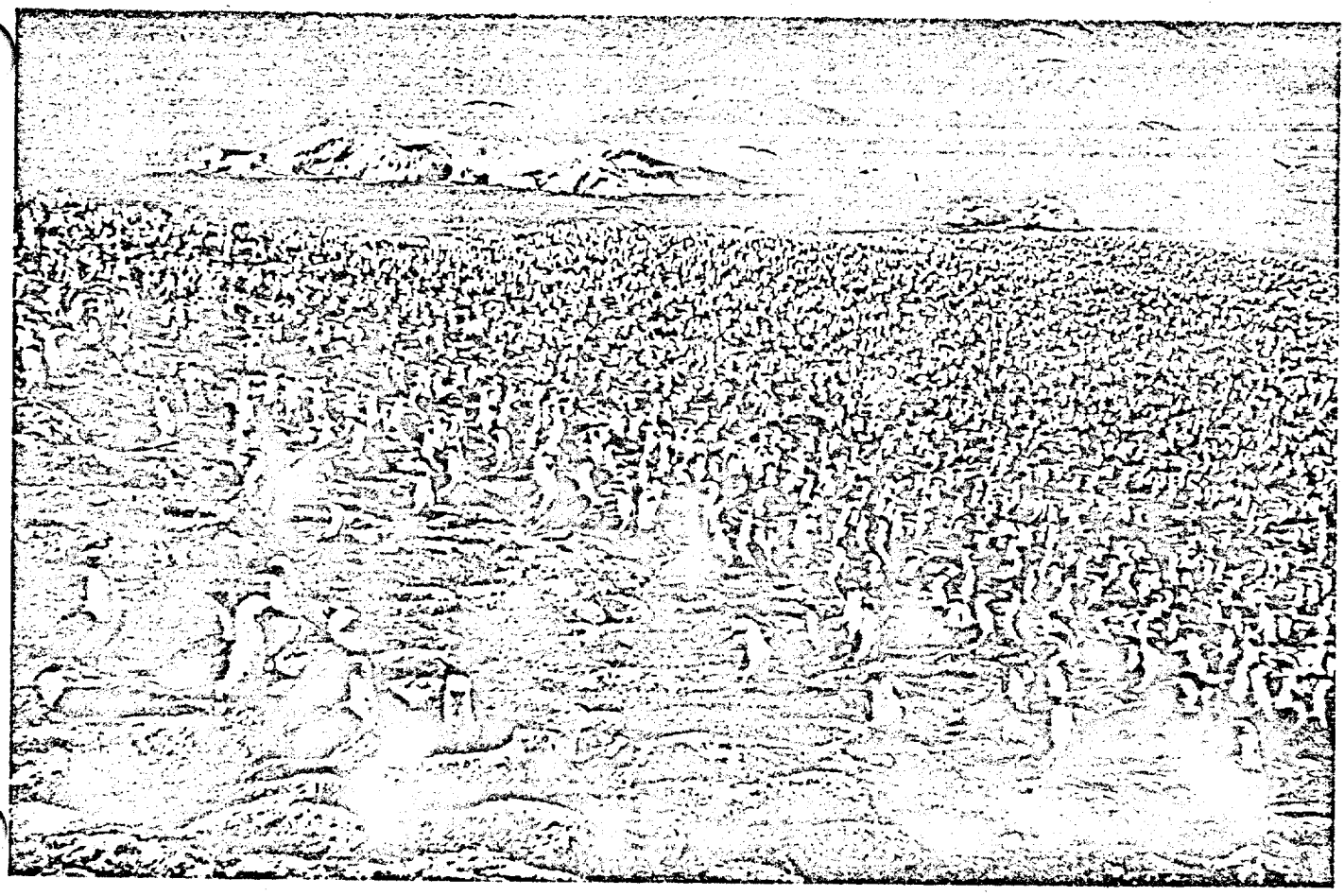
Fig. 1. Gas chromatograph of sterols of non-saponifiable matter of seafood.
A) Salmon. A = squalene; B = cholesterol.
B) Clam. A = C_{26} sterol; B = dehydrocholesterol; C = cholesterol; D = brassicasterol; E = 24 methylene cholesterol; F, G, M, = C_{29} sterols.

Quite apart from the sea change, however, the Peruvian commercial fishery must accept a share of the blame. The 1970 catch of 12.3 million tons considerably exceeded the 10-million-ton level that fishery biologists had estimated to be the maximum sustainable yield of the Peruvian stock. Several economic and political stresses were responsible for the excessive catch. Foremost among these harsh realities is that there are many more fishing boats and fish-meal factories in Peru than are needed to harvest and process the catch. The anchovy fleet is so large that it could harvest the equivalent of the annual U.S. catch of yellowfin tuna in a single day or the annual U.S. salmon catch in two and a half days. The fleet could be reduced by more than 25 percent and still comfortably harvest a rational quota of 10 million tons of anchovies a year. Moreover, the record 1970 catch figure does not measure the full toll the fishery took of the anchovy stock that year. Conservative estimates of losses from spoilage at sea and unloading and processing ashore raise the commercial total to some 13 or 14 million tons.

At this writing the future of the Peruvian anchovy is uncertain. The gloomy forecasts based on biological sampling in 1972 have been confirmed by additional observations early this year and by the results of trial fishing allowed at that time. For three weeks in March the anchovy fleet went to sea and about one million tons of fish were caught. During that brief period the catch per unit of fishing effort (a statistic that provides a measure of the size of the stock) declined rapidly. This suggests that the fleet had caught a significant proportion of all the fish that were available. Most of the catch consisted of fish recruited since July, 1972. There will not be any substantial additions to the stock until this coming October, when the progeny of the present population, much reduced by the fishing in March, have grown big enough to enter the fishery. Even so, fishing was authorized again in April with the quota set at 800,000 tons. Only 400,000 tons were taken. At present the 1973 catch is forecast at no more than three million tons.

If things are as bad as the worst prognostications indicate, the anchovy fishery may, like the California sardine industry and the Hokkaido herring industry, collapse forever. Many aspects of the history of the Peru fishery bear a disturbing resemblance to the events that brought about these earlier disasters.

Nature being what it is, the Peruvian coast will sooner or later once again have normal winds and ocean currents. If the anchovy population has not been too severely reduced, the fishery will then begin to recover. On the other hand, human nature being what it is, difficulties may arise in enforcing soon enough and strictly enough the moderate catch quotas required to avoid overexploitation of the diminished population. Unless such a policy of moderation is achieved, not only will the fish stock suffer but also the world of the Peruvian anchovy will be permanently changed. The guano-bird population will be further reduced and perhaps even eliminated. There will also be enormously complex effects among the many other animals that depend to a greater or lesser degree on the presence of the little fish. Finally, if the anchovies' world is allowed to go awry, the biggest loser will be man. He will have lost not only a rich natural resource but also some of the quality of his own world.



GUANO ISLAND off the coast of Peru is a nesting ground for a vast population of piqueros, or boobies. Cormorants, pelicans and boobies are the principal guano birds. Although now reduced in numbers, they once ate some 2.5 million tons of anchovies per year.

J Am oil chem Soc 42 246-249, 1965

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

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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 o and linseed oil.

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

Chang (10), however, has made a clear distinctio between the two flavors and carried out investigation into the hardening flavor of hardened soybean oi By means of a degassing technique Chang (11), ob tained a flavor conc from hardened soybean oil whic conc then was separated into fractions. He found tha the characteristic hardening flavor is present in th fractions of the saturated aldehydes and ketones (12)

For a number of years the problem of the hardenin flavor has been investigated in our laboratory and i has been established that the hardening flavor is pres ent in the mixture of aldehydes which can be conc from hardened soybean oil and linseed oil.

Procedure and Discussion

Since both hardened soybean and linseed oils de velop hardening flavors on storage, it was first in vestigated what unsaturated fatty acid is responsibl for this flavor. Elias (13) has found that on harden ing ethyllinolenate using a nickel 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 it 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 conc 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 conc was carried

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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 compounds(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:

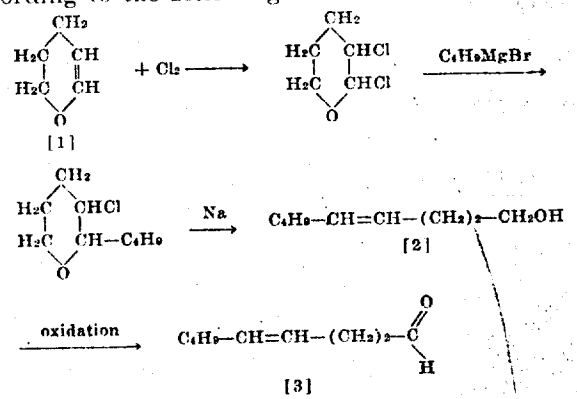
a) A small portion was oxidized with moist silver oxide for 1 hr at 60C (17,18) after which the hardening flavor disappeared completely. From this finding the conclusion was drawn that an aldehyde was present which is even very likely to have the following group $R-CH_2-CH_2-CH_2-\overset{\overset{O}{\|}}{C}-H$, 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.

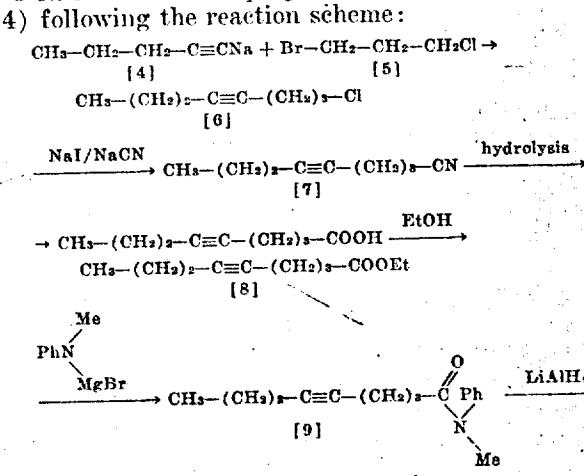
Therefore, it was assumed that an unsaturated C_9-aldehyde was present, having a double bond which is further removed from the carbonyl group than in the case of 2-nonenal. By synthesis of 4-,5- and 6-nonenals and comparing their flavor, flavor potency and R_v of their DNPHs, it was found that 6-*cis*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

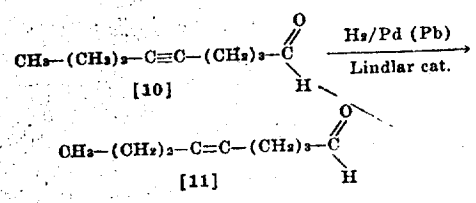
Synthesis of 4-,5- and 6-Nonenals. 4-Nonenal was synthesized starting from dihydropyran (I) (20) according to the following reaction scheme:



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:

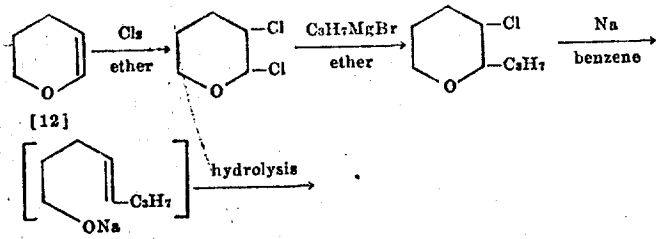




1-Chloro-3-bromopropane [5] was prepared from allyl chloride with HBr under the influence of UV radiation (28). 1-Chloro-4-octyne [6] was converted into the cyanide [7] via the iodide, and the cyanide [7] hydrolyzed and esterified with ethanol. To prevent formation of a *trans* double bond in the final 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 tetrahydrido 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_v 5-nonynal-DNPH = 250 ml, R_v 5-nonenal-DNPH = 112 ml.

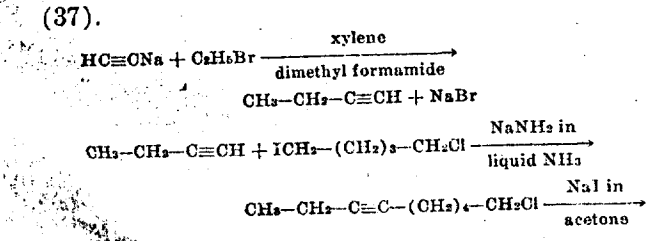
In an attempt to hydrogenate [7] directly to [10] by means of lithium tetrahydrido aluminate, no results were obtained.

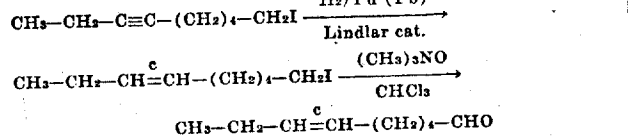
5-trans-Nonenal was prepared in accordance with the following reaction scheme:



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.

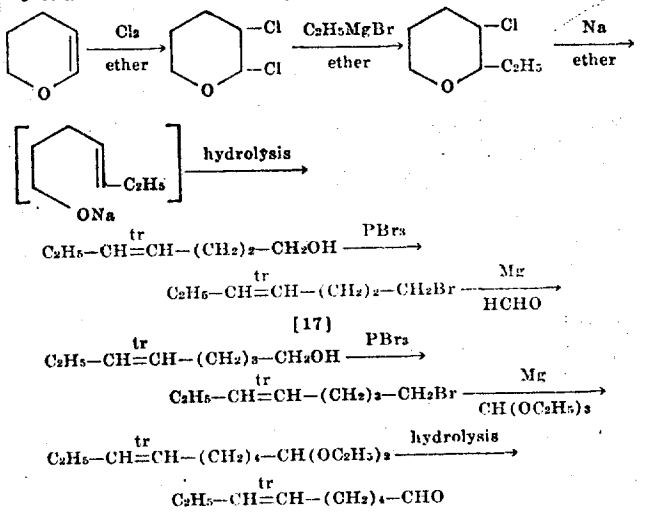
6-cis-Nonenal was prepared according to the following scheme (34–36). The last stage in the synthesis was carried out by the method of Franzen and Otto (37).





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 analogou to that for 5-*trans*-compound with the exception of th 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. Th yield of 6-*trans*-nonenal was 70%, purity being 99%

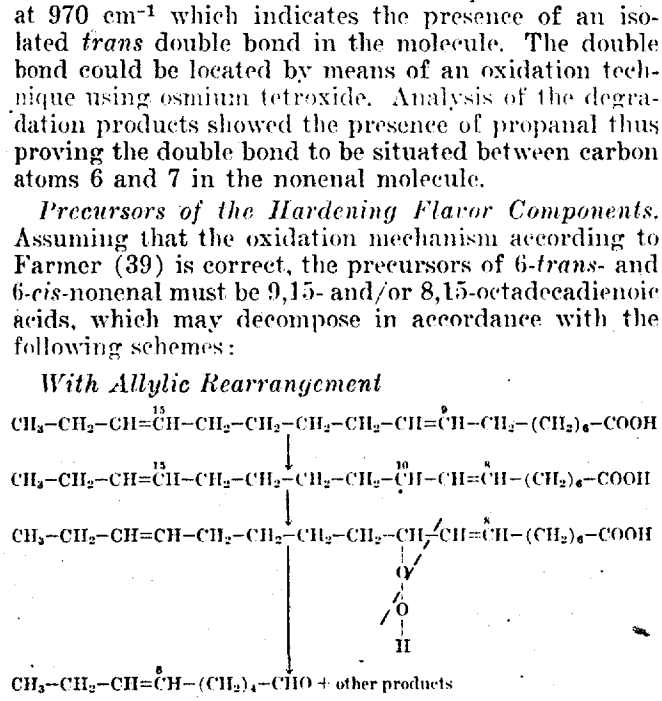
Both 6-*cis*- and 6-*trans*-nonenal have a typical flavo reminiscent of green melon. The flavor potency of th *trans*-form dissolved in paraffin oil is ca. 10 time stronger than that of the synthesized *cis*-form (to b published). Although the type of flavor of 5-*tran* nonenal is identical with that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

Location of the Double Bond. In order to verif the results obtained so far and to obtain addition proof of the location of the double bond, we applic a modification of the technique of De Jong et al. (4 using TLC with silica impregnated with silver nitra (41) for the separation of unsaturated DNPHs in various classes. A cone of the hardening flavor w obtained by degassing 50 kg of hardened linseed (I.V. 87.6) and by preparative GLC (apparatu F and M 770, temp 170C, press. 20 lbs/sq in, colun length 4.80 m) using polyethylene glycol adipa (20%) as immobile phase. The fraction with t typical hardening flavor and with the same retenti time as synthetic 6-*trans*-nonenal (carbon number 9. (42) was trapped in a condenser which was cool with liquid nitrogen. The volatile material was tak up in carbonyl-free light petroleum (bp 40–60C) a the carbonyls converted into their DNPHs (43). T DNPHs were purified by passing over a neut alumina column using a mixture of light petrole and an increasing amt of diethyl ether as eluent a then separated on chromatoplates using silica i pregnated with silver nitrate as adsorbent (to be p lished). From these plates two fractions were covered with the same elution rates as those of 6-*tra* and 6-*cis*-nonenal. Samples of both fractions displa the typical hardening flavor after regenerati whereas in the other fractions this flavor was present.

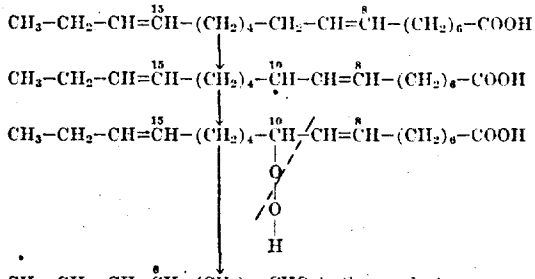
Subsequently, the two DNPH fractions were separated by TLC using silicagel G (ex Merck) (44), and the spots having the same elution rates as the model substances were recovered. After regeneration of the carbonyls from part of the DNPHs, the hardening flavor in question could be perceived again. Finally, 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

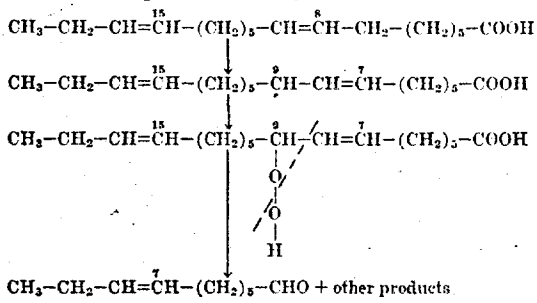


Without Allylic Rearrangement

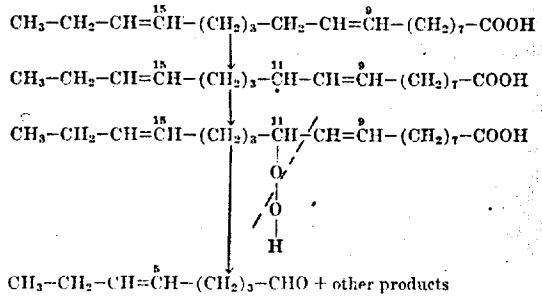


Besides the formation of both 6-*cis*- and 6-*trans*-nonenal from 9,15- and 8,15-octadecadienoic acids other monoenal degradation products may also be formed from the peroxides of these acids:

With Allylic Rearrangement



Without Allylic Rearrangement



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

ACKNOWLEDGMENT

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

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[Received July 13, 1964—Accepted October 26, 1964]

Gangliosides and Glycerophospholipids in Multiple Sclerosis White Matter

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HISTOLOGICALLY, multiple sclerosis is characterized by focal necrobiosis 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,[2-6] as well as selective loss of particular fatty acids.[5-8] 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 *mono*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 obtained 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-

Submitted for publication July 30, 1966; accepted Sept 23.

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lows: 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 about 16 hours after death.

CASE MS 2.—A 34-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 cachexia (28.6 kg [63 lb]), aspiration pneumonia, severe chronic pyelonephritis without uremia but with anemia (6.4 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 diabetes mellitus treated with tolbutamide (Orinase) 1 gm daily. Her neurological status prior to death was as follows: confined to bed; moderate dementia with aphasia; temporal pallor with blindness; ptosis; vertical and dissociated nystagmus; scanning speech; dysphagia (feeding tube required); intention tremor (jaw, head, and extremities); spastic quadraparesis (legs > arms); pallanesthesia; and incontinence (catheter). She died with a fever of 4.4 F (3.4 C), mild pulmonary congestion and edema, focal chronic pyelonephritis 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 50-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 50-year-old woman died with bronchopneumonia 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 23 hours postmortem.

Control 3.—A 65-year-old woman had at the time of death hypertensive arteriosclerotic cardiorenal vascular 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 these lines. 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.

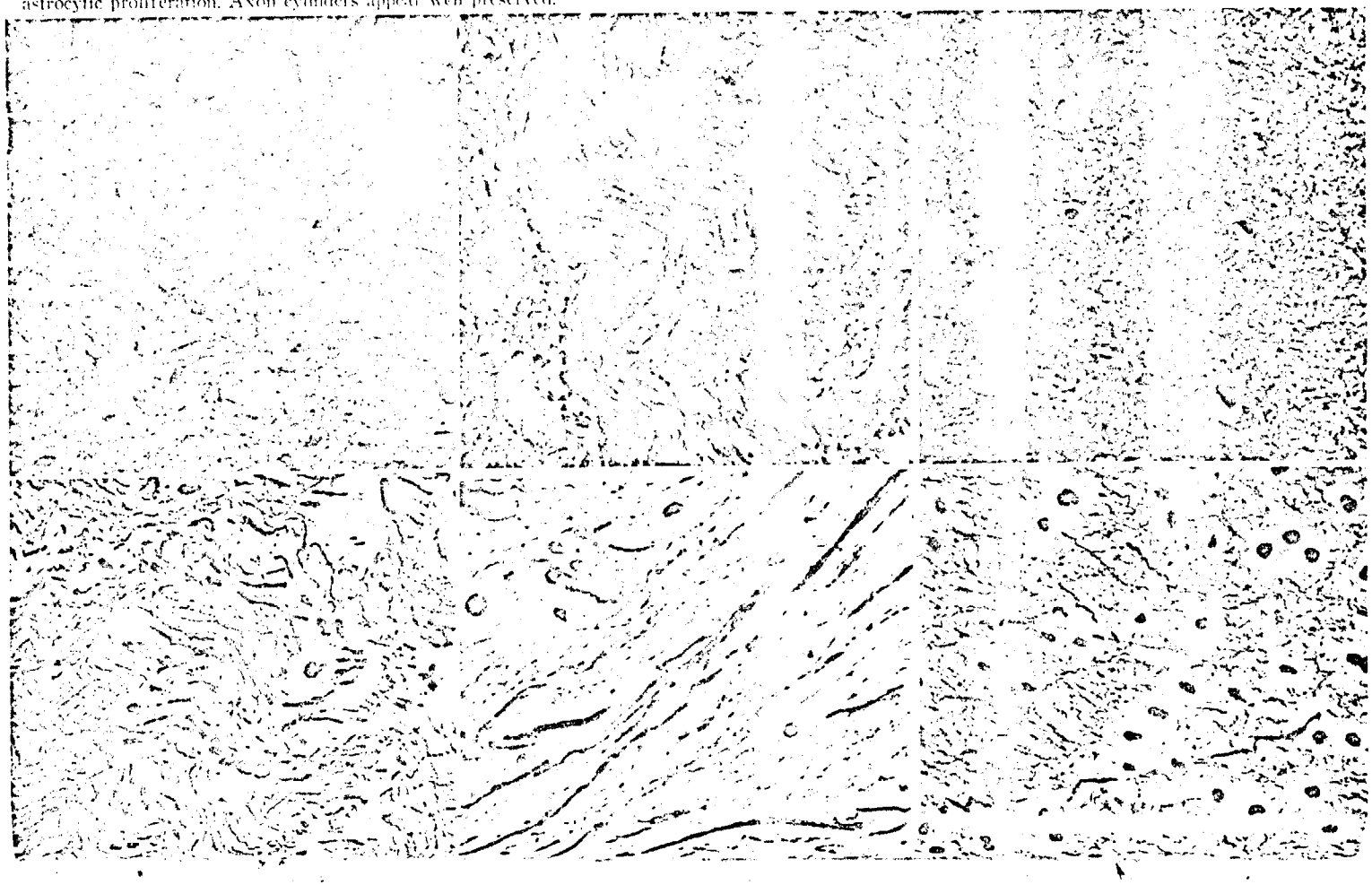
(The sheets from the plaques, incidentally, do not seem to have been noted by other workers. Possibly, it is denatured proteolipid protein liberated during myelin degeneration or a conglomeration of fibers derived from proliferated astrocytes.)

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 acids 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 Florisil chromatography and analyzed by gas chromatography. A

468

Fig 2.—Photomicrographs from frontal lobe white matter, as in Fig 1. *Right column*, control 2, index=139. *Middle column*, MS 2 NAWM, index=61. 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.



Combined Methods. Adsorption chromatography of lipid mixtures yields classes of compounds that may be further resolved by standard methods of partition chromatography. In our experience thin-layer chromatography gives separations that are more discrete and more rapid than those obtained by column chromatography. Thin layer adsorption chromatography on silicic acid is an ideal supplement and often a desirable preliminary for other separation techniques on a micro-scale, such as paper chromatography and gas-liquid chromatography.

Lipid classes, after having been isolated on silicic acid, were scratched off the plate and eluted from the adsorbent. All substances presented in Figure 1 were eluted with diethyl ether except the phospholipid, which was partially recovered with methanol. The appropriate eluant for lipids may be determined by chromatographing these substances with different solvents on silicic acid. Solvents that carry a compound with the solvent front are suitable as eluants for that substance.

Thin-layer adsorption chromatography on silicic acid was used for the purification of small amounts of lipids. About 10 mg. of impure linolenyl aldehyde, for example, were dissolved in diethyl ether, and the solution was applied along one side of a plate, 2 cm. from the edge. The aldehyde was separated within 20 min. from linolenic acid, nonadecatrienol-2, and nonadecatrienediol-1,2 with the solvent system of 95 volumes of n-hexane and 5 volumes of diethyl ether at room temperature. A narrow vertical strip of the chromatogram was sprayed with dichlorofluorescein solution. All contaminants were located close to the starting-point. The pure aldehyde, R_f 0.65, was scratched off the plate and eluted from the adsorbent with diethyl ether. Thin-layer chromatography of the recovered aldehyde indicated that it was pure.

The combination of thin-layer chromatography with reversed-phase paper chromatography is illustrated by the following example. Ratfish liver oil was saponified and acidified, and the glyceryl ethers were separated as a class from the fatty acids, Vitamin A, sterols, and hydrocarbons. The system of 70 volumes of petroleum ether, 30 volumes of diethyl ether, and 1 volume of acetic acid was used as the developing solvent. The substances were located with dichlorofluorescein. After elution from the adsorbent with diethyl ether, the glyceryl ethers were resolved into their constituents by chromatography on siliconized paper with the solvent system of 60 volumes of tetrahydrofuran and 40 volumes of water at 30 C. for 18 hrs. (6). Batyl alcohol, R_f 0.38, and selachyl alcohol, R_f 0.45, were identified as the major constituents of this fraction.

Fatty acids were analyzed by combining thin-layer chromatography with gas-liquid chromatography. A Beckman "GC-2 Gas Chromatograph"[8] was used in this investigation. Methyl esters of fatty acids derived from menhaden oil were separated from the unsaponifiable constituents by thin-layer adsorption chromatography on silica gel with a mixture of 90 volumes of petroleum ether and 10 volumes of diethyl ether. The esters appeared, after exposure to iodine vapors, as two spots, R_f 0.80 and 0.89, which were eluted with diethyl ether and analyzed by gas chromatography (1). Saturated constituents were found mainly in the upper spot whereas highly unsaturated esters were enriched in the lower. The gas-chromatogram of the total methyl ester fraction agreed in all details with a chromatogram obtained from a large-scale preparation of these methyl esters.

Many vegetable oils and some waxes and bacterial lipids contain hydroxylated acids that are altered during gas-liquid chromatography (9) so that an analysis by this method alone is difficult and often unsatisfactory. Classes of hydroxylated acids may be separated by thin-layer chromatography from the nonhydroxylated acids, and the latter may be eluted from the chromatogram and analyzed by gas-liquid chromatography. About 10 mg. of the total acidic fraction from castor oil, for example, were separated into dihydroxystearic acid, ricinoleic acid, and nonhydroxylated acids with the solvent system of 70 volumes of petroleum ether, 30 volumes of diethyl ether, and 1 volume of acetic acid. The nonhydroxylated acids, which comprise about 10% of the total, were eluted, esterified with diazomethane, and further resolved into their constituents by gas-liquid chromatography. Palmitic, stearic, and three unsaturated C_{18}-acids were identified as major components of this fraction.

In all kinds of column chromatography one depends upon analysis of the eluant, and substances remaining on the column are not detected. In thin-layer chromatography ("open columns") and in paper chromatography all components can be visualized and measured.

Conclusion

Further work has been done on the application of thin-layer adsorption chromatography to the fractionation of complex lipid mixtures into classes.

New methods, the use of siliconized silicic acid plates and the application of thin-layer adsorption chromatography combined with the complementary techniques of gas-liquid chromatography and paper chromatography, are presented for the resolution of classes of lipids into their constituents.

In contrast to such elaborate conventional techniques as column chromatography, analyses using the methods reported in this paper can be performed rapidly in large numbers on a routine basis.

Acknowledgment

The authors wish to express their appreciation to E. J. Gauglitz Jr. of the Technological Laboratory, Bureau of Commerical Fisheries, Seattle, Wash., for his help in gas-chromatographic analyses.

[8] Beckman Scientific and Process Instruments Division, Fullerton, Calif.

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[Received July 13, 1960]

Fish Oils, M.E. Stansby (Ed.), 1967

CHAPTER 5

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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.

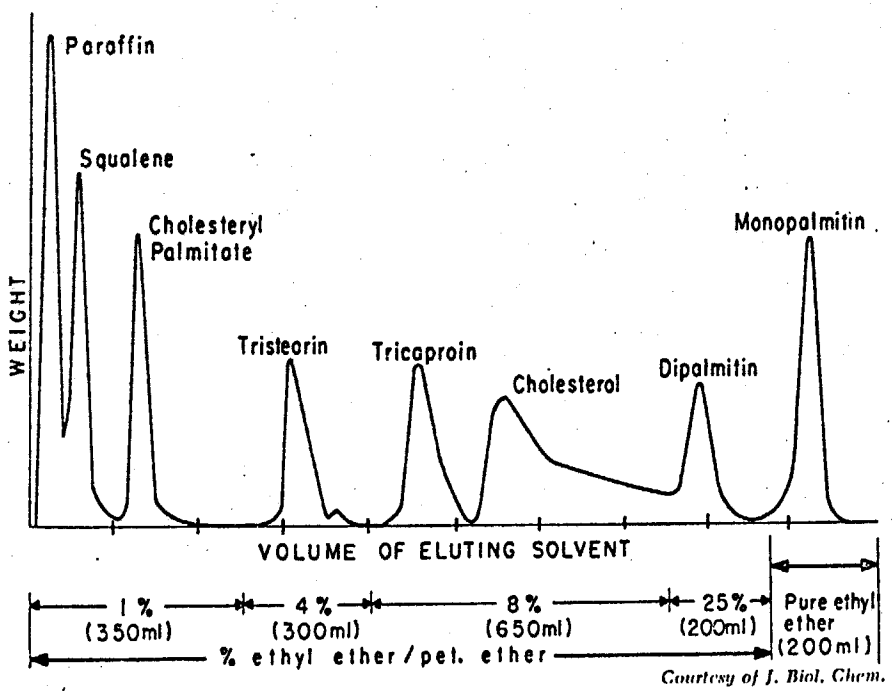
Column chromatography is widely used for the fractionation of complex mixtures of lipids, particularly those of biological origin. Best results with fish oils are generally obtained by employing carefully controlled conditions (e.g., temperature, flow rate, and particle size) and gradient elution techniques.

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 is generally 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. ADSORPTION COLUMN CHROMATOGRAPHY OF LIPIDS

lipids; and lecithins were obtained. The lecithin fraction usually contained small percentages of sphingomyelin and phosphatidyl 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 (*Anthropleura 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.

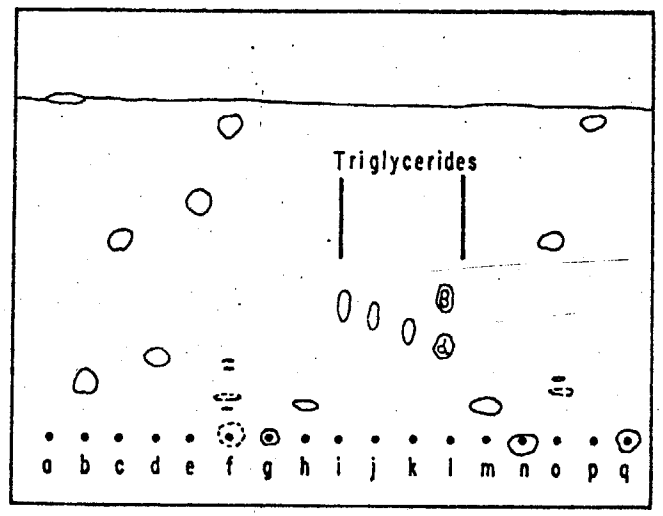


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FIG. 6. THIN-LAYER ADSORPTION CHROMATOGRAPHY OF LIPID CLASSES ON SILICA GEL

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.

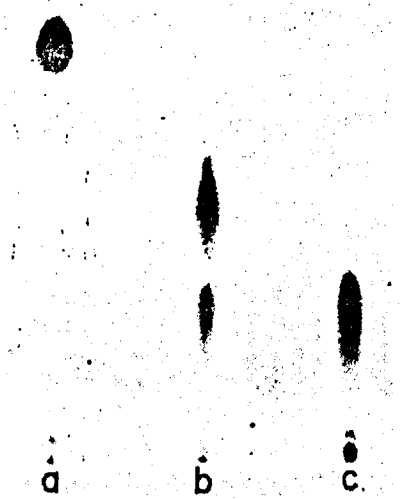

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FIG. 7. THIN-LAYER CHROMATOGRAPHY OF FISH OILS HAVING VASTLY DIFFERENT CLASS COMPOSITIONS

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 *Ruvettus 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 and co-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 *homol*

ous series according 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.

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

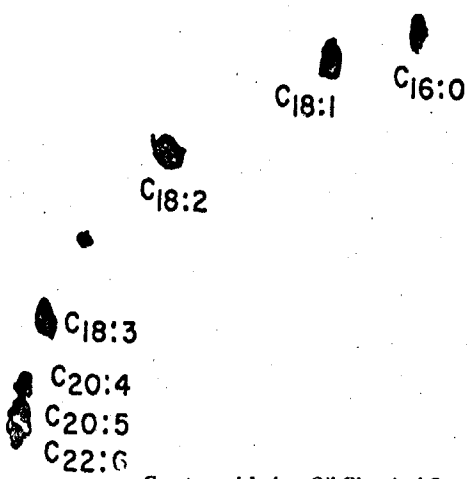

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FIG. 8. TWO-DIMENSIONAL SILVER NITRATE TLC SEPARATION OF A MIXTURE OF REFERENCE STANDARDS OF FATTY ACID METHYL ESTERS

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

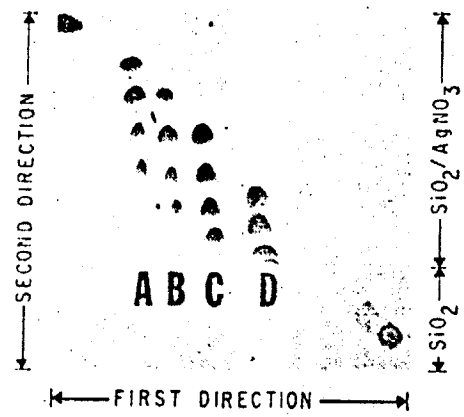


FIG. 9. TWO-DIMENSIONAL TLC EMPLOYING SILICA GEL AND SILVER NITRATE IMPREGNATED SILICA GEL ON A SINGLE PLATE

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

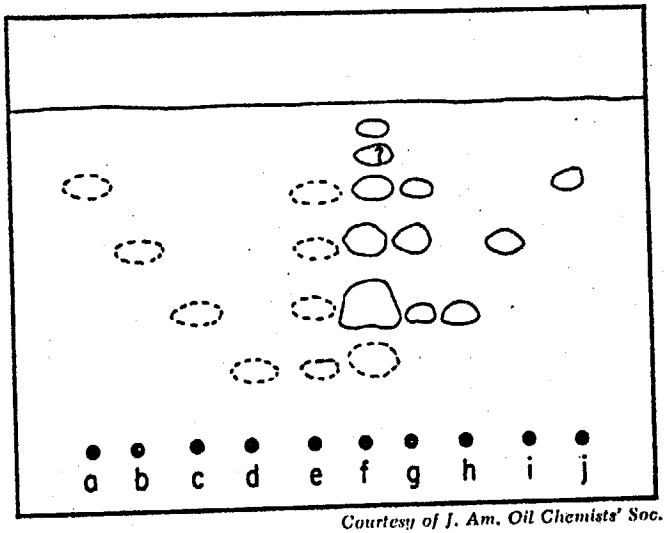
Separations *within* classes can be effected by reversed-phase partition chromatography. In such a liquid-liquid partition system, the stationary phase is a nonpolar liquid (e.g., silicone oil) and the mobile phase is a polar solvent that usually contains water (e.g., acetronitrile-water). As a general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



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.

tography for the resoluton of the odd and even chain-length acids of the mullet (*Mugil cephalus*). Kayama and co-workers (1963) in studies of the biogenesis of the polyenoic acid in kelp bass (*Paralabax clathratus*) also utilized this type of chromatography for the isolation and purification of C_{14}-labelled eicosapentaenoic acid and docosahexaenoic acid.

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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(Puget Sound) is remarkably similar, both qualitatively and quantitatively, to that obtained by Hallgren and Larsson (1962) in the analysis of *Squalus acanthias*, the Greenland shark (*Somniosus microcephalus*), and the ratfish (*Chimaera monstrosa*) from Scandinavian waters.

Because of advances in chromatographic methods for separating complex biological mixtures, it recently became possible to resolve the diacyl glyceryl ethers from other glycerolipids, notably the triglycerides that differ only slightly in structure (Mangold and Malins 1960). Malins and coworkers (1965) isolated the diacyl glyceryl ethers from dogfish (*Squalus acanthias*) flesh and liver oils and then analyzed the fatty acids. The diacyl glyceryl ethers from the flesh were characterized by high percentages of the typical C_{20} and C_{22} polyenoic acids. In contrast to the flesh, diacyl glyceryl ethers from the liver contained remarkably low percentages of these acids and high percentages of C_{20} and C_{22} monoenoic acids. It appears that the fatty acid composition of the diacyl glyceryl ethers may be quite variable and dependent upon their location in the fish. A need obviously exists for further studies relating to the composition of the individual glycerolipids, such as the ether-containing lipids.

Alkenyl ethers having a double bond between the 1- and 2-carbon atoms of the alcohol radical have been detected in the diverticula of the starfish (*Asteria forbesi*) by Gilbertson and Karnovsky (1963). The only structural difference between these compounds and the diacyl glyceryl ethers is the vinyl ether structure, but their chemical behavior is quite different.

WAX ESTERS

Wax esters are found, often in high percentages, in the oils of a number of marine species. These compounds, which presumably serve as an energy reserve, comprise a fatty alcohol (usually hexadecanol and octadec-9-enol) and a fatty acid, usually of low unsaturation.

The sea anemone (Bergmann *et al.* 1956) and crustaceans (Lovern 1935) are examples of invertebrates that contain wax esters. These lipids are also major constituents of the depot fat of a variety of fish, such as *Ruvettus pretiosus* (Nevenzel *et al.* 1965); *Xenogramma carinatum* (Matsumoto *et al.* 1955); the coelacanth, *Latimeria chalumnae* (Nevenzel and Mead 1965); *Lampanyctus ritteri* (Nevenzel and Mead 1965); *Hoplostethus islandicus* (Kaufmann and Gottschalk 1956); *Mugil japonicus* (Tsujimoto and Koyanagi 1937); *Lotella phycis* (Agawa *et al.* 1953); and *Laemonema morosum* (Ueno *et al.* 1955). Wax esters are also present in large amounts in some marine mammals, such as the dolphin (species not ascertained) (Lovern 1964), bottlenose whale (*Berardius bairdii*) (Saiki *et al.* 1958–1959), and sperm whale (*Physeter macrocephalus*) (Hilditch and Lovern 1929).

A recent analysis by Nevenzel and co-workers (1965) of the lipids from the muscle and liver of the gempylid fish, *Ruvettus pretiosus* ("castor oil fish") revealed that the muscle contained 91.5% and the liver 3.9% of wax esters. The composition of the alcohol and fatty acid components from the liver and muscle are presented in Table 6.

TABLE 6

COMPOSITION OF THE FATTY ACID AND LONG-CHAIN ALCOHOL MOIETIES OF *Ruvettus pretiosus* LIPIDS

Carbon Numbers of Components	Fatty Acids				Alcohols
	Triglycerides	Liver Free Fatty Acids	Phospholipids	Muscle Wax Esters	Muscle Wax Esters
	%	%	%	%	%
14:0	0.5	0.3	0.5	0.1	2.0
16:0	20.0	12.2	46.6	0.9	57.6
16:1	3.9	2.6	0.8	1.4	5.9
17:1	0.1	0.3	Trace	0.3	0.5
18:0	3.7	2.0	5.6	0.9	2.9
18:1	62.6	72.9	34.1	76.7	29.0
18:2	0.3	0.4	—	1.1	—
20:1	5.9	3.7	3.1	11.0	0.9
20:4	—	1.6	1.6	—	—
20:5	0.2	0.6	0.6	—	—
22:1	1.2	—	—	3.6	0.1
22:6	0.5	2.0	4.1	—	—
24:1	0.2	—	0.4	1.0	—
24:3	—	—	0.7	0.3	—
26:1	—	—	—	0.8	—

From Nevenzel et al. (1965).

PHOSPHOLIPIDS

The main phospholipids of marine animals are lecithins (phosphatidyl cholines) and phosphatidyl ethanolamines. Lecithins are usually present in the highest concentration. A number of other components common to land animals are also present in lesser amounts. In addition to lecithins and phosphatidyl ethanolamines, Silk and De Koning (1964) demonstrated the presence of cardiolipins, inositol phosphatides, cerebrosides, sphingomyelins, lysolecithins, and plasmalogens in the flesh of the South African pilchard (*Sardina ocellata*).

Several well-executed studies on the composition and positional distribution of the fatty acids in individual phospholipids have been undertaken. Shuster and co-workers (1964) analyzed by GLC the fatty acids present in the lecithins and phosphatidyl ethanolamines of tuna (*Thunnus alalunga* and *Thunnus albacares*) white muscle. The characteristic C_{20} and C_{22} polyunsaturated acids, particularly docosahexaenoic acid, were present in relatively large amounts. Other observations would suggest that the leci-

558

TABLE 7

SPECIFIC DISTRIBUTION OF THE PRINCIPLE FATTY ACIDS FROM THE LECITHINS OF FISH

Fatty Acids	Weight Per Cent of Specific Fatty Acid Isolated From									
	Salmon			Menhaden				Tuna		
	Total	α′	β	Total	α′	β	Total	α′	β
14:0	0.7	1.9	0.6	0.5	Tr	1.3	0.7	Tr	1.0
14:1	—	—	—	Tr	Tr	Tr		Tr	1.8
16:0	22	37	10	45	78	6.2	19	36	5.0
16:1	8.0	1.4	3.8	Tr	0	Tr	2.1	Tr	2.3
18:0	2.0	2.7	Tr	3.2	5.9	0.9	5.0	7.9	1.0
18:1	6.7	8.2	23	7.4	2.3	13	7.6	6.4	16
18:2	Tr	Tr	Tr	1.0	Tr	2.4	0.7	Tr	2.2
18:3	Tr	Tr	Tr	Tr	Tr	Tr	0.8	Tr	1.1
20:4	1.0	0.6	0.3	2.2	Tr	0.9	2.7	1.3	5.0
20:5	12	14	17	13	1.7	29	7.1	8.4	15
22:1	0	0	0	0	0	0	0	0	0
22:4	Tr	Tr	Tr	Tr	Tr	Tr	Tr	0	0.5
22:5	Tr	Tr	Tr	Tr	Tr	Tr	Tr	Tr	Tr
22:6	43	33	46	28	12.5	42	55	39	48

From Menzel and Olcott (1964).

thins and phosphatidyl ethanolamines of fish generally contain relatively high percentages of these acids (Lovern 1964).

Until the last few years, the positional distribution of the fatty acids in the individual phospholipids was not known. Recent studies have revealed, however, considerable definitive information on this subject. Brockerhoff and co-workers (1963) determined the positional distribution of the acids in the lecithins and phosphatidyl ethanolamines of cod (*Gadus callarias*), lobster (*Homarus americanus*), and scallop (*Platopecten magellanicus*) by the use of snake venom phospholipase A and GLC. Menzel and Olcott (1964) similarly investigated the lecithins of tuna (*Thunnus alalunga*), menhaden (*Brevoortia tyrannus*), and salmon (*Oncorhynchus tshawytscha*) muscle. The polyunsaturated acids characteristic of fish oils are located primarily on the 2-position of glycerol as was found to be the case with the triglycerides. Table 7 shows the relative distribution of the fatty acids between the 1- and 2-position of the lecithins of salmon, menhaden, and tuna muscle.

Phosphorylated plasmalogens have been detected in several marine species (Bergmann and Landowne 1958; Rapport and Alonzo 1960). Present evidence would suggest that most of these compounds are similar in structure to phosphatidyl choline and phosphatidyl ethanolamine, except that the α, β-unsaturated ether (vinyl ether) linkage replaces the ester group on the 1-position of glycerol. A careful study by Bergmann and Landowne (1958) indicated that the sea anemone contains a choline plasmalogen having an acetal structure. Later work by Rapport and Alonzo (1960) with a wide variety of marine animals indicated that 80% or more of the plasmalogens contain the vinyl ether structure. Undoubtedly, further work on plasmalogens would be most desirable to verify and elucidate the various structures presumed to be present.

The structures of several other phospholipids from marine animals have been reported in recent years. Bergmann and Landowne (1958) have isolated sphingomeyelin from the sea anemone (*Anthropleura elegantissima*). In a later investigation, the same workers identified an unusual sphingolipid that differed from sphingomeyelin by having the hydroxyl group of the 3-carbon of sphingosine acylated. Choline was not detected and the phosphate radical was present as a monoester. Palmitic and palmitoleic acids predominated.

Other novel phospholipids have also been recently identified in marine animals: Nakazawa (1959) isolated an unusual glycolipid from an oyster and Igarashi and co-workers (1958) detected a threonine-containing lipid in tunny (*Thynnus orientalis*) muscle. Rouser and co-workers (1963) also demonstrated the presence of ceramide aminethylphosphonate in the sea anemone (*Anthropleura elegantissima*) and Quin (1965) found com-

pounds having a carbon to phosphorus bond in the phospholipid fraction of a variety of marine invertebrates. Predictably, further investigations will reveal a number of new and interesting phospholipids of marine origin.

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J. Am. Oil Chem. Soc 37: 576-578, 1960

Analysis of Complex Lipid Mixtures by Thin-Layer Chromatography and Complementary Methods

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THIN-LAYER ADSORPTION chromatography on silicic acid according to Stahl (11) has been used for the resolution of lipid extracts of blood (12), acetylated lipids (3,4), and fats, oils, and waxes (7). The present contribution is concerned with further applications of this technique to lipid separations. In addition, the preparation and some applications of "siliconized chromatoplates" for the fractionation of classes into their constituents by reversed-phase partition chromatography in conjunction with thin-layer chromatography are also described.

Experimental

Preparation of Chromatoplates. Thin layers (250 to 275 μ) of silicic acid on glass (20 x 20 cm.) were prepared according to Stahl (11), using "Silica Gel G"[2] a fine grade (60μ or about 250 mesh) of silica gel containing 1% of plaster of Paris. "Silica Gel G" and the apparatus for its application were purchased from C. Desaga G.m.b.H., Heidelberg, Germany.[3]

Siliconized chromatoplates were obtained by slowly immersing the silicic acid plates at room temperature into a solution of 5% silicone[4] ("Dow Corning 200 fluid," viscosity 10 cs.) in diethyl ether. Plates may be used immediately after evaporation of the solvent.

Solvents and Indicators. Thin layers of silicic acid were developed with mixtures of petroleum ether, B.P. 60-70°C., diethyl ether, and acetic acid.

Separation within classes of lipids on siliconized chromatoplates was achieved with systems of acetonitrile-acetic acid-water or acetic acid-water. After having been sprayed with 0.2% 2′,7′-dichlorofluorescein[5] in ethanol, all lipids on untreated silicic acid films were manifested as a bright yellow-green fluorescence by ultraviolet light.[6] Unsaturated lipids were detected as brown spots on a light yellow background with iodine vapors.

Dichlorofluorescein was found to be unsatisfactory for locating the position of lipids on siliconized chromatoplates because the entire plates fluoresced. The indicators α-cyclodextrin-iodine however revealed monochain saturated lipids as white spots on a purple background (8). Unsaturated lipids were detected, in the usual manner, with iodine vapors alone. The substances were amenable to further fractionation since no evidence was found which suggested that the indicators reacted chemically with the lipids to any marked extent.

Separation of Lipids into Classes. Complex lipid mixtures may be fractionated rapidly into classes of compounds by thin-layer chromatography on silicic acid. Figure 1 demonstrates the different migration rates of a variety of lipids, ranging from hydro-

[1] Supported in part by the U. S. Public Health Service National Institutes of Health Research Grant 5817, and the National Science Foundation.

[2] E. Merck A. G., Darmstadt, Germany.

[3] "Silica Gel G" may be obtained in the U.S.A. from Terra Chemicals Inc., 500 Fifth avenue, New York 36, N. Y., or from C. A. Brinkmann and Company Inc., 115 Cutter Mill road, Great Neck, L. I., N. Y. The later firm is also the U. S. representative of C. Desaga G.m.b.H., Heidelberg, Germany.

[4] Dow Corning Corporation, Midland, Mich.

[5] Eastman Kodak Company, Rochester 3, N. Y.

[6] "Mineralight," Ultra-Violet Products Inc., San Gabriel, Calif.

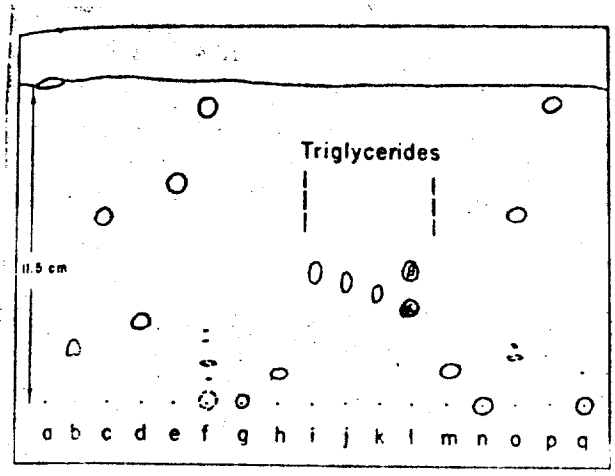

FIG. 1. Thin-layer adsorption chromatography of lipid classes on silicic acid.

Solvent system: 90 volumes of petroleum ether, B.P. 60–70°C., 10 volumes of diethyl ether, 1 volume of acetic acid.
Development time: 40 min.
Indicator: Dichlorofluorescein, 0.2% in ethanol.
Amounts: 20 γ, each.
a—Octadecene-9, 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.

carbons to phosphatides. The separation of these substances on silicic acid is effected by virtue of their differences in polarity, which depend primarily upon the type and number of functional groups in the molecules. Subfractionation of triglycerides derived from fatty acids of widely different chainlengths (1) and from unsaturated acids of the C_{18}-series (i,j,k,) is also presented. Homologous compounds, such as triglycerides, are only slightly separated from each other. Unsaturation however has a more pronounced effect on the subfractionation of lipid classes. The spots of triolein, trilinolein, and trilinolenin demonstrate the diminution in migration rate because of increasing unsaturation. These subfractionations, although perceptible with pure compounds, usually are not sufficient to interfere with the separation of classes. Larger amounts of a substance result in a slightly increased migration rate. This increased rate and the undetermined factors account for a lack of close reproducibility of R_f values. The pattern of separation of a mixture however is reproducible and reliable.

Polar substances, such as monoglycerides, glycerylethers, and phospholipids, which do not migrate under the conditions stated in Figure 1, were fractionated with a more polar solvent. A mixture of 30 volumes of petroleum ether, 70 volumes of diethyl ether, and 1 volume of acetic acid served to segregate monoolein or selachyl alcohol, both R_f 0.27, from dioleoyl lecithin, which remains at the starting point.

Resolution Within Classes on Siliconized Chromatoplates. Classes of lipids were resolved into their constituents by reversed-phase partition chromatography on siliconized silicic acid plates. Figure 2 demonstrates the separation of reference mixtures and of a C_{18}-fraction from a distillation of methyl esters derived from menhaden oil. By comparison of the spots with those of reference compounds the components of this fraction were assumed to be octadecanoic, otadecenoic, octadecadienoic, octadecatrienoic, and octadecatetraenoic acids. After iodine vapors were used for staining, the unsaturated esters were scratched off the plate and eluted from the adsorbent with diethyl ether. The identity of the polyunsaturated fatty acids was confirmed by their ultraviolet spectra after alkaline isomerization. A constituent of unknown structure was evident in the chromatogram, and comparison with reference compounds, alkaline isomerization, and gas-liquid chromatography all failed to reveal the identity of this substance.

As in chromatography on siliconized paper (8) and on columns of silicone-Celite (2), aqueous acetic acid yielded favorable separations of lipids on thin layers of siliconized silicic acid. The system of 85 volumes of acetic acid and 15 volumes of water was used to separate C_{18}-acids from each other within 3 to 4 hrs. at room temperature. The corresponding methyl esters were resolved under the same conditions. Acids and esters of chainlengths other than C_{18} were separated in solutions of acetic acid and water in different proportions.

In reversed-phase partition chromatography at ambient temperatures the rate of migration of a lipid with one double bond is about equal to that for a saturated analog shorter by two methylene groups. Consequently palmitic acid is not separated from oleic acid. Such pairs were resolved by low-temperature chromatography (10) on siliconized chromatoplates. Palmitic acid, R_f 0.0, and oleic acid, R_f 0.1, were separated in the solvent system of 40 volumes of formic acid, 40 volumes of acetic acid, and 20 volumes of water within 8 hrs. at 4–6°C.

Saturated and unsaturated acids or methyl esters were also resolved by the use of solvents in which part of the acetic acid was replaced by peracetic acid (5). By this procedure saturated substances were still separated from each other on siliconized chromatoplates in 3 to 4 hrs. at room temperature while all unsaturated compounds migrated to the solvent front as oxygenated derivatives. A mixture of 10 volumes of peracetic acid,[7] 75 volumes of acetic acid, and 15 volumes of water served as the developing solvent.

[7] Becco Chemical Division, Food Machinery and Chemical Corporation, Buffalo 7, N. Y.

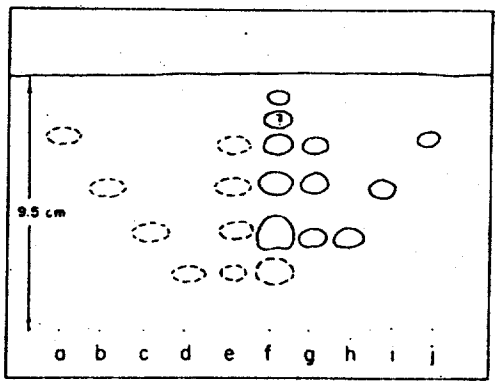

FIG. 2. Reverse-phase partition chromatography of methyl esters on a siliconized chromatoplate.

Solvent system: 70 volumes of acetonitrile, 10 volumes acetic acid, 25 volumes of water.
Development time: 40 min.
Indicators: Iodine (solid lines), followed by α-cyclodextrin 1% in 30% aqueous ethanol and iodine (dotted lines).
Amounts: 20 γ of individual compounds.
a—Methyl laurate, b—Methyl myristate, c—Methyl palmitate, d—Methyl stearate, e—Mixture of saturated methyl esters, f—Fraction derived from menhaden oil (150 γ), g—Mixture of unsaturated C_{18} esters, h—Methyl oleate, i—Methyl linoleate, j—Methyl linolenate.

With one or more carboxyl groups present in the molecule, even one hydroxyl group is effective. Glycollic $OH{\cdot}CH_2{\cdot}CO_2H$, lactic $CH_3{\cdot}CH(OH){\cdot}CO_2H$, and malic $CO_2H{\cdot}CH_2{\cdot}CH(OH){\cdot}CO_2H$, acids all possessed marked protective properties. In the case of malonic acid $CH_2(CO_2H)_2$, the two carboxyl groups plus a reactive methylene group produce a moderately powerful antioxidant, although succinic acid, in which the carboxyl groups are further separated and the methylene groups inactive, does not confer protection. A combination of two or more carboxyl groups with one or more hydroxyl groups as in tartaric $CO_2H{\cdot}CH(OH){\cdot}CH(OH){\cdot}CO_2H$, or even more so in citric acid $CO_2H{\cdot}CH_2{\cdot}C(OH)(CO_2H){\cdot}CH_2{\cdot}CO_2H$, produces a powerful antioxidant.

Aliphatic amino-acids appear to be even more effective than the corresponding hydroxy-compounds. Glycine $NH_2{\cdot}CH_2{\cdot}CO_2H$, with only one amino- and one carboxyl group, is of the same order of efficiency as citric acid. Aspartic acid $CO_2H{\cdot}CH_2{\cdot}CH(NH_2){\cdot}CO_2H$, glutamic acid $CO_2H{\cdot}CH_2{\cdot}CH_2{\cdot}CH(NH_2){\cdot}CO_2H$, and asparagine $CO_2H{\cdot}CH(NH_2){\cdot}CH_2{\cdot}CO{\cdot}NH_2$, are all powerful antioxidants. All of these substances are effective at concentrations as low as 0·01%. In some cases increasing the concentration in the aqueous phase above this value increased the protective effect (Table II), in others (*e.g.*, glycine) it did not.* Proteins (albumin and gelatin) were found to possess quite marked antioxidant properties, which suggests that natural oxidation inhibitors may in some cases be of a protein nature. Di- and tri-ethanolamine $NH(C_2H_4{\cdot}OH)_2$ and $N(C_2H_4{\cdot}OH)_3$ possessed some activity, but less than might have been anticipated from their structure.

Among inorganic substances, phosphoric acid $PO(OH)_3$ is active and boric acid $B(OH)_3$ practically inactive. No data appear to have been published on the use of phosphoric acid as an antioxidant, but since its activity was observed,[15] patents covering the use of ortho- and meta-phosphoric acid, sulphuric acid, and their salts having an acid reaction have appeared.[21] The author finds pyrophosphoric acid $P_2O_3(OH)_4$ considerably more active than the ortho-acid, particularly at low concentrations. Phosphite $HPO(OH)_2$ also seems to be better than phosphate. The use of acid phosphites for the prevention of rancidity in cereal products, flours, etc. has been patented.[22] Phosphite has the advantage of being a reducing agent which on oxidation yields phosphoric acid, itself an antioxidant. Phosphites, however, are rather unstable. Several commercial samples examined, after removal of the large amount of copper originally present, were found to contain only about 20% of phosphite, the remainder being phosphate.

Until quite recently it was generally assumed that a substance to be an antioxidant must also be a reducing agent, or at least a fairly readily oxidisable substance. Instances, however, have been accumulating for autoxidative reactions outside the field of fat chemistry in which this relationship does not hold, and phosphoric and pyrophosphoric acids, as shown in the present paper, are similar exceptions in the case of fats.

The majority of the antioxidants examined are non-toxic substances suitable for addition to foodstuffs. Pyrophosphoric or citric acid or their salts, or glycine, for example, might be incorporated into lard, margarine, or shortening fats. Glutamic acid and protein, though fairly effective, are probably less suitable because of their tendency to encourage microbial spoilage. On the basis of the results given in Table VII it seems doubtful whether addition of antioxidants of the type here investigated to fat–water systems already containing an appreciable quantity of soluble protein, will produce any marked stabilising effect. This point requires further investigation.

SUMMARY

The effects of water-soluble substances on the rate of oxidation of lard in contact with an aqueous phase have been examined. It has been found that:

(1) The oxidation of lard in glass vessels in contact with water is much more rapid at an alkaline than at an acid p_H. It is suggested that this effect is due to dissolution of copper from the glass.

(2) At p_H values below 5 nitrite is a powerful pro-oxidant. Its action at a concentration of 0·03% is not inhibited by 5% of various antioxidants.

(3) Copper and iron in aqueous solution accelerate the oxidation of lard. Copper is approximately 20 times as active as iron, its effect being perceptible at concentrations as low as 0·01 part per million. Iron in neutral or alkaline solution, *i.e.*, colloidal ferric hydroxide, is inactive.

(4) Aliphatic polyhydroxy-compounds such as glycerol and the sugars are weak antioxidants.

(5) Aliphatic hydroxy-acids, *e.g.*, lactic and glycollic, the ethanolamines, and maleic acid are moderate, and polybasic hydroxy-acids, *e.g.*, tartaric and citric, powerful, antioxidants. Malonic acid is also effective.

(6) Aliphatic amino-acids, *e.g.*, glycine, aspartic and glutamic acids, and asparagine, are all powerful antioxidants. Protein has also considerable antioxidant activity and may be partly responsible for the stabilisation of crude natural oils and fats.

(7) The pro-oxidant effect of copper at low concentrations (up to approximately 1 p.p.m. under the conditions here employed) is completely inhibited by protein, cyanide, and other antioxidants. At higher concentrations of the metal, even 4% of protein fails to prevent a powerful acceleration of oxidation.

(8) Orthophosphoric acid is a fairly good, and pyrophosphoric a very good, antioxidant. Phosphorous acid is also effective.

(9) These water-soluble antioxidants still inhibit oxidation when the water content of the fat is as low as 0·25%.

(10) They are active at p_H 7, certainly in more acid and probably in more alkaline solutions. In the latter

* These conclusions apply to the two-phase system. Soluble antioxidants added directly to the fat in some cases prolong the induction period for a time roughly proportional to the amount added.[23]

[21] E. W. Eckey, U.S.P. 1,982,907, 1934; U.S.P. 1,993,152, 1935. A. S. Richardson, F. C. Vibrans, and J. T. R. Andrews, U.S.P. 1,993,181, 1935.

[22] E. Cox, U.S.P. 1,938,999, 1933.

[23] R. B. French, H. S. Olcott, and H. A. Mattill, Ind. Eng. Chem., 1935, 27, 724.

case the effect obtained in glass vessels will be reduced by (1).

(11) Under the conditions of these experiments the more powerful of the antioxidants examined were effective at concentrations as low as 0·01%, producing an extension of the induction period, measured at 20° in complete absence of copper, of from 2 to 6 times. The magnitude of the factor obtained varied with the sample of fat, and in presence of traces of copper was sometimes as high as 20. For some of the substances used, increase in the concentration in the aqueous phase beyond 0·01% produced an increased effect; for others it did not. These inhibitors produce an effect over and above that of the natural antioxidant, and in two cases examined an appreciable advantage was secured by using a mixture of two antioxidants. This, however, did not appear to be the case when protein was one component of the mixture.

(12) It has been shown that a number of these antioxidants do not function by inhibiting the pro-oxidant effect of traces of copper. An exception is cyanide, which has no antioxidant properties in the absence of active metal. Whether or not copper and other pro-oxidants function only by destroying natural antioxidants is not known.

The author is much indebted to Prof. Hilditch for hydrogenating the herring oil used in part of this work.

The Low Temperature Research Station,
Cambridge

Received August 4, 1936

J. Am. Oil Chem. Soc., 49:44-47, 1972

Analysis of Arsenic and Bromine in Marine and Terrestrial Oils

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ABSTRACT

Samples of marine and terrestrial oils of both plant and animal origin have been analyzed for arsenic and bromine content. Two oil samples (cod liver oil and oil extracted from mackerel fillets) were fractionated on silica gel columns and bromine was determined in the different fractions. The results obtained indicate that lipid soluble bromine and arseno organic compounds are characteristic components of marine animal and marine plant oils (seaweed). The results also show that the bromine is not localized in any particular compound or type of compounds. The bromine-containing compounds seem to be relatively stable, but the arseno-containing compounds are not. When oils containing arsenic and bromine were saponified, some of the arsenic and bromine compounds were found in the fatty acid fraction while others appeared in the water soluble fraction.

INTRODUCTION

It has been shown in previous work (1-3) that lipids extracted from various marine organisms contain arsenic and bromine as organic compounds. In analyses made on lipids from whale and fish, the contents of these elements were found to vary between 3-25 ppm for arsenic and 1-50 ppm for bromine. It has also been shown that arsenic is present as two or more lipid soluble arseno organic compounds in herring (4). Bromine and arsenic are likewise found in the lipid phase of fresh water fish (3,5). Bromine is also present in lipid extracts from fresh and salt water algae, 20-150 ppm and 10-15000 ppm respectively (3). Arsenic was not determined in these samples.

The results obtained so far indicate that the presence of arseno and bromine organic compounds is characteristic of oils produced from marine and fresh water fish. The purpose of this work was to analyze oils from other species of both terrestrial and marine origin in order to see if this assumption has a more general validity. In addition to analyzing, these oils some in which both arsenic and bromine were present were saponified so that the fatty acids could be isolated and analyzed separately.

It was also of interest to ascertain whether bromine was present as one or more specific bromine organic compound. Such a study was undertaken by fractionating some of the oil samples and analyzing the different fractions. By calculating the bromine as parts per million (μg Br/g oil), it should be possible to obtain an indication as to whether this element was present as one or a few discrete compounds, i.e., present in one or a few fractions, or whether it is more distributed in several or all of the different compounds in the oil.

EXPERIMENTAL PROCEDURE

Methods

The production and saponification of the oils were carried out using conventional methods. In some samples where little material was available and where the yield of oil was low, the saponification was carried out directly on the raw material.

Analyses for bromine and arsenic were made using neutron activation. Such analyses may be made without chemical treatment of the sample (oil and fatty acid) after the activation, providing that after activation they do not contain trace elements that will interfere with the detection of the induced radioactivity of the arsenic and bromine. Among elements which do interfer, sodium should be emphasized. The content of sodium and other inorganic ions may, however, be reduced by washing with distilled water. When little material is available, a solution of the material in a suitable solvent may be washed. Some solvents appear to have an affinity to inorganic ions, and may carry the ions into the oil phase where they are left behind when the solvent is evaporated. Chloroform seems to be such a solvent, and should be avoided (unpublished results obtained in the author's laboratory). Among the solvents which may be used are diethylether and toluene. By this method it is also possible to analyze small amounts of material (milligram amounts) without taking any special precautions. Transfer of the samples before and after the neutron activation should be accomplished with the aid of a suitable solvent. Further details concerning activation analyses of oils are discussed elsewhere (3,4,6).

The fractionation of the two oil samples was made on silica gel columns according to the directions given by Barron and Hanahan (7).

Materials

The raw materials used for the production of the oil samples were obtained from the local fish and meat markets and also through the kind assistance of other laboratories.

TABLE I

Eluting Agent and Characterization of the Main Fractions From Chromatography of Fish Lipids on Silicic Acid

Fraction	Eluting agent	Amount, ml	Characterization of main components
A	Hexane (H)	150	Hydrocarbons
B	15% Benzene in H	400	Sterol esters
C	5% Diethylether in H	1330	Triglycerides and free fatty acids
D	15% Diethylether in H	950	Free sterol
E	30% Diethylether in H	600	Diglycerides
F	50% Diethylether in H	600	Diglycerides–monoglycerides
G	Diethylether (D)	600	Monoglycerides
H	3% Methanol in D	250	Phospholipids ?
I	5% Methanol in D	150	Phospholipids
J	80% Methanol in D	250	Phospholipids
K	Methanol	100	Phospholipids

TABLE II

Bromine and Arsenic in Oil and Fatty Acids Extracted From Marine Fishes and Invertebrates

Sample			Locality	Br, ppm		As, ppm	
				Oil	Fatty acid	Oil	Fatty acid
Capelin	*Mallotus villosus*	whole fish	Northern Norway	9.2	8.1	12.1	6.3
Herring	*Clupea harengus*	whole fish	Western Norway	5.8	2.2	13.8	9.2
Herring	*Clupea harengus*	mature whole fish	Skagerak	2.6	2.7	19.3	12.1
Mackerele	*Scomber scomber*	fillet	Southern Norway	2.8	3.1	8.2	4.1
Mackerel	*Scomber scomber*	liver	Southern Norway	16.5	8.5	13	6.2
Cod[a]	*Gadus morrhua*	liver	Western Norway	34	12	8.4	6.1
Cod[a]	*Gadus morrhua*	liver	Western Norway	36	9.2	9.9	7.2
Cod	*Gadus morrhua*	liver	Western Norway	26	6.8	10	6.0
Plaice	*Pleuronectes platessa*	fillet	Southern Norway	17.3	7.6	6.1	5.2
Clam	*Pecten maximum*	whole animal	Northern Norway	15	7.8	4.8	1.9
Squid	*Ommatostrephes sagittatus*	whole animal	Northern Norway	---	3.9	---	0.7
Starfish	*Asterias rubens*	whole animal	Oslo Fiord Norway	14.6	6.4	9.1	7.5
Shrimp	*Pandalus borealis*	whole animal	Oslo Fiord Norway	17	13.0	10.1	4.8
Lobster	*Homarus vulgaris*	whole animal	Southern Norway	50	≈3	4.7	≈3
Mussel	*Mytilus edulis*	whole animal	Western Norway	137	35	18	22
Snail	*Littorina littorea*	whole animal	Western Norway	294	82	84	32

[a]From the same locality.

Some of the vegetable raw oils were factory produced.

Treatment of the Samples

All samples were homogenized in a mechanical blender, and extracted with two volumes of chloroform-methanol 2:1. The extractions were carried out for 2-4 hr at 50 C on a waterbath, under reflux. The extract was then filtered, and the extraction was continued with a similar amount of solvent for another 2-4 hr. The two extracts were combined, the chloroform phase was removed in a separatory funnel, and finally removed under reduced pressure at 35-40 C. If a sufficient amount of oil was available, the oil phase was washed twice with distilled water. Usually it was necessary to add magnesium sulphate as a deemulsifying agent. The oil was then centrifuged. Where the oil yield was small, the oil was dissolved in toluene or diethylether and washed with water. After two washing processes the organic phase was separated by centrifuging and the solvent evaporated.

The saponification of the oils was performed in an alcoholic potassium hydroxide solution (2 N KOH in C_2H_5OH). The solution was subsequently made acidic with sulphuric acid and extracted with diethylether. The ether solution was then washed with water and the ether evaporated. Some seaweed samples with a low oil content were saponified directly. The fatty acids were separated as described for the other samples.

Fractionation of Oil

Oil produced from mackerel fillets and from cod liver were used for the fractionation experiments. The silica gel (Merck AG, Darmstadt, 0.2-0.5 mesh) used as solid phase was prepared in the following way: The gel was first dried at 110 C and then treated with the following solvents: diethylether, 15% benzene in *n*-hexane, and *n*-hexane. About 90 g of gel was added to a column with diameter 2.5 cm. Approximately 20 mg oil per gram of gel was used. The different eluting agents used and the amounts of each are given in Table I. The absorption of the eluate at 254 mμ was obtained with an Uvicord-Unit (LKB, Sweden). From the elution curves obtained, and information given by Barron and Hanahan (7), the eluate was devided into different fractions and the solvents were removed by evaporation.

Neutron Activation

The oil and fatty acid samples were neutron activated in quartz ampoules. The quartz ampoules were first washed with warm nitric acid and rinsed with distilled water. Standards (PA Chemicals: Merck AG, Darmstadt) of ammonium bromide and arsenic dissolved in hydrochloric acid (0.1 N) were neutron activated under the same conditions as the samples. The activation took place in the nuclear reactor JEEP 2, Kjeller, Norway, with a neutron flux of approximately 5×10^{12} n/cm^2 sec for 2 hr. The quartz ampoules were cooled to -196 C before opening in order to reduce the pressure from volatile compounds formed during the irradiation. The samples were then transferred to inactive glass vials. The standards were treated in the same way.

TABLE III

Bromine and Arsenic in Oil and Fatty Acids Extracted From Seaweed

Sample[a]	Br, ppm		As, ppm	
	Oil	Fatty acid	Oil	Fatty acid
Laminaria digitata	566	640	221	36
Laminaria saccharina	496	477	155	7.5
Laminaria hyperborea	368	385	197	16
Ascophyllum nodosum (1968)	61	27	7.8	5.2
Ascophyllum nodosum (1969)	56	25	49	21
Fucus vesiculosus	43	44	35	5.1
Fucus Serratus	40	39	27	6.1
Fucus spiralis	12	19	5.7	5.0
Pelvetia canaliculata	34	41	10.8	7.3

[a]The samples were collected at the west coast of Norway.

TABLE IV

Bromine in Oil Extracted From Birds, Terrestrial Mammals and Vegetables

Sample		Locality	Bromine, ppm
Hare	*Lepus timidus*	Inland Norway	<0.1
Reindeer	*Rangifer torandus*		0.12
Elk	*Alces alces*		<0.01
Cow	*Bos taurus*		<0.05
Wood-grouse	*Tetrao urogallus*		0.01
White-grouse	*Lagopus lagopus*		0.03
Lesser black-backed gull	*Larus fuscus*	West coast Norway	1.0
Common gull	*Larus canus*		0.6
Herring gull	*Larus argentatus*[a]		11.0
Herring gull	*Larus argentatus*[a]		13.2
Soybean oil			0.06
Olive oil[b]			0.03
Peanut oil			0.09
Linseed oil[b]			0.12
Cocoa fat			0.18

[a]Traces of arsenic present.
[b]Commercially produced raw oil.

Registration of the Samples

After neutron activation, measurement of the induced activity in the samples was made with the aid of a 2 x 2 in. sodium iodide detector coupled to a multichannel γ-spectrometer. The samples were not treated chemically before these measurements. Some of the samples contained traces of sodium and the registration of their activity was therefore postponed for 4-5 days to ensure that the activity from sodium -24 would not disturb the registration of the induced bromine and arsenic activity. The sensitivity for the analysis of bromine and arsenic when using this method is approximately 0.005-0.01 μg and 0.002-0.005 respectively. The sensitivity is reduced when the registration after activation is postponed, or if activities from other radioactive isotopes increase the "background" activity.

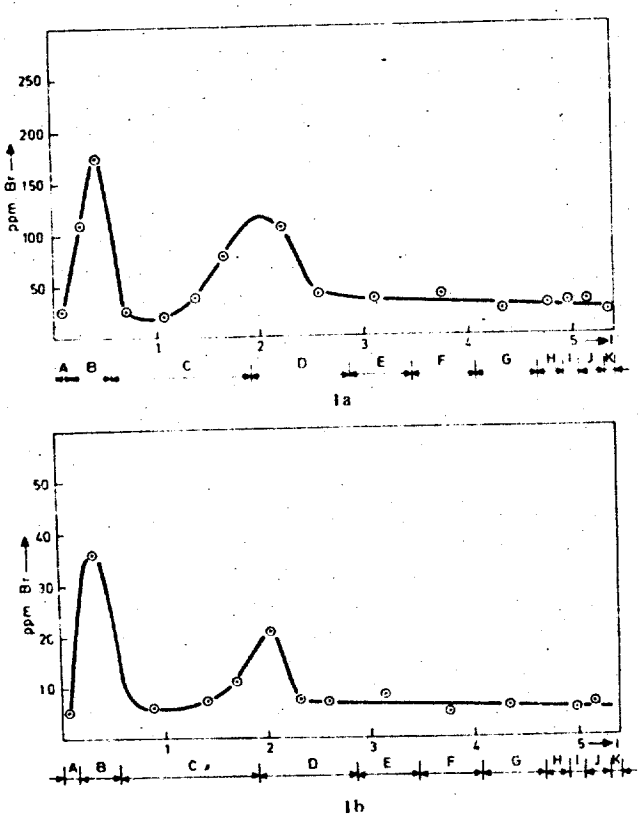

FIG. 1. The Bromine content in different fractions of cod liver oil (a) and oil extracted from mackerel fillet (b) fractionated in a silica gel column (see Table I for characterization of fractions).

RESULTS AND DISCUSSION

The results of the bromine and the arsenic analysis in the different oils and in the corresponding fatty acids are shown in the Tables II-IV. They confirm the earlier findings that lipid soluble arseno and bromine organic compounds are characteristic of oils of marine origin. This conclusion applies to lipids extracted from fish, different invertebrates, seaweed and algae. The amount of bromine in vegetable oils and in some oils extracted from terrestrial animals is significantly lower than in marine oils, and it is probably present as inorganic bromine which has not been removed from the oils by the washing process.

The only oil from organisms of terrestrial origin in which bromine was detected came from seabirds, and the bromine-organic components were here partly present in somewhat reduced amounts. Fish constitutes the main part of the diet of these birds and is most likely the source of the bromine-containing compounds found in them. The analytical results indicate both a gradual reduction in the content of bromine organic compounds through the feed chain from algae to fish and to seabirds, and that at least some of these components are quite stable. No arsenic was found in any of the terrestrial oils, except for some traces in the oil from seabirds.

When the arsenic content in marine oils is compared with the arsenic content in the corresponding fatty acids, the results show that at least two groups of arseno organic compounds are present in the marine oils. One type consist of an arsenic-containing acid which follows the fatty acid during the saponification process while the other type is converted to a water soluble compound during this process.

It is interesting to note that the lipid phase in seaweed also contains arseno organic compounds as do the lipids from the marine animals studied. The amount following the fatty acids in a saponification is, however, relatively lower than for the other marine oils studied. These compounds are probably formed from inorganic arsenic absorbed from the sea water, synthesizing first a water soluble arseno organic compound. Compared with the total amount of arsenic present in seaweed (30-110 ppm) (8,9), the amount of arsenic found in the oil represents a relatively small amount of the total arsenic present in the seaweed. The yield of extractable lipids here is usually from 3-6%. However it should be noted that the contents of arsenic particularly in oil extracted from some *laminaria* species exceeds that in any other marine raw material analyzed.

The analysis of bromine in the marine oils and in the

rresponding fatty acids shows that this element also is alized in the fatty acid fraction, but that the content is somewhat lower here than in the oils. This would indicate that the bromine organic compounds are not formed solely by an addition of bromine to double bonds in the fatty acids.

The results of the bromine analysis in the fractions from the two oils fractionated on a silica-gel column (see Figs. 1a and b) give nearly the same picture of the bromine distribution in the different fractions, although the absolute amount of bromine is considerably higher in the cod liver oil than in the mackerel oil. Both diagrams show that the highest bromine concentration is located in fractions B and C-D. Fraction B (15% benzene in hexane) contains mainly sterolesters while the fractions C-D (5-15% diethylether in hexane) contains triglycerides, free fatty acids and sterols. It is also possible that the bromine may be present in other compounds which are eluted under these conditions, but which are present in smaller amounts and not found in this study. Although the results indicate that the bromine is randomly added to the main components of the oil, there may be one or more specific bromine organic compound located in fractions B and C-D.

ACKNOWLEDGMENT

Mr. Shistad contributed technical assistance.

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[Received March 15, 1971]

Fish Oils M.E. Stansby (Ed.), 1967

CHAPTER 2

Donald C. Malins

The Classes of Lipids in Fish

INTRODUCTION

The present chapter is a discussion of the main classes of lipids in marine animals, particularly fishes. For additional coverage, the reader is referred to the comprehensive review on the lipids of marine organisms by Lovern (1964).

The classes of lipids most commonly found in marine animals are hydrocarbons, wax esters, triglycerides, phospholipids, diacyl glyceryl ethers, and neutral plasmalogens (Fig. 1). Sterols, steryl esters, carotenoids, and vitamins, usually present as minor components of marine oils, will not be discussed in this brief review.

The lipid class composition of a marine species may vary considerably, depending upon the location in the animal (e.g., the liver versus the muscle). Also, although one class sometimes predominates, various proportions of two or more classes are usually present. Observations so far suggest that most fish oils contain primarily triglycerides, but a great diversity of class composition exists.

HYDROCARBONS

Hydrocarbons are readily obtained by chromatography (e.g., columns of silicic acid eluted with light petroleum) of the original oil or the nonsaponifiable fraction. Evidence so far suggests that a wide variety of saturated and unsaturated straight-chain and branched-chain hydrocarbons are present.

Some marine animal oils contain less that 0.1% hydrocarbons; others contain as much as 90% (Heller *et al.* 1957). Lambertsen and Holman (1963), employing current techniques of thin-layer chromatography (TLC) and gas-liquid chromatography (GLC), were able to demonstrate the presence in herring oil (0.05% hydrocarbons) of 21 straight-chain and 8 branched-chain paraffins (Table 4). Of the straight-chain paraffins, odd chain compounds predominated and it was suggested that these hydrocarbons may have arisen from the enzymatic decarboxylation of fatty acids. The branched-chain hydrocarbons were dominated by pristane and squalene, both of which have been isolated previously from other marine animals oils (Hilditch and Williams 1964).

High percentages of hydrocarbons are found in several species of shark

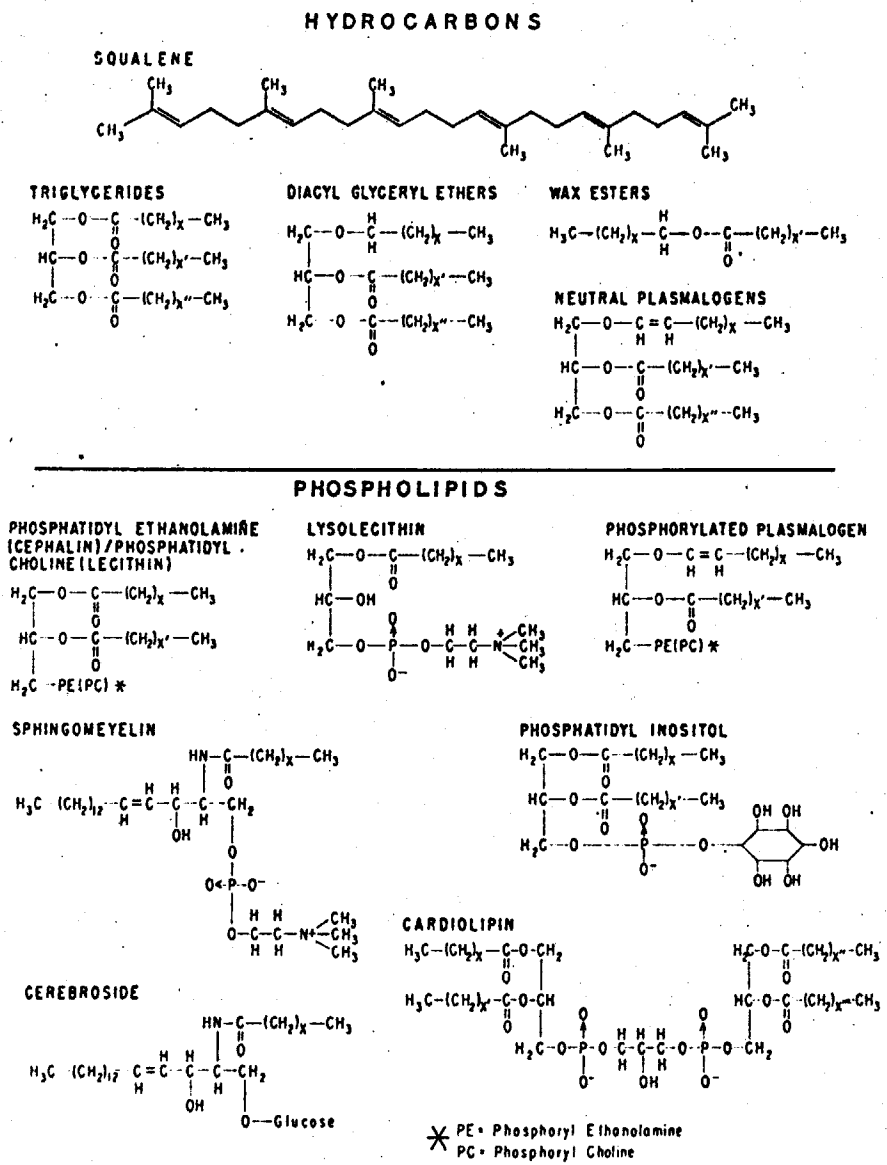

FIG. 1. STRUCTURE OF SOME COMMON LIPIDS OF FISH

(Hilditch and Williams 1964), such as *Centrophorus uyato* which contains up to 90% squalene (Heller *et al.* 1957). This hydrocarbon generally predominates, but smaller amounts of pristane and its unsaturated analog zamene (2,6,10,14-tetramethylpentadec-1-ene) are usually present (Christensen and Sørensen 1951).

TABLE 4

HYDROCARBONS OCCURRING IN HERRING OIL AS DETERMINED BY GAS-LIQUID CHROMATOGRAPHY

Straight-Chain Components		Branched-Chain Components	
Carbon Number	% Area	Carbon Number	% Area
14.0	0.3	15.4	3.5
15.0	7.5		
16.0	0.6	16.8 (pristane)	70.0
17.0	9.0		
17.6	2.2	17.9	5.0
18.0	1.7		
19.0	10.5	19.0	0.5
20.0	0.3		
21.0	6.0	19.9	1.0
22.0	0.3		
23.0	0.8	21[1]	3.5
24.0	0.3		
25.0	9.5	22.2	3.5
26.0	Trace		
27.0	33.0	26.3 (squalene)	13.0
28.0	Trace		
29.0	9.0		
30.0	Trace		
31.0	~7		
32.0	(Trace)		
33.0	~1		

[1] Flat and broad peak.
From Lambertsen and Holman (1963).

TRIGLYCERIDES

Triglycerides are generally the principal sources of fatty acids in marine oils. Until recently, interest in the saponifiable fraction was usually oriented toward the composition of the fatty acids liberated by hydrolysis of the whole oil. These studies, however, afforded no insight into the composition of individual glycerolipids, such as the triglycerides. The composition of the fatty acids and their positional distribution in the triglycerides was still incompletely understood until recently. In the last few years, micro methods for determining glyceride structure have enjoyed notable advances and it is now possible to ascertain the positional distribution of the fatty acids on the 2- and 1,3-positions of glycerol. Furthermore, there is hope that the fatty acids on both the 1- and 3-positions can be studied by employing pancreatic lipase and phospholipase A in conjunction with derivative techniques (Brockerhoff 1965). Because of these noteworthy advances, it is now possible to obtain a better picture of the structure of glycerides of marine species.

Although more evidence must still be acquired, it appears that the fatty acids in the triglycerides of land animals generally obey certain rules: that is, the characteristic polyenoic acids (usually linoleic acid) are preferen-

558

tially bound at the 2-position of glycerol (Hanahan *et al.* 1960). Marine species, with the probable exception of most mammals, show a similar positional specificity for polyenoic acids. Using pancreatic lipase hydrolysis, Brockerhoff and co-workers (Brockerhoff *et al.* 1963; Brockerhoff *et al.* 1964A; Brockerhoff and Hoyle 1965) have presented strong evidence to indicate that the characteristic polyenoic acids (notably eicosapentaenoic acid and docosahexaenoic acid) of the triglycerides of cod, scallop, lobster, halibut, skate, and trout are preferentially bound to the 2-carbon of glycerol.

The origin(s) and biochemical significance of this characteristic distribution pattern is still not understood. Several studies by Brockerhoff and co-workers (Brockerhoff *et al.* 1963; Brockerhoff *et al.* 1964A; Brockerhoff *et al.* 1964B), however, have indicated that the "2-monoglyceride" structure may have its origin in the photoplankton. It has been shown, for example, that the common diatom, *Skeletonema costatum*, and a mixed fraction of zooplankton caught off the coast of Nova Scotia exhibit this characteristic structure (Brockerhoff *et al.* 1964B).

It has also been demonstrated, employing triglycerides labeled C^{14} in the 2-position of glycerol, that the characteristic "monoglyceride structure" of the lipids of certain marine invertebrates and fish is retained for as long as 28 days after ingestion (Brockerhoff *et al.* 1964A). Furthermore, it would appear that the pancreatic lipases of fish resemble those of land mammals: that is, they hydrolyze predominantly the acids on the 1,3-position of glycerol (Brockerhoff and Hoyle 1965). This evidence suggests that the "2-monoglyceride" structure is carried through the food chain.

In contrast to marine fish, marine mammals, such as the seal and pilot whale, appear to store the C_{20}:5 and C_{22}:6 acids in the 1- and 3-positions (Brockerhoff and Hoyle 1963). The significance to the animal of this structural specificity is yet to be elucidated.

Dolev and Olcott (1965), for example, studied the composition of the triglycerides of the sablefish. These workers first fractionated the glycerides according to unsaturation by the use of silver nitrate-silica gel chromatography, then analyzed the individual components by the use of pancreatic lipase hydrolysis and GLC. The triglyceride types were found to be highly complex and several hundred fractions were obtained. Predictably, future investigations will be oriented toward a determination of the specific structures of individual glycerides.

Furthermore, asymmetry (optical activity) can be predicted[1] for the tri-

[1] This topic was discussed in a presentation by W. Schlenk at the Spring meeting of the American Oil Chemists' Society, 1965.

glycerides of certain marine animal oils, notably those of dolphin oil which contain high percentages of *iso*-valeric acid (Hilditch and Williams 1964). Studies of the possible optical activity of triglycerides of known structures might well contribute to a better understanding of the role these compounds play in the live animal.

LIPIDS CONTAINING AN ETHER GROUP

Evidence accumulated so far suggests that the ether-containing lipids of fish occur primarily as diacyl glyceryl ethers in which the 1- and 2-positions of glycerol are esterified with fatty acids (Lovern 1951). Other alkanyl ethers, such as glyceryl ether phospholipids (Hanahan and Thompson 1963), have been identified in a number of terrestrial animals, but so far their presence in fish can only be predicted.

Karnovsky and co-workers (Karnovsky *et al.* 1946, 1948), surveying the composition of a number of marine fish and mammals, reported the presence of high percentages of glyceryl ethers in the nonsaponifiable fractions of the liver and body oils. Chimyl, batyl, and selachyl alchols were assumed to be the principal constituents. In recent years, the glyceryl ethers of several species of fish have been analyzed with the aid of GLC and a more comprehensive picture of their composition is now available.

The hydrocarbon chains of the glyceryl ethers of fish are almost entirely either saturated or monounsaturated and contain an even number of carbon atoms from C_{14} to C_{22}. Odd, branched, and polyunsaturated chains are present in only small amounts (Hallgren and Larsson 1962; Hilditch and Williams 1964; Malins *et al.* 1965). The main constituents are, as previously suggested (Karnovsky *et al.* 1946, 1948), chimyl, batyl, and selachyl alcohols. An analysis by GLC of the glyceryl ethers of the dogfish (*Squalus acanthias*) is presented in Table 5. The glyceryl ether composition of this species, obtained from the Pacific Coast of the United States

TABLE 5

GLYCERYL ETHERS IN THE FLESH AND LIVER OF THE DOGFISH (*Squalus acanthias*)

Sample	Composition of Long-Chain Ether Group							
	14:0	15br	15:0	16br	16:0	16:1	17br	17:0
								wt. %
Flesh	4.8	0.9	1.0	0.8	22.9	13.2	0.5	0.8
Liver	2.6	0.6	0.7	0.7	13.4	10.2	0.9	0.3
	17:1	18br	18:0	18:1	18:2	19	20:0(?)	20:1
Flesh	0.8	0.3	2.8	47.3	0.7	0.3	0.1	1.9
Liver	1.1	0.7	3.9	61.4	tr.	0.8	0.2	2.3

From Malins *et al.* (1965).

558

JOURNAL of THE SOCIETY OF CHEMICAL INDUSTRY

TRANSACTIONS AND COMMUNICATIONS

OCTOBER 16, 1936

ANTIOXIDANTS AND THE PRESERVATION OF EDIBLE FATS

By COLIN H. LEA

In common with other autoxidative processes the oxidation of an unsaturated fat exposed to the air may be either accelerated or retarded by the presence of traces of foreign substances termed "pro- or anti-oxidants." It is now believed that substances of the latter class are present in plant and animal tissues and, being to some extent extracted with the fat, are responsible for the initial lag phase or "induction period" which in the majority of fats precedes the stage of rapid oxidation. Little progress has yet been made towards the isolation and identification of these natural anti-oxidants, and at present the stabilisation of oils by direct addition of small quantities of substances of known composition appears to be more promising.

Numerous aromatic phenols and amines have been shown to possess in varying degree the property of retarding atmospheric oxidation of unsaturated organic compounds, but such substances are undoubtedly toxic when taken in any appreciable quantity. Only recently have substances of a definitely non-toxic character been proposed as antioxidants, and in practically every case the evidence so far obtained as to their usefulness is conflicting. Lecithin has been stated to possess protective properties,[1] and to be inactive.[2] The observation that lecithin and cyanide are powerful antioxidants in presence of a metallic catalyst (cobalt)[3] is, as shown in the present paper, by no means a certain indication that they are active in absence of the metal. Carotene has been stated to accelerate[4, 5, 6] and to retard[7, 8] oxidation. Maleic acid (which is reputed to possess a toxicity similar to that of oxalic acid[9]) has been described as a powerful antioxidant[6, 10] and as completely inactive.[2, 11]

Since the completion of a number of the experiments described in the present report, two papers containing references to some of the substances here described have been published. Greenbank and Holm[6] found antioxidant properties, though less marked than with maleic acid, for several organic acids including tartaric and citric. They state, however, that the salts are ineffective, and that protection is much reduced in presence of water. Olcott,[11] on the other hand, failed to obtain any protective effect whatever from employment of these acids. Some of the contradictory results mentioned above are undoubtedly due to different experimental conditions, but the whole situation is somewhat confusing.

Estimation of the efficiency of antioxidants

In practically the whole of the published work on pro- and anti-oxidants the protective effect has been measured by addition of the substance to the pure, dry fat. The majority of foods, however, contain fat in contact with an aqueous phase, and information regarding protection from oxidation under these more complicated conditions is entirely lacking. That anti-oxidants do not necessarily behave similarly under the two sets of conditions is shown by the fact that polyphenolic substances such as pyrogallol, which for dry fats are among the most powerful antioxidants known, are of little use in presence of water, particularly at a slightly alkaline p_H.

Measurement of the efficiency of antioxidants has almost invariably been made at some fairly high temperature (usually 70—100°), or in presence of light or a metallic salt as accelerator. In the present paper it has been considered more satisfactory to work at ordinary temperatures (20°) in presence of an aqueous phase and in absence of light. Oxidation has been followed by determination of peroxide-oxygen content by the method described in previous papers,[12] using 0·5 g. of fat and 20 ml. of 2 : 1 mixture of glacial acetic acid and chloroform. The ratio of the induction periods in absence and in presence of the substance has been used as a "protection factor" to measure the efficiency of the substance as an antioxidant. A negative protection factor indicates that the substance accelerates oxidation, and a factor of 1·0 or —1·0 that it has no effect.

EXPERIMENTAL

This investigation was originally undertaken with the idea that antioxidants might prove of value in the curing of bacon, a foodstuff in which rancidity arising from atmospheric oxidation of the unsaturated constituents of the fat is the chief obstacle to successful

[1] E. W. Koehenderfer and H. Gregg-Smith, Proc. Iowa Acad. Sci., 1932, 39, 169.
[2] H. O. Triebold, K. E. Webb, and W. J. Rudy, Cereal Chem., 1933, 10, 263.
[3] E. I. Evans, Ind. Eng. Chem., 1935, 27, 329.
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[7] R. C. Newton, Oil and Soap, 1932, 9, 247.
[8] B. R. Monaghan and F. O. Schmitt, J. Biol. Chem., 1932, 93, 357.
[9] G. W. Monier-Williams, "Food and the Consumer," 1935.
[10] G. R. Greenbank, Science, 1933, 77, 6.
[11] H. S. Olcott, J. Amer. Chem. Soc., 1934, 56, 2492.
[12] C. H. Lea, Report of the Food Investigation Board for the Year 1929, p. 30; Proc. Roy. Soc., 1931, B, 108, 175.

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Journal of the Society of Chemical Industry

storage for prolonged periods.[13] For this reason in earlier experiments the rate of oxidation of lard in contact with a synthetic pickle of composition, sodium chloride 26·0 g., potassium nitrate 3·5 g., potassium nitrite 0·03 g., distilled water to 100 ml., was investigated. Later the work developed along more general lines.

Chemically, the aromatic amines and polyhydric phenols provide some of the best known inhibitors for the autoxidation of unsaturated organic compounds, though by no means all such substances are active. It therefore seemed possible that some of the aliphatic amino- and polyhydroxy-compounds might also possess this power in some degree.

Oxidation of lard at 20° in presence of hydroxy- or amino-compounds

Lard extracted in the laboratory from the leaf fat of a freshly killed animal was used. The fat was pipetted into small flat-bottomed glass dishes to a depth of 1·5 mm., allowed to solidify, and covered with pickle to a depth of 3·75 mm. Six dishes were stored in a stoppered glass jar at a constant temperature of 20° and removed one by one for examination. The aqueous layer was poured off, the fat dissolved in peroxide-free ether, washed free from all trace of nitrite, and dried over sodium sulphate, after which the solvent was removed under vacuum at 50° and the fat examined for peroxide content. In this, as in subsequent experiments, care was taken to prevent contamination with traces of

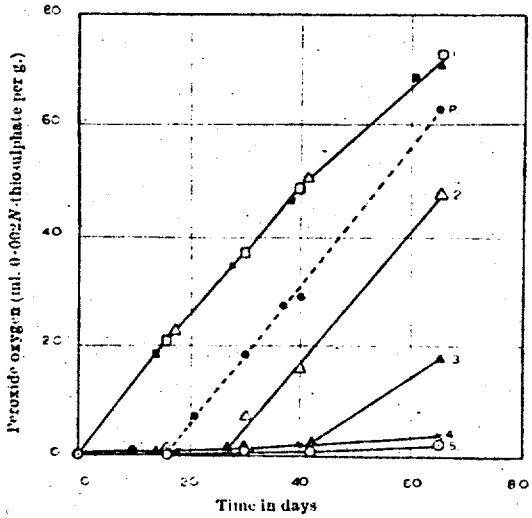

FIG. 1

The oxidation of lard in pickle containing 5% of hydroxy-compound. (1) *Acetic, citric, or tartaric acid.* (2) *Glycerol.* (3) *Glucose.* (4) *Sodium tartrate.* (5) *Sodium citrate.* (*P*) *Control*

oxidised fat, which cling tenaciously to glass apparatus and tend to increase the rate of oxidation of subsequent samples. Treatment with hot, concentrated sodium hydroxide solution prior to the customary washing with chromic acid sufficed to eliminate trouble from this cause.

[13] C. H. Lea, J.S.C.I., 1931, 50, 343 T; 1933, 52, 57 T.

The resulting curves (Fig. 1) show that all three of the acids examined eliminated the induction period, but that some of the other substances, notably the tartrate and citrate, possessed quite marked protective properties. The quantity of antioxidant used (5% of the aqueous phase) is, however, too high for most practical purposes.

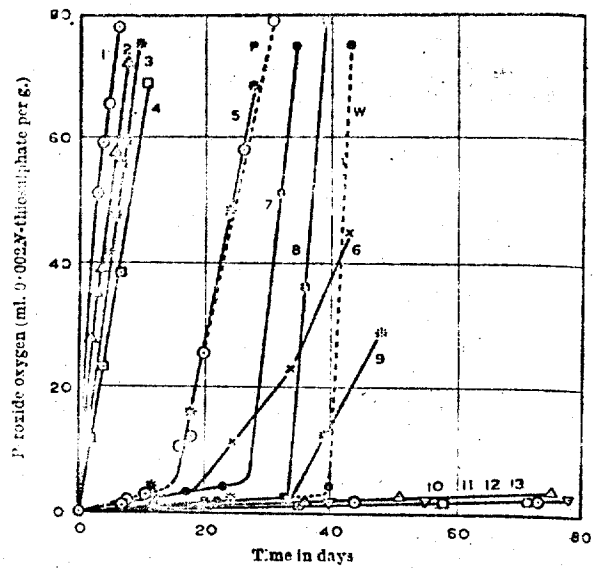

FIG. 2

The oxidation of lard emulsified with pickle containing 2% of hydroxy- or amino-compound. (1) *Lactic acid.* (2) *Citric acid.* (3) *Tartaric acid.* (4) *Succinic acid.* (5) *Mannitol.* (6) *Glucose.* (7) *Sodium maleate.* (8) *Ammonium lactate.* (9) *Sodium tartrate.* (10) *Glycine.* (11) *Sodium citrate.* (12) *Asparagine.* (13) *Sodium malonate.* (*P*) *Control.* (*W*) *Water*

A second series of samples was therefore put down with a smaller proportion of active substance, the melted fat in this case being emulsified with 15% of its volume of synthetic pickle containing 2% (0·3% calculated on the fat) of antioxidant, and solidified before separation could occur. The thickness of the fat layer was again 1·5 mm., and the temperature 20°. The results are summarised below.

Pro-oxidants (P.F. —20)	Succinic, tartaric, citric, and lactic acids.
Inactive (P.F. 1·0) ...	Mannitol.
Weak antioxidants (P.F. 1·0—2·0)	Glycerol, glucose, sucrose, fructose, sodium maleate, sodium succinate.
Moderate antioxidants (P.F. 2·0—3·0)	Sodium malate, ammonium lactate, sodium tartrate, sodium glycollate, sodium lactate.
Powerful antioxidants (P.F. > 4·5)	Glycine, asparagine, sodium citrate, sodium malonate.

Fig. 2 conveys some idea of the enormous range in rate of oxidation covered by these figures.

These two experiments proved the existence of a wide variety of water-soluble substances capable of acting as inhibitors for the autoxidation of lard, but

closer study of the results indicated that some disturbing factor was present. It was observed, for example, that while smooth and fairly reproducible oxidation curves could be obtained in many cases, in others, particularly those of water or pickle alone, duplicate experiments gave erratic results or parallel curves with varying induction periods.

Investigations on the mechanism of yellowing in lard, also in progress at this time,[14] indicated that traces of alkali markedly accelerated the oxidation of lard stored in thin films in glass bottles. These data, in conjunction with the unexpected pro-oxidant effect of all the acids tested, now directed attention to the question as to whether the rate of oxidation of the fat might not be influenced by the p_H of an aqueous phase in contact with it.

Effect of p_H on the oxidation of lard

In a preliminary trial, uniform thin films (0·4 mm.) of freshly rendered lard were spread on microscope slides immersed to a depth of 1 cm. in pickle contained in jars which were stored at 20° under atmospheres containing different partial pressures of carbon dioxide. The results are tabulated below.

Atmosphere	p_H	Induction period (days)
Air	7·8	12
Air 98·7%, carbon dioxide 1·3%....	6·5	22
Air 50%, carbon dioxide 50%	4·8	21
Air 50%, nitrogen 50%	7·8	11

Oxidation of the sample in air diluted with 50% of nitrogen was quite as rapid during the induction period as that of the control in air, but later the rate became decidedly less. Owing to the presence of the aqueous phase oxygen pressures above the fat would be quite small—of the order of 0·0005 and 0·001 atmosphere in the two cases.

These figures indicate a considerable sensitivity towards p_H of the aqueous phase, but cannot be considered as more than a rough guide to the quantitative effect. The experiments were therefore repeated using standard buffer systems to cover a wider p_H range. Under these conditions a considerable range of oxidation rates was observed,[15] but it was now apparent that the nature of the buffer salts was having some influence on the oxidation rate. Later work showed this to be due to the fact that phosphate possesses marked antioxidant properties,[16] whilst borate is a very weak pro-oxidant.

In subsequent experiments, therefore, the mixed phosphate, borate, phenylacetate buffer system (in water) of Prideaux and Ward[17] was used, by means of which it was possible to cover the range from p_H 2 to 10 simply by variation of the concentration of $Na^{\cdot}$ or Cl', neither of which at the low total concentration employed is likely to affect the oxidation in any marked degree. Under these conditions several series of curves were obtained for different specimens of fat spread on slides and immersed in buffer in the manner already described. In Fig. 3 the initial rate of oxidation, considered in this case as the reciprocal of the number of days required by the fat to reach 5 ml. of 0·002*N*-thiosulphate per gram (induction period), is plotted against p_H. In all cases a sharp decrease in stability occurred as the solution became alkaline. The actual magnitude of the change, however, varied considerably from sample to sample, being greater in the water-extracted and charcoal-treated lards, which contain less of the natural antioxidant, than in the untreated ether-extracted specimens. When nitrite was present (0·03 g. per 100 ml.) stability also decreased sharply as the p_H fell below 5. This behaviour, which accounts for the marked accelerating effect of acids on the oxidation of

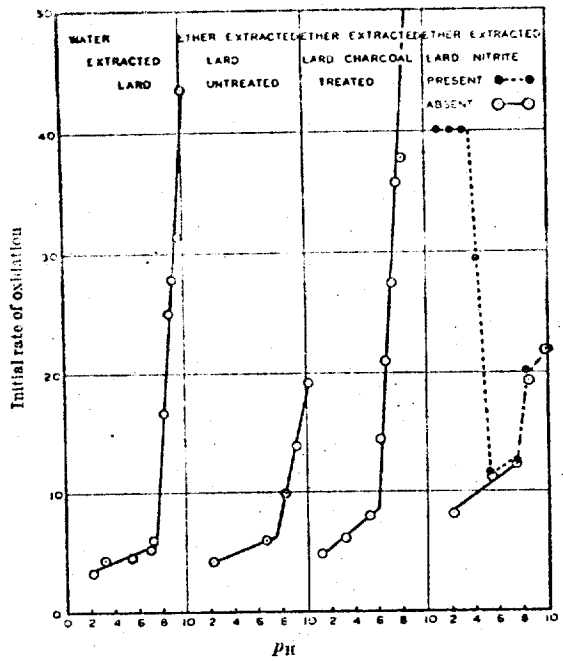

FIG. 3

The effect of p_H on the oxidation of lard in glass

lard in pickle, already observed, is presumably due to a powerful pro-oxidant effect of free nitrous acid. Colorimetric determination by the Ilosvay method did, in fact, show that the nitrite radical quickly disappeared from those solutions in which rapid oxidation occurred, but remained undestroyed for long periods in the others.

The similarity of these curves to the known variation in solubility of glass with change in p_H, coupled with the results of other experiments on the catalytic effect of minute traces of copper, now suggested that the accelerating effect of an alkaline p_H might possibly be due to dissolution of copper from the glass, rather than to any direct action on the fat. The area of glass (which was of the ordinary "soft" variety) exposed to the liquid in each jar was fairly large—of the order of 1·3 sq. cm. per ml. of fluid.

To test this hypothesis, the interiors of small Pyrex beakers were completely covered with a thin film of

[14] C. H. Lea, J.S.C.I., 1934, 53, 182 T.
[15] C. H. Lea, Report of the Food Investigation Board for the Year 1934, p. 41.
[16] C. H. Lea, J.S.C.I., 1934, 53, 388 T.
[17] J.C.S., 1924, 125, 426.

charcoal-treated lard (average thickness 0·04 mm.), and filled with buffer solution. To one series, cyanide (0·001% as HCN) was added in order to inhibit the effect of any copper already present in the chemicals used. Under these conditions the fat oxidised rapidly and rather erratically, but now the p_H effect (Table I, Expt. 1) appeared to be much reduced or, in case of the cyanide, almost eliminated. The difference in rate between the two series seemed to point to the presence of copper in the buffers, and analysis showed 0·2 and 0·5 parts of copper and iron respectively per million of solution.

TABLE I

The initial rate of oxidation of lard in buffer without contact with glass*

Experiment 1			Experiment 2		
p_H	Cyanide absent	Cyanide present	p_H	Cyanide absent	Cyanide present
2·2	25	—	1·9	6	4
3·9	25	10	3·3	6	5
4·8	25	10	4·1	6	5
6·2	19	11	5·1	4	6
7·1	25	11	6·5	5	6
8·1	35	13	7·2	5	7
9·1	33	11	8·5	5	5
10·0	41	10	10·1	6	5

* Reciprocal of the number of days required to reach 25 and 5 ml. per g. respectively in experiments 1 and 2.

The boric, phenylacetic, and phosphoric acids used were therefore purified until the buffer made up in water distilled twice from Pyrex contained less than 0·005 part of copper per million. Water-extracted lard was used without charcoal treatment. The result (Table I, Expt. 2) showed that now the fat oxidised in presence and absence of cyanide at approximately the same rate, presumably indicating complete removal of significant amounts of copper. The spread of the points in both of these experiments is unusually high, owing partly to the thinner and much less uniform films obtainable by this technique, and partly to the increasing effect of chance contamination, as the amount of catalyst hitherto controlling the oxidation rate was reduced.

From consideration of these data it appears that, although the rate of oxidation of lard in glass vessels is very sensitive to the p_H of an aqueous phase in contact with it, the effect is in fact a secondary one due probably to dissolution of copper from the glass, and does not take place when contact between aqueous phase and glass is prevented.

Effect of antioxidants in buffered solution

It was apparent at an early stage in the experiments described above that if reliable comparisons were to be made between the efficiencies as antioxidants of a series of substances in aqueous solution in contact with films of lard and with glass, it would be necessary to maintain the p_H at some fixed value throughout, or alternatively, as the later data suggested, to prevent contact between aqueous phase and glass.

Actually much of the later work was carried out in a borate buffer adjusted accurately to p_H 7·0 by means of the glass electrode, after addition of the substance under examination. At this p_H dissolution of the glass is relatively slight, and possible traces of copper derived from this source would in any case be the same for both antioxidant solution and control. Fig. 4, obtained by use of the slide technique already described, is, however, of interest in that it shows that antioxidants of the type under investigation retain their activity even in an alkaline solution, but it is obvious that the alkaline p_H

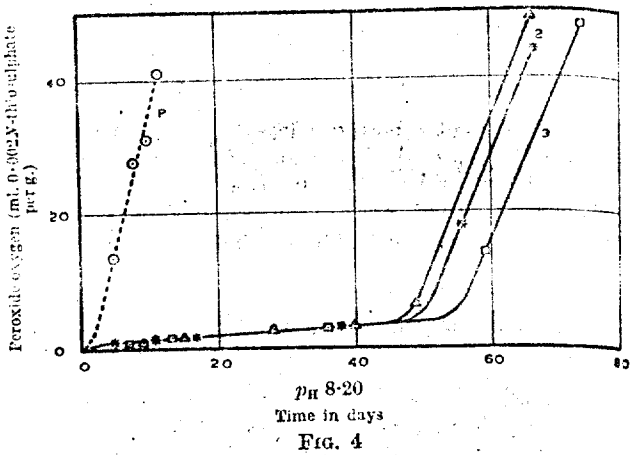

FIG. 4

The effect of antioxidants (1%) in borate buffer at p_H 8·2. (P) Pickle control. (1) Glycine. (2) Citrate. (3) Malonate

has greatly reduced stability in the control solution, probably owing to dissolution of copper from the glass.

Fig. 5 and Table II give results obtained in a similar manner from charcoal-treated, ether-extracted lard at p_H 7·0 with several antioxidants at three concentrations,

TABLE II

The effect of concentration on the activity of inhibitors

Substance	Protection factors 0·01%	0·1%	1·0%
Buffer at p_H 7·0	1·0	1·0	1·0
Buffer + lactate	1·3	2·1	3·1
Buffer + maleate	2·0	2·5	4·7
Buffer + phosphate	1·7	3·9	7·5
Buffer + malonate	4·3	> 8·0	> 8·0
Buffer + glutamate	6·6	> 8·0	> 8·0
Buffer + citrate	7·7	> 8·0	> 8·0
Buffer + glycine	> 8·0	7·3	—

the latter calculated, as in all other cases, as the free acid. Protection factors shown in the table as > 8·0 varied from 8·0 to 11·0, the precise figure possessing little significance owing to the growth of mould in some of the solutions after prolonged storage. Two other samples of lard showed much smaller protection factors for 0·01% of antioxidant, the figures being: for urea —1·1, —1·1; phosphate 1·0, 1·1; citrate 1·5, 1·7; and glycine 1·8, 2·2.

Pro-oxidant effect of copper and iron

It is well known that a number of metals are able to function as accelerators in various oxidative reactions. Cobalt, vanadium, copper, manganese, iron, and lead, for example, are all capable of accelerating the atmospheric oxidation of unsaturated fatty acids, cobalt, manganese, and lead finding commercial application as driers for paints. Copper and iron are the metals most likely to be of importance in the oxidative deterioration of the fat of foodstuffs, and here again, though their

action on pure fats has repeatedly been demonstrated, but little information is available as to the effect of the metals when present in a system containing both fat and aqueous phase.

To investigate the effect of metallic salts in aqueous solution, a slightly different technique was employed. The fat, in this case herring oil extracted by ether from the fresh material, was dissolved (10%) in carbon tetrachloride, and pipetted into flat-bottomed Pyrex crystallising dishes to a depth of 4 mm., beneath a 2-cm. layer of buffer at p_H 7·0 containing the metal in solution.* Glass-distilled water, and salts recrystallised until the

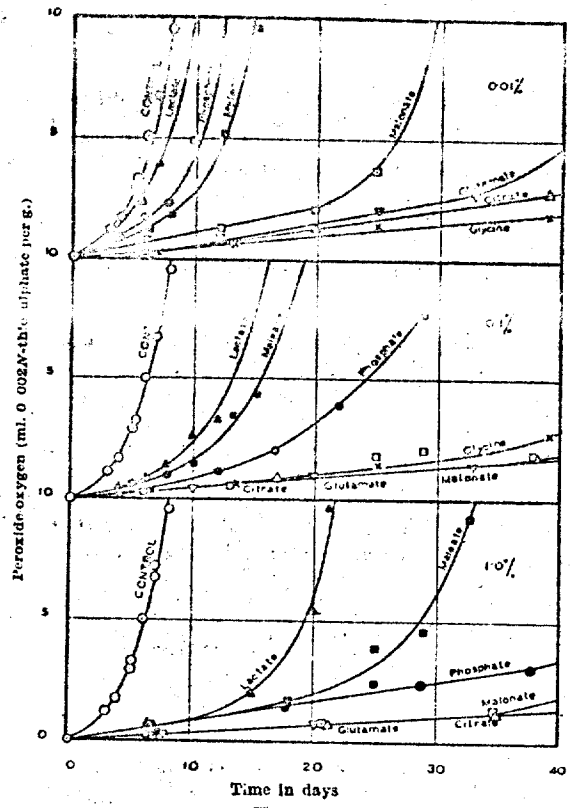

FIG. 5

The effect of antioxidants (0·01, 0·1, and 1·0%) in borate buffer at p_H 7·0

control buffer and sodium chloride solutions at double concentration failed to give any reaction for copper, were used.† The presence of the solvent considerably retarded oxidation of the oil, but this does not affect the result.

The observed rates of oxidation (Fig. 6) indicate that the presence of one part of copper in ten million parts of the solution in contact with the fat increased the rate of oxidation of the latter to about 2·5 times its original value. Iron present at ten times the concentration was only about half as effective. The effect of copper was detectable even at a dilution of one part in one hundred million of solution.

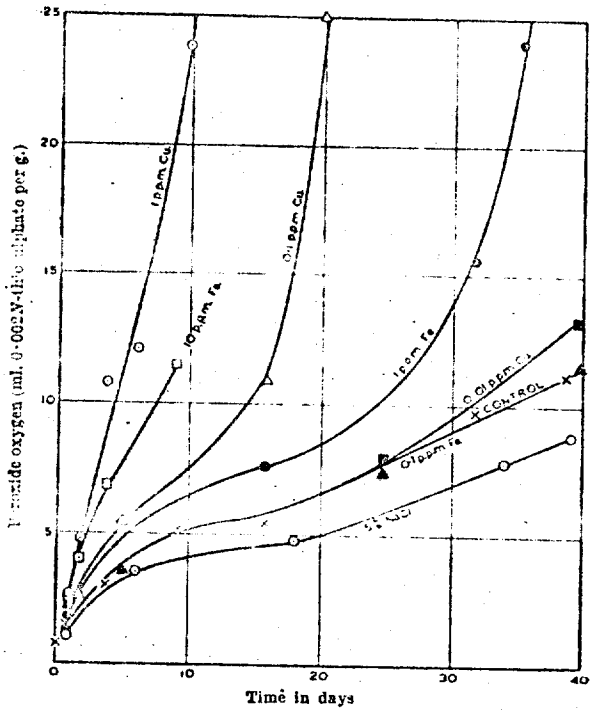

FIG. 6

The effect of metals on the oxidation of herring oil at 20°

Several experiments with films of lard immersed in borate buffer at p_H 7·0 containing 5 parts of iron per million of solution proved that the metal under these

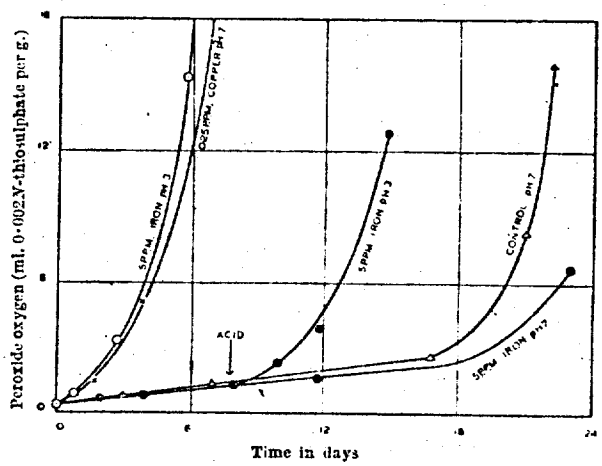

FIG. 7

The effect of p_H on the pro-oxidant activity of iron

conditions was completely inactive. In acid solution, on the other hand, it appeared to possess about one twentieth of the activity of copper (Fig. 7). The p_H at

* In case of solutions containing iron it was necessary to add a little acid in order to prevent precipitation of the metal.

† Since the limit of sensitivity of the method employed for determination of copper (diethyl dithiocarbamate with extraction by carbon tetrachloride) is of the order of 0·01 part per million, the solutions used in experiments described as with "copper-free" reagents contained less than 0·005 p.p.m. of this metal.

which iron loses its activity (apparently owing to precipitation of the hydroxide) was not determined, nor were any tests made of the activity of the metal in the presence of protein.

Sodium chloride at a concentration of 5% not only failed to accelerate oxidation, but actually slightly retarded it (Fig. 6). This observation is in agreement with other experiments in which 26% of sodium chloride buffered at various p_H values also failed to produce any appreciable acceleration. The earlier result in which an increased rate of oxidation was observed in presence of sodium chloride[14] was probably due either to the effect of traces of alkali in the salt on the p_H of the unbuffered solution or to the presence of metallic impurities.

Action of antioxidants in complete absence of copper

The majority of the results so far reported concerning the effect of antioxidant substances in aqueous solution on the oxidation of lard were obtained in presence of traces of copper derived from the distilled water, buffer salts, or glass vessels. The amounts of active metal involved were extremely small but, in view of the data already presented, could not be ignored. Experiments with buffered solutions had attempted to overcome this difficulty by ensuring that control and antioxidant solution should always be under the same conditions as regards copper content. The possibility, however, still remained that these antioxidants might be acting indirectly via the copper rather than directly on the fat; in fact, it seemed reasonable to suspect that some or all of the substances under investigation might perhaps function as antioxidants only by inhibiting the positive catalytic action of copper.

In order to test this hypothesis it was necessary to oxidise the fat in contact with a copper-free aqueous phase in presence and absence of antioxidant. The method used was similar to that already described for investigation of the effect of p_H. The bottom and 1 cm. of the wall of small Pyrex beakers were covered with a thin film of fat, and the "copper-free" buffer solution containing the substance under investigation and adjusted to p_H 7·0 was added in quantity just sufficient to cover the fat. Films produced in this way were very much less uniform in thickness than those obtained by means of the glass-slide technique previously used. At 20°, however, oxidation is relatively slow, and the rate is not very sensitive to variation in thickness of the film.

In addition to lard, freshly extracted herring oil transformed into a solid of suitable consistency by admixture with half its weight of the fully hydrogenated oil was used. The latter, after treatment several times with boiling dilute acid, gave negative tests for both iron and copper. The data listed in Table III were obtained from films of average thickness 0·323, 0·179, and 0·175 mm. at 30°, 20°, and 20° respectively. In experiments 2 and 3 the oil was boiled with dilute acid in an inert atmosphere for some time before use, in order to destroy or remove as much as possible of the natural antioxidant.

As was to be expected from its higher degree of unsaturation (iodine value about 136), the herring oil oxidised much more rapidly than lard under similar conditions, and without any very sharply defined induction period. For this reason the length of time required for the controls to reach 15 ml. of 0·002*N*-thiosulphate per gram (calculated on the herring oil present) was taken as the unit (its actual magnitude was 4·0, 4·5, and 3·25 days), and the protection factors listed in Table III were obtained by comparison with this.

TABLE III

The effect of antioxidants on the oxidation of herring oil in presence and in absence of copper (p_H 7)

Solution	Protection factors Expt. 1	Expt. 2	Expt. 3
Buffer at p_H 7·0	1·0	1·0	1·0
Buffer + copper 0·2 p.p.m.	—	−7·7	−3·1
Buffer + cyanide 0·001*M*	—	−1·1	−1·1
Buffer + copper + cyanide	—	−1·1	—
Buffer + albumin 1%	2·0	1·7	—
Buffer + albumin 1% + copper	—	1·7	—
Buffer + albumin 10%	2·5	—	—
Buffer + triethanolamine 0·1%	—	—	2·0
Buffer + triethanolamine + copper	—	—	1·0
Buffer + glycine 0·01%	—	—	2·5
Buffer + glycine 0·1%	—	—	2·5
Buffer + glycine 0·1% + copper	—	—	2·5
Buffer + iron 5 p.p.m.	—	1·0	1·1
Buffer + iron + cyanide	—	1·0	—
Buffer + iron + albumin	—	−1·1	—

These data prove that cyanide in the absence of copper or similar metal does not possess antioxidant properties but may, if anything, slightly accelerate oxidation*. It does, however, completely inhibit the pro-oxidant influence of copper (and presumably also of iron). Absence of any retardation by cyanide can therefore be used as a criterion of the absence of significant quantities of these metals. Results of similar experiments with water-extracted, acid-treated lard are given in Table IV.

TABLE IV

The effect of antioxidants on the oxidation of lard in "complete" absence of copper (temp. 20°)

Solution	Induction period*	Protection factor
Buffer at p_H 7·0	5·6	1·0
Buffer + diethanolamine 0·01%	7·1	1·3
Buffer + ,, 0·1%	14·0	2·5
Buffer + phosphate 0·1%	16·7	3·0
Buffer + malonate 0·1%	16·8	3·0
Buffer + citrate 0·01%	23·8	4·3
Buffer + phosphite† 0·1%	25·0	4·5
Buffer + pyrophosphate 0·1% } Buffer + citrate 0·1% .... } Buffer + glycine 0·01% .... }	ca. 31·0	ca. 5·5

* Days to reach 6 ml. per g.
† Contains also 0·41% phosphate.

It appears from these figures that the antioxidant effect of the substances tested is not dependent on the presence of copper in the solution, but that they are capable of retarding oxidation in absence of significant quantities of active metal. The fact that some of these antioxidants are also capable of completely inhibiting the effect of small amounts of copper (presumably by formation of very slightly ionised compounds, *e.g.*, with proteins, or to incorporation of the metal in complex ions) indicates that in presence of traces of active metal

* The difference between −1·1 and 1·0 is, in this case, of the same order as the experimental error.

the protection factor found for substances of this type might be greatly increased (Fig. 4).

When the rate of oxidation of the oil was measured in presence of both protein and cyanide, the protective effect of the protein was found to be reduced. For example, 0·001*M*-cyanide reduced the protection factor for 1% albumin from 2·0 to 1·4 and from 1·7 to 1·0 in two cases. Similar results were obtained with gelatin. This effect may perhaps be due to combination of the cyanide at antioxidant-active centres of the protein molecule.

Effect of large amounts of copper

The results presented above indicate that the pro-oxidant effect of copper at a concentration of 0·2 p.p.m. (calculated on the aqueous phase, 6·6 p.p.m. on the fat) is completely inhibited by a number of antioxidants including protein. Milk, which has the approximate composition fat 3·5%, protein 4%, carbohydrate 5%, and water 87%, is known to develop an oxidised flavour

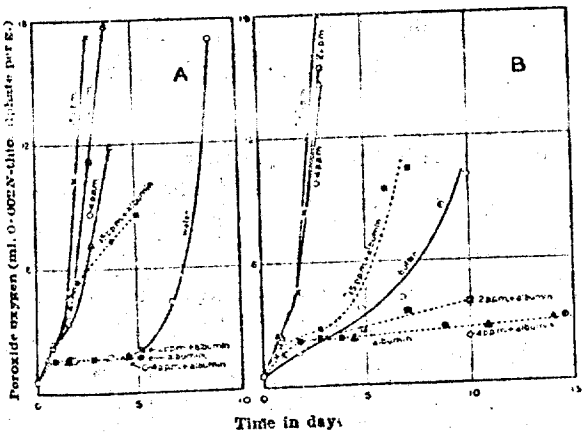

FIG. 8

The pro-oxidant effect of copper in presence of protein, (A) in water, (B) in borate buffer at p_H 7·0

in about 24 hours at 0—5° when 1·5 p.p.m. of copper is present.[18] Similarly butter (fat 86%, protein 0·7%, water 13%) containing 2 p.p.m. of copper develops incipient tallowiness in about 6 days at room temperature.[19] The aqueous phases of tallowy milk and butter would therefore contain at least 1·7 and 15 p.p.m. of copper respectively, in addition to 4—5% of protein. Calculated on the fat these figures correspond to 40 and 2·3 p.p.m., and represent quantities of metal much higher than those used in the present experiments, where the relative proportions of fat, protein, and water, but not the area of surface of the fat exposed, approximated to those in milk.

Experiments similar to those already described, but using an aqueous phase containing 4% of albumin and 0·4, 2·0, and 15·0 p.p.m. of copper (as lactate), were therefore carried out. The fat was water-extracted, acid-treated lard, and the temperature 20°. It was necessary to duplicate the samples, using water in one case and buffer in the other, since a large proportion of the metal precipitated from buffer solutions containing 15 p.p.m. of copper. On the other hand, the presence of borate was essential when readings extended beyond 4—5 days in order to inhibit the growth of micro-organisms. The results (Fig. 8) show that although protein completely inhibits the pro-oxidant action of small amounts (0·4 p.p.m.) of copper, larger quantities of the metal (15 p.p.m.) powerfully accelerate oxidation in spite of the presence of the protein.

The rate of oxidation after conclusion of the induction period was slightly greater in presence of the larger amounts of copper, but the acceleration produced, except perhaps at very low concentrations, did not increase in proportion to the amount of copper added. It is possible that in this case the limiting rate of oxidation, as fixed by diffusion of oxygen into the fat, was being approached, but this explanation cannot account for the failure of the large amount of copper to reduce the induction period below about 1·7 days. Much more rapid oxidation has been observed for solidified herring oil under identical physical conditions.

Action of water-soluble antioxidants in presence of very little water

The majority of the experiments on the effect of water-soluble antioxidants were carried out in presence of a volume of the aqueous phase large by comparison with the fat. In only one case (Fig. 2) was the proportion of water present relatively small (15%, *i.e.*, of the same order as in butter), and in this, reduction of the volume of water by 200—300 times had apparently failed to impair the activity of the antioxidants used. Some natural fats, however, occur in the tissue in presence of still smaller amounts of water, and commercial lards contain only 0·2—0·3%. It seemed doubtful whether antioxidants of the water-soluble type would be effective under such conditions.

Aqueous solutions of several antioxidants, adjusted approximately to p_H 6 but unbuffered, were therefore prepared of such concentration that addition of 0·25% of the solution to the fat (water-extracted, acid-treated lard) gave a concentration of 0·02% of antioxidant in the fat. Under these conditions the antioxidants were still effective in stabilising the lard (Table V).

TABLE V

The effect of water-soluble antioxidants in presence of very little water (temp. 32°)

Added to the lard	Induction period*	Protection factor
Dry lard	6·7	—
Water 0·25%	5·4	1·0
Water 0·25% + albumin 0·02%	ca. 7·7	1·4
Water 0·25% + phosphate 0·02%	10·6	2·0
Water 0·25% + glycine 0·02%	20·7	3·8
Water 0·25% + pyrophosphate 0·02%	22·0	4·1
Water 0·25% + citrate 0·02%	22·0	4·1

* Days to reach 4 ml. per g.

The combined effect of two antioxidants

The results of experiments already described had shown that, under the conditions used, a concentration of antioxidant was soon reached beyond which further increase gave no increase in protection; in fact in case

[18] W. L. Davies, Milk Ind., 1933, 13, 55.

[19] W. L. Davies, "The Chemistry of Milk," London, 1936, p. 83.

of glycine concentrations greater than 0·01% usually gave inferior results. On the other hand some, at least, of the antioxidants tested were capable of prolonging the induction period of the fat beyond that due to the natural antioxidant (Table VI). The data given for acid-treated lard in Table VII show that citrate plus

TABLE VI

The effect of antioxidants in aqueous solution on the oxidation of ether-extracted, untreated lard at 32°*

Solution	Induction period†	Protection factor	Induction period‡	Protection factor
Water	7—11	1·0	·9—13	1·0
Glycine 0·01%	16	1·8	19	1·7
Pyrophosphate 0·01%	18	2·0	22	2·0
Citrate 0·01%	20	2·2	24	2·2

* p_H 6 but unbuffered. † Days to 4 ml. per g.
‡ Days to 6 ml. per g.

TABLE VII

The combined effect of two antioxidants on lard at 20°

Solution	Induction period*	Protection factor
Buffer at p_H 7·0	6	1·0
Buffer + albumin 4%	14—20	2·8
Buffer + glycine 0·02%	15	2·5
Buffer + pyrophosphate 0·02%	19	3·2
Buffer + citrate 0·02%	23	3·8
Buffer + albumin + citrate	15	2·5
Buffer + albumin + pyrophosphate	14—19	2·7
Buffer + citrate + glycine	28	4·7
Buffer + glycine + pyrophosphate	31	5·2

* Days to 5 ml. per g.

glycine, or glycine plus pyrophosphate, protected the fat considerably more efficiently than did either component of the pair alone. Unfortunately the same was not true when one of the substances was protein, neither citrate nor pyrophosphate increasing the protective effect over the normal figure for the protein alone. These results must be confirmed with higher concentrations of antioxidant and extended to cover other substances, before it can be assumed that non-toxic antioxidants of the types studied are in general ineffective in presence of protein.

DISCUSSION

The majority of the experiments described here have been carried out in presence of a borate buffer at p_H 7·0. This, even in the "copper-free" condition, acts as a very weak pro-oxidant, reducing, for example, the induction period of an untreated lard (at 20°) from 27 days in water to 22 days in buffer. Measurements of the efficiency of antioxidants in water confirmed those obtained in buffer, but were more erratic, even when contact with glass was minimised by deposition of the fat on the walls of the beaker (Table VI). The case in which only 0·25% of water was present was exceptional in giving curves as smooth as in the buffer experiments.

Treatment of lard by (*a*) heating with 2½% by volume of 30% sodium hydroxide at 100° for 8 minutes, (*b*) boiling with 8% hydrochloric acid under steam for 1 hour, or (*c*) heating with 2% of adsorbent charcoal at 100° under nitrogen for 15 or 30 minutes, all reduced the induction period (measured at 100° by a method previously described[16]) to approximately half its previous value. The maximum effect obtained by boiling several other samples of lard and two of herring oil with dilute hydrochloric acid was a similar decrease, the effect in case of lard being often much less than this. Hilditch and Sleightholme,[20] on the other hand, reduced the induction period of olive oil at 100° from 260 to 20 minutes by this means. It follows that the properties of antioxidants present in different natural fats vary considerably. Even the numerous samples of leaf lard used in the present experiments varied both in susceptibility to oxidation and in case of stabilisation by antioxidants.

Several substances were found to produce a protective effect over and above that due to the natural antioxidant. Sometimes the peroxide-oxygen content increased steadily throughout the induction period, first at a constant rate, and then more rapidly as the induction period drew to an end. In other cases the peroxide value rose rapidly at first, slowed down again or even remained practically constant for a more or less prolonged period, and then rose again as rapid oxidation set in (Figs. 6 and 8). This behaviour could be due either to an initial oxidation of some labile constituent of the fat, or, perhaps more probably, to attainment of a temporary equilibrium between the rates of formation of peroxide and of its reduction by the antioxidant. In the latter process the antioxidant itself would be destroyed and rapid oxidation of the fat would follow.

Since the samples containing added antioxidants began to oxidise rapidly at approximately but not exactly the same peroxide value, the protection factors obtained by comparison of the times required for the stabilised and unstabilised samples to reach 4, 5, or 6 ml. per g. give only an approximate comparison of the activities of antioxidants. These figures also fail to take into account the depressing action still occasionally in evidence after the end of the induction period proper (*e.g.*, curve 9, Fig. 2), and when this is present the protection factor gives an estimate of efficiency which is too low.

The water-soluble antioxidants examined appear to retain some of their activity on the alkaline side of the neutral point, but if tested on lard in glass the p_H effect already described will result in an increased rate of oxidation as compared with the same antioxidant in acid solution, although the protection factor obtained may actually be higher (Fig. 4). These observations possibly explain the insistence on an acid reaction in the patents cited below. A second reason is that the salts of the various antioxidants are probably insoluble, or much less soluble in the dry fat than the corresponding acids.

Relationship between antioxidant activity and structure

Of the organic substances examined, polyhydroxy-compounds such as glycerol $C_3H_5(OH)_3$ and the sugars—glucose $C_6H_7O(OH)_5$, fructose $C_6H_7O(OH)_5$, and sucrose $C_{12}H_{14}O_3(OH)_8$—were all active in 2% aqueous solution (0·3% calculated on the fat). They were not tested at higher dilutions. Mannitol $C_6H_8(OH)_6$ was inactive, but the sample used contained 9 p.p.m. of copper (0·18 p.p.m. on the aqueous solution), which probably accounts for the apparent discrepancy.

[20] J.S.C.I., 1932, 51, 39 T.

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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[Received December 16, 1970]

J. Food Sci. 31: 48-52, 1966

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The Fatty Acid Composition of Commercially Hydrogenated Fish Oils

SUMMARY

Five samples of industrially hydrogenated fats of marine origin were analyzed for fatty acid composition by the combined methods of group-separation by thin-layer partition chromatography followed by gas-liquid chromatography of each group.

The general fatty acid composition was 30–45% saturated acids, 45–55% monoenoic acids, and 10–20% polyenoic acids. A substantial part of the polyenoic acids were trienoic acids, the rest dienoic. The presence of tetraenoic acids could be ascertained in the low-melting samples (mp 30–32°C).

INTRODUCTION

The bulk of the world production of marine oils is hydrogenated for human consumption as margarine and shortening. Since the marine oils are characterized by a high content of polyenoic acids with 4–6 double bonds, extensive hydrogenation is necessary. The process results in a fat of specific, desired melting point, usually in the range of 30–45°C. The ultimate melting point is a function not only of the degree of saturation, but also of the degree of trans-isomerization during the process. An extensive hydrogenation may therefore lead to a fat with a melting point approaching that of lard but still containing substantial amounts of polyenoic acids.

The literature on this problem is concerned almost entirely with hydrogenated vegetable oils. The literature on hydrogenated marine oils seems limited. Pedersen (1938) studied partly hydrogenated whale oils by iodine values, thiocyanogen values, bromine absorption, etc., and stated that polyenoic acids of up to four double-bonds were present. Schilling (1959) has discussed problems of determination of fatty acids in hydrogenated marine oils. Pokorny *et al.* (1963) determined the fatty acid composition of hydrogenated vegetable and marine oils and in margarines. Their results, based on gas-liquid chromatography, show a complicated pattern including several polyenoic acids.

Analytical methods available for the determination of fatty acid composition are all of limited value when applied to hydrogenated fats. Particular problems result from the extensive isomerization which takes place during catalytic hydrogenation. These isomerization reactions have been studied in detail by Dutton (1963) and his group. Besides the formation of trans-isomers there is also found a complicated mixture of positional isomers. An example of this is given by Scholfield *et al.* (1963) for hydrogenated methyl linolenate. The polyenoic acids include isomers containing conjugated double bonds and isomers with more than one methylene group between the double bonds. The technique of alkaline isomerization followed by spectroscopy thus fails to give a correct picture of the content of polyenoic acids in hydrogenated fats.

Methods of gas-liquid chromatography presently dominate fatty acid analysis. Unfortunately, positional isomers show small differences in retention times and cannot be satisfactorily separated by direct gas-liquid chromatography (Scholfield *et al.*, 1961). Two closely related acids, i.e. C_{20} with 2 and 3 double bonds, have each broad bands of elution which overlap and cannot be quantitated (Fig. 1).

The partition chromatographic methods described by Kaufmann (1958) will separate fatty acids into groups of "critical partners." The principle can be applied to thin-layer chromatography of methyl esters of fatty acids, thus giving separation into groups which can be directly resolved by GLC. The group separation depends upon the major differences in the over-all unsaturation and the molecular size, and is not influenced by the presence of isomers. The principles are illustrated by Fig. 1 and 2.

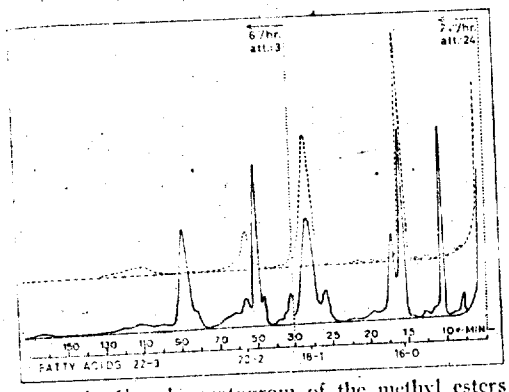

Fig. 1. Gas-chromatogram of the methyl esters from a hydrogenated fat (mp 30–32°C), full line, and of fraction 4 from thin-layer chromatography of these esters, broken line 15% BDS; column, 190°C.

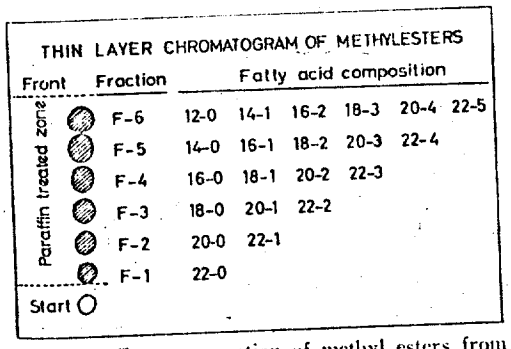

Fig. 2. Group separation of methyl esters from hydrogenated fats by thin-layer chromatography. System: paraffin/acetonitril-isopropanol-water 80:15:5.

METHODS

Sampling and preparation of methyl esters. Samples of hydrogenated fats of marine origin were received from three different Norwegian refineries. Two samples had specified melting points of 30–32°C, and three had 31–33, 36, and 38–40°C. Five-gram portions were saponified for 30 min with 50 ml methanol (aldehyde free) + 5 ml 60% (w/w) KOH in the presence of pyrogallol and ascorbic acid for protection. The methanol was quickly removed under vacuum, thereby cooling the mixture. The saponification mixture was transferred to a separating funnel with 100 ml ethyl ether (peroxide-free) and 100 ml distilled water. The unsaponifiable matter was extracted into the ethyl ether, and the extract washed with 30 ml distilled water to which was added 1 ml KOH solution. The water phase was added to the soap solution, and the ether phase discarded. The fatty acids were set free by 50 ml of 15% (v/v) HCl and extracted three times with 50 ml ethyl ether. The combined extracts were washed four times with 50 ml distilled water. The ethyl ether was evaporated in vacuum at approx. 40°C. Twenty ml of 12% (v/v) boroutrifluoride (Fluka) in methanol was added quickly, and the solution boiled for 5 min. The methyl esters were transferred to a separating funnel with 25 ml distilled water and extracted three times with 25 ml ethyl ether. The combined extracts were washed four times with 25 ml distilled water. The ethyl ether was evaporated at low temperature in vacuum. The methyl esters were dissolved in a few ml of hexane (bp 67–72°C). Oxydation products were removed by passing the solution through a column (ID 12 mm) of soft alumina (40 g Al_2O_3 Brockmann + 10% water). The methyl esters were eluted with 50 ml of hexane, which was removed in vacuum at low temperature. The esters were dissolved in ten times their weight of methylhexanoate, and stored in the freezer.

Hydrogenation. To 3 ml of the hexanoate solution of the methyl esters was added a pinch of palladium on activated carbon (Fluka) as a catalyst. The mixture was shaken in a small flask for 3–4 hr under approx. 20 psi of hydrogen, and then filtered.

Preparative thin-layer chromatography. Sets of nine glass plates (20 × 10 cm) were prepared with 5 g Kieselguhr G (Merck) in 15 ml distilled water. The plates were dried for 3–4 hr at 120°C. The plates were impregnated by allowing a solution of 10% paraffin (bp 180–200°C) dissolved in pentane (bp 45–50°C) to ascend to within 2.5 cm of the top edge. The pentane was evaporated by drying in air. On each plate was applied 7–8 spots each of 5 μl of the methyl ester solution on the unimpregnated strip. The elution was carried out with a mixture of acetonitrile (Merck)–isopropanol–water (80:15:5). The mixture was saturated with paraffin prior to use. Normal elution time was 45 min. The plates were dried under nitrogen at room temperature, and developed by spraying with a solution of 0.001% of sodium fluoresceinate in water. The spots were plotted by UV-light (360 mμ). The rows of spots were carefully scraped off the plates and transferred to centrifuge tubes containing 20 ml hexane and 10 ml distilled water. The tubes were shaken, centrifuged, and frozen. The hexane phases were removed, concentrated in vacuum, and transferred to small tubes with conical bottoms. The rest of the hexane was removed by a current of nitrogen, and the methyl esters were left in the accompanying paraffin solution.

Gas-liquid chromatography. The fractions were chromatographed in a PYE-argon chromatograph with Sr^{90} ionization detector. The columns were 120 cm long, with an internal diameter of 4 mm.

They were provided with a flash heater (230°C). The stationary phases were 10% of SE-30 silicone gum on silanized Kieselguhr (80–100-mesh), and 15% of butanediol succinate polyester on the same adsorbent. The first column was run at 205°C, the second at 190°C, both with a gas velocity of 40 ml per min. The hydrogenated fractions were chromatographed on the silicone column, whereas the original methyl esters and the fractions from the thin-layer chromatography were chromatographed on the polyester column. Each analysis consisted of three parallel runs.

Calculation of results. Each peak was quantitated by calculation of the area from the formula height × width at half height. The polyenoic acids of groups C_{20} and C_{22} were generally of a shape corresponding to two overlapping peaks. This was probably caused by the presence of several positional isomers. Such composite peaks were calculated as one.

Chromatograms from the fully hydrogenated fraction were used for calculation of chain-length percentages and the percentages of odd-numbered and branched fatty acids. The sum was calculated to 99.5%, thus giving 0.5% as the sum of uncalculated peaks.

From each of the six fractions (Fig. 2) obtained by thin-layer chromatography, a major fatty acid peak was chosen as representing the fraction. In the present investigation these fatty acids were: C_{22}:0 for fraction 1; C_{22}:1 for fraction 2; C_{20}:1 for fraction 3; C_{18}:1 for fraction 4; C_{14}:0 for fraction 5; C_{16}:1 for fraction 6.

These six peaks were quantitated on the chromatograms of the original methyl esters to obtain relative values between the thin-layer fractions. The peaks from the chromatograms of each of the thin-layer fractions could then be calculated on the basis of their representative fatty acid. All areas summed up according to the relations found in the chromatogram of the original methyl esters.

Lastly, the acids from each chain length were summed up and corrected according to the values found in the chromatograms of the fully hydrogenated fraction.

RESULTS AND DISCUSSION

The results of the analyses are reported in Tables 1 and 2. The methods described for the preparation of methyl esters gave recoveries above 95% by weight. The methylation by boron trifluoride was performed mainly as described by Metcalfe and Schmitz (1961). The thin-layer chromatography was adopted from Mangold (1962). The principle of calculation of gas-liquid chromatograms on the basis on fully hydrogenated samples is generally adopted, and has been used by Ackman and Burgher (1963) and Gruger *et al.* (1964). The areas are summarized as weight-percentages, and this has been controlled by calculation and determination of the mean molecular weight of the fully hydrogenated methyl esters. The results agreed within ±2.5%. Identification of the fatty acid peaks was carried out by the principles discussed by Ackman (1963), and by Iverson *et al.* (1965). The different principles of analyses used in the present work are discussed in detail by Lambertsen (1965).

Table 1. Fatty acid composition of hydrogenated oils of marine origin. Complete list of fatty acids recorded and calculated (percentages).

Fatty acid designation	Samples				
	A-1 30–32°	A-2 31–33°	A-3 30–32°	B 38–40°	C 36°
14:0	7.8	7.8	9.7	8.0	7.9
14:1	0.2	0.5	0.2	0.4	0.4
15:br	0.6	0.3	0.4	0.7	0.4
15:0+1	0.6	0.3	0.4	0.6	0.4
16:br	—	—	—	0.1	0.1
16:0	12.6	13.8	18.4	12.4	17.7
16:1	6.8	8.5	11.0	8.7	6.2
16:2	0.8	1.5	0.5	1.1	0.2
17:brI	0.4	0.1	0.1	0.4	0.2
17:brII	0.4	0.2	0.1	0.4	0.2
17:0+1	0.9	0.4	0.4	0.7	0.5
18:br	—	0.3	0.1	0.3	0.3
18:0	3.5	3.8	3.2	6.8	7.8
18:1	19.9	15.5	17.0	14.8	15.3
18:2	2.0	2.5	2.5	1.5	1.2
18:3	0.1	0.6	0.2	0.1	—
19:0+1	0.4	0.4	0.2	0.3	0.5
20:0	1.2	1.9	0.8	3.4	3.9
20:1	10.5	11.4	9.8	10.3	10.3
20:2	4.0	4.2	4.4	4.4	3.5
20:3	4.5	3.2	4.5	2.3	1.6
20:4	0.1	0.3	0.2	—	—
21:0+1	0.4	0.6	0.2	0.5	0.6
22:0	1.1	2.0	1.0	3.9	4.1
22:1	13.5	13.6	8.0	11.9	10.7
22:2	1.6	2.8	1.7	2.9	2.8
22:3	4.3	2.2	2.7	1.8	1.9
22:4	0.8	—	1.1	—	—
24+	0.5	0.7	0.6	0.8	0.8
Others*	0.5	0.6	0.6	0.5	0.5
Totals	100.0	100.0	100.0	100.0	100.0

* By difference.

Table 2. Fatty acid composition of hydrogenated oils of marine origin. Summation of groups according to chain lengths and unsaturation.

	Samples					
	A-1 30—32°	A-2 31—33°	A-3 30—32°	B 38—40°	C 36°	Av.
C_{14}	8.0	8.3	9.9	8.4	8.3	8.58
C_{16}	20.2	23.8	29.9	22.2	24.1	24.04
C_{18}	25.5	22.4	22.9	23.2	24.3	23.66
C_{20}	20.3	21.0	19.7	20.4	19.3	20.14
C_{22}	21.3	20.6	14.5	20.5	19.5	19.28
C_{24}	0.5	0.7	0.6	0.8	0.8	0.68
Others	4.2	3.2	2.5	4.5	3.7	3.62
Totals	100.0	100.0	100.0	100.0	100.0	100.0
Saturated	29.1	31.5	34.9	38.0	44.1	
Monoenoic	52.7	51.2	47.3	47.9	44.7	
Polyenoic						
Dienoic	8.4	11.0	9.1	9.9	7.7	
Trienoic	8.9	6.0	7.4	4.2	3.5	
Tetraenoic	0.9	0.3	1.3	—	—	
Totals	18.2	17.3	17.8	14.1	11.2	
Iodine value (Wijs)	83.9	73.6	87.6	66.0	63.6	

Tables 1 and 2 show that the general fatty acid composition as well as the iodine values classify these hydrogenated oils as rather similar in unsaturation to many vegetable oils (palm oil: 40% sat., 50% oleic, 10% linoleic I.V. 65; olive oil: 20% sat., 70% oleic, 10% linoleic I.V. 80). This can be seen in relation to the experiments on the effect of different oils on blood cholesterol levels. Hydrogenated rats fall in the "neutral" group in this connection (Nicolaysen and Ragård, 1961). The Tables give all the details with regard to the distribution of the different fatty acids, but certain relations may be emphasized.

The monoenoic acids constitute about half of the total fatty acids in the present samples. The same concentration is normally present in the corresponding nonhydrogenated marine oils. Evidently hydrogenation from polyenoic to monoenoic acids balances the hydrogenation from monoenoic to saturated acids. Somewhat higher values for C_{22}:1 and somewhat lower values for C_{16}:1 characterize the hydrogenated oils as compared with the raw oils (Lambertsen, 1965).

The saturated acids increase during hydrogenation and constitute 30–45% of the total fatty acids. The main increase falls on C_{16}:0. It is of particular interest to note that the C_{20}:0 and the C_{22}:0 are present in relatively small concentrations. Only in oils hydrogenated to higher melting points is a notable increase of these acids observed to about 4%.

The major polyenoic acids of marine oils are C_{18}:4, C_{20}:5, C_{22}:5, and C_{22}:6 (Gruger *et al.*, 1964). These acids have all completely disappeared during the hydrogenation process. Up to 1.3% of the tetraenes could be found in the low-melting samples, and none in the higher-melting samples. Trienes, however, are important constituents of these oils. They were found in concentrations from 3.5 to 8.9% in the samples. These acids are partly masked by the dienes in methods applied for the analysis of fatty acid composition because of the many positional isomers after hydrogenation. They can be clearly identified by thin-layer partition chromatography, as in the present study. The percentage of trienes is notably lower in the high-melting fats than in the low-melting ones, respectively 3.9 and 7.4%. The dienes are present in total quantities from 7.7 to 11.0%. These acids show no relation to the degree of hydrogenation, the

average value being 9.5% for low-melting fats and 8.8% for high-melting fats.

Generally, the polyenoic acid may be considered an important part of hydrogenated marine oils, with values ranging from 11.2 to 18.2 in the present samples.

Finally may be mentioned that the gas chromatographic methods have firmly established the presence of many acids of odd-numbered and branched carbon chain. Such acids are particularly prominent in marine oils, many in quantities of the order from 0.5 to 1% of the total fatty acids. The group of C_{17}-acids must be given particular attention in analyses of marine oils. Small quantities of C_{12} and lower acids may be present, but the methods applied in the present study did not record these short-chain compounds.

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Ms. rec'd 5/25/65.

This study was made possible by a grant from the Research Fund of Norwegian Margarine Producers.

Table 2. Percentage of sterols in nonsaponifiable fraction of seafood (determined by gas-liquid chromatography).

Compound	R_c[a]	Oyster	Haddock	Pollock	Salmon	Shrimp	Lobster	Clam	Scallop	Crab
Squalene	0.49	.....	1.0	5.9	3.9	0.4	.....	.....	.....	.....
C_{28} sterol	0.64	5.6	.....	.....	.....	.....	.....	4.8	4.4	.....
22-Dehydrocholesterol	0.90	2.9	.....	.....	.....	.....	.....	7.9	13.4	3.9
Cholesterol		41.4	93.1	94.1	96.1	95.6	99.2	36.7	25.7	57.4
Brassicasterol	1.13	16.0	4.7	.....	.....	.....	.....	14.1	14.1	36.7
24-Methylene cholesterol	1.31[b]	25.9	1.2	.....	.....	.....	0.8	20.2	19.5	2.0
C_{29} sterol	1.45	.....	.....	.....	.....	.....	.....	4.4	3.0	.....
C_{29} sterol	1.69	8.2	.....	.....	.....	.....	.....	11.8	5.5[c]	.....

[a] R_c = Identification based upon retention time relative to cholesterol (R_c = 1.00).
[b] Dehydrobrassicasterol exhibits an R_c of 1.29.
[c] 9.3% of component with R_c = 1.64; 2.3% of component with R_c = 2.04.
The percentages given in this table are based on measurement of areas under GLC peaks and are subject to the errors inherent in such measurements).

these data that oyster, clam, scallop and crab contain considerable amounts of sterols other than cholesterol. The content of 24-methylenecholesterol reported for clam and oyster in these studies is somewhat less than that found by Idler and Fagerlund (1955).

The R_c of brassicasterol and desmosterol is identical, but thin-layer chromatography has shown that the former is the sterol present in the tissues we examined.

The apparent cholesterol content of various seafoods has been reported by a number of authors; their findings are summarized in Table 3. Despite variations in methodology, there is fair agreement among the results of most of the workers except Thompson. She subjected the tissues to a short (30 sec) extraction by chloroform-methanol, 1:2. We compared her extraction method with that used by us, and found that the tissues were not completely extracted by either method of chloroform-methanol extraction, but the shorter extraction left most of the sterol in the tissue residue. The discrepancy between our present data and our earlier results (Kritchevsky and Tepper, 1961) is due to the complete saponification of tissue and digitonin precipitation of the sterols in this study.

Some of the variations in the data presented in Table 3 may be due to a combination of seasonal variation (Idler *et al.*, 1964; Bourcart *et al.*, 1964) and species differences. In seafoods which contain many sterols other than cholesterol, some of the apparent variation may be an artifact due to the color yield of the several sterols when a cholesterol standard is used. It has been shown that sterols differing from cholesterol in only one nuclear or side-chain double bond give considerably different color yields in the Liebermann-Burchard reaction (Idler and Baumann, 1953; Steinberg *et al.*, 1960). The C_{28} and C_{29} sterols, even those with a nuclear configuration identical to that of cholesterol, also will give different color yields, either slightly lower, as sitosterol, or higher, as ergosterol (Kritchevsky and Tepper, 1961; Idler and Baumann, 1953; Brieskorn and Herrig, 1959). In our hands, brassicasterol gave about half the color yield of cholesterol at either 560 or 620 mμ. Thus the actual weight of the nonsaponifiable material present in oyster, clam, scallop, and crab may be considerably different from that adduced by extraction and colorimetric analysis. The actual weight of nonsaponifiable matter present in these seafoods was (mg/100 g): oyster, 219; clam, 176; scallop, 161; crab, 142.

The presence of a variety of sterols other than cholesterol in certain seafoods does not make their inclusion in a low-cholesterol diet more desirable, since dihydrocholesterol (Cook *et al.*, 1954; Nichols *et al.*, 1953; Curran and Costello, 1956; Schwenk *et al.*, 1961), dehydrocholesterol lathosterol (Cook *et al.*, 1954), soy sterols (Curran and Costello, 1956), and desmosterol (Avigan and Steinberg, 1962) have all been shown to be atherogenic.

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Table 3. Apparent cholesterol content[a] of seafood (mg/100g).

Sample	This study	Kritchevsky and Tepper (1961)	Kaucher *et al.*[b] (1943)	Okey (1945)	Pihl (1952)	Achard *et al.* (1934)	Thompson (1961)
Oyster	150	112	.....	230–470	.....	215	37–58
Haddock	90	45	.....	.....	64	.....	.....
Pollock	75	.....	.....	.....	.....	.....	.....
Salmon	95	65	38	.....	57	25	
Shrimp	200	138	156	.....	226	116	157
Lobster	170	.....	.....	.....	208	.....	.....
Clam	190	122	.....	.....	.....	.....	82
Scallop	175	.....	.....	.....	.....	.....	60
Crab	140	.....	.....	145	.....	.....	52–98

[a] Based on cholesterol standard.
[b] Calculated from dry-weight data, assuming 80% moisture in the tissue.

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This work was supported in part by Research Grant HE-03[illegible] and Research Career Award 5-Ko-HE-734 from the National He[illegible] Institute, USPHS.

Acta Chem. Scand. 17: 281-282, 1963

Partial Characterization of the Hydrocarbons of Herring Oil

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Shark liver oils are known to contain high concentrations of hydrocarbons, and squalene is present in as high as 80 % of the oil of the basking shark. Other well characterized hydrocarbons in fish oils are pristane and zamene, belonging to the C_{19} aliphatic terpenoids [1,2]. Others have been named, but their structures are uncertain [3].

A preliminary gas chromatographic study of several vegetable oils and fish oils revealed the presence of complicated mixtures of hydrocarbons in many of them. In general the vegetable oils contained fewer hydrocarbons than did the fish oils. Rapeseed oil contained several major hydrocarbon constituents, whereas squalene was the principal hydrocarbon in olive oil.

Herring oil was studied in most detail. This oil contained 0.05 % hydrocarbons, about 5 % of the unsaponifiable matter. 500 g of herring oil were saponified [4] using hydroquinone and ascorbic acid as antioxidants. The unsaponifiable fraction was extracted quickly with ethyl ether and resaponified. The unsaponifiable fraction was chromatographed on alumina with hexane and the eluate was concentrated and rechromatographed in the same way on a smaller column. Upon evaporation of the solvent, the fraction gave a near-infrared spectrum corresponding to a pure hydrocarbon.

Table 1. Hydrocarbons occurring in herring oil as determined by gas chromatography.

Straight chain components		Branched chain components	
Carbon Number	% area	Carbon Number	% area
14.0	0.3	15.4	3.5
15.0	7.5		
16.0	0.6	16.8 (pristane)	70.0
17.0	9.0		
17.6	2.2	17.9	5.0
18.0	1.7		
19.0	10.5	19.0	0.5
20.0	0.3		
21.0	6.0	19.9	1.0
22.0	0.3		
23.0	0.8	~21*	3.5
24.0	0.3		
25.0	9.5	22.2	3.5
26.0	trace		
27.0	33.0	26.3 (squalane)	13.0
28.0	trace		
29.0	9.0		
30.0	trace		
31.0	~7		
32.0	(trace)		
33.0	~1		

* Flat and broad peak.

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The Journal of Biochemistry, Vol. 41, No. 5, 1954

STUDIES ON THE TOXICITY OF FISH OIL. II.*

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(Received for publication, July 15, 1954)

In the foregoing paper, the author reported that ethyl ester of highly unsaturated fatty acid freshly obtained from cuttle fish oil showed quite the same good nutritive effect as oleic acid ester for rat, while the acid exposed to air was proved to be extremely toxic.

Since it seems to be most plausible that the toxicity is concerned with the formation of peroxide by autoxidation of unsaturated fatty acid, the author determined the amount of peroxide. The result proved this was just the case, the details of which will be reported here.

EXPERIMENTAL

The determination of peroxide was carried out following the Wheeler's method improved by Nakamura (*1*). It runs as follows.

An adequate portion of the sample, usually 0.1-0.5 g., was exactly weighed out into conic flask of 300 ml. capacity, mixed with 20 ml. of carbon tetrachloride, 30 ml. of glacial acetic acid and finally 1 ml. of saturated potassium iodide solution. After well shaken for 1 minute, 100 ml. of distilled water was added to and thus free iodine liberated in the solution was titrated by 0.01 *N* thiosulphate solution. 1 ml. of this thiosulphate solution corresponds to 0.00008 g. of oxygen.

The acid ester used here was prepared from cuttle fish oil as described in the foregoing paper and had the following properties.

TABLE I

n_D^{20}	Iodine value. (Wijis)	Sap. value	Unsap. matter %	Peroxide *mg./100 g.*
1.4815	342.1	168.6	Trace	2.

About 50 g. of this ester was poured into Petri-dish and submitted for autoxidation

* The resume of this experiments was reported at the 26th Annual Meeting of the Japanese Biochemical Society, in April, 1954.

by contact with air at room temperature (2–18°). The determinations of peroxide were carried out at a few days interval over 200 days. The results are illustrated in Fig. 1.

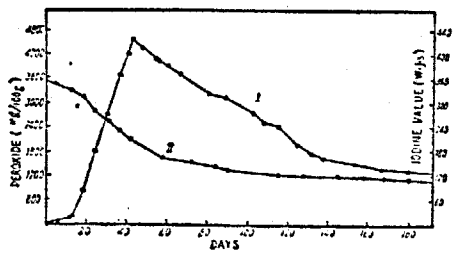

Fig. 1. The change of peroxide (I) and iodine values (II) of the fatty acid ester exposed to air at room temperature (2–18°).

It is interesting to note that during initial 2 weeks the formation of peroxide was almost negligible and then abruptly increased. On the 42nd day, it reached the maximum showing the peroxide value 4400 and iodine value 208. Then it decreased gradually until the peroxide value dropped to 1200, and the iodine value to 120. In the course of this autoxidation the ester became gradually viscous and tinted yellowish brown. On the samples corresponding to various phase of autoxidation, the toxicities were tested by feeding rat with the diet containing 5 per cent of the sample. One which showed the peroxide value 3112 and the iodine value 137 was found fatal to rat, while one with the decreased peroxide value such as 85 and the iodine value such as 76.6 showed no more toxic action.

When the process of autoxidation was allowed to proceed at 30°, the formation of peroxide was accelerated, but its maximal amount was much reduced compared with that at room temperature.

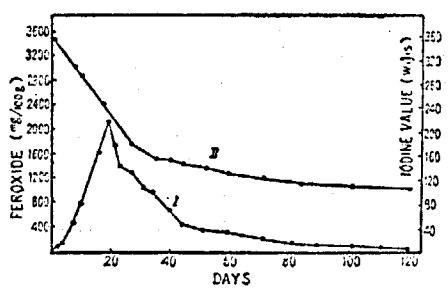

Fig. 2. The change of peroxide (I) and iodine value (II) of the fatty acid ester exposed to air at 30°.

This tendency of the effect of the heat was more apparent at 100°, the maximal amount of peroxide being only 240 in this case. Further it was noticed that at higher temperatures the decomposition of once preformed peroxide occurred more rapidly and thoroughly.

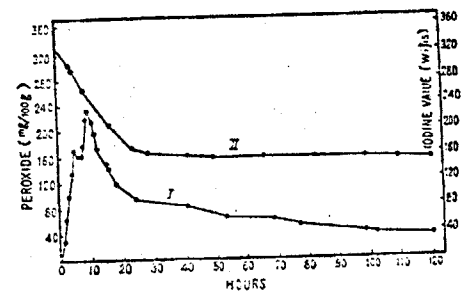

Fig. 3. The change of peroxide (I) and iodine values (II) of the fatty acid ester exposed to air at 100°.

The sample heated at 100° and possessing the peroxide value 240 and the iodine value 245.5 was found fatal for rat, causing abrupt decrease of body weight, which led to death within 7 days, as shown in Fig. 4.

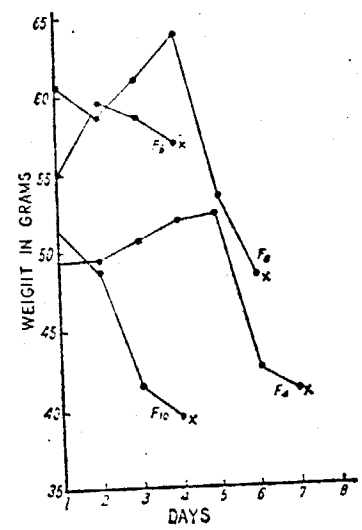

Fig. 4. The effect of the heated fatty acid ester possessing peroxide value of 240 and the iodine value 245.5.

585

To the contrary, the sample heated further and possessing the peroxide value 30 and the iodine value 156.1 showed no more toxicity. Moreover, its nutritional effect was fairly good as shown in Fig. 5.

This fact suggests an important mean to eliminate the toxicity of fish oil.

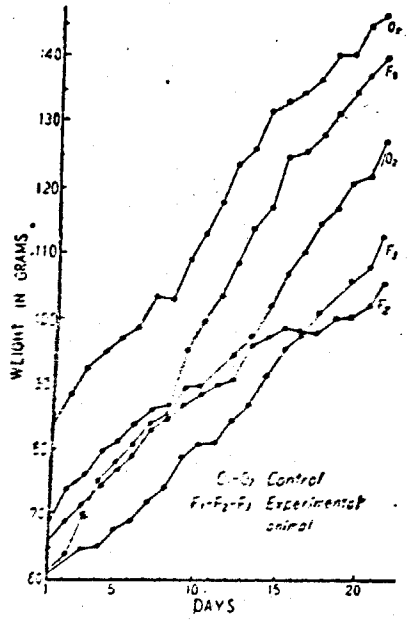

FIG. 5. The growth curve of rat fed on the diet containing 5% of the heated fatty acid ester with the peroxide value 30 and the iodine value 156 in basal diet.

DISCUSSION

From the experimental results above mentioned it is almost certain that the toxic action of fish oil is due to the peroxide formed by autoxidation at the site of double linkage in the molecule of unsaturated fatty acid.

The chemism involved in peroxide formation is pictured as follows.

$$-CH=CH-CH_2-CH=CH- \xrightarrow{+O_2} -\underset{\vdots\,O}{CH}-\underset{\vdots\,O}{CH}-CH_2-CH=CH-$$

According to the recent theory advanced by Gunstone and his co-worker (2) as well as by Lundberg and his associates, (3) the peroxide radical thus formed has tendency to become hydroperoxide as follows.

$$-\underset{\vdots\,O}{CH}-\underset{\vdots\,O}{CH}-CH_2-CH=CH- \longrightarrow -\underset{\vdots\,OOH}{CH}-CH=CH-CH=CH-$$

The resulted hydroperoxide compound may easily enter into ether bond with a double bond of other fatty acid molecule in the following way (4, 5, 6).

$$-CH-CH=CH-CH=CH- + -CH=CH- \longrightarrow -CH-CH=CH-CH=CH- \quad (\text{with } -\underset{|}{O}-\underset{|}{CH}-\underset{|}{CH}-\ \text{and } OOH)$$

It is thought also that the presence of -OOH radical activates the double bond close to it, so that the double bond shifts in the following way (7).

$$-CH-CH=CH-CH=CH- \longrightarrow -\overset{\overset{\oplus}{|}}{C}-CH=C-CH_2-CH_2- \quad (\text{with } \overset{\ominus}{O}\cdots OH)$$

And such dipolarized molecules combine each other as follows.

$$-C-CH=C-CH_2-CH_2- \quad (\text{with } O\cdots OH \text{ and } OH\cdots O)$$
$$-CH_2-CH_2-C=CH-C- \quad (\text{with } OH \text{ and } O)$$

Anyhow, in this way the peroxide radical may be gradually decomposed inducing polymerization of fatty acid molecules.

This is also the reason why the sample, which lost its peroxide activity by standing or heating for longer time gained high viscosity.

SUMMARY

1. When ethyl ester of highly unsaturated fatty acid from cuttle fish oil was submitted to autoxidation in the presence of air, peroxide was formed. Its amount increased gradually, reached maximum and then decreased.

2. The toxicity of oxidized fatty acid ester ran parallel to the content of peroxide. Even the fatty acid, which once showed a high

585

toxicity, turned out to be non toxic when its peroxide contents were reduced by further heating or by long standing.

3. The rate of formation of peroxide and its amount were much influenced by temperature. At low temperature the rate was slow but the amount was large, while at higher temperature the rate was rapid, at the same time its decomposition was also quick, so that remaining peroxide content was much less.

4. It is of prime importance to examine the peroxide content in fish oil when this is applied for dietary use.

The author expresses his heartful thanks to Prof. Keizo Kodama for his continuous guidance through the course of this research, and at the same time, also wishes to thank Prof. S. Misaka and Dr. G. Shibamoto for their encouragement for this work and T. Yoshikawa for his assistance in carrying out the experiments.

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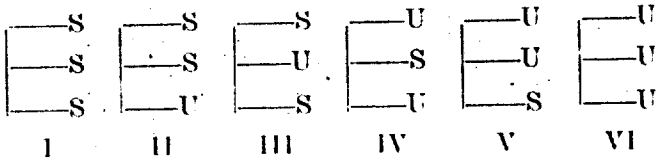
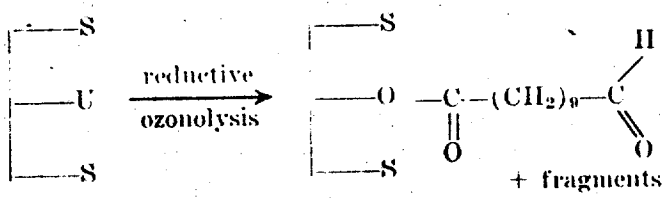
Privett and Blank (149) have published an elegant method for the determination of mono-, di-, and triglycerides varying in fatty acid constituents in regard to unsaturation. It is based upon reductive ozonolysis of these types of compounds, followed by separation and quantification of the glyceryl residues by TLC. This ozonization-reduction-thin-layer chromatographic method (149) of analysis permits analyzing the four possible types of monoglycerides, six of the seven diglyceride types, and four of the six triglyceride types. Since this method has such wide potential applications, it is explained here in more detail, with the example of the structural analysis of triglycerides.

There are six types of triglycerides, *viz.*,



S and U in these models denote saturated (S) and unsaturated (U) fatty acid moieties. Reductive ozonolysis will leave triglycerides of type I untouched, whereas all other triglyceride types will yield glyceryl residues—aldehydic cores—of the kind demonstrated with a triglyceride of type III:



The saturated fatty acid constituents of a type III triglyceride are not affected by ozonolysis, whereas the long chain of the unsaturated acid is ruptured to yield a structure as depicted above, i.e., the unsaturated-disaturated triglyceride has become a compound of a polarity which is widely different from that of the unchanged fully saturated triglyceride (type I). Consequently, the aldehydic cores resulting from triglycerides of type III and from the other triglycerides containing one or more unsaturated fatty acid moieties, are easily resolved, as classes, by TLC on Silica Gel G. An example of such separations is presented in Figure 13. Saturated and unsaturated triglycerides give very different standard curves, but the aldehydic cores resulting from unsaturated triglycerides by reductive ozonolysis yield identical curves as saturated triglycerides, if correction is made for the different percentages of fissioned carbon lost in the various types of compounds. The quantitative evaluation of chromatograms is demonstrated in Figure 14.

Privett (151) utilized TLC in studies on the autoxidation of unsaturated fatty acids and esters. Figure 15 serves as an example to illustrate the kind of separations possible.

TLC has been used to check the course of synthesizing procedures and the purities of final products (49, 50, 94, 100, 217). In checking by means of adsorption-TLC it is not necessary to start a sequence of reactions with an individual compound, say oleic acid, as is necessary with reversed-phase paper partition chromatography (106). The use of adsorption-TLC permits working with a lipid class, e.g., a mixture of fatty acids derived from fish oils (49, 50).

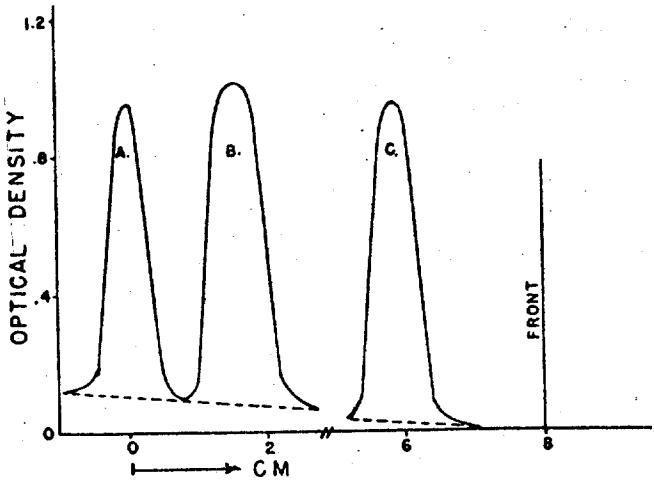


FIG. 13. Densitometric analysis of the aldehyde cores derived from A. Triolein, B. Palmitodiolein, C. Oleodipalmitin (149). Solvent: Petroleum hydrocarbon-diethyl ether, 65/35, v/v. Development time: 40 min. Indicator: Charring with aqueous sulfuric acid, 1/1, v/v.

Methods for analyzing polar lipids of industrial importance, such as amines, amides, and nitrils—also alkyl sulfates and sulfonates, alkyl phosphates and phosphonates—have recently been worked out (111).

Several investigators have applied TLC to check the efficiency of other separation techniques (59, 129, 211). Morris, Holman, and Fontell (127) have found by the use of TLC, that the methyl esters of some long-chain fatty acids are altered during GLC. Conjugated trienoates undergo *cis-trans* isomerization. The esters of vicinally unsaturated hydroxy acids are dehydrated and unsaturated hydroperoxides are similarly altered to more highly unsaturated derivatives. Mangold and Kammereck (110) fractionated mixtures of the methyl esters of saturated fatty acids and the acetoxymercuri-methoxy compound of unsaturated ones by TLC into groups, according to the degree of unsaturation in the original esters (Fig. 9). Each group of esters was recovered and after cleavage of the addition compounds, it was resolved into its components by GLC, according to chain length. Pairs of esters which are not resolved by GLC are well separated by TLC of their adducts and *vice versa*. The successive application of TLC and GLC results in more reliable identification of the various compounds.

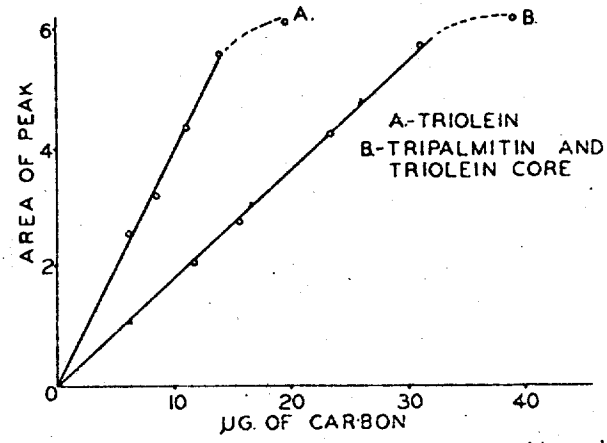


FIG. 14. Standard curves, thin-layer chromatographic analysis of triolein, tripalmitin, and the aldehydic core derived from triolein (corrected for fissioned carbon) (149).

recorded in GLC. In addition, this combination of methods yields more trustworthy quantitative analyses than does GLC alone.

Phospholipids, Sulfolipids, and Glycolipids

Application of TLC in this domain have been especially fruitful (30, 52, 64, 71, 72, 87, 89, 111, 164, 219, 220, 223). The solvents applied are usually the same as those used for resolving phospholipids by chromatography on silicic acid-impregnated paper (113) or on columns of silicic acid (95, 113). The capacity of the chromatoplates is much less with such polar developing solvents than with mixtures of petroleum hydrocarbon and diethyl ether.

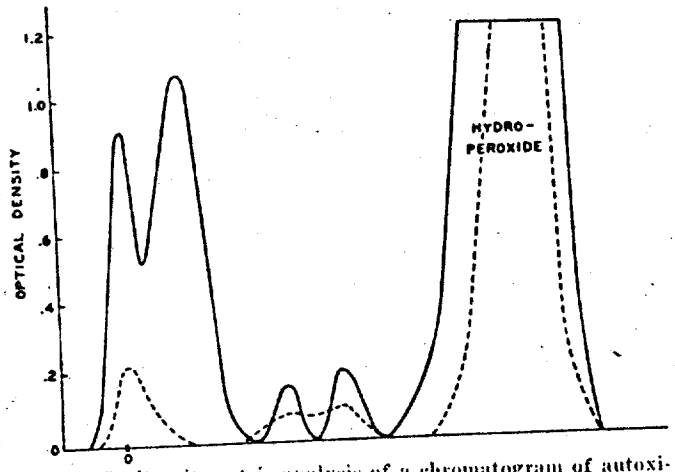


FIG. 15. Densitometric analysis of a chromatogram of autoxidized methyl linoleate (151). Solvent: Petroleum hydrocarbon-diethyl ether, 4/1, v/v. Development time: 40 min. Indicator: Charring with aqueous sulfuric acid 1/1, v/v. Unchanged methyl linoleate migrates to the solvent front.

Figure 16 shows a chromatogram of various phospholipids, as presented by Wagner (219) and Wagner, Hörhammer, and Wolff (220).

Privett and Blank (152) have extended their ozonization-reduction-thin-layer chromatographic method by applying it to lecithins. The four types of lecithins can be distinguished and quantitatively determined. In this case the aldehydic cores derived by reductive ozonolysis are separated by reversed-phase partition TLC on siliconized Silica Gel G plates (101).

Figure 17 shows photodensitometer curves obtained from chromatograms of aldehydic cores derived from various lecithin preparations as obtained by Privett and Blank (152).

TLC has become an important tool in research concerned with the structure of gangliosides (87, 89, 219, 220). With this method Kuhn, Wiegandt, and Egge (89) isolated four crystalline gangliosides from beef brain, each containing one mole of galactosamine and three moles of glucose plus galactose.

Steroids and Bile Acids, Triterpenoic Acids

Linford (97) reported that sterols are chemically altered when they are chromatographed on columns of dry silicic acid, and he recommended that sterols should be separated by partition chromatography on silicic acid containing between 35 and 40% of water. Other workers (e.g., 15, 204) had previously observed that sterols are altered when chromatographed on alumina. Reference is made to a comprehensive discussion of the problem by Neher (138).

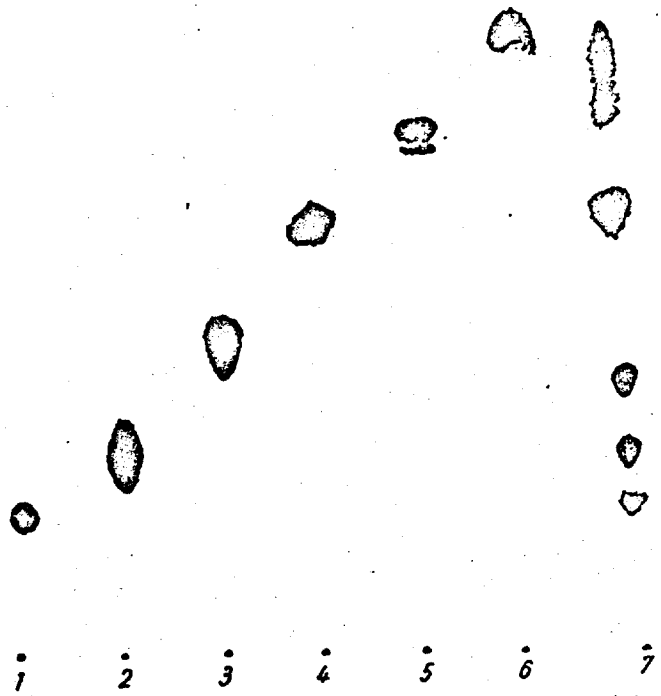


FIG. 16. Separation of phospholipids on Silica Gel. (219, 220). Solvent: chloroform-methanol-water, 65/25/4, v/v/v. Development time: 2 hours. Indicators: Rhodamin B and Dragendorff reagent. Amounts: 50-100 γ, each. 1) lysolecithin, 2) sphingomyelin, 3) lecithin, 4) cephalin, 5) cerebroside, 6) cardiolipid, 7) mixture.

Several recent publications described the use of TLC for resolving steroids by adsorption or partition chromatography on silicic acid; alumina was used as an adsorbent also (1, 7, 25, 31, 32, 69, 100, 139, 201, 202, 221).

Especially remarkable is the resolution of fifteen different cholesteryl esters by two-dimensional reversed-phase partition TLC on paraffin-impregnated Silica Gel G plates, as reported by Kaufmann, Makus, and Deicke (80).

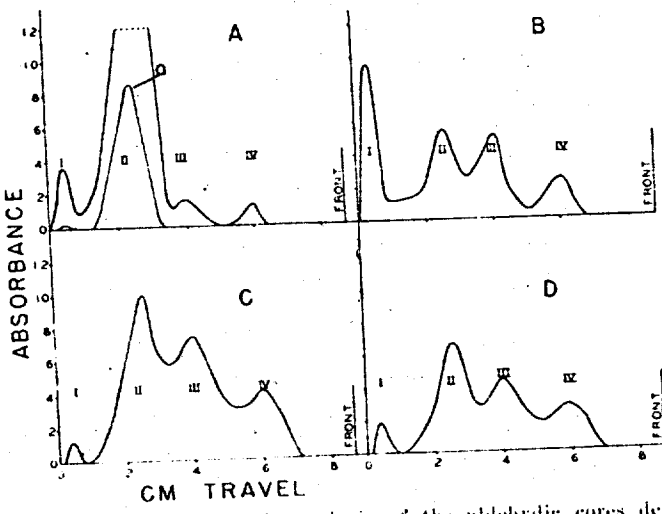


FIG. 17. Densitometric analysis of the aldehydic cores derived from A) egg lecithins, B) beef spinal cord lecithins C) soybean lecithins, D) wheat lecithins (152). I. Disaturated lecithins, II. aldehydic cores derived from α-saturated-β-unsaturated lecithins, III. aldehydic cores derived from α-unsaturated β-saturated lecithins, IV. aldehydic cores derived from diunsaturated lecithins. Solvent: acetic acid-water, 85/15, v/v on siliconized Silica Gel G.

Waldi and Munter (221) developed an early pregnancy test which is based upon a semiquantitative determination of pregnandiol in the urine.

Gänshirt, Koss, and Morianz (48) worked out a simple and rapid method for the quantitative determination of bile acids by adsorption TLC. The bile acids were eluted from the plates with aqueous sulfuric acid (65%) and they were quantitatively determined by colorimetry of the red solutions that formed upon heating. The above authors encountered a standard error of only ± 3% in analyses of mixtures of cholic, deoxycholic, and glycodeoxycholic acids, and of glycocholic, taurocholic, and taurodeoxycholic acids (48). TLC of bile acids was also reported by Hofmann (60).

TLC has been extensively applied in work with triterpenoic acids by Tschesche and his co-workers (207, 208, 209, 210). Vioque and Morris (214) reported the isolation of two triterpenoic acids from orujo oil. Stahl (186) demonstrated the use of TLC for the fractionation of tars and naturally occurring balsams and rosins.

Fat-Soluble Vitamins

Winterstein and Hegedüs (227, 228) and Winterstein, Studer, and Rüegg (229) have applied both adsorption and reversed-phase partition TLC to the analysis of vitamin A and the corresponding aldehyde, *viz.*, retinene and other polyenals C_{20}–C_{40}. Winterstein and Hegedüs (227, 228) prepared deeply colored rhodanine derivatives of these aldehydes on the plates, and with this very sensitive indicator technique they were able to detect as little as 0.03 γ of an aldehyde on a plate. The ubiquitary occurrence of retinene in a plant kingdom and its presence in liver was demonstrated. A thorough review on this work has already appeared (230).

Mangold and Malins (108) demonstrated vitamin A esters in various fish oils and free vitamin A in the nonsaponifiable fractions therefrom. Both vitamin A and its ester can readily be recognized by the blue color they yield by heating with sulfuric acid.

Janecke and Maas-Goebels (69) investigated the behavior of vitamins D_2 and D_3 under the conditions usually applied for their assay and they concluded that the D-vitamins are less stable than had generally been believed.

Methods for the quantitative analysis of the E-vitamins were published by Seher (171, 172, 173). The β and γ tocopherols could not be resolved by TLC, but both were well separated from α and from γ tocopherols.

Stoffel and Martius (196) and Billeter and Martius (13) have used TLC in studying the metabolism of K-vitamins. Martius (114) has summarized the results of these investigations in a recent review.

Food Coloring Agents

TLC was applied for analyzing vitamin A, carotenes, and other food coloring agents by Lagoni and Wortmann (91, 92) and also by Mottier (131, 132, 134). Isler, Rüegg, and Schudel (66) have presented a most interesting survey on the use of synthetic carotenoids as food-coloring agents, and in this review they also discussed in detail applications of TLC and other methods in analyzing such substances.

Stanley and Vannier (192) used chromatostrips for detecting biphenyl.

Antioxidants, Prooxidants

Methods for the analysis of synthetic and natural antioxidants by TLC have been described by Seher (171, 172, 173). Complex mixtures of antioxidants in commercial preparations and in oils have been analyzed by two-dimensional TLC (17), mixtures of tocopherols have been quantitatively determined (172, 173). Naturally occurring quinones, which are of significance in this connection, may also be detected by TLC (6). Procedures for quantitative determination of p-hydroxy benzoates, widely used preservatives, have been described by Gänshirt and Morianz (47).

Organic peroxides have been separated by Maruyama, Onoe, and Goto (115) and by Stahl (183). Reference is made again to the work of Privett (151) on the use of TLC for the analysis of autoxidation products (Fig. 15), and to the publications by Seiler and Seiler (174, 175), Seiler and Rothweiler (176) and Seiler and Kaffenberger (177) on the separation by TLC of inorganic cations and anions, many of which are prooxidants.

Minor Constituents of Fats

Because of its sensitivity, TLC is ideal for detecting minor lipid constituents, such as naturally occurring steroids, diterpenoids, triterpenoids, including saponins, aromatic rosins, and fat-soluble vitamins, or additives such as food coloring agents, antioxidants, and insecticides (8, 187).

Methods for analyzing non-lipid material in fats are scarce. The author believes that TLC can be effective in identifying carbohydrates (191, 235), amino alcohols and amino acids (16, 133), as well as short-chain alcohols and aldehydes (37, 143), acids, amines (203), mercaptans and other odoriferous compounds, plasticizers (144), and trace amounts of metals (174, 175, 176).

Thin-Layer Chromatography in Lipid Radio-Chemistry

Methods for analysis of trace amounts of lipids by the use of the isotopic derivative technique in conjunction with TLC and PC have been described by the author (107). The lipid substances were labeled by reacting their functional groups with C^{14}-labeled diazomethane—H^3-labeled diazomethane cannot be used (10, 96)—or with C^{14}- or H^3-labeled acetic anhydride. "Hot" methyl esters, acetylated long-chain alcohols, monoglycerides, diglycerides, glyceryl ethers, amines, and hydroxy ethyl amides of higher fatty acids were fractionated from each other as classes by adsorption-TLC. Their ratios were determined by radiometry. Consecutively, each of the classes of labeled lipid derivatives was further resolved by reversed-phase paper partition chromatography. The ratios of the components of each class were then determined by scanning their respective activities with a radio chromatograph or by evaluating autoradiograms in a photodensitometer. Mangold, Kammerer and Malins (112) have recently presented an analysis of the fatty acids of castor oil, using these methods.

The same authors have analyzed commercial preparations of radioactively labeled lipids and found that most of them are very impure (112, see also, e.g., 9). Because their indiscriminate use must lead to erroneous results, TLC should be used for purifying such "hot" lipids prior to their application in chemical and metabolic studies (112). Figure 18 shows

autoradiograph of various commercial fatty acids labeled in the carboxyl group with C^{14}, and Figure 19 represents an autoradiograph of a chromatogram of cholesterol-4-C^{14}. The radioactive sterol preparation was applied in a row across the plate to permit recovering the pure cholesterol. The isolation of C^{14}-labeled phytol from the nonsaponifiable fraction of the alga *Chlorella pyrenoidosa*, grown in an atmosphere of radioactive carbon dioxide (104), was also reported (112).

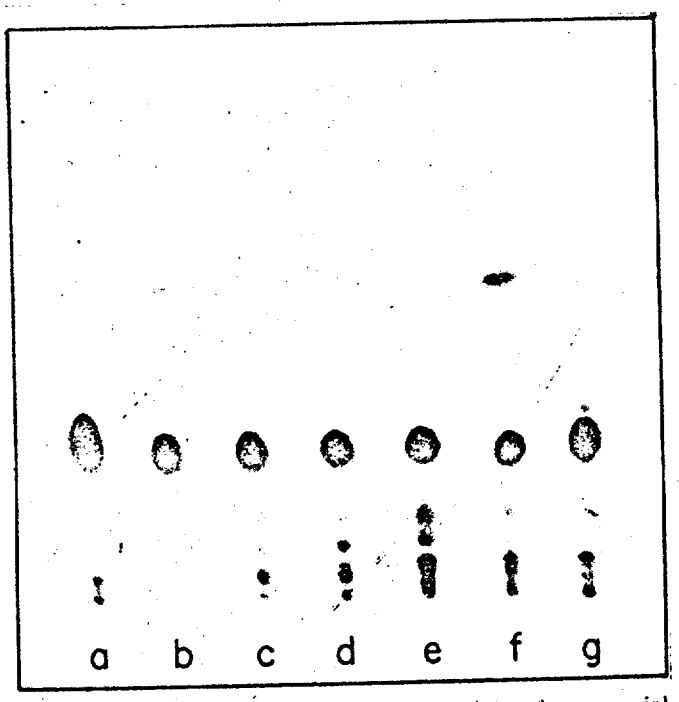


FIG. 18. Autoradiograph of a chromatoplate of commercial fatty acids -1-C^{14}. Solvent: petroleum hydrocarbon-diethyl ether-acetic acid, 90/10/1, v/v/v. Development time: 40 min. a) lauric acid, b) myristic acid, c) palmitic acid, d) stearic acid, e) oleic acid, f) linoleic acid, g, linolenic acid.

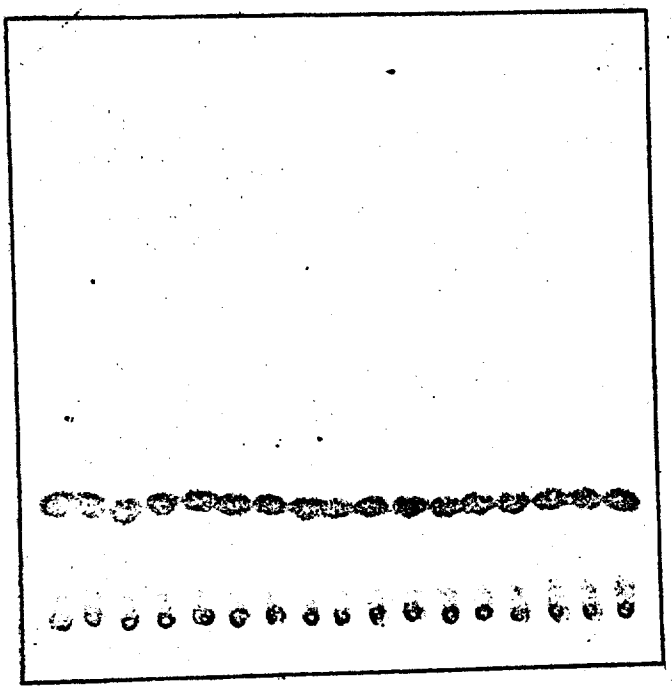


FIG. 19. Autoradiograph of a chromatoplate of cholesterol-4-C^{14}. Solvent: petroleum hydrocarbon-diethyl ether-acetic acid, 70/30/2, v/v/v. Development time: 40 min.

Summary and Discussion

The technique of thin-layer chromatography and its numerous uses in the lipid field have been summarized. The most significant advantages of TLC over existing methods of lipid analysis are, in the reviewer's opinion, the following:

Simplicity: The equipment required can be handled by inexperienced workers.

Speed: Complex lipid mixtures can be separated in 30 min. Less complicated samples can often be resolved in 5 min. All other chromatographic methods require hours or days to achieve similar separations.

Efficiency: Thin-layer chromatography yields separations which are much sharper than the fractionations obtained by column or paper chromatography.

Sensitivity: In many instances, the sensitivity of the detection methods used in paper chromatography are increased one hundred fold when applied on thin-layer chromatograms, because in the latter the individual spots are more discrete and not as diffuse as on paper chromatograms.

Another advantage of thin-layer chromatography is that several indicators may be applied consecutively, on one plate, also corrosive spray reagents, such as chromic-sulfuric acid may be used to char all organic material on the chromatoplate. In other chromatographic techniques frequently the indicator employed for the detection of spots is blind in regard to one component.

Capacity: Up to 50 mg. per plate can be separated and isolated by thin-layer chromatography, whereas only microgram amounts are available, in a reasonable time, through paper chromatography.

Thin-layer chromatography is fully exploited only when it is used in conjunction with other methods. Especially rewarding is the combined use of thin-layer chromatography with the following methods:

- Gas-Liquid Chromatography
- Paper Chromatography
- Column Chromatography
- Radioactive Tracers
- Derivatization, i.e., the application of chemical reactions prior to chromatography.

Although thin-layer chromatography has been widely applied to lipid analysis and preparation, its usefulness is certainly not yet exhausted.

Refinements of TLC are being designed and new techniques are being developed. Honegger (63) has described ionophoresis on chromatoplates which he combined with TLC in two-dimensional technique for the resolution of amines and amino acids. He found this combination of methods in some respects superior to the separation of amines and amino acids on paper.

Only relatively small amounts of material can be separated on a plate by reversed-phase partition TLC. It may be useful to arrange several impregnated chromatoplates in a block, in a manner similar to that for large-scale paper chromatography recently described by Hall (53).

The isotopic derivative technique involves fractionation of the labeled derivatives on a microscale. TLC is especially applicable to the resolution of such complex lipid mixtures. TLC should be applied in combination with existing methods of labeling sterols (5, 11, 61) and other hydroxy and amino compounds (107, 112) with acetic anhydride-1-C^{14} (or -2-H^3), or

with 3-chloro-anisoyl chloride-3-Cl^{36} (179), or with 4-iodobenzoylchloride-I^{131} (197). Several publications describe procedures for labeling acids and their analysis as methyl-C^{14} esters after esterification with radioactive diazomethane-C^{14} (10, 105, 112, 167, 178, 198), or 4-chloroaniline-Cl^{36} (180). Unsaturated fatty acids were labeled by addition of radio-iodine I^{131} (22, 76, 77). The speed of TLC will be appreciated in work with short-lived isotopes, especially if apparatus can be constructed which will perform automatic advancement of the chromatoplate and graphic or numerical recording of the radioactivity, as in the quantitative evaluation of paper strips and two-dimensional paper chromatograms (148).

Autoradiography of paper chromatograms (43) has in recent years become a prominent method in studies utilizing radioactive tracers (160). Because of its high capacity, adsorption-TLC will certainly also find wide application in isotope studies.

Strickland and Benson (e.g., 200) have been very successful in using neutron activation of phospholipids and sulfolipids on paper chromatograms. It would be desirable to develop techniques for neutron activation TLC analysis. As both calcium and sulfur atoms are activated, calcinated calcium sulfate cannot be applied, and starch will have to be used as a binder. For similar reasons, plastic plates will have to be used instead of glass.

Lipids are well protected against autoxidation if they are chromatographed on silicone-impregnated paper (165). Mahadevan (99) has investigated to what extent unsaturated lipids autoxidize during adsorption TLC, and even with the most sensitive methods he could not find any sign of alteration of unsaturated lipids during chromatography. He demonstrated, though, that TLC is well suited for rapidly purifying oxidized lipids.

This review is concluded with a hint as to the potentials of TLC as a teaching method. The chromatoplate shown in Figure 10 was prepared by a high school student on the first day of his adventures with TLC. It is obvious that this method will find wide application in the training of students.

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TLC of derivatives of short-chain alcohols, aldehydes and ketones, acids, amines and mercaptans.

Thin-layer chromatograms of radioactively labeled lipids are recorded by taking autoradiographs on x-ray film, as described below. Still, it is to be emphasized that chemical indicatars should always be used also. Application of both photographic and chemical detection methods yields information regarding both radiochemical and chemical purity of a substance.

Documentations of Chromatograms

Because thin-layer chromatograms are relatively small, they can readily be copied by tracing them with a pencil directly into a laboratory journal.

Thin-layer chromatograms can be preserved if a stable indicator was used for visualizing the individual spots. This may be done by covering the chromatoplates with a glass plate of the same dimensions; the two plates protecting the adsorbent layer are sealed along the edges with tape. Barrollier (9) recommended dipping chromatoplates into a dilute solution of label glaze. The adsorbent layer is firmly held together by the label glaze and can be pulled off the glass as a film. The reviewer has found Fisher Label Glaze[23] suitable for Barrollier's procedure. This technique requires, of course, that an indicator be used which is insoluble in the solvent used for diluting the label glaze. Meinhard and Hall (120) demonstrated that the adsorbent layer can be stripped off on adhesive tape and pasted into a laboratory journal.

Black and white or color photography of chromatoplates is easily done in daylight or in U.V. light. Bürki and Bolliger (23) recommend Sylvania F8T5/BLB, Blacklite Blue tubes[24] and a yellow filter for taking black and white photographs in U.V. light. A photograph of a thin-layer chromatogram taken in daylight is shown in Figure 10.

Chromatoplates may be copied by exposing them to sheet film or photographic paper in an enlarger. Seher (e.g. 171) and Morris, Holman, and Fontell (e.g. 126) prepared photostats[25] of chromatoplates (Fig. 11). This technique has been applied also for quantitative analysis (74, 172, 173). It is simple and rapid, but it has the disadvantage that small or weakly stained bigger spots are easily lost. Hefendehl (55) recommended spraying the chromatoplates with a solution of paraffin in ether (1/1, w/v) to render them transparent. These plates yield better photostats than do untreated ones.

Chromatoplates containing radioactively labeled substances yield fine x-ray prints (107, 112). Autoradiographs are obtained by exposing the plates in the dark to No-Screen Medical X-Ray Safety Film[26] for a few hours to one week, depending upon the type of radiation and the activities of the resolved compounds. The films are developed with Supermix Developer[26] for 4 to 6 min., and they are fixed for 30 min. with Acid Fixer.[26] Autoradiographs of commercial preparations of C^{14}-labeled lipids are presented in Figures 18 and 19.

Elution and Recovery of Lipids

One of the striking features of TLC is the high capacity of the plates. It is possible to separate by adsorption TLC up to 50 mg. of a lipid mixture on a single chromatoplate, 20 x 20 cm., coated with a thin layer of Silica Gel G of 250–275 μ thickness. About 5 mg. of lipid may be separated on such a plate by partition and reversed-phase partition TLC. Lipids, after having been isolated by TLC, may be scraped off the plates and extracted from the carrier material. The appropriate eluans for lipids may be determined by chromatographing these substances with different solvents on plates. Solvents that carry a compound with the solvent front are suitable as eluans for that substance (101). Care must be taken, however, that the eluans does not dissolve large amounts of the calcium sulfate, and thus aqueous solvents cannot be used.

A novel technique of recovering substances from a chromatoplate is to sublime the material from the adsorbent layer onto a cooled glass plate held 1 mm. above it (39).

TLC has been used to analyze commercial preparations of lipids labeled with C^{14}, H^3, or I^{131}, and for the purification and isolation of milligram amounts of compounds (112). Winterstein, Studer, and Rüegg (229) isolated unusual carotenoids by TLC, stating that no other method allowed the preparation of some of these materials (230).

With polar lipids, the yields of recovered material are usually very poor. This may be due to the presence of calcinated calcium sulfate in the solid adsorbent layer; and if so, better yields may be expected with loose layers of plain adsorbents. The use of sugar (75) as an adsorbent may be helpful. Even though it has a rather low capacity, its solubility in water may facilitate recovery of lipids. Thicker layers of adsorbents may be applied to preparative work. However, the thickness of the coating should not exceed 1 mm. to avoid distortion by uneven migration rates on the surface and inside the adsorbent layer.

The yields are improved by using the less polar derivatives of lipids containing free functional groups. Thus, alcohols and acids are more easily recovered as acetates and methyl esters, respectively (112). The nonpolar derivatives are especially important in TLC with isotopically labeled lipid derivatives *(vide infra)* where quantitative recovery is crucial.

Recovering various substances from a chromatostrip (194) or from a chromatoplate (17) is also possible by letting the developing solvent run over.

Esters of unusual fatty acids are often found by GLC in mixtures of methyl esters derived from natural lipids. Curves from such analyses can be correctly interpreted only if the sample subjected to GLC was free of compounds that are not methyl esters of fatty acids. The absence of such contaminants is not routinely checked. It appears to be necessary to analyze each sample of methylation products by TLC prior to GLC for substances other than methyl esters. Pure methyl esters may be isolated by TLC (241).

Quantitative Thin-Layer Chromatography

The methods that have been suggested for the quantitative evaluation of thin-layer chromatograms are summarized in Table V.

Few of the above methods are exact and the better ones are particularly impractical. Four of these methods involve elution of the various lipids from the plate. A suitable eluans for a given lipid is found by

[23] Fisher Scientific Company, 633 Greenwich Street, New York 14, New York.
[24] Sylvania Electric Products Inc., 500 Fifth Avenue, New York, New York.
[25] Copease Corporation, 425 Park Avenue, New York, New York.
[26] General Electric X-Ray Department, Milwaukee 1, Wisconsin.

TABLE V

Methods of Quantitative Thin-Layer Chromatography

Method	Compounds analyzed	Range of sample size (per spot)	Error %	Ref.
Eluting, weighing	Neutral lipids from feces and fecaliths	20–50 mg. (per plate)	semi-quant-	(225)
Eluting and U.V. spectrophotometry	Esters of p-hydroxy benzoic acid	20–70 γ	2–3	(47)
Eluting, colorimetry of hydroxamic acid-Fe complexes	Methyl esters of fatty acids and of oxygenated acids, mono-, di-, and triglycerides	0.1–1 mg.	± 5	(216)
Eluting with aqueous sulfuric acid, colorimetry after heating	Bile acids	10–60 γ	± 3	(48)
Eluting and radiometry	Labeled derivatives of acids, hydroxy and amino compounds	0.1–100 γ	± 5	(107, 112)
Charring followed by photodensitometry	Mono-, di-, and triglycerides	2–30 γ	± 5	(150)
	Glycerides and "aldehydic cores" derived from unsaturated glycerides	2–30 γ	± 5	(149)
	Lecithins and "aldehydic cores" derived from unsaturated lecithins	2–30 γ	± 5	(152)
Measuring of spot area	Tocopherols	30–100 γ	± 5	(172,173)
	Industrial lipids	30–130 γ	± 5	(74)
Mineralization, colorimetry of phosphate	Phospholipids	0.2–25 γ	± 3	(52)

chromatographing it in various polar solvents as described in the preceding paragraphs.

Semiquantitative analysis by weighing eluted material is possible only with nonpolar lipids that can easily be recovered from the adsorbents. The same limitations apply to the hydroxamic acid method; however, the colorimetric determination is much more exact than weighing of small amounts. In view of its general applicability to a wide range of compounds having various functional groups (28) the method will, in the reviewer's opinion, become a preferred procedure for the quantitative evaluation of thin-layer chromatograms. So far, the hydroxamic acid method has been applied only to the quantitative analysis of esters of common and oxygenated fatty acids derived from seed oils. Extension of the method to the analysis of mono-, di-, and triglycerides has been suggested (216). The method described for the elution and quantitative colorimetry of bile acids with sulfuric acid may be of general applicability. The isotopic derivative technique is, in the author's experience, very precise, one reason being that the lipids are separated as derivatives which are more easily quantitatively recovered than are the more polar original compounds.

Direct quantitative determination of lipids on the plates would be desirable, especially if a method could be found that would not involve destruction of the compounds measured. Most methods suggested so far require the use of destructive reagents for visualizing the lipids on the plate, or derivatization before chromatography. Photodensitometry after charring of the lipid materials as applied by Privett and Blank (149), Privett, Blank, and Lundberg (150) gives good results in a range of 5–35 γ of lipid This method has been most extensively applied by the last-mentioned authors, and has yielded valuable information. The spots are measured in a Photovolt photodensitometer Model 520A, 52-C,[27] with a stage attached for semi-automatic plotting of curves. The areas under the densitometer curves are directly proportional to the amount of sample in a range of about 5–30 μg. of carbon (Fig. 14). This applies to saturated compounds. Different classes of saturated lipids give different curves. Also, unsaturated lipids are more deeply stained than are the saturated members of the same class. Therefore, individual standard curves must be prepared for all compounds that do not give spots of the same densitometrically determined response (Fig. 14) (149). Mixtures of known composition should be run alongside the sample to be analyzed. The densitometer mentioned is easily adapted to fit square plates. Complex mixtures of natural lipids containing both saturated and unsaturated fatty acid moieties can be measured only if the whole mixture is measured after catalytic hydrogenation. Unfortunately, hydrogenation often involves not only the desired saturation of the double bonds, but changes the functional groups, and thus also the pattern of the mixture to be analyzed.

The method of measuring spot areas on photostats of chromatoplates supplements the photodensitometric method, as it can be applied to amounts of 30–130 γ of lipid per spot. In the author's laboratory this method has been found useful for the analysis of synthetic lipid mixtures with an error of ± 5% (174).

Preparation of Radioactively Labeled Lipid Derivatives

The isotopic derivative technique, i.e., the method for detecting and analyzing components of mixtures after reaction of their functional groups with a radioactively labeled reagent, has so far found little application in the lipid field, due to lack of efficient fractionation procedures. TLC allows the rapid separation of complex lipid mixtures and thus constitutes an ideal auxiliary tool for the analysis of lipids by the isotopic derivative technique.

Fatty acids may be labeled by esterifying them with radioactive diazomethane, whereas lipids containing hydroxy or amino groups are labeled by acetylation with radioactive acetic anhydride. p-Tolylsulfonyl methyl-C^{14}-nitrosamide (198), a precursor for the preparation of "hot" diazomethane, and acetic anhydride-1-C^{14} and acetic anhydride-H^3 are all commercially available. The latter two reagents are available in license-exempt packages.

Radioactive diazomethane, $C^{14}H_2N_2$, is prepared by reacting a solution of 10 mg. (0.05 millimoles) of p-tolylsulfonyl-methyl-C^{14}-nitrosamide, specific activity of 0.6 millicuries per millimole[28] in 1 ml. diethyl ether with 2 ml. of an ice-cold solution of 0.1 g. of NaOH in ethanol-water, 10/1, v/v, in a micro gas generator. Diazomethane and ether are distilled in a stream of nitrogen by heating the reaction vessel to 60–70°C. The diazomethane is collected in two test tubes in series, each containing 1–2 ml. of ether which is maintained at 0–5°C. The diazomethane solutions are combined at the end of the distillation and aliquots are added to solutions of 2–20 mg. fatty acids (0.01–0.1 millimoles) in diethyl ether methanol 9/1, v/v (166, 198). Excess diazomethane is avoided in order to conserve this expensive reagent and also to prevent the formation of polymethylenes that may interfere

[27] Photovolt Corporation, 95 Madison Avenue, New York 16, New York.

[28] New England Nuclear Corporation, 575 Albany Street, Boston 18, Massachusetts.

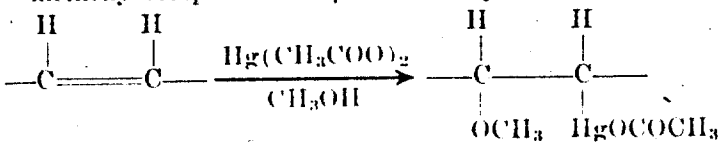
in subsequent analyses. The diazomethane can also be distilled directly into a solution of the fatty acids to be esterified in diethyl ether-methanol, 9:1, v/v (166).

Lipids (10–20 mg.) containing hydroxy or amino groups may be labeled by reaction with a 1/10 solution of acetic-1-C^{14} anhydride, specific activity of 0.6 millicuries per millimole[29] in pyridine. The reaction is conducted in a 5 x 150 mm. sealed tube at 100°C. for 30–60 min., using about 20% excess of reagent. After cooling, the tubes are opened, the reaction mixture is diluted with 10 ml. *N*-sulfuric acid, the acetylated lipids are extracted with diethyl ether, washed with water, and dried over sodium sulfate. Monoglycerides and diglycerides undergo acetolysis to a small extent; and therefore, if quantitation of lipids containing ester-bonds is desired, this method must be applied with caution (107).

Reaction with radioactive ketene (2) may yield cleaner acetylation products.

Preparation of Mercuric Acetate Addition Compounds

Unsaturated lipids react with mercuric acetate in methanol, at room temperature, to give acetoxymercuri-methoxy compounds in quantitative yields:



cis-Compounds react ten times faster than their *trans* isomers (70). The original lipid may be regenerated by cleaving the addition compound with hydrochloric acid.

Chromatography of acetoxymercuri-methoxy compounds of unsaturated lipids on silicic acid effects their fractionation according to degree of unsaturation.

The acetoxymercuri-methoxy compounds of lipids are best obtained by the method published by Jantzen and Andreas (70) for the preparation of adducts of methyl esters of unsaturated fatty acids. This procedure is described here:

The reagent is a solution of 14 g. mercuric acetate in 250 ml. methanol, 2.5 ml. water, and 1 ml. glacial acetic acid. About 25 to 50 ml. of this solution is added to 1 g. of esters and allowed to react in a stoppered flask in the dark at room temperature. After 24 hr., the methanol is evaporated at <30°C. *in vacuo* or by a stream of nitrogen, and the dry residue is taken up with 50 ml. chloroform. This chloroform solution is washed with five 25-ml. portions of water to remove excess mercuric acetate, and then dried with sodium sulfate. Saturated lipids do not react with mercuric acetate, unless they contain a reactive functional group.

The unsaturated lipids are recovered after chromatography by shaking the silicic acid containing the adducts in a test tube with 10 ml. methanol and 0.5 ml. conc. HCl. After most of the adsorbent has settled, the supernatant is decanted and filtered. The residual silicic acid is treated once more with methanol-hydrochloric acid. The combined filtrates of the two extractions are diluted with 25 ml. water and extracted consecutively, once with 25 ml., and four times with 10 ml. of diethyl ether. The combined ether extracts are washed with water, dried over sodium sulfate, and evaporated.

[29] Radioactive Acetic Anhydride, either labeled with C^{14} or H^3, is available from several companies. The author has used preparations of the Picker X-Ray Corporation, 25 South Broadway, White Plains, New York.

Applications

In the lipid field, the following types of separations have been performed by TLC:

Fractionations according to classes of compounds (Fig. 6). Long-chain hydrocarbons, alcohols, aldehydes, acids, monoglycerides, diglycerides, triglycerides, and other lipids are separated as classes according to the type and number of functional groups, irrespective of chain lengths and degree of unsaturation, by adsorption TLC on silicic acid. Subfractionations within classes are perceptible but generally do not interfere with class separations.

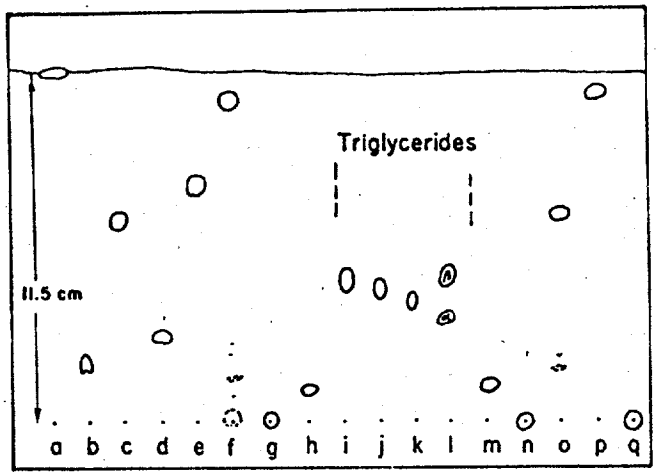


FIG. 6. Thin-layer adsorption chromatography of lipid classes on Silica Gel (101). Solvent: Petroleum hydrocarbon-diethyl ether-acetic acid, 90/10/1, v/v/v. Development time: 40 min. Indicator: Dichlorofluorescein. Amounts: 20 γ, each. a) octadecene-9, b) oleylalcohol, c) oleylaldehyde, d) oleic acid, e) methyl oleate, f) cholesteryl oleate, g) monoolein, h) diolein, i) triolein, j) trilinolein, k) trilinolenin, l) tricaproin (α) and tristearin (β), m) cholesterol, n) selachyl alcohol, o) selachyl diolein, p) oleyl oleate, q) dioleoyl lecithin.

Fractionations within homologous series (Fig. 7 and 8). a) Compounds of low chain lengths (C_1–C_6) are separated within classes by TLC of their derivatives. Alcohols, for example, are separated as 3,5-dinitrobenzoates on silicic acid. With the same adsorbent, aldehydes and also ketones are fractionated as 2,4-dinitrophenyl hydrazones, and acids are separated as anilides.

b) Long-chain compounds (C_{12}–C_{20} lipids) are fractionated within classes by reversed-phase partition TLC on, e.g., siliconized silicic acid. Higher fatty acids, for example, are separated according to chain length and unsaturation.

Overlapping critical partners, such as palmitic and oleic acids, are well separated by chromatography at low temperatures, by chromatography with oxidizing solvents, or by the following technique:

Fractionation according to degree of unsaturation (Fig. 9). Saturated and various unsaturated members of a homologous-vinylogous series are separated according to their degree of unsaturation by TLC of the mercuric acetate addition compounds formed with unsaturated lipids. As an example, mixtures of methyl esters of naturally occurring fatty acids are rapidly and quantitatively separated by TLC, in the

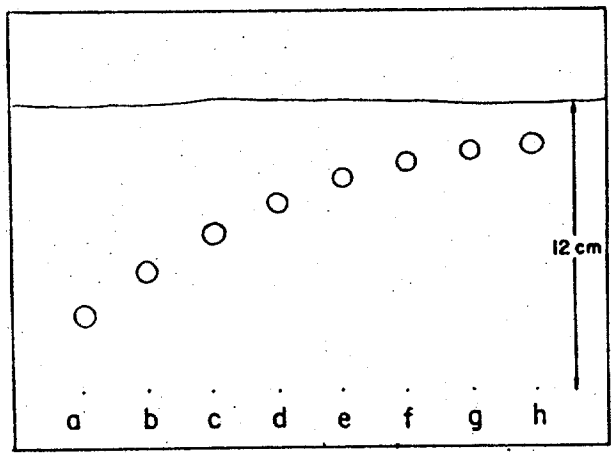


FIG. 7. Thin-layer adsorption chromatography of derivatives of short-chain compounds of a homologous series. Solvent: Petroleum hydrocarbon-diethyl ether, 70/30, v/v. Development time: 40 min. Indicator: 1% of "Fluorescent Chemical No. 601"[22] in the silica gel layer. Observed in U.V. light. Amounts: 5 γ, each. 2,4-dinitrophenyl derivatives of a) methyl amine, b) ethyl amine, c) propyl amine, d) butyl amine, e) amyl amine, f) hexyl amine, g) heptyl amine, h) octyl amine.

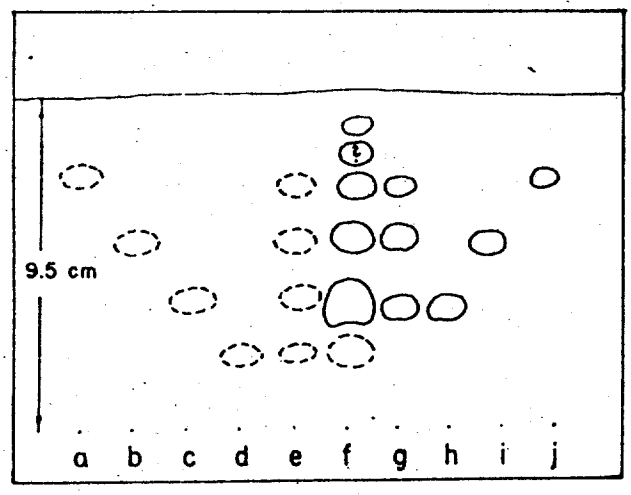


FIG. 8. Reversed-phase partition chromatography on a siliconized chromatoplate (101). Solvent: Acetonitrile acetic acid-water, 70/10/25, v/v/v. Development time: 40 min. Indicators: Iodine (solid lines), followed by α-cyclodextrin-iodine (dotted lines). Amounts: 20 γ of individual compounds. a) methyl laurate, b) methyl myristate, c) methyl palmitate, d) methyl stearate, e) mixture of saturated methyl esters, f) C_{18}-Fraction derived from menhaden oil, g) mixture of unsaturated C_{18} methyl esters, h) methyl oleate, i) methyl linoleate, j) methyl linolenate.

form of their acetoxymercuri-methoxy derivatives, into saturated esters, monoenoates, dienoates, and trienoates. The original esters can be recovered after separation of their addition compounds.

Fractionation of positional isomers and fractionation of cis-trans *isomers is also possible in certain cases (128, 147) (Fig. 11).*

The fractions obtained by one of these methods are amenable to further resolution by either or several of the other techniques, or by complementary methods. Combinations of adsorption-TLC with reversed-phase PC and GLC have proven to be especially useful. Lipids which cannot be resolved by adsorption chromatography (TLC) may well be separated by partition chromatography (PC, GLC).

Fats, Oils and Waxes

Procedures for the fractionation of neutral lipids—naturally occurring crude vegetable fats, marine oils, and insect waxes, and their hydrolysis products, such as fatty acids, and also alcohols, glyceryl ethers and other nonsaponifiables have been described. Adsorption-TLC effects sharp separations of chemically very similar classes of lipids, such as long-chain alkyl esters, steryl esters, and polyenol esters of fatty acids; even triglycerides and glyceryl ether diesters may be resolved.

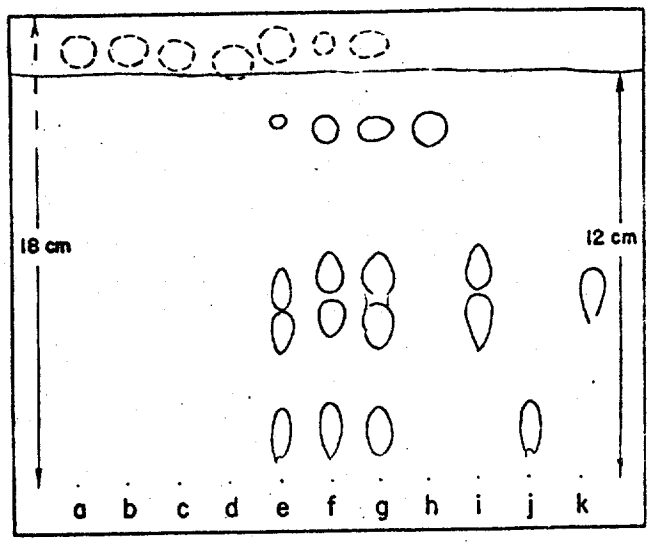


FIG. 9. Thin-layer chromatography of mercuric acetate adducts on Silica Gel (See 110). First solvent: Petroleum hydrocarbon-diethyl ether, 4/1, v/v. Second solvent: n-Propanol-acetic acid, 100/1, v/v. Development times: First Solvent, 1.5–2 hours, Second Solvent, 3–4 hours. Indicators: s-diphenyl carbazone (solid lines), followed by iodine (dotted lines). Amounts: 20 γ of esters, 50–100 γ of mercuric acetate adducts. a) methyl stearate, b) methyl oleate, c) methyl linoleate, d) methyl linolenate; e–j) Mercuric acetate adducts of e) C_{18} fraction from *Chlorella*, f) C_{18} fraction from *Chlorella*, g) total methyl esters from *Chlorella Pyrenoidosa*, h) methyl oleate, i) methyl linoleate, j) methyl linolenate, k) mercuric acetate.

Among the vegetable lipids analyzed are jojoba oil, castor oil, olive oil, oiticica oil (108, 112), and wheat (140). The following animal lipids have been investigated: lanolin; liver oils of soupfin shark, basking shark, and ratfish, also fur seal blubber oil (108) and dogfish liver oil (102) (Fig. 10).

The last-mentioned publication refers to work of Malins on the analysis of the constituent fatty acids of each of the triglyceride and the glyceryl ether diester fractions of dogfish liver oil. This commendable work would not have been possible by any other means than TLC and GLC.

Weicker (222), Huhnstock and Weicker (65), and van Dam (32) applied TLC to the analysis of human serum lipids. Williams, Sharma, Morris, and Holman (225) analyzed human feces and fecaliths by TLC and GLC. The same two techniques have also been applied, consecutively, to the analysis of human tissue lipid extracts, by Mangold and Tuna (109). The total lipids of human serum and of the following tissues were analyzed: bone marrow, liver, kidney, perinephric fat, spleen, and atheromatous plaques of the aorta. TLC was used to obtain lipid classes, the fatty acid

patterns of which were then determined by GLC of the methyl esters. Lipid classes not previously found in man were detected in several human tissues. Some of these lipids, such as methyl esters of fatty acids, are most likely artefacts produced during extraction. Others, like glyceryl ether diesters, must be regarded as genuine constituents of human tissues (211). Stein and Stein (195) used TLC in studying the selective *in vivo* labeling of epididymal fat of the rat with radioactively labeled fatty acids. Dhopeshwarkar and Mead (38), and Fulco and Mead (46) used TLC extensively in nutritional and metabolic investigations.

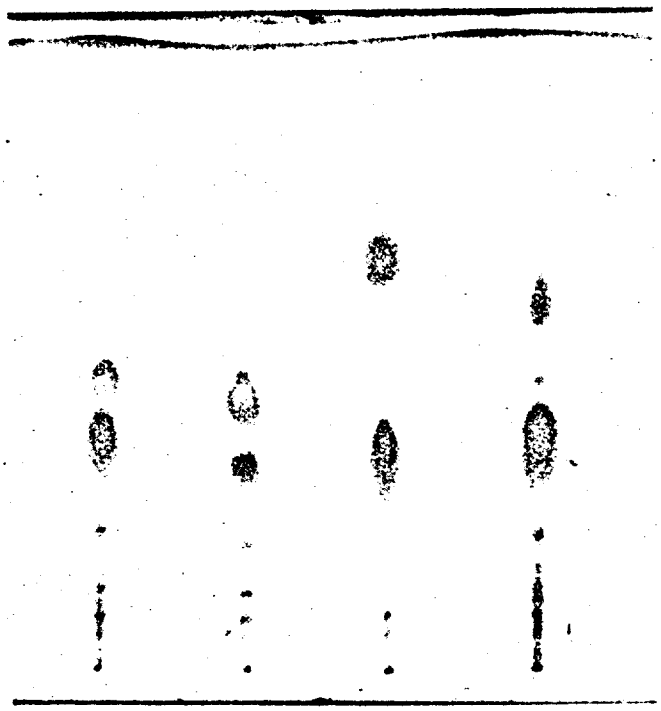


FIG. 10. Fractionation of shark liver oils on silica gel. Solvent: Petroleum hydrocarbon-diethyl ether-acetic acid, 90/10/1, v/v/v. Indicator: Iodine, reproduced by photographing. Amounts: 300 γ, each. Liver oils of a) Dogfish, b) Ratfish, c) Basking shark, d) Soupfin shark. The chromatoplate was developed in saturated atmosphere.

Naturally occurring oxygenated fatty acid were studied by Morris, Holman, and Fontell (126, 128), and by Morris, Hayes, and Holman (129). These workers found two isomeric acids, namely 9-hydroxy-*trans*-10-*cis*-12- and 13-hydroxy-*cis*-9-*trans*-11-octadecadienoic acids, and also at least three distinct epoxy acids in various seed oils (Fig. 11). Vioque, Morris, and Holman (215) found *trans*-9,10-epoxystearic acid in orujo oil. Chalvardjian, Morris, and Holman (27) applied TLC in nutritional studies with epoxy acids. TLC of oxygenated fatty acids was recently also reported by Kaufmann and Makus (79) and by Applewhite, Diamond, and Goldblatt (4).

A method, based on TLC, for the quantitative determination of mono-, di-, and triglycerides in mixtures has been reported by Privett, Blank, and Lundberg (150) (Fig. 12). These workers could quantitatively estimate as little as 0.1% of tripalmitin in monopalmitin.

Procedures for the quantitative analysis of α- and β-monoglycerides after splitting of the former with periodate, and for the analysis of 1,2- and 1,3-diglyce-

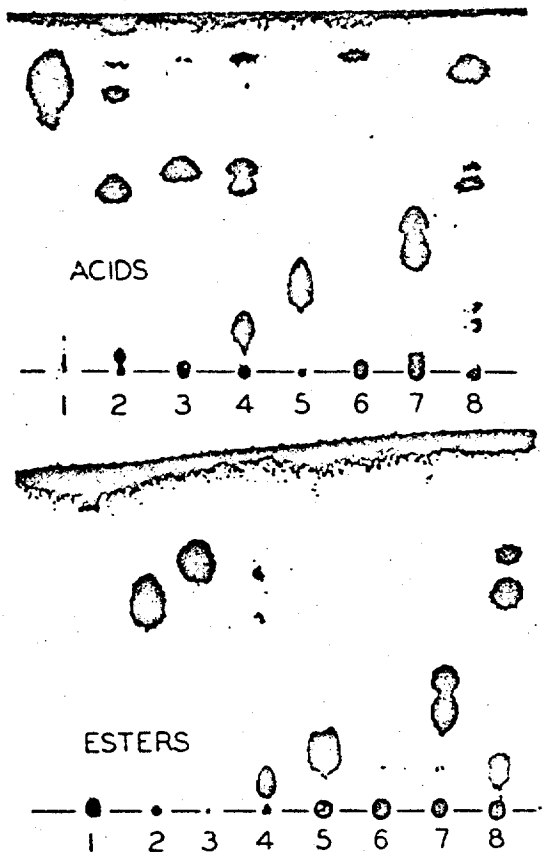


FIG. 11. Thin-layer chromatography of oxygenated fatty acid and their methyl esters (128). Solvent: Petroleum hydrocarbon diethyl ether, 9/1, v/v (for esters). Petroleum hydrocarbon diethyl ether-acetic acid, 90/10/1, v/v/v (for acids). Development time: 40 min. Indicator: Charring with aqueous sulfuric acid, 1/1, v/v. Reproduced by photocopying. Amounts: About 50 γ, each. 1) palmitoleic and oleic, 2) *cis*-9:10 epoxystearic, 3) *cis*-12:13-epoxyoleic, 4) *cis-cis*-9:10, 12:13-diepoxystearic plus two monoepoxy impurities, 5) 12-hydroxyoleic, 6) *threo*-12,13-dihydroxyoleic, 7, *threo*-12,13-chlorohydroxyoleic and *threo*-13,12-chlorohydroxyoleic, 8) *Artemisia absinthium* mixed acids and mixed esters.

erides were also presented. These new methods yielde exact results with synthetic mixtures and were the further applied to the analysis of monoglyceric preparations from safflower oil, menhaden oil, an lard. Periodate titration and molecular distillatio served for checking the accuracy of these analyses.

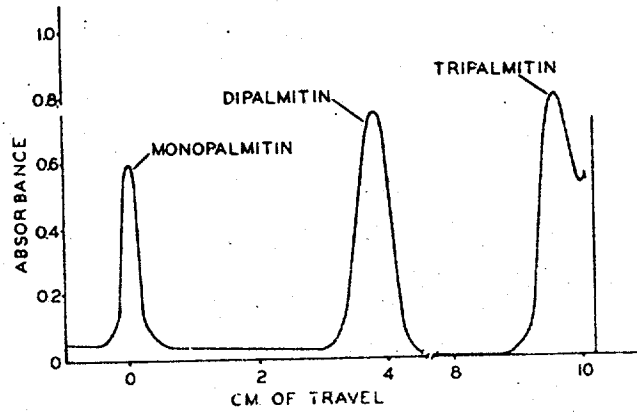


FIG. 12. Densitometric analysis of a mixture of mono-, and tripalmitin (150). Solvent: Petroleum hydrocarbon-diet ether, 7/3, v/v. Development time: 40 min. Indicator: Ch ring with aqueous sulfuric acid, 1/1, v/v.

glass plates which are coated with a thin layer of urea or another clathrate-forming material. Molecular sieves should be tried also.

The Sample [15]

Neutral lipids such as fats, oils, and waxes are applied to the plates with a micropipette or better, with a microsyringe, 10–50 λ,[16,17] as 0.1–1% solutions in petroleum hydrocarbon, b.p. 60–70°C. More polar solvents such as diethyl ether should be avoided as they effect chromatographic separations at the point of application which results in wider spots and poor resolution. Phospholipids, sulfolipids, glycolipids, and other very polar compounds are applied as chloroform or chloroform-methanol solutions, 1/1, v/v. Mercuric acetate adducts of unsaturated lipids *(vide infra)* are also dissolved in chloroform.

The samples must always be applied as single spots, not in a streak across the plate, as is frequently done in paper chromatography, because the developing solvent will not migrate across such a cut in the adsorbent layer. To avoid irregularities in migration of the solvent, the chromatoplates should always be developed against the direction of application of the layer. To achieve optimal resolutions the spots should not spread more than about 0.5 cm. in diameter. This is accomplished by applying the solutions in several small portions in a stream of nitrogen. Several firms [2,3,4] offer special transparent template with side rails that permit covering a chromatoplate without touching the adsorbent layer. Such a template facilitates spotting of samples; it may also be used to draw a line across the plate, 10–15 cm. from the starting points, to mark the level to which the solvent will be allowed to travel. The use of the template as designed by Stahl is schematically illustrated in Figure 5.

In adsorption chromatography, between 10 and 20 different samples, 50–500 γ of each, may be applied on one plate in a row of spots along one side of a plate, about 2 cm. from the edge. Several mg. of one complex lipid mixture may be fractionated on one plate by applying its solution along one side of a plate. Even up to 50 mg. of less complicated mixtures may be separated on one plate if the constituents are widely different in polarity, such as fatty acids and their methyl esters.

Only 1–10 mg. of lipids or lipid derivatives like mercuric acetate adducts of unsaturated lipids can be separated on one plate through partition chromatography by the thin-layer technique.

Selection of Solvents

Lipids may be separated by adsorption into classes of compounds, and classes may be resolved by reversed-phase partition chromatography. Fractionation is also possible according to degree of unsaturation. The choice of solvents depends on the type of separation desired. Solvent mixtures, rather than individual solvents, are usually applied, but the mixture should be kept as simple as possible for better reproducibility. Also, multicomponent systems tend to produce secondary solvent fronts, due to demixing during chromatography. A binary or ternary mixture of solvents can always replace a developing system consisting of five or six components.

Separating Lipids Into Classes. Brockmann and Volpers (19) found that a solvent elutes organic compounds from adsorbent columns according to the type and number of functional groups in these compounds. Hydrocarbons leave the column first, followed by ethers, ketones, alcohols, and acids, in this sequence. Within each class, saturated compounds are eluted before unsaturated ones. Polyunsaturated compounds with isolated double bonds are more easily eluted than are those containing a conjugated system of double bonds; *cis*-compounds are more easily eluted than their *trans*-isomers.

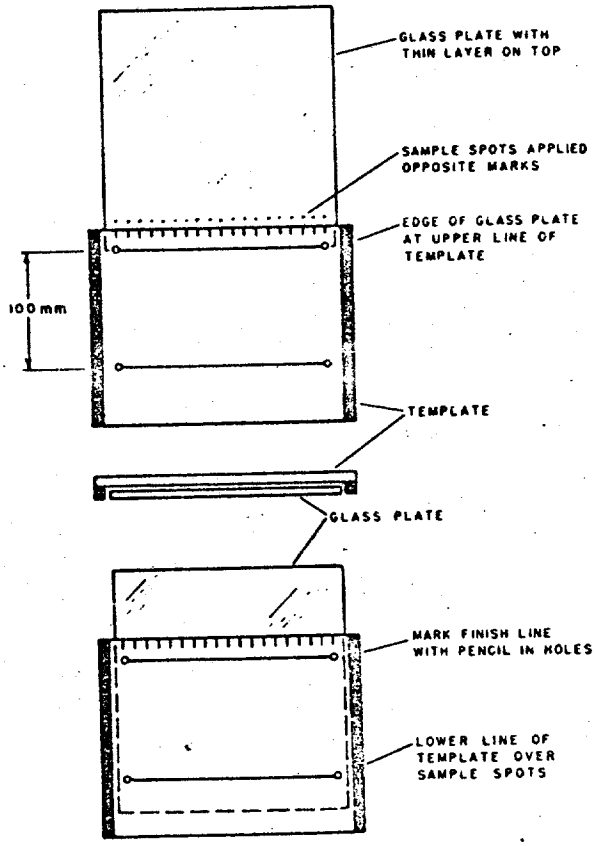


FIG. 5. Use of the template.[4]

These rules apply also in TLC: hydrocarbons migrate further on a chromatoplate than do ethers, ketones, or alcohols, and within each spot the saturated components of a class are concentrated in the front part of this spot. These subfractionations are quite pronounced with, e.g., the methyl esters of fatty acids derived from fish oils, where great differences exist in chain length and degree of unsaturation of the individual constituents. However, they never interfere with separation according to classes.

Trappe (204, 205, 206) established a series of solvents of increasing elution power. This Eluotropic Series of Solvents was extended, and other series were described, e.g., by Strain (199), and by Knight and Groennings (88). The tabulation of eluotropic series of solvents (Table 1) is very helpful in choosing suitable solvents for adsorption chromatography on columns or on thin layers. Izmailov and Shraiber (68) and other workers (145, 183) have described a spot

[15] Lipid standards for TLC may be obtained from The Hormel Foundation, Austin, Minnesota.
[16] Hamilton Company, Inc., Whittier, California.
[17] Disposable self-filling, self-measuring dilution micropipettes (Gerarde Pipettes) are recommended for work with radioactive lipids. "Unopette," Becton-Dickinson, Rutherford, New Jersey.

test for choosing a suitable solvent for chromatography: drops of a solution of the sample to be fractionated are placed on a thin layer of the adsorbent to be used and different solvents are applied to these spots. The various solvents will yield concentric rings of the constituents of the mixture and the solvent effecting the clearest separation by this test is also most suitable for chromatographing the mixture in a larger scale on a chromatoplate or on a column.

TABLE I

Eluotropic Series of Solvents

Trappe (204)	Strain (199)	Knight and Groennings (88)
Light petroleum	Light petroleum 30–50°	Heptane
Cyclohexane	Light petroleum 50–70°	Diisobutylene
Carbon tetrachloride	Light petroleum 70–100°	Benzene
Trichloroethylene	Carbon tetrachloride	Isopropyl chloride
Toluene	Cyclohexane	Diisopropyl ether
Benzene	Carbon disulfide	Diethyl ether
Dichloromethane	Anhydrous diethyl ether	Ethyl acetate
Chloroform	Anhydrous acetone	sec. Butyl alcohol
Diethyl ether	Benzene	Ethyl alcohol
Ethyl acetate	Toluene	Water
Acetone	Esters of organic acids	Acetone
n-Propanol	1,2-Dichloroethane	Methanol
Ethanol	Alcohols	Pyruvic acid
Methanol	Water	
	Pyridine	
	Organic acids	
	Mixtures of acids or bases, water, alcohols, or pyridine	

Mixtures of two or three solvents of different polarities may be chosen for optimum resolution, instead of a single solvent. Schroeder (169) found mixtures of petroleum hydrocarbon with diethyl ether to be especially useful. This solvent pair is now preferred in column chromatographic separations of natural lipid mixtures according to classes of compounds (e.g. 42, 58, 118, 119, 181) and also in TLC (e.g. 107, 108, 126, 149). Ethyl acetate may be used to replace diethyl ether in such mixtures.

Classes of non-polar lipids, such as hydrocarbons, ethers, cholesteryl esters, and alkyl esters are best separated, as classes, by TLC with nonpolar solvents such as petroleum hydrocarbons, carbon tetrachloride or benzene or mixtures of two solvents containing largely one of these solvents (Table II). More polar lipids are resolved with mixtures of petroleum hydrocarbon and diethyl ether in different ratios (Table II). The same solvent mixtures with 1–2% of glacial acetic acid serve for resolving complex mixtures of natural lipids (Table II). The addition of acetic acid is essential if free fatty acids are present, in order to prevent their streaking.

Very polar nitrogenous lipids of industrial importance, like amines, amides, and nitriles, are well resolved with a mixture of chloroform-methanol-aqueous ammonia. Chloroform is equilibrated, at 20°C., with aqueous ammonia, 10/1, v/v, and 97 volumes of this ammoniacal chloroform and 3 volumes of methanol are used as the developing solvent system (111).

Acidic lipids, such as sulfates and sulfonates, phosphates and phosphonates, are separated by TLC on Silica Gel G containing 10% of ammonium sulfate, with mixtures of chloroform-methanol-aqueous sulfuric acid. Methanol containing 5% of 0.1 *N* sulfuric acid is mixed with chloroform, depending upon the polarity of the lipids to be resolved (111). Typical solvent systems are chloroform-acidic methanol-sulfuric acid, 97/3 and 8/2, v/v. The latter solvent mixture is especially suitable for the separation of natural phospholipids, such as lecithins, cephalins, and sphingomyelins on modified Silica Gel G plates. This acidic system will split, however, any aldehydogenic phospholipids.

Solvent systems for the separation of phospholipids, sulfolipids, and glycolipids on Silica Gel G plates are listed in Table II. Typical solvent systems for the separation of steroids, of bile acids, and of triterpenoic acids are listed in the same table.

Solvents for Fractionating Classes by Reversed-Phase Partition TLC. The solvent systems used in chromatography on silicone-impregnated paper or on paraffin-impregnated paper can also be applied in reverse-phase partition TLC. Chakrabarty (26) ha

TABLE II

Solvents for Thin-Layer Chromatography of Lipids on Silica Gel[a]

	References
Fats, Oils, and Waxes, Oxygenated Acids and Their Esters	
Tetralin-Hexane, 1/1, 1/3	(80
Petroleum hydrocarbon[b]-diethyl ether, 95/5, 90/10, 80/20, 50/50	(101,108,149
Petroleum hydrocarbon-diethyl ether-acetic acid, 90/10/1, 80/20/1, 70/30/2	(101,108,128
Diethyl ether	(79
Diisopropyl ether	(79,80
Diisopropyl ether-acetic acid, 100/1.5	(79,80
n-Propanol-conc. aqueous ammonia, 2/1, followed by chloroform-benzene, 3/2, followed by carbon tetrachloride	(65,222
Phospholipids, Sulfolipids, and Glycolipids	
Chloroform-methanol-water, 70/22/3, 65/30/5, 65/25/4, 60/35/8	(161,219,229
Chloroform-methanol-aqueous ammonia, 3/1 with 40 ml. of conc. aqueous ammonia/liter; 1/3 with 40 ml. of conc. aqueous ammonia/liter	(30,64,111
Chloroform-methanol-aqueous sulfuric acid Chloroform-methanol (containing 5% of 0.1 *N* sulfuric acid), 97/3, 4/1	(111
n-Propanol-water, 7/3	(89
n-Propanol-aqueous ammonia (12%), 4/1	(72,223
n-Propanol-conc. aqueous ammonia, 7/3	(72
n-Propanol-aqueous ammonia (12%), 4/1, followed by ethylene chloride-methanol, 49/1, followed by chloroform-acetic acid (96%), 95/5	(72
n-Butanol-pyridine-water, 3/2/1	(87
Stercids	
Benzene, toluene, cyclohexane, hexane, dichlorethane	(25,31,56
Benzene-ethyl acetate, 9/1, 2/1	(31
Benzene-ethanol, 49/1, 95/5, 9/1	(25
Cyclohexane-benzene, 4/1, 1/1	(31
Cyclohexane-ethyl acetate, 19/1, 17/3, 9/1, 7/3	(7,31
Diethyl ether	(56
Bile Acids	
Upper phase of the system, toluene-acetic acid-water 10/10/1, 5/5/1	(48
n-Butanol-acetic acid-water, 10/1/1	(48
Triterpenoic Acids and Esters	
Benzene, cyclohexane, methylene chloride, diisopropyl ether, ethyl acetate, butyl acetate	(207,210
Cyclohexane-toluene, 4/1, saturated with formic acid	(210
Diethyl ether-hexane, 1/1	(214
Diisopropyl ether-acetone, 5/2	(210
Diisopropyl ether-acetone, 5/2, with 5% of pyridine	(210
Ethyl acetate-methanol-diethyl amine, 14/4/3	(210
Chlorobenzene-acetic acid, 9/1	(210
Methylene chloride-pyridine, 7/2	(210

[a] All ratios are v/v.
[b] Usually petroleum hydrocarbon, b.p. 60–70°C. (mainly hexane).
[c] On silica gel G containing 10% of ammonium sulfate.
[d] On alumina.

used the same developing solvents on silicic acid plate that had been impregnated with squalane.

Suitable solvent systems for reversed-phase partition PC or TLC of a given class of lipids can be chosen after studying the behavior of this lipid in adsorption chromatography (111). Typical solvent systems are given in Table III.

Compounds differing by two methylene groups and one double bond, such as palmitic and oleic acids, or palmitonitrile and oleonitrile, myristonitrile and linoleonitrile, lauronitrile and linolenonitrile, overlap in reversed-phase partition chromatography. Such critical pairs are separated by low-temperature chromatography. Palmitic and oleic acids may be resolved with a mixture of acetic acid-formic acid-water, 2/2/1

TABLE III

Solvents for Reversed-Phase Partition Thin-Layer Chromatography

Solvents[a]	Ratios v/v	Stationary phases[b]	Lipid classes fractionated	Ref.
Methanol	.....	Higher paraffins	Carotenals	(229)
Acetic acid-water	24/1	Undecane	Fatty acids	(79)
.....	3/1	Silicone	Fatty acids and their methyl esters	(101)
.....	17/3	Silicone	Fatty acids and their methyl esters and aldehydic cores derived from lecithins	(101) (152)
.....	17/3	Squalane	Fatty acids and their methyl esters	(26)
Acetic acid-acetonitrile	1/1	Undecane	Fatty acids	(79)
Acetic acid-acetonitrile water	2/14/5	Silicone squalane	Methyl-esters of fatty acids	(101) (26)
Methyl ethyl ketone-acetonitrile	7/3	Higher paraffins	Cholesteryl esters of fatty acids	(80)
Chloroform methanol-water	5/15/1	Undecane	Diglycerides	(79)
Acetone-acetonitrile	7/3	Undecane	Triglycerides	(79)
Acetone-ethanol-water	6/1/3	Polyethylene	Methyl esters of fatty acids	.....
Acetic acid formic acid water[c]	2/2/1	Silicone	Fatty acids saturated/unsaturated	(101)
Acetic acid-peracetic acid-water	15/2/3	Silicone	Fatty acids saturated/unsaturated	(101)

[a] All solvent mixtures must be saturated with the stationary phase.
[b] Silica Gel G served as a carrier for the stationary phases. Only polyethylene was applied directly to the plates.
[c] Chromatographed at 4–6°C. (All others at room temperature.)

v/v/v, at 4–6°C., on siliconized Whatman #1 paper[12] (165) or on a siliconized Silica Gel G plate (101). An alternative method of resolving critical pairs is to chromatograph with oxidizing solvents (101, 105, 107). Saturated and unsaturated nitriles can be resolved on siliconized paper or on a siliconized chromatoplate with solvent systems consisting of 75 volumes of acetic acid and 25 volumes of water, or 70 volumes of acetonitrile and 30 volumes of water. A mixture of 65 volumes of acetic acid, 10 volumes of peracetic acid[18] and 25 volumes of water, or of 70 volumes of acetonitrile, 10 volumes of perhydrol, and 20 volumes of water separates saturated nitriles from one another, whereas all unsaturated lipids are quantitatively oxidized and migrate in bulk to the solvent front.

Solvents for Separating Lipids According to Unsaturation. Saturated and unsaturated lipids can be separated by chromatography after reacting the latter with mercuric acetate in methanol. A procedure for forming acetoxymercuri-methoxy compounds of unsaturated lipids is described later in this text.

Saturated and the acetoxymercuri-methoxy derivatives of unsaturated lipids, e.g., hydrocarbons, methyl esters, or nitrils, are resolved by TLC on Silica Gel G with a mixture of petroleum hydrocarbon-diethyl ether, 4/1, v/v. The derivatives of the unsaturated lipids are then fractionated according to the degree of unsaturation, regardless of chain length, in a solvent consisting of n-propanol-glacial acetic acid, 100/1, v/v. After isolation by TLC, the original monounsaturated, diunsaturated, and triunsaturated lipids can each be recovered by treating their complexes with methanolic hydrochloric acid.

Developing

Chromatostrips and chromatoplates are usually developed at room temperature in ascending technique, i.e., they are placed on edge in a covered jar containing a 0.5–1 cm. layer of solvent which then travels up the length of the plate until it reaches a pre-set line. Jars for use with standard chromatoplates are commercially available.[2,4,19] Several chromatoplates may be simultaneously developed in one jar. To obtain straight solvent fronts, the adsorbent should be scraped off the edges of the plates before chromatography, and the chromatoplates should not touch the sides of the jar. For the same reason, it is advantageous to line the inside of the jars with filter paper which acts as a wick to assure that they are uniformly saturated with the solvent vapors (185). This gives a straight solvent front, rounder spots and it shortens the developing time by about ⅓. It may be noted that chromatography in lined jars requires somewhat more polar solvent than in plain jars. For example, to obtain the same kind of separation one will have to use a system of petroleum hydrocarbon, b.p., 60–70°C., diethyl ether, glacial acetic acid, 80/20/1, on a jar lined with filter paper, but the same solvents in a ratio of 90/10/1, v/v/v, in a jar that does not contain such a wick. Generally the separations achieved in a saturated chamber are not quite so sharp as those obtained in plain chambers.

Chromatoplates may also be developed step-wise running the same solvent once or twice or different solvent systems consecutively in the same direction (multiple development). Stahl (184) has utilized Matthias' wedge strip technique (116) in TLC, by scratching a row of wedge-tipped strips in the adsorbent layer.

Dhopeshwarkar and Mead (38), also Chakrabarti (26), have used a gradient-elution scheme for TLC of lipids. The developing solvent mixture is continuously being diluted with a more polar solvent. This technique permits fractionation of lipids of widely different polarity on a single plate.

Brenner and Niederwieser (17) extended the scope of TLC by developing a simple arrangement which forces solvents to run over. This method was applied to the separation of substances that could not be separated otherwise. The same technique should be useful for collecting fractions from an overflowing chromatoplate. An apparatus for use with chromatostrips has been devised by Stanley, Ikeda, and Cook (194).

Chromatoplates may be run in two-dimensional technique. A single sample is placed in a corner of the plate, and the plate is then developed, consecutively in two directions with two different solvent systems. Using the same solvent in both directions yields little improvement in resolution but indicates alteration of components if the resulting spots are not uniform on a line bisecting the plate. For example, in TLC of tissue lipids it can be observed that the phospholipid fraction remains where it had been after developing in the first dimension (211). Stahl (187) has applied two-dimensional TLC for studying chemical reactions on the plate of the compounds fractionated by chromatography in the first direction (Separation-Reaction-Separation; SRS Technique). Miller and Kirchner (124) had already described carrying out oxidations, reductions, dehydration, and hydrolysis reactions, and formation of derivatives on the plate. Kaufmann and Makus (79) have performed adsorption chromatography and reversed-phase partition chromatography

[18] Becco Chemical Division, Food, Machinery and Chemical Corp., Buffalo 7, New York.

[19] A. H. Thomas Company, Vine Street at Third, Philadelphia 5, Pennsylvania.

of a sample, consecutively, on the same plate. The lipid mixture was first fractionated according to classes by adsorption chromatography. Then the unused major portion of the chromatoplate was impregnated, and classes were resolved into individual compounds by reversed-phase partition chromatography in the second direction.

The possibility of reacting parts of a complex mixture in the course of chromatography has been illustrated by chromatography in oxidizing solvents (101, Table III).

Chromatoplates may be developed horizontally. This is mandatory if loose layers of adsorbent, free of binding agent, are applied (e.g., 25, 56, 91, 92, 131, 153). It is also used in radial development, i.e., Circular-TLC. The sample is placed in the center of a plate and developed by slowly dripping solvent onto it from a pipette (91, 92, 226) or by supplying solvent from a reservoir through a wick (184). Chakrabarty (26) has found centrifugally accelerated TLC useful.

Visualization and Identification of Lipids

Most indicators used in PC of lipids (103, 113) may also be applied in TLC, provided washing of the chromatograms is not required. These indicators are usually non-destructive, thus permitting the individual fractions to be eluted from the plates for further fractionation and identification. In addition, corrosive spray reagents, such as oxidizing acids, may be used for charring organic matter on the chromatoplates over a hot plate. Such indicators have the great advantage that all compounds that may not be detected by more specific reagents are visualized unless they are volatile. The author prefers chromic sulfuric acid solution. With this agent, unsaturated lipids appear as light brown spots on a white background, even before heating. Heating the plates yields further information on the chemical nature of the various compounds as different colors appear at increasing temperatures.

A list of indicators used in TLC of lipids is presented in Table IV.

The consecutive use of two or several indicators on one chromatoplate increases the probability that no substance remains undetected. The author prefers using iodine vapors on one plate, and after evaporation of the iodine (5 min.), dichlorofluorescein, followed by chromic sulfuric acid solution (111). The dichlorofluorescein on the plate does not interfere with the subsequent charring process, neither is silicone on reversed-phase plates charred when heated with chromic-sulfuric acid. Dichlorofluorescein, however, cannot be used on reversed-phase layers, as the whole plate fluoresces when sprayed with this indicator (101).

Fluorescent chemicals may be incorporated in the adsorbent layer for visualizing spots in U.V. light after chromatography. Reitsema (157) added about 0.004% of Rhodamine 6 G to his chromatoplates. Stahl (183) used 0.04% sodium fluorescein, Černy, Joska, and Lábler (25) mixed morin into loose layers of alumina. Kirchner, Miller, and Keller (82), following a suggestion by Sease (170), incorporated about 0.75% of each zinc silicate and zinc cadmium sulfide as fluorescent agents into silicic acid layers on glass. The reviewer adds 1% of the commercial Luminescent Chemical #601 [22] to Silica Gel G plates, in

[20] Eastman Kodak Company, Rochester 3, New York.
[21] "Mineralight," Ultra-Violet Products Inc., San Gabriel, California.
[22] E. I. du Pont de Nemours & Co. (Inc.), Photo Products Department, Wilmington 98, Delaware.

TABLE IV
Indicators Used in Thin-Layer Chromatography of Lipids

Reagent	Color of spot	Substances visualized	Sensitivity	Ref.
Daylight or U.V. light	Various colors, white background	Colored compounds. Fluorescent substances	0.1–10 γ	(37) (225) (228)
Fluorescent dye or mineral in adsorbent layer	Various colors, colored background	Aromatic derivatives of lipids. Conjugated unsat. fatty acids	1 γ	(47) (82) (157)
Radioisotopes	An autoradiograph is taken	All radioactive labeled materials	<0.1 γ	(107) (112) (195)
Iodine vapors or iodine, 1% in methanol *	Brown, white or yellow background	All unsaturated lipids, also some saturated nitrogenous lipids	<1 γ	(101) (107) (108)
α-Cyclodextrin, 1% in aqueous ethanol, 30% followed by iodine vapors	White, purple background	Monochain lipids	5 γ	(101) (107)
2′,7′-Dichlorofluorescein, 0.2% in ethanol, 96% [20]	Green, purple background in U.V. light	Nonpolar lipids, saturated and unsaturated	1–5 γ	(101) (108) (111)
Rhodamine B, 0.05% in ethanol, 96%	Purple, rose background	Most lipids	1 γ	(79) (80) (220)
Bromothymol blue 0.04 mg. in 100 ml. 0.01 N aqueous NaOH	Yellow on blue background	Most lipids	0.1–1 γ	(52) (71) (72)
Fluorescein-bromine 0.04% sodium fluorescein in water followed by bromine vapors	Yellow, pink background, best in U.V. light	Unsaturated compounds	1 γ	(4) (82) (183)
Wet hydroxylamine impregnated paper, followed by 5% ferric chloride in 0.5 N aqueous HCl	Purple, white background	Esters	5–10 γ	(35) (40)
Antimony trichloride or antimony pentachloride or stannic chloride. Saturated solution in chloroform or carbon tetrachloride	Various colors in daylight and U.V. light	Aliphatic lipids, steroids	1–10 γ	(7) (31) (222)
o-Phosphoric acid 40% in water	Gray-blue	Pregnandiol	.....	(221)
Phosphomolybdic acid, 5–20% in ethanol, 96%	Blue, white, or yellow-green background after heating to 100°C.	Neutral lipids Antioxidants	<0.5 γ 1 γ	(7) (80) (171)
Aqueous sulfuric acid, 1:1, v/v, or conc. sulfuric acid, or saturated chromic sulfuric acid solution	Various colors during heating eventually gray or black	Most nonvolatile organic compounds	1 γ	(82) (112) (128)
Ninhydrin * 0.2% in n butanol aqueous acetic acid 10%, 95:5	Red purple, white background after heating to 105°C.	Aminophosphatides	10 γ	(164) (219) (220)
Dragendorff reagent a) 1.7 g. basic bismuth nitrate in 100 ml. aqueous acetic acid (20%); b) 40 g. potassium iodine in 100 ml. water. Reagent: 20 ml. of a) with 5 ml. of b) and 70 ml. of water	Orange or red orange	Cholin phosphatides	10–20 γ	(164) (219) (220)
Diphenyl amine 20 ml. 10% diphenyl amine in ethanol, 96%, plus 100 ml. conc. HCl and 80 ml. acetic acid	Blue gray, light gray background	Glycolipids	4–10 γ	(87) (219) (220)
2,4-Dinitrophenyl hydrazine, 0.5% in 2 N-HCl	Yellow or orange	Aldehydes and ketones	1–5 γ	(7) (157) (158)
Potassium iodide starch, 5 ml. 4% KOH with 20 ml. acetic acid, after 5 min., 1% aqueous starch solution	Blue, white background	Peroxides	.....	(183)
Ammonium thiocyanate 0.2 g. in 15 ml. acetone and 0.4 g. ferrous sulfate in 10 ml. water	Red and red-brown, white background	Peroxides	.....	(183)
2,6-Dichloroquinone chloromide 1% in ethanol	Various colors, white background	Antioxidants	1 γ	(171)
s-Diphenylcarbazone 0.1% in ethanol, 95%	Purple and blue, light rose background	Mercuric acetate adducts of unsaturated lipids	10–20 γ	(110)

* Available as aerosol package.

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Lectures of the 1961 Short Course on Newer Lipid Analyses

conducted by

The American Oil Chemists' Society

at the

University of Rochester, Rochester, New York, July 24-26

J. Am Oil Chem Soc. 38: 708-727, 1961

Under the sponsorship of the Education Committee, J. C. Cowan, chairman, with N. H. Kuhrt as local chairman and Raymond Reiser as program chairman

Part III

Thin-Layer Chromatography of Lipids

HELMUT K. MANGOLD,[1] University of Minnesota, The Hormel Institute, Austin, Minnesota

A METHOD for chromatographic adsorption analysis on thin layers of adsorbent was described as early as 1938 by Izmailov and Shraiber (68). A decade later Williams (226) used a layer of adsorbent between horizontal glass plates, the upper one containing a small hole through which samples and solvents were introduced. Meinhard and Hall (120) developed "surface chromatography" on a powdered adsorbent, fixed rigidly to a microscope slide by means of a suitable binder. At the same time, Kirchner and Keller (81) described a procedure for impregnating filter paper with silicic acid. In 1951 Kirchner, Miller, and Keller (82) extended the use of adsorption chromatography to glass strips, 0.5 x 5.25 in., or sheets coated with a layer of adsorbent which contained starch or plaster of Paris as binding agent. Miller and Kirchner (125) developed an apparatus for coating glass strips with the adsorbent more rapidly than could be accomplished by hand. The same group of workers applied the new technique extensively to the separation and identification of terpenes and oxygenated terpenes and to their isolation from essential oils (82, 83, 84, 85, 86, 122, 123, 124).

Through the work of Kirchner, Miller, and Keller the chromatostrip method became well known and it was also used by other workers for the analysis of terpenes (21, 51, 67, 90, 142). With this method Vannier and Stanley (212) were able to detect the addition of grapefruit oil to lemon oil. These workers (193) and Bernhard (12) applied the chromatostrip technique to the detection and isolation of substituted coumarins from lemon oil. Stanley and Vannier (192) used chromatostrips for the quantitative estimation of biphenyl in citrus fruits. Onoe (143) utilized the same technique for the fractionation of 2,4-dinitrophenyl hydrazones of aliphatic aldehydes; Fukushi and Obata (45) analyzed azulenes; and Maruyama, Onoe, and Goto (115) separated organic peroxides.

A modification and extension of the chromatostrip method was developed by Reitsema (157, 158). He prepared chromatoplates, 5 x 7 in., to characterize various essential oils by chromatography. The use of chromatoplates instead of narrow strips permits running reference compounds alongside the mixtures of unknowns. Rigby and Bethune (159) used chromatoplates for separating and characterizing hop oils, and Wagner (218) analyzed phenols similarly.

Further application of the chromatostrip and chromatoplate techniques was reported by Lagoni and Wortmann (91, 92), who developed a method for the detection of fat-soluble pigments. These techniques were also used by Mottier (131, 132, 133, 134, 135), who carefully described methods for the analysis of food colors and of amino acids. Demole (34) separated phytol and isophytol, and Röchelmeyer (161) described the fractionation of alkaloids on thin layers of adsorbent.

It is amazing that the elegant technique of chromatographing on open columns, i.e., on thin layers of an adsorbent, was not applied to other lipids after it had become such a prominent method in terpene research.

[1] The author's work is being supported by the U. S. Public Health Service, National Institutes of Health Research Grant 5817, by The Bureau of Commercial Fisheries, Fish and Wildlife Service, U. S. Department of the Interior (Contract No. 14-17-008-127), by The National Science Foundation, and by The Hormel Foundation.

Index to Part III

708 THIN-LAYER CHROMATOGRAPHY OF LIPIDS, by Mangold
728 CRYSTALLIZATION OF FATTY ACIDS, by Schlenk

Actually the method remained in obscurity until 1956 when Stahl (182, 183, 184) described equipment and procedures for the preparation of chromatoplates, and demonstrated the potential usefulness of thin-layer chromatography in the fractionation of substances other than terpenes. Thin-layer chromatography (TLC) has suddenly gained recognition and the technique is now being applied to the analysis of a great variety of substances. Simultaneously the method itself has been developed further and supersedes all other separation techniques in many laboratories, especially those engaged in lipid research.

Adequate equipment for work with thin-layer chromatography, such as spreaders for the uniform coating of glass plates, and standardized adsorbents, have become commercially available.

Several reviews on the application of TLC have appeared recently (35, 36, 73, 130, 168, 188, 190, 232, 233). Vioque (213) and Fontell, Holman and Lambertsen (44) discussed specific applications of TLC for the analysis of lipids. Brenner and Pataki (18) studied some theoretical aspects of TLC. (See also 40, 117, 234.)

The present review provides detailed descriptions of apparatus and techniques used in TLC. Applications of the method to lipids and related substances are summarized briefly.

Lipid fractions resolved by TLC may be isolated and further analyzed by complementary methods, such as paper chromatography (PC) and gas-liquid chromatography (GLC). Examples of the application of TLC in conjunction with PC and GLC are presented here.

Apparatus and Techniques

The Applicator and Other Equipment. The uniform coating of glass plates with a thin layer of powdered material requires the use of a special applicator.

At present there are three types of applicators on the market.[2,3,4] The equipment designed by Stahl (182, 183, 186) is the oldest and most widely used, and therefore it is described in detail.

A plastic board 22 x 113 cm., with retaining ledges 1.8 cm. wide along a short and a long side (Fig. 1), is placed on a laboratory bench so that the long edge faces the worker. The short retaining ledge is now on the right side of the board. A glass plate, 5 x 20 cm., and five carefully cleaned square glass plates of uniform thickness, 20 x 20 cm., and another plate, 5 x 20 cm., are placed closely together in a row, on the board. A drop of water under each plate will prevent the glasses from sliding on the board. The applicator (Fig. 1) is placed on the small plate in the "open" position. The operator should grasp the applicator with both hands and move it slowly, in one stroke, across the plates to assure that they are well aligned. In the author's laboratory it was found useful to attach a piece of cork about 0.5 cm. high with adhesive tape to the leading edge of the applicator's guide bar. This permits one to move the applicator more smoothly across the row of plates.

A slurry of adsorbent is made by thoroughly mixing in a mortar 25 g. of Silica Gel G, a commercial

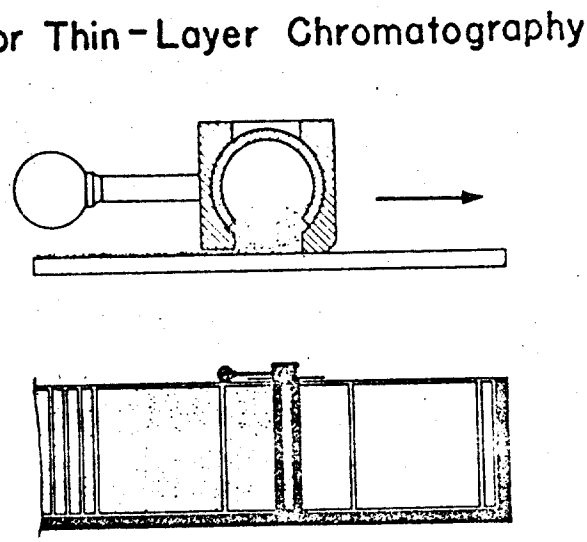


FIG. 1.

preparation containing calcinated calcium sulfate (or another adsorbent containing the same binding material[5,6]), with 50 ml. of distilled water. The slurry must be of uniform consistency and free of air bubbles. The time required for preparing the slurry should not exceed 1–1½ min. The mixture is poured into the applicator and the lever turned 180° in the direction of the red arrow on top of the instrument, to permit the slurry to run out of the slit as the applicator is moved smoothly across the row of glass plates on the board. The entire operation, from the addition of water to the silica gel until the spreading of the thin layer, must be finished in about 4 min. before the slurry hardens. The chromatoplates are air-dried for 10–20 min. to allow the binder to set and then are placed in a rack and activated by heating in an oven to 110–120°C. for 1–2 hr. The adsorbent layer thus produced is about 250–275 μ thick and very uniform in appearance in both reflected and transmitted light. According to Stahl (186) the activity of the adsorbent layer corresponds to grades II to III of the Brockmann activity scale (20). The chromatoplates may be kept in a storage cabinet to protect them from dust and laboratory fumes.

The adsorbent layer absorbs water from the air and thus slowly becomes deactivated. Adsorbents of different activities produce different rates of migration of various substances during chromatography. Therefore, R_f values usually are not highly reproducible in thin-layer adsorption chromatography. The pattern of separation, however, is constant and reproducible.

Plates, whose efficiency of separation is affected as a result of too much moisture, may be reactivated by heating. In very humid laboratories it is advisable to store the activated chromatoplates in a desiccator over freshly activated silica gel. Seiler and Seiler (174), working on the separation of inorganic cations, recommend further drying of the air-dry chromato-

[2] C. Desaga G.m.b.H., Hauptstrasse 60, Heidelberg, Germany; U. S. representative: C. A. Brinkmann and Company, Inc., 115 Cutter Mill Road, Great Neck, Long Island, New York.

[3] Camag A. G., Homburger Str. 24, Muttenz, B.L., Switzerland; U. S. representative: Kensington Scientific Corporation, 1717 Fifth Street, Berkeley 10, California.

[4] Research Specialties Co., 200 South Garrard Blvd., Richmond, California.

[5] E. Merck, A. G., Darmstadt, Germany; U. S. representative: Terra Chemicals Inc., 500 Fifth Avenue, New York 36, New York.

[6] Fluka, A. G., Buchs, S. G., Switzerland; U. S. representative: Gallard-Schlesinger Chemical Manufacturing Corp., 1001 Franklin Avenue, Garden City, New York.

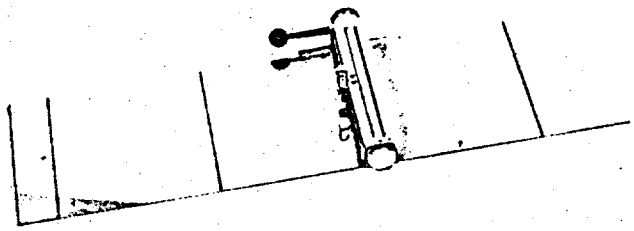


FIG. 2. Applicator "Model S" according to Stahl. Desaga G.m.b.H.[2]

plates over calcium chloride in a desiccator overnight. Thereafter the plates should be stored in a cabinet.

Recently a versatile model of Stahl's applicator has been produced which permits one to obtain uniform thin layers of any desired thickness between 250 μ and 2 mm. This applicator, Model S, as shown in Figure 2, is commercially available.[2] Stahl's new spreader may find application especially in preparative work where the use of plates of higher capacity is desired.

A rather different applicator for the preparation of chromatoplates has been designed by Mutter and Hofstetter (137). This relatively new apparatus[3] and its use have been adequately described by Wollish, Schmall, and Hawrylyshyn (232). They have given detailed instructions for the building of a similar instrument, as well as auxiliary equipment, in any small machine shop. The instrument is shown in Figure 3. It produces uniform layers of adsorbent in adjustable thicknesses on glass plates, 3.5 x 4.5 in.

An important advantage of this instrument, according to Wollish *et al.* (232), is that the glass plates used need not be uniform in thickness; thus one can easily buy inexpensive window glass cut to size in any glass shop.

Another new applicator for thin-layer chromatography has just become commercially available.[4] It is a simplified version of Stahl's original applicator, as shown in Figure 4. It yields fine layers about 275 μ thick. Layers of different thickness cannot be produced with this apparatus.

In many cases inexpensive, self-made applicators may be adequate, such as those described by Barbier, Jäger, Tobias, and Wyss (7), Černy, Joska and Lábler (25), Machata (98), Miller and Kirchner (125), Mottier (134), and Vioque (213). The author, however, believes that the purchase of a commercial applicator, standardized and uniform glass plates, and commercially available adsorbents is a good investment because they will save the many hours of experimentation that would be required to build a satisfactory instrument and produce a suitable adsorbent.

Peifer (145) has recently developed a method for TLC on lantern slides, 8 x 10 cm., on microscopic slides, 2.5 x 7 cm., and even on microscope cover slips, 2.5 x 3 cm. The glass plates are covered with adsorbent by dipping them into a suspension of Silica Gel G in chloroform or chloroform-methanol. The microchromatoplates may be used after drying over a hot plate for 1–3 min. This rapid and simple technique does not require the use of applicators, glass plates of specific dimensions, or prolonged drying periods. Therefore, it may become a useful modification of TLC even though it is not suited for resolving complex mixtures or larger samples.

Adsorbents, Stationary Phases for Partition Chromatography, and Ion Exchangers

Silica Gel (Silicic Acid). This adsorbent is most frequently used for the separation of neutral and acidic lipids (234) in columns (29), on impregnated cellulose paper (163), or on impregnated glass fiber paper (54). Fine grade (about 80 μ or 200 mesh) silica gel, containing up to 15% calcinated calcium sulfate (gypsum, plaster of Paris) as a binder, is widely used in TLC. Such preparations may be purchased as "Silica Gel G for thin-layer chromatography according to Stahl."[2,5] If very fine layers are desired, the commercial product should be run through a 200-mesh sieve before use. Silica Gel G is slurried with distilled water in a ration 1/2, w/v, and applied to the glass plates as described above.

For special purposes it may be necessary to remove small amounts of ferric ion from the adsorbent. Seiler and Seiler (174) accomplished this by washing 500 g. of Silica Gel G with 1000 ml. of concentrated hydrochloric acid-water, 1/1, v/v. After removing of the acid by decanting, the adsorbent was washed with three to four portions of 1000 ml. of water, and filtered through a Büchner funnel. After drying in a stream of air the adsorbent was washed with 300 ml. of benzene and then dried in an oven at 120°C. for 1–2 hr. Because the acid treatment removes a great part of the calcium sulfate, it is necessary to add 2 g. of this material to the 500 g. of washed adsorbent before preparing the chromatoplates.

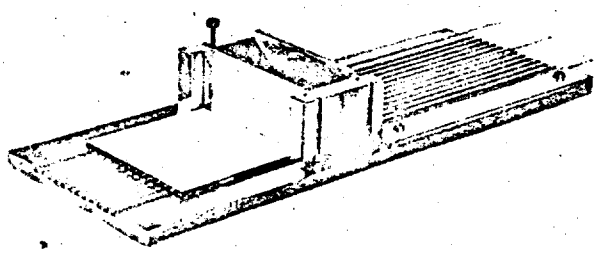


FIG. 3. Applicator according to Mutter and Hofstetter. Camag A.G.[3]

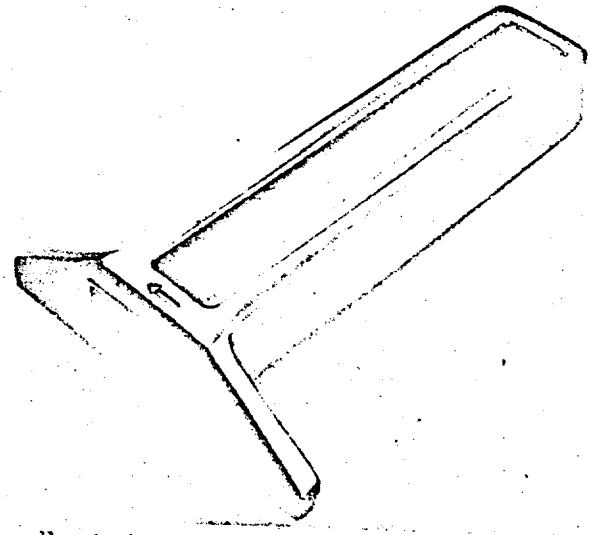


FIG. 4. Applicator of Research Specialties Co.[4]

A product closely resembling the commercial materials may be obtained by thoroughly mixing; e.g. Mallinkrodt Silica Gel,[7] 200 mesh, (154) with 10–15% calcinated sulfate of the same grain size (145). Onoe (143) prepared thin layers of silicic acid on glass strips from a slurry of 23 g. of silica gel, 200 mesh, and 30 ml. of a 2.5% solution of polyvinyl alcohol to which a few drops of ethanol were added to prevent foaming.

Several workers have recommended solvents other than water for preparing slurries of silicic acid. Müller and Honerlagen (136) claim that more uniform layers are obtained if 16 g. of Silica Gel G is mixed with 30 g. of acetone. Peifer (145) and Peifer, Muesing and Janssen (146) suspended 35 g. of Silica Gel G in 100 ml. of either chloroform or a mixture of chloroform-methanol, 3/2, v/v, for the preparation of microchromatoplates by the dipping technique. The use of nonaqueous solvents results in shorter drying periods for the wet chromatoplates. The adsorbent layer does not adhere very well to the glass and therefore, it may be necessary to expose the plates to steam (145).

It may be advantageous, especially in preparative work, to chromatograph on thin layers of powdered silica gel which do not contain calcinated calcium sulfate or other binders. Such loose layers can easily be obtained by applying the dry adsorbent with any spreader which will produce layers of any specific thickness from 0.5 to 2 mm.

Hernandez, Hernandez, Jr., and Axelrod (57) have described procedures for activating and standardizing silicic acid preparations used for chromatographing so that all Brockmann activity grades are obtainable and reproducible with this adsorbent. In TLC it is usually not necessary to know the activity of the adsorbent layer, as long as satisfactory separations can be obtained. Since the R_f-values are not reproducible in TLC, they may be referred to the position of standard dyes on the chromatogram. Stahl (183) recommends developing a mixture of 50 mg. each of p-dimethyl amino benzene (butter yellow), sudan red G, and indophenol, in 50 ml. of benzene,[2] concurrently with the samples to be separated. However, this test mixture can be applied only when benzene, methylene chloride, chloroform, or solvent mixtures of similar eluting power are used. Azulene is used with less polar solvents, such as petroleum hydrocarbon, toluene, or carbon tetrachloride.

Seher (171) has found that constant R_f values are obtained on Silica Gel G plates if the adsorbent is conditioned by developing the plate with chloroform before the sample itself is chromatographed.

Modified Silicic Acid Layers. For the separation of acidic compounds, Stahl (185) prepared acidic silicic acid chromatoplates, by using aqueous 0.5 *N* oxalic acid solution instead of water in making the silicic acid slurry. Peifer (145) and Peifer, Muesing, and Janssen (146) added 2.5 ml. of concentrated sulfuric acid to 100 ml. of chloroform-methanol, 3/2, v/v. This mixture was slurried with 35 g. of Silica Gel G, as described above, for the preparation of microchromatoplates. The sulfuric acid in the adsorbent layer has the added advantage that the spraying of the plates after chromatography can be circumvented. The microchromatoplates may be placed on a hot plate right after developing, and the sulfuric acid in the adsorbent suffices for charring the organic matter in the individual spots.

Stahl (185) also described the preparation of alkaline chromatoplates. He used aqueous 0.5 *N* KOH solution instead of water in making the slurry of Silica Gel G. The use of such alkaline silica gel layers has not yet been described for TLC of lipids. This author assumes that such plates might be suitable for achieving separations of the kind described by Rouser, Bauman, Kritchevsky, Heller, and O'Brien (162) on silicic acid-silicate-water columns.

Winterstein, Studer, and Rüegg (229) separated carotenals on chromatoplates that were prepared from a slurry of 5 g. Silica Gel G and 20 g. calcium hydroxide in 50 ml. of water.

Honegger (63), in his work on thin-layer ionophoresis and combined thin-layer ionophoresis-TLC, described the use of adsorbent layers containing sodium citrate buffer. Honegger prepared chromatoplates with a slurry of 25 g. of Silica Gel G (or Kieselguhr G, or Aluminum Oxide G) in 50 ml. of 0.1 M sodium citrate buffer of pH 3.3. Spraying of chromatoplates with buffer solution also yielded useful layers. Nürnberg (141) added a buffer to the developing solvent instead of incorporating it into the adsorbent layer.

Mangold and Kammereck (111) used chromatoplates of Silica Gel G containing about 10% ammonium sulfate for the separation of phospholipids and strongly acidic fatty acid derivatives. Such weakened plates were prepared from a slurry of 25 g. of Silica Gel G in a solution of 2.5 g. ammonium sulfate in 60 ml. of water. This slurry has to be applied to the glass plates very quickly, as it hardens even faster than do slurries of plain Silica Gel G.

Adsorbent layers containing acids, bases, or salts do not adhere well to the glass plates and tend to flake off. It is recommended that such plates be dried at room temperature for several hours.

Diatomaceous Earth (Kieselguhr). This adsorbent may be purchased already mixed with calcinated calcium sulfate as Kieselguhr G, Merck, for thin-layer chromatography according to Stahl.[25] Diatomaceous earth may be useful for separations of polar lipid where a very weak adsorbent is desired. It is applied like Silica Gel G, as a slurry in water, 25 g. per 50 ml. Stahl and Kaltenbach (191) used it for the fractionation of sugars by TLC.

Aluminum Oxide (Alumina). This adsorbent is rarely used for the chromatography of complex lipid mixtures because it causes hydrolysis of ester linkages and isomerization of double bonds (14, 15, 204, 205, 206). Still, alumina is superior to any other adsorbent for the chromatographic fractionation of the A-vitamins, also of hydrocarbons and other classes of lipids, especially of basic ones. It is often used for chromatographing sterols (138).

Two brands of alumina for TLC are commercially available: Aluminum Oxide G Merck, for thin-layer chromatography according to Stahl,[5] and Fluka Alumina for coated-glass chromatography.[6] Both preparations contain around 5% of calcinated calcium sulfate as a binder. The product manufactured by Merck is slurried with water in a ratio of 25 g./50 ml. whereas Fluka Alumina is mixed in a ratio of 20 g./6[illegible] ml. of water. The manufacturer of the latter material states that a high activity stage, approximately corresponding to Brockmann activity grade II (20), is obtained by heating the chromatoplates for at least 4 hr. to 200–220°C. The plates are cooled and stored

[7] Mallinckrodt Chemical Works, St. Louis 7, Missouri.

in a desiccator containing alumina of Brockmann activity grade I (Fluka Alumina with moisture indicator[6]). If plates with an adsorbent layer of lower activity (III or IV) are desired, the plates are dried at a temperature of 150–160°C. for 4 hr. In any case, the activated plates must be stored for at least 24 hr. over alumina of the corresponding Brockmann grade for setting the required activity, according to the producer's[6] specifications.

Alumina may also be obtained by mixing, e.g., Alcoa Activated Alumina[8] 200 mesh with 5% of calcinated calcium sulfate of the same grain size (108).

Hermánek, Schwarz, and Čekan (56) applied a layer, about 0.6 cm. thick, of plain alumina powder to glass plates using Mottier's procedure (134) and they separated a great many steroids on such chromatoplates. Černy, Joska, and Lábler (25) used loose alumina layers, 3–4 mm. thick, for TLC of steroids also.

Stahl (189) has recently described a convenient technique for determining the activity of alumina according to the Brockmann scale (20). The gadget and the test dyes required for this procedure are now commercially available.[2]

Other Adsorbents. All adsorbent materials used in column chromatography may also be applied in TLC, either as a loose layer of powdered material (153), or, with a binder, as a solid film (121). Kirchner, Miller, and Keller (82) have already described the preparation of thin layers of magnesium oxide, magnesium carbonate, calcium hydroxide, calcium carbonate, dicalcium phosphate, Anex, Filtrol, Florisil, Talc, and Starch. These workers used starch (5%) or calcinated calcium sulfate (20%) as a binding agent.

Florisil,[9] which has recently been adapted for the fractionation of complex lipid mixtures on columns (24), appears to be especially promising. Peifer (145) has described a procedure for coating microchromoplates with a thin layer of this adsorbent.

Sodium carbonate (156), charcoal (62), and sugar (75) have been used in paper and column chromatography of lipids but the author is not aware of any applications of these adsorbents in TLC.

Cellulose. The preparation and use of chromatoplates covered with cellulose was first described by Teichert, Mutschler, and Röchelmeyer (203) who separated alkaloids. Several brands of cellulose powder for TLC are now commercially available.[10,11,12] The oldest product of this kind[10] contains calcinated calcium sulfate as a binder. Only 8 g. of this material, slurried with 55 ml. of water, suffice for coating 5 standard size square plates with a fine layer of cellulose. The coated plates are dried in air for a few minutes, and then in an oven for ½ hr. at 100°C. Plain cellulose powder[12] can also be applied as thin layers on glass plates (145).

To this reviewer's knowledge, cellulose chromatoplates have not yet been used successfully in the lipid field. They might become very helpful for the separation of lipids from non-lipids by adapting the procedures described by Lea and Rhodes (95) and by Westley, Wren, and Mitchell (224).

[8] Aluminum Ore Company, East St. Louis, Illinois.
[9] Floridin Co., Tallahassee, Florida.
[10] Excorna o.H.G., Mainz, Germany; U. S. representative: C. A. Brinkmann and Company, Inc. (see 2).
[11] Macherey, Nagel & Co., Düren, Rhld., Germany; U. S. representative: C. A. Brinkmann and Company, Inc. (see 2).
[12] H. Reeve Angel & Co., Ltd.; U. S. representative: H. Reeve Angel & Co., Inc., 52 Duane Street, New York 7, New York.

Teichert, Mutschler, and Röchelmeyer (203) impregnated cellulose chromatoplates with a 20% solution of formamide in acetone. Such plates may be useful for TLC of phospholipids (219).

Layers for Reversed-Phase Partition TLC. Thin layers of Silica Gel G can be impregnated with silicone (101), undecane (79) or higher paraffins (80, 229) or with squalene (26). They are then hydrophobic and can be used for the resolution of a lipid class into its individual constituents by reversed-phase partition TLC. Malins and Mangold (101) coated thin layers of Silica Gel G by slowly immersing the plates at room temperature into a 5% solution of silicone (Dow Corning 200 fluid viscosity 10 cs)[13] in diethyl ether. Such a silicone solution is now also commercially available in an aerosol package[4] for impregnating chromatoplates by spraying. Winterstein, Studer, and Rüegg (229) dipped Silica Gel G plates for 2 min. into a 5% solution of paraffin oil in petroleum hydrocarbon. The same procedure was later applied by Kaufmann and Makus (79) and by Kaufmann, Makus, and Deicke (80). These authors also used undecane as a temporary impregnating agent. In all these procedures it is important that the chromatoplates to be impregnated be of the same temperature as the silicone or paraffin solutions and, also, that the plates be immersed into these solutions very slowly. Disregarding these precautions will lead to crumbling of the thin layers. The impregnated plates may be used immediately after evaporation of the solvent.

The author has impregnated thin layers of cellulose[10] and of diatomaceous earth[5] with silicone. Such plates, however, proved to be inferior to impregnated Silica Gel G plates for lipid separations.

Polyethylene powder (Hostalen S)[14] as used in columns (231) has also been used successfully for the preparation of chromatoplates which yield favorable separations of fatty acids or their methyl esters. Hostalen S, 20 g. 250 mesh, is slurried with 50 ml. of aqueous ethanol (96%), 1/1, v/v, and applied to glass plates with a commercial spreader. The chromatoplates are dried at room temperature for several hours and are then ready for use. Davídek and Davídeková (33) have recently reported the preparation of thin layers of polyamide on glass plates, and the separation of flavonoids on such chromatoplates. Stahl (188) mentioned the use of Perlon as a stationary phase in TLC.

Ion Exchangers. Only one publication has appeared so far on the use of ion exchangers in TLC. Randerath (155) used Ecteola Cellulose[12] bound to the glass plates by collodion to separate various purine and pyrimidine nucleotides. Undoubtedly, materials like the various modified cellulose ion exchangers applied on columns by Rouser, Bauman, Kritchevsky, Heller, and O'Brien (162) will also be useful if applied in the TLC technique.

Ion exchange chromatography has so far found little application in the lipid domain. It can be expected that the current wide use of TLC will also lead to extensive utilization of ion exchange materials for the separation of polar lipids.

Various Coating Materials. Urea has been used for separating straight-chain from branched-chain fatty acid in columns (62) and on paper (3). It can be expected that similar separations are possible on

[13] Dow Corning Corp., Midland, Michigan.
[14] Farbwerke Hoechst A. G., Frankfurt-M., Germany.

tion. One ml. of this thiosulfate solution corresponds to 0.00008 gm. of oxygen.

The acid ester used here was prepared from cuttlefish oil and had the properties shown in Table 69.

TABLE 69

PROPERTIES OF ETHYL ESTER OF HIGHLY UNSATURATED FATTY ACID

n_D^{20}	Iodine Value, Wijs	Saponification Value	Non-saponifiable Matter, Per Cent	Peroxide Value, Mg./100 Gm.
1.4815	342.1	168.6	Trace	2

About 50 gm. of this ester was poured into a petri dish and subjected to autoxidation by contact with air at room temperature (2° to 18°C.). Measurement of peroxide was made at a few days' interval over 200 days. The results are illustrated in Fig. 77.

It is interesting to note that during the initial two weeks the formation of peroxide was almost negligible and then increased abruptly. On the 42nd day, it reached the maximum, showing a peroxide value of 4,400 and an iodine value of 208. Then it decreased gradually until the peroxide value became 1,200 and the iodine value 120. In the course of this autoxidation the ester became gradually viscous and yellowish brown. The toxicity of samples corresponding to various phases of autoxidation was surveyed by feeding the rats a diet containing five per cent of the oxidized oil.

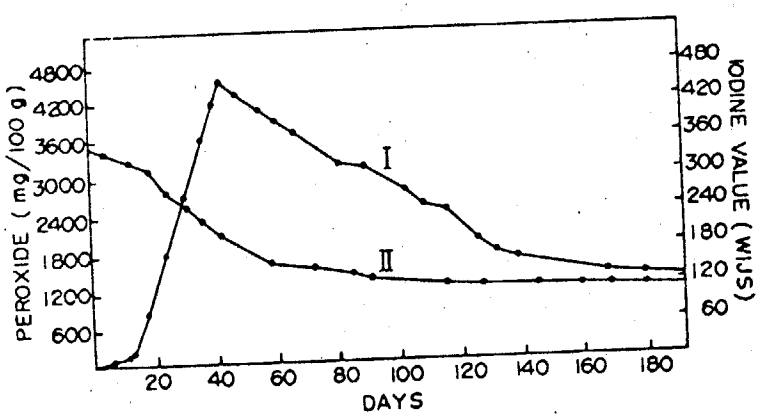


FIG. 77. THE CHANGE OF PEROXIDE (I) AND IODINE VALUES (II) OF THE FATTY ACID ESTER EXPOSED TO AIR AT ROOM TEMPERATURE (2–18°C.)

When the process of autoxidation was allowed to proceed at 30°C., the formation of peroxide was accelerated, but its maximum amount was much reduced compared with that at room temperature (Fig. 78).

At 100°C. the effect of heat was even more apparent, the maximum amount of peroxide being only 240 in this case (Fig. 79). Further, it was noticed that at higher temperatures the decomposition of the peroxide formed occurred more rapidly and thoroughly.

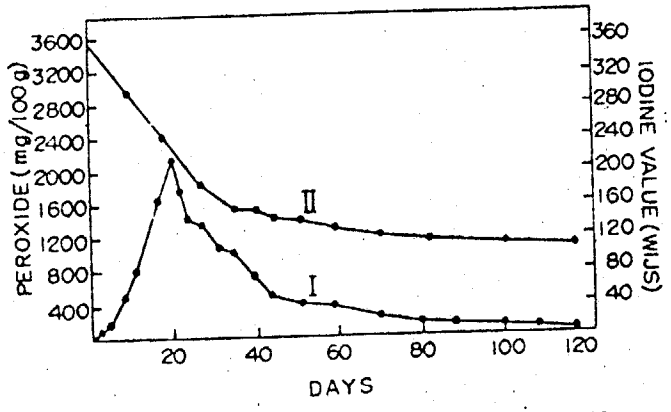


FIG. 78. THE CHANGE OF PEROXIDE (I) AND IODINE VALUES (II) OF THE FATTY ACID EXPOSED TO AIR AT 30°C.

Experiments using rats showed that the toxicity is proportional to the amount of peroxides. In the case of ethyl linolenate and ethyl linoleate, their autoxidized products were also found to be toxic.

When peroxides were removed from the autoxidized product prior to feeding, the rats grew well. As for cuttlefish oil itself, the rats grew normally when they were given unoxidized oil, while autoxidized oil showed toxicity (Figs. 80 and 81).

From the experimental results mentioned above, it may be concluded that the toxic action of fish oil is due to the peroxide formed by autoxidation (Matsuo 1954B).

Meanwhile, no toxicity appeared when peroxide content was 100 mg./100 gm. or less. Crampton *et al.* (1953) are of the same opinion on this point.

Toxicity of Autoxidized Ester Applied to Back Skin of Rats

Autoxidized ethyl ester of highly unsaturated fatty acid (peroxide value: 1,700 mg./100 gm.) was applied in an amount of 1 gm. once each day to a depilated skin area of about 2.5 to 3 cm. in diameter on the back of rats weighing about 80 gm. each. It was found, in this case too,

that all of them died in a few days. Fig. 82 gives the curves depicting the change in the body weight of the rats.

Another experiment was conducted by applying the original ester (not oxidized) to the back skin of rats, expecting that such ester will change into the oxidized ester by undergoing gradual autoxidation and show similar toxicity although it may take a longer time. The result was just as expected. It was found that all rats died in 14 to 19 days in this case, while they died in 7 to 10 days in the case of applying the autoxidized

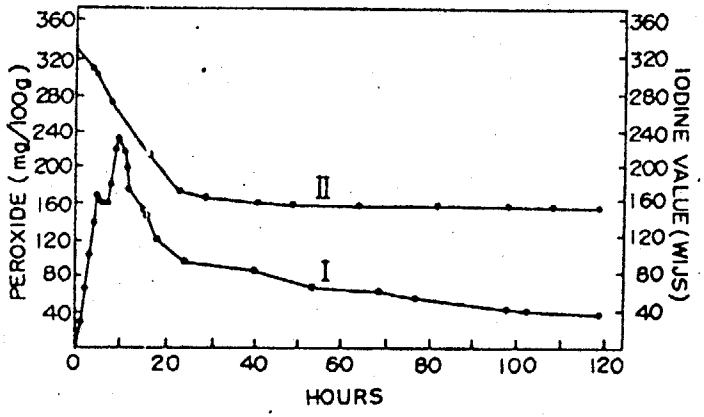


Fig. 79. The Change of Peroxide (I) and Iodine Values (II) of the Fatty Acid Ester Exposed to Air at 100°C.

ester. Curves showing the change in the body weight of the rats in this experiment are given in Fig. 83.

It is notable that toxicity is quite remarkable when the esters are applied on the back skin of the animal, which eventually caused death (Matsuo 1957A).

Effect of Autoxidized Ester on Proteins, Amino Acids, and Enzymes

In order to ascertain the origin of the toxicity, the effect of autoxidized ethyl ester of highly unsaturated fatty acids on proteins, amino acids, and enzymes was examined.

Effect of the Autoxidized Ester on Protein

A mixture of 40 ml. of a two per cent aqueous solution of the ovoalbumin and 5 ml. of the ethyl ester of highly unsaturated fatty acid or its autoxidized product, showing a peroxide value of about 1,800 mg./100 gm., was left at 37°C., with occasional shaking (Fig. 84). The solution to which the original ester (not oxidized) was added showed little noticeable change. The solution to which autoxidized ester was added,

on the other hand, gradually turned yellowish brown. As time passed, the precipitate increased and became more deeply colored. At suitable intervals of time, the precipitate was filtered off and washed thoroughly in water. To the filtrate and the washings, the same volume of a ten per cent aqueous solution of trichloroacetic acid was added, producing a second precipitate.

The appearance of the precipitate I is shown in Fig. 85.

Precipitate II was obtained when an equivalent volume of ten per cent trichloroacetic acid was added to filtrate I, which was obtained by standing at 37°C. and then filtering the ovoalbumin solution to which original

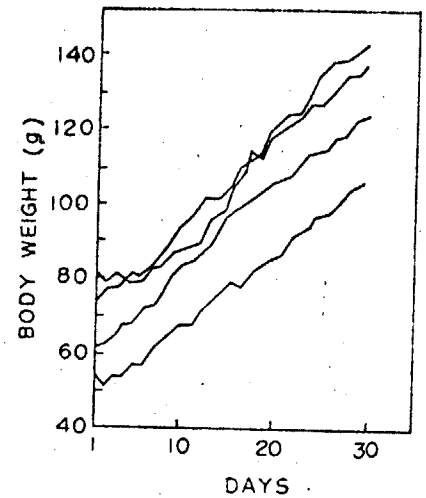


Fig. 80. Growth of Rats Fed on Diet Containing 20 Per Cent Raw Cuttlefish Oil.

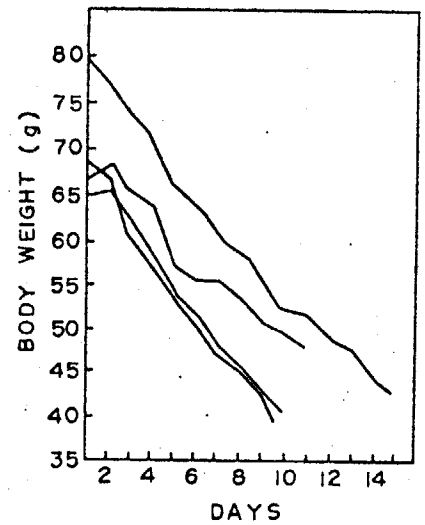


Fig. 81. Growth of Rats Fed on Basal Diet Containing 20 Per Cent Autoxidized Cuttlefish Oil

Peroxide value: 912 mg./100 gm.; all rats died at the end of the curves

ester (not oxidized) was added. On the other hand, there was no precipitation when ten per cent trichloroacetic acid was added to the same volume of filtrate I, which was obtained by standing for over 100 hours at 37°C. and then filtering the ovoalbumin solution to which autoxidized ester had been added. This means that protein, when left for a long time, has been completely denatured and precipitated by the autoxidized ester.

Fig. 86 represents the change in the percentage of the nitrogen content in precipitate I for all nitrogen of the ovoalbumin solution (40 ml.) plotted against time.

When an autoxidized ethyl ester of highly unsaturated fatty acid was added to the aqueous solution of ovoalbumin, almost all the ovoalbumin was denatured and precipitated in about 100 hours. When the original (not oxidized) ester was added, the nitrogen content of the precipitate I was only 6 to 7 per cent. When autoxidized ester was added to the aqueous solution of ovoalbumin, the ratio of the quantity of nitrogen contained in filtrate II to that contained in 40 ml. of the original aqueous solution of the ovoalbumin, as shown in Fig. 87, remained below one per cent, irrespective of a lapse of time.

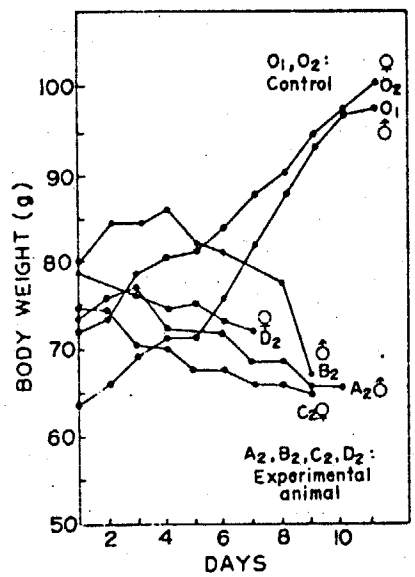


FIG. 82. GROWTH OF RATS WHEN AUTOXIDIZED ETHYL ESTER OF HIGHLY UNSATURATED FATTY ACID WAS APPLIED TO THEIR DEPILATED BACK SKIN ONCE EVERY DAY

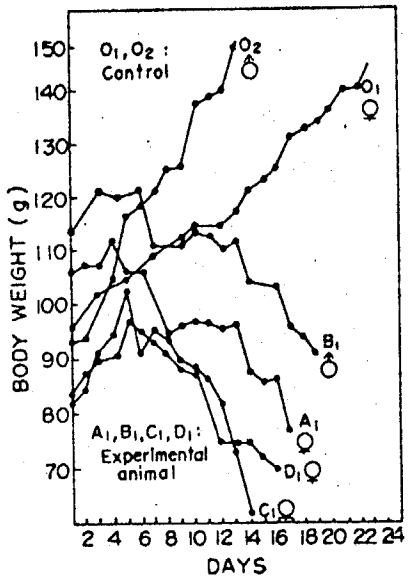


FIG. 83. GROWTH OF RATS WHEN ORIGINAL ESTER (NOT OXIDIZED) WAS APPLIED TO DEPILATED BACK SKIN ONCE EVERY DAY

Terminal amino acid was more or less oxidized, but it became clear that the greater part of ovoalbumin precipitates by forming an adduct with the autoxidized ester (Matsuo 1957B).

The Effect of Autoxidized Fatty Acid Ester on Amino Acids

The autoxidized ethyl ester of highly unsaturated fatty acid shows a notable action on protein as mentioned. In order to investigate its action on amino acids, experiments were conducted using glycine, glycylglycine, and cystine. Cystine, for example, was oxidized into cysteic acid by

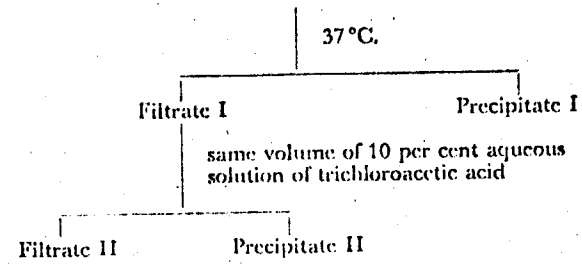
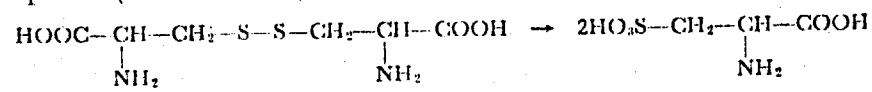


FIG. 84. REACTION OF OVOALBUMIN WITH UNSATURATED FATTY ACID ESTERS

Forty ml. of two per cent aqueous solution of ovoalbumin plus five ml. of the ethyl ester of highly unsaturated fatty acid or its autoxidized product.

autoxidized ester as shown in the following formula with the –S–S– bond ruptured (Matsuo 1957B):



Effect of Autoxidized Ester on Enzymes

As proteins are markedly denatured by autoxidized ester, it is natural to expect that enzymes will also be affected to a greater extent. To confirm this point, experiments were conducted on succinodehydrogenase, salivary amylase, and sweet potato amylase (Matsuo 1957C).

Succinodehydrogenase.—Three rats weighing 132, 202, and 234 gm., respectively, were fed a basal diet containing five per cent of autoxidized ethyl ester of highly unsaturated fatty acid (peroxide value: about 1,800 mg./100 gm.) for a few days. The animals were killed when they became critically weak.

Succinodehydrogenase activity of their muscle and liver was measured and compared with that of the control rats. The results are shown in Table 70, where the time required for decolorization of methylene blue is given.

TABLE 70

COMPARISON OF SUCCINODEHYDROGENASE ACTIVITIES OF MUSCLE AND LIVER OF RATS FED WITH AUTOXIDIZED ETHYL ESTER OF HIGHLY UNSATURATED FATTY ACID WITH THOSE OF CONTROL RATS

Rat No.		I	II	III	Control I	Control II
Time required for decolorization of methylene blue	Muscle	15′20″	17′0″	17′20″	8′0″	8′40″
	Liver	16′30″	17′20″	16′40″	7′10″	7′0″

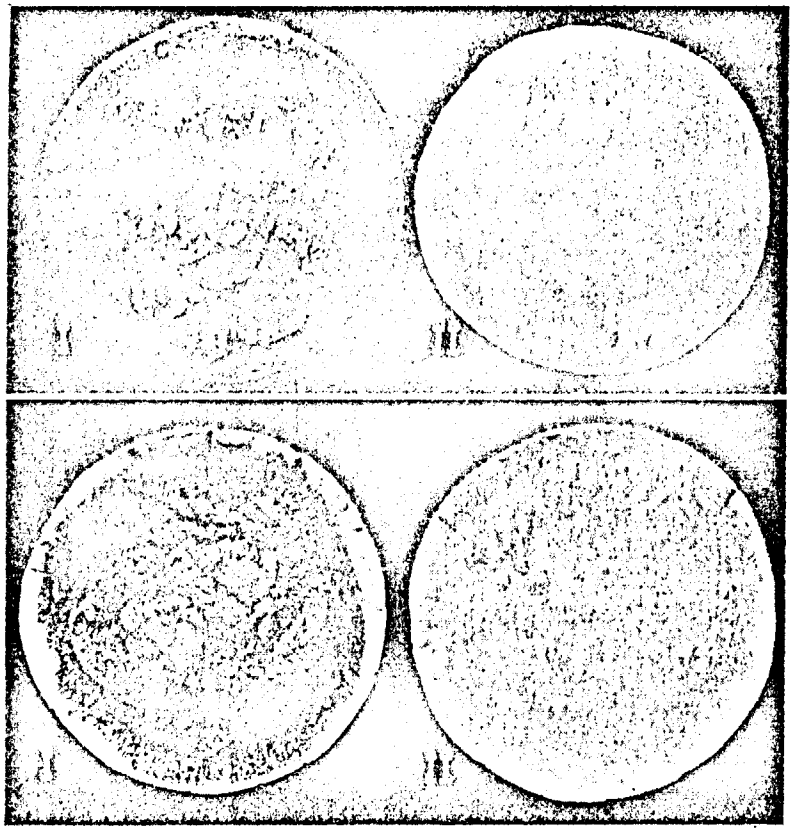


FIG. 85. DENATURATION OF PROTEIN BY AUTOXIDIZED ETHYL ESTER OF HIGHLY UNSATURATED FATTY ACID

Upper figure after 51 hrs.
Lower figure after 75 hrs.

Salivary Amylase.—Three kinds of solutions of the following compositions (in ml.) were prepared:

	Buffer Solution pH 6.81	Saliva	Oxidized Ester (Peroxide Value: 2152)	Original Ester	Water
I	20	5	5	—	—
II	20	5	—	5	—
III	20	5	—	—	5

The above-mentioned mixtures were thoroughly shaken and kept at 37°C. At regular intervals, 0.5 ml. of a one per cent starch solution was added to each 2.5 ml. aliquot of these mixtures, left for 30 minutes at

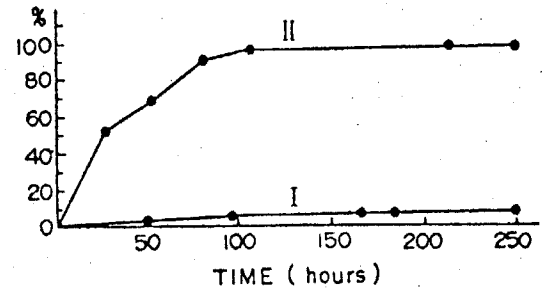


FIG. 86. NITROGEN CONTENT OF PRECIPITATE I

I: Ovoalbumin solution plus ethyl ester of highly unsaturated fatty acid. II: Ovoalbumin solution plus autoxidized ethyl ester of highly unsaturated Fatty acid.

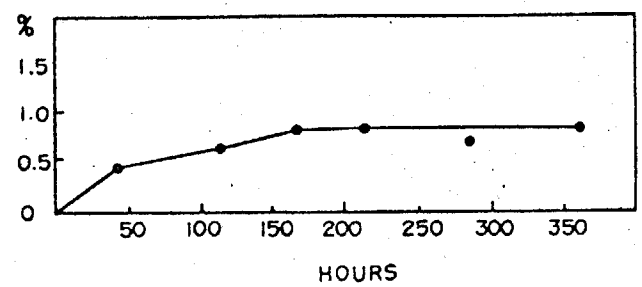


FIG. 87. NITROGEN CONTENT IN FILTRATE II WHEN AUTOXIDIZED ESTER IS ADDED

37°C., and each solution was subjected to examination by the iodine-starch reaction.

In the case of II and III, in which original (not oxidized) ester or distilled water was added, as shown in Table 71, salivary amylase activity was noticed even after a lapse of 20 hours. When oxidized ester (peroxide value 2,152 mg./100 gm.) was added, on the other hand, disappearance of the activity of salivary amylase was evident 22 hours after the mixing.

The same results were obtained with sweet potato amylase.

Pathological Studies.—An attempt was then made to determine whether autoxidized ethyl esters of highly unsaturated fatty acid are as toxic to rabbits as they are to rats.

Once a day, 0.8 ml. of autoxidized ester was given orally to four healthy rabbits each weighing about 1.5 kg. As shown in Table 72, all these rabbits died. Upon autopsy, it was found that the mucous membrane of the stomach was seriously affected and in one a stomach ulcer had formed (Fig. 88).

TABLE 71

SCHEMATIC REPRESENTATION OF LAPSE OF TIME AND IODINE-STARCH REACTION WHEN 5 ML. OXIDIZED ESTER OR ORIGINAL ESTER IS MIXED WITH 5 ML. SALIVA AND LEFT AT 37°C.

Time Elapsed, Hours	I	II	III
10	−	−	−
14	−	−	−
18	−	−	−
22	+	−	−
26	+	−	−

Plus and minus signs represent positive and negative iodine starch reaction, respectively.

TABLE 72

FEEDING RECORD OF RABBITS FED AUTOXIDIZED ESTERS

	Sex	Initial Weight, Gm.	Final Weight, Gm.	Wt. Decreased, Gm.	
A_1	♀	1500	1290	−210	Died on 5th day
A_2	♀	1570	1350	−220	Died on 12th day
A_3	♂	1500	1520	+ 20	Died on 15th day
A_4	♂	1720	1575	−145	Died on 5th day

The intestines were found to be less pliable and apt to break when stretched.

An autopsy on the rabbits revealed that fairly severe deterioration and destruction had occurred in the mucous membranes of the stomach and intestine. This finding suggests that the unsaturated fatty acids, when autoxidized, become a protein poison.

Detailed pathological examination of the internal organs of the rats fed with autoxidized ester is being made by Professor Ogata and others (1955) in the Department of Pathology, Faculty of Medicine, Tokushima University, Japan.

Toxicity of Thermally Polymerized Fish Oils

Sakurai *et al.* (1951) conducted research on the nutritive value of whale oil and found that the polymerized oil of the fin whale (*Balaenoptera physalus*) showed considerable toxicity when rats were fed a diet containing this oil at a level of 20 per cent. In this case, the rats died in about three weeks, but if the percentage content is reduced to below ten per cent they grew well, utilizing the oil as a caloric source without developing any undesirable symptoms.

Crampton *et al.* (1951A, 1951B, 1951C, 1953, 1956) reported that heat-polymerized oil is toxic to rats despite the extremely low peroxide content. Kaunitz *et al.* (1955, 1956A, 1956B) also announced that the oil obtained by heating cottonseed oil at 95°C. for 200 to 300 hours was toxic to rats.

FIG. 88. INSIDE OF THE STOMACH OF A RABBIT WHICH DIED AFTER TAKING 0.8 ML. OF THE AUTOXIDIZED ESTER ONCE EVERY DAY

The coats of the stomach are ulcerous at the points marked a and b.

Johnson *et al.* (1956, 1957A, 1957B) and Perkins and Kummerow (1959A, 1959B) also reported on the physiological effects of feeding polymerized oils.

Judging from the mechanism of heat polymerization, it is believed that the degree of unsaturation of oil is most closely related to polymerization. Since fish oil is more unsaturated than vegetable oil, it may polymerize more easily. Examinations were therefore made to see whether such a product from refined cuttlefish oil is toxic to rats.

Toxicity of Thermally Polymerized Cuttlefish Oil

The cuttlefish oil used in this experiment is a light yellow, transparent liquid, whose properties are shown in Table 73.

Fig. 89 gives the growth curves of rats fed the basal diet shown in Table 74. All curves show normal growth. When the experiment was over, the

TABLE 73

PROPERTIES OF RAW CUTTLEFISH OIL

n_D^{20}	Iodine value, Wijs	Saponification Value	Acid Value	Peroxide value, Mg./100 Gm.	Molecular weight, Rast
1.4847	194.6	180.2	3.31	0	680

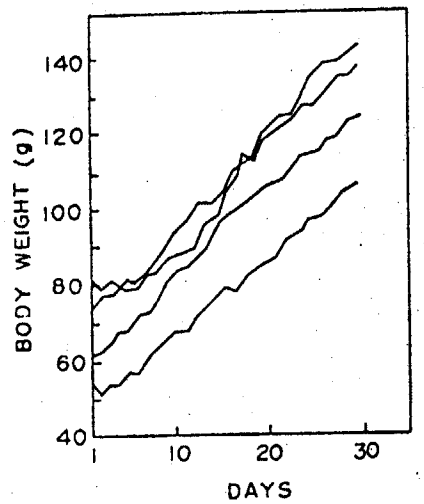


FIG. 89. GROWTH OF RATS FED ON BASAL DIET CONTAINING 20 PER CENT RAW CUTTLEFISH OIL

peroxide value of the cuttlefish oil used was measured. The increase was found to be extremely small.

TABLE 74

COMPOSITION OF BASAL DIET

	Per Cent
Starch (rice powder)	65
Casein (ether extracted)	9
McCollum salt mixture	3
Yeast	3
Liver oil	One drop a day
Sample oil	20

Raw cuttlefish oil was heated at 250°C. for ten hours in an oil bath in a stream of carbon dioxide. Some properties of the polymerized oil are shown in Table 75.

Animal experiments were conducted using rats each weighing about 60 to 80 gm. They were fed the same basal diet but containing 20 per cent of

TABLE 75

PROPERTIES OF THE POLYMERIZED OIL HEATED AT 250°C. FOR TEN HOURS

n_D^{20}	Iodine Value, Wijs	Saponification Value	Acid Value	Peroxide Value, Mg./100 Gm.	Molecular Weight, Rast
1.4947	121.9	171.1	2.42	0	1320

polymerized oil (Table 75). All the rats succumbed within 15 days after the start of the experiment (Fig. 90).

When rats were fed on a basal diet containing five per cent of thermally polymerized oil, no particular toxicity appeared, though the nutritive value of the oil was somewhat lower than the original cuttlefish oil.

Extensive research under way in Japan and abroad demonstrated the intra- and inter-molecular cyclic compounds are formed in the course of thermal polymerization.

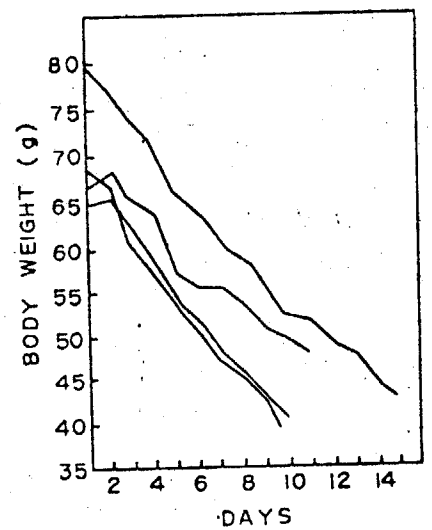


FIG. 90. GROWTH OF RATS FED ON BASAL DIET CONTAINING 20 PER CENT OF POLYMERIZED OIL HEATED AT 250°C. FOR TEN HOURS

The ethyl ester was prepared by ethanolysis of the oil thermally polymerized in a carbon dioxide stream at 250°C. for ten hours. An animal experiment using rats was conducted on straight-chain and cyclic esters separated from this ethyl ester by the urea-adduct method. The results confirm those reported by Crampton and others, that when rats were given 20 per cent of straight-chain ester they grew well (Fig. 91), while feeding with 20 per cent of cyclic ester resulted in marked loss of weight. In the latter case, all the rats died in 2 to 7 days (Fig. 92). Similar results were obtained when only ten per cent was fed (Matsuo 1957C).

The properties of the straight-chain and cyclic esters are shown in Table 76.

Experiments using cuttlefish oil which had been thermally polymerized in air at 250°C. for ten hours give similar results (Matsuo 1958).

TABLE 76

PROPERTIES OF STRAIGHT-CHAIN AND CYCLIC ESTERS

	n_D^{20}	Iodine Value, Wijs	Saponification Value	Acid Value	Peroxide Value, Mg./100 Gm.	Molecular Weight, Rast
Straight-chain ethyl ester	1.4624	75.1	170.7	0.36	0	330
Cyclic ethyl ester	1.5059	175.2	148.3	1.30	0	380

Toxicity of Thermally Polymerized Ethyl Linolenate

For the purpose of elucidating the properties and structure of cyclic products, experiments were designed to investigate the toxicity or thermally polymerized ethyl linolenate (Matsuo 1959A).

Separation of Linolenic Acid from Linseed Oil.—Refined linseed oil was saponified by the ordinary method and the separated fatty acid was dis-

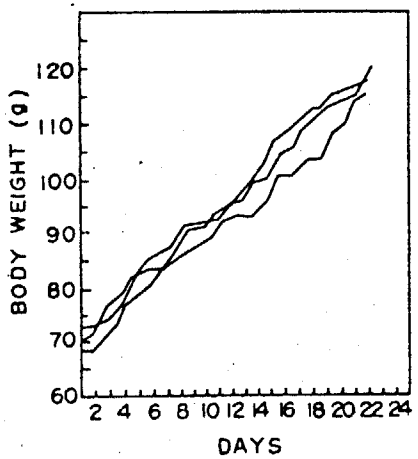


FIG. 91. GROWTH OF RATS FED ON BASAL DIET CONTAINING 20 PER CENT OF STRAIGHT CHAIN ESTER

solved in ether. Bromine was dropped into the cooled ether solution. The precipitated bromide was collected by filtration, washed in cold ether, and dried. For purification, the bromide was dissolved in a large quantity of benzene and the solution was boiled on a hot water-bath. The hexabromide of linolenic acid was obtained as a white precipitate on cooling. The precipitate was collected, washed in cold ether, and dried. The hexabromide thus obtained was treated with zinc, ethanol, and hydrochloric acid to effect simultaneous debromination and esterification.

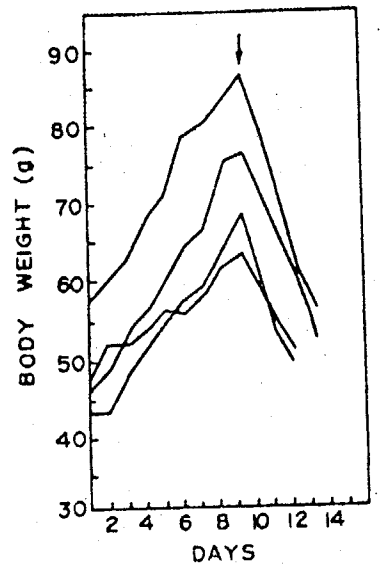


FIG. 92. GROWTH OF RATS FED ON BASAL DIET CONTAINING 20 PER CENT OF CYCLIC ESTER

All rats died at the end of the curves.

The ester mixture was distilled in vacuum (1.5 mm. Hg) and the fraction boiling at 167° to 170°C. was used in this experiment. The properties of ethyl linolenate and linseed oil are shown in Table 77.

TABLE 77

PROPERTIES OF LINSEED OIL AND ETHYL LINOLENATE

	n_D^{20}	Iodine Value, Wijs	Saponification Value	Acid Value	Molecular Weight, Rast
Linseed oil	1.4833	191.8	186.9	0	—
Ethyl linolenate	1.4688	242.5	183.1	1.0	302

Thermal Polymerization of Ethyl Linolenate and Separation of Cyclic Monomer.—Thermal polymerization of ethyl linolenate was carried out by heating at 250°C. in a stream of carbon dioxide for 40 hours. During the process, the iodine value decreased from 242.5 to 157.5, as shown in Fig. 93.

Straight-chain ester and cyclic esters of the polymerized ethyl linolenate were separated by the urea-adduct method. The yields of the straight-chain ester and the cyclic ester were 21.2 per cent and 63.3 per cent

The author expresses his heartfelt thanks to Dr. Keizo Kodama, President of Tokushima University, for his continuous guidance throhgh the course of this research.

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[Tokushima Journal of Experimental Medicine, 8, 90-95; 1961]

STUDIES ON THE TOXICITY OF FISH OIL (III)*

TOXICITY OF AUTOXIDIZED HIGHLY UNSATURATED FATTY ACID ETHYL ESTER APPLIED TO RAT

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(Received for publication, April 28, 1961)

In the preceding papers[1,2], the author reported that the ethyl ester of highly unsaturated fatty acid separated from cuttlefish oil showed as high nutritive value as that of oleic acid ester, while the said ethyl ester autoxidized in the air showed severe toxicity in accordance with the amount of peroxide formed.

This paper will describe the results of experiments on the toxicity shown when low boiling-point fractions of the ethyl ester of highly unsaturated fatty acids obtained from cuttle-fish oil are oxidized and applied to the back skin of rats or given orally to rabbits.

Experimental and Results

The raw cuttle-fish oil was treated by the soda-acetone process[3] and fractionated by distillation under 2 mm Hg. The fractions and their properties are indicated in Table I.

Table I. Properties of Fraction of Highly Unsaturated Fatty Acid Ethyl Ester

Fraction No.	Temp. C/2 mm Hg	%	% for Raw Oil	Iodine Value (Wijs)	n_D^{20}	Sap. Value
1.	~180*	10.8	3.0	232.3	1.4652	179.2
2	180~192	16.0	4.4	276.3	1.4700	176.0
3	192~205	21.3	5.8	320.0	1.4771	168.7
4	205~215	20.0	5.5	358.7	1.4823	163.9
5	215~227	19.6	5.4	384.9	1.4861	160.2
6	227~	5.0	1.4	386.1	1.4902	155.7
7	Residue**	7.3	2.0	—	—	—

* Temp. of first drop: 140 C ** Solid at room temp.

The first fraction was subjected to autoxidation in the air at 7~13°C. As the result, the iodine value decreased from 232.3 to 156.6 and the peroxide value

* A brief account of this paper was reported at the Kanto regional meeting of the Japan Biochemical Society held in November, 1954. The full text written in Japanese appeared in *Seikagaku*, 29, 769 (1958).

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increased from 0 to 1461 mg per 100 g after a lapse of 22 days. The properties of the autoxidized ester are shown in Table II.

Table II. Properties of the Autoxidized Highly Unsaturated Fatty Acid Ethyl Ester

n_D^{20}	Iodine Value (Wijs)	Sap. Value	Unsap. Matter (%)	Peroxide (mg/100 g)
1.4727	156.6	190.3	Trace	1461

The effect of this autoxidized ester on the growth of rat was tested by feeding the animal on the diet with the following composition.

Starch (rice powder)	80%	Yeast	3%
Casein (ether extracted)	9%	Liver oil	1 drop/day
McCollum salt mixture	3%	Autoxidized fatty acid ester or ethyl oleate	5%

As the result, the white hair of the rats turned yellowish brown, and they all died in a few days. The growth curves are given in Fig. 1.

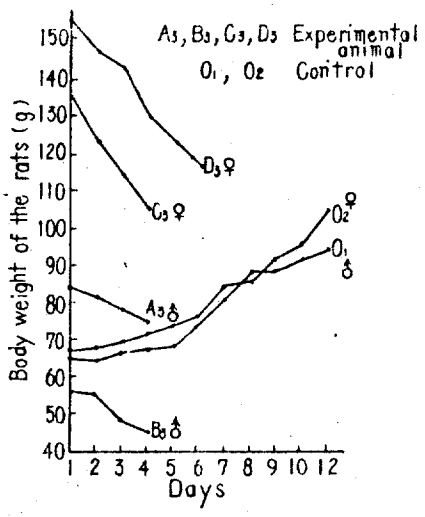

Fig. 1. Growth curves of rats fed on diet containing 5 per cent of autoxidized product of No. 1 fraction.
Control: 5 per cent of non-oxidized No. 1 fraction.

Another sample of autoxidized fatty acid ester containing 1716 mg of peroxide per 100 g as a result of autoxidation in the air at 7~13°C for 25 days, was applied in an amount of 1 g once every day to a depilated skin area of about 2.5 to 3 cm in diameter on the back of rats weighing about 80 g each. It was found, in this case too, that the hair of these rats turned yellow and that all of them died in a few days.

Fig. 2 shows an example of a rat which died in this way, and Fig. 3 gives curves depicting the change in the body weight of the rats.

Fig. 2. Rat which died after autoxidized ethyl ester of highly unsaturated fatty acid was applied to back skin in an amount of 1 g once every day.

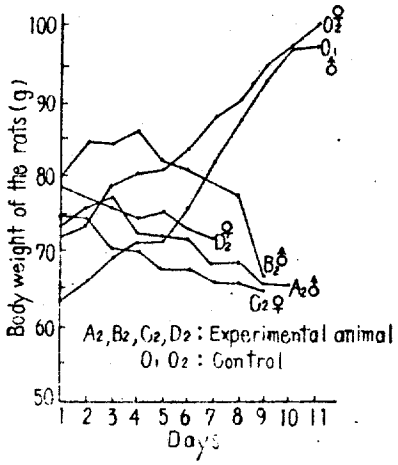

Fig. 3. Change in the body weight of the rats when autoxidized ethyl ester of highly unsaturated fatty acid was applied to back skin once every day.
(Diet containing 5 per cent of ethyl oleate).

Next the author conducted another experiment by applying original ester (not oxidized) to the back skin of rats, expecting that such ester will change into the oxidized ester by undergoing gradual autoxidation and show toxicity alike, although it may take a long time.

The result was just as expected. It was found that all rats died in 14 to 19 days in this case, while they died in 7 to 10 days in the case of applying the

autoxidized ester. Fig. 4 shows an example of a rat a few hours before it died in this way.

It is notable that the whole body appears quite seboraceous. Curves showing the change in the body weight of the rats are given in Fig. 5.

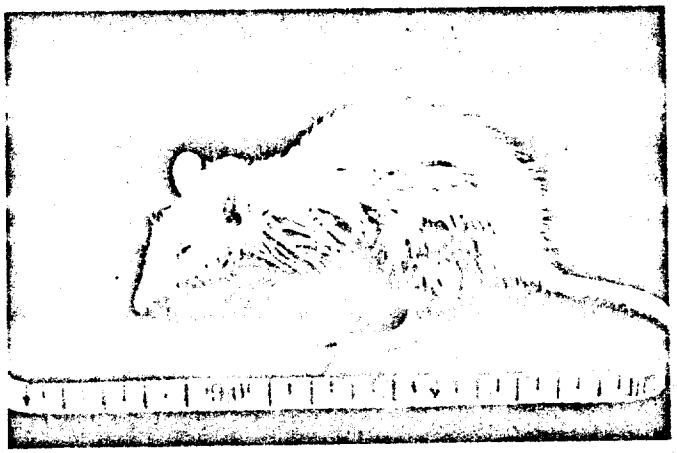

Fig. 4. A rat a few hours before it died after having 1 g of original ester applied to back skin once every day.

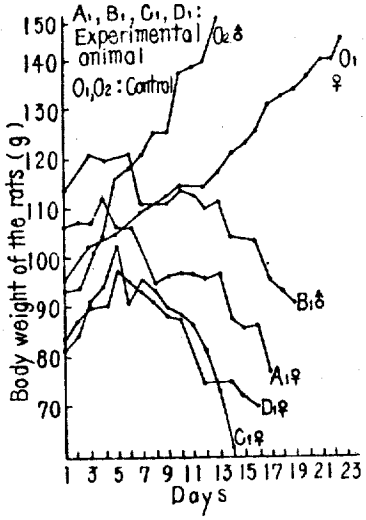

Fig. 5. Change in the body weight of rats when 1 g of original ester was applied to back skin once every day. (Diet containing 5 per cent of ethyl oleate).

The author next attempted to determine whether autoxidized ethyl ester of highly unsaturated fatty acid will show such toxicity to rabbits as to rats.

Once a day, 0.8 ml of autoxidized ester was given by mouth to each healthy

rabbit weighing about 1.5 kg. As shown in Table III, all these rabbits were found to die. Upon autopsy of the carcasses, it was found that mucous membrane of the stomach had been seriously affected and in one of them a sort of stomach ulcer occured (see Fig. 6).

The intestines were found to be less flexible. It was liable to be torn off when stretched. The liver partially turned yellowish brown in color.

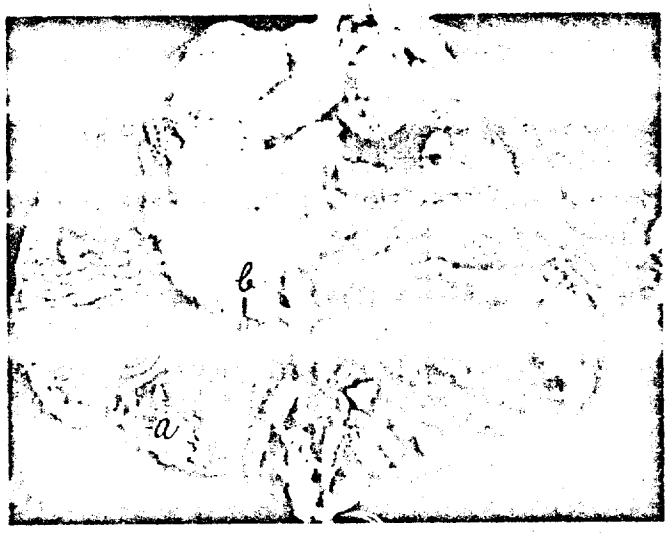

Fig. 6. Inside of the stomach of a rabbit which died after taking 0.8 ml of the autoxidized ester once every day. The coats of the stomach are ulcerous at the points marked a and b.

Table III. Feeding Record of the Rabbits

	Sex	Initial Weight (g)	Final Weight (g)	Wt Decreased (g)	
A	♂	1500	1290	−210	died on 5th day
A_2	♀	1570	1350	−220	died on 12th day
A_3	♂	1500	1520	+ 20	died on 15th day
A_4	♀	1720	1575	−145	died on 5th day

Discussion

The soda-acetone process[3] is an efficient method for obtaining a good yield of highly unsaturated fatty acids. As pointed out by Toyama and Tsuchiya[4], however, the product naturally contains acids of two or three double bonds.

According to Tsujino's report[5], cuttle-fish oil contains a small amount of linolic and linolenic acid. The fatty acid esters obtained as the No. 1 fraction in this experiment are considered to be a mixture of ethyl linolate and linolenate, judging from their iodine value. The iodine values of ethyl linolenate and ethyl

linolate are 247.4 and 164.9 respectively. The iodine value of the No. 1 fraction was 232.8, just between the above two values.

It is rather unexpected that the linolenate and linolate show such a high toxicity when they are autoxidized, as they are claimed to be essential for living organism. Further it is notable that the toxicity is quite apparent when the esters are applied to the back skin of the animal, bringing finally to death 7-10 days after application.

The cause of death is not clear at present, but the autopsy on rabbit as mentioned above revealed that fairly severe deterioration and destruction occurred in mucous membrane of stomach and intestine. This finding suggests that the unsaturated fatty acids, when autoxidized, act as protein poison.

Summary

1. It was made clear that the ethyl ester of unsaturated fatty acid of low iodine value, when autoxidized and made thereby to contain a large amount of peroxide, shows as marked toxicity as in the case of highly unsaturated fatty acid.

2. When nonoxidized ester was applied to the back skin of rat, toxicity was also apparent as in the case of the autoxidized ester, though a little longer time was taken for its manifestation.

3. When 0.8 ml of the autoxidized ester was administered orally to rabbit once every day, the animal succumbed after a few days. It was found by dissection that stomach, intestines and liver suffered profound damage.

The author expresses his heartful thanks to Dr. Keizo Kodama, President of Tokushima University, for his continuous guidance through the course of this research.

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SYMPOSIUM ON FOODS: LIPIDS AND THEIR OXIDATION
AVI PUBLISHING CO; WESTPORT, CONN. 1962

CHAPTER 17

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Nutritional Effects of Oxidized and Thermally Polymerized Fish Oils

The fish oils obtained from sardines, cuttlefish, mackerel, etc. are extremely unstable to oxidation and heating, since they contain larger amounts of the glycerides of the highly unsaturated fatty acids. Recent research made it clear that autoxidized or thermally polymerized fish oil shows acute toxicity as compared with other oils and fats.

This paper deals with the toxicity of autoxidized fish oil and of thermally polymerized fish oil, with special reference to the results of the research the author conducted primarily on cuttlefish oil.

TOXICITY OF AUTOXIDIZED FISH OILS

Detailed studies were made by Ozaki (1926A, 1926B, 1927A, 1927B, 1932) on the nutritive values of various kinds of fatty acids. He reached the conclusion that highly unsaturated fatty acids are toxic and that such toxicity is attributable to the presence of many double bonds. Accordingly, the toxicity of fish oils was thought to be due to the highly unsaturated fatty acids contained in the fish oils.

It was later made clear that autoxidized oil destroys vitamins (Holman 1941; Kaunitz *et al.* 1952) and inhibits some kinds of enzymatic actions (Bernheim *et al.* 1952; Ottolenghi *et al.* 1955). Kaneda *et al.* (1953, 1954A, 1954B), of the Higashi Laboratory of the Tokai Regional Fishery Research Institute, fed rats the ethyl esters of highly unsaturated fatty acids separated from sardines, taking strict care to prevent autoxidation, and reported that no toxicity was found, but that when the esters were once autoxidized simply by being exposed to air, they became toxic. This fact was confirmed with highly unsaturated fatty acids separated from cuttlefish oil, and an attempt was made to locate the origin of toxicity in the autoxidized products, with reference to the biological effect of the toxic substances and their relationship to proteins, amino acids, enzymes, etc.

Oils, especially fish oils, which contain such highly unsaturated fatty acids in the form of glycerides, as shown in Table 63, are unstable to autoxidation and heating.

Toxicity of Autoxidized Ethyl Ester of Highly Unsaturated Fatty Acids

The properties of the cuttlefish oil used are shown in Table 64.

The highly unsaturated fatty acids were separated by the soda acetone

TABLE 63

HIGHLY UNSATURATED FATTY ACIDS DERIVED FROM FISH OILS

Molecular Formula	Fatty Acid	Number of Double Bonds	Source
$C_{18}H_{32}O_2$	Moroctic acid	4	Sardine oil
$C_{20}H_{32}O_2$	Arachidonic acid	4	Sardine and whale oil
$C_{22}H_{34}O_2$	Clupanodonic acid	5	Sardine oil
$C_{24}H_{38}O_2$	Scolidonic acid	5	Tuna oil
$C_{26}H_{42}O_2$	Shibinic acid	5	Tuna oil
$C_{24}H_{36}O_2$	Nisinic acid	6	Sardine oil
$C_{26}H_{40}O_2$	Thynnic acid	6	Tuna oil

TABLE 64

PROPERTIES OF RAW CUTTLEFISH OIL

d_4^{15}	Iodine Value (Wijs)	Acid Value	Saponification Value	Non-saponifiable matter, Per Cent
0.9305	182.5	2.03	189.3	2.19

TABLE 65

PROPERTIES OF THE FRACTIONS OF HIGHLY UNSATURATED FATTY ACIDS (ETHYL ESTERS)

Fraction No.	Temperature, °C./2 mm. Hg	Yield, Per cent	Yield, Per cent of Raw Oil	Iodine Value, Wijs	n_D^{20}	Saponification Value
1	–180[1]	10.8	3.0	232.3	1.4652	179.2
2	180–192	16.0	4.4	276.3	1.4700	176.0
3	192–205	21.3	5.8	320.0	1.4771	168.7
4	205–215	20.0	5.5	358.7	1.4823	163.9
5	215–227	19.5	5.4	384.9	1.4861	160.2
6	227–	5.0	1.4	386.1	1.4902	155.7
7	Residue[2]	7.3	2.0	—	—	—

[1] Temp. of the first drop of the distillate: 140°C.
[2] Solid at room temperature.

process (Toyama and Tsuchiya 1925) as shown in Fig. 71. The properties of fractions of ethyl ester of highly unsaturated fatty acids are shown in Table 65.

Raw cuttlefish oil was saponified with an ethanolic solution of sodium hydroxide, mixed with acetone, left overnight at 0° to –10°C. and filtered. From the filtrate, which contained acetone-soluble sodium salts of highly unsaturated acids, acetone was distilled off and its residue was decomposed by dilute hydrochloric acid.

The free acids were dissolved in petroleum ether (b.p. below 65°C.); the solution was washed several times with water and dried over anhydrous sodium sulfate. Petroleum ether was then distilled off, and highly unsaturated acids were left as the residue.

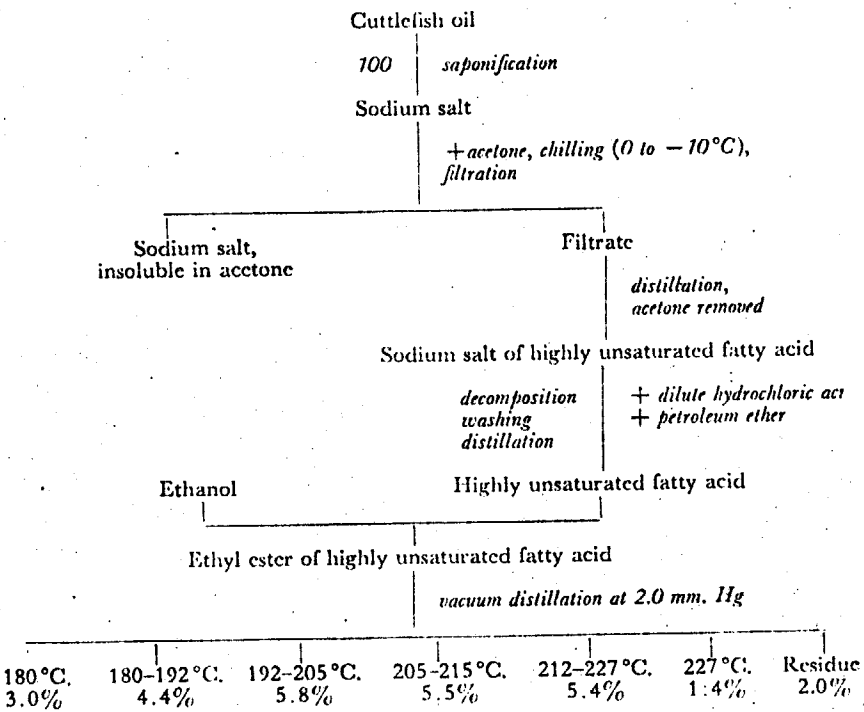

FIG. 71. SEPARATION OF HIGHLY UNSATURATED FATTY ACIDS BY SODA-ACETONE METHOD

Since the sample contained some unsaponifiable matter which was present in the original oil, it was desirable to remove as much of it as possible. In order to accomplish this, the sample was dissolved in twice its volume of absolute ethanol containing 2.5 per cent hydrochloric acid, and heated on a water bath for about three hours. By this process the ethyl esters of the acids were prepared. The ethyl esters were fractionated in a vacuum at 2 mm. of Hg at various temperatures. The fraction with a b.p. 205° to 215°C. was chiefly used for the experiment. This fraction had a light yellow color and no odor. Since these esters are liable to be rapidly oxidized when they are in contact with air, special precautions were taken throughout the process.

The container of the esters was always filled with carbon dioxide gas and was kept in dry ice during the experiment. An aliquot of the sample thus prepared was put in a petri dish, and was brought into contact with air at room temperature. As the oxidation proceeded, the esters developed a light yellow color. At a certain point of oxidation the reaction was stopped and the ester was again kept in dry ice to avoid further change.

Ethyl oleate was used as control. Some chemical properties of the esters and of their oxidized compounds are shown in Table 66 together with those of oleic acid ester for comparison.

TABLE 66

ANALYSIS OF THE SAMPLES USED IN EXPERIMENT

	d_4^{15}	n_D^{20}	Iodine Value	Saponification Value	Non-saponifiable Matter
Ethyl esters of highly unsaturated fatty acids	—	1.4823	358.70	163.90	Trace
Oxidized esters of highly unsaturated fatty acids	0.9568	—	264.82	201.49	Trace
Ethyl oleate	0.8822	—	82.96	187.69	0

Rats of nearly the same size and weight (about 60 gm.) were divided into three groups, each comprising four rats. The first group was given the feed containing five per cent of unsaturated fatty acid esters in basal diet, that of the oxidized esters to the second group, and that of oleic acid ester, which was considered as the control, to the third. The composition of the basal diet is given in Table 67.

TABLE 67

COMPOSITION OF BASAL DIET[1]

	Per Cent
Starch (rice powder)	80
Casein (ether extracted)	9
McCollum salt mixture	3
Yeast	3
Liver oil	One drop a day
Ester	5

[1] Total amount of diet per day was 10 gm. (from 1st to 5th day), 12.5 gm. (from 6th to 10th day), 15 gm. (from 11th to 30th day).

The experimental results are recorded in Table 68 and also illustrated in the accompanying graph (Fig. 72) and in the photographs (Fig. 73, 74, and 75) (Matsuo 1954A). It is apparent that the rats fed on the diet containing unsaturated fatty acid ester showed as satisfactory a growth as those fed on the oleic acid ester diet, but it is remarkable that all rats fed on the oxidized ester diet not only showed retarded growth but died within a few days after the beginning of the experiment. It was also noticed in this group that the white fur had turned brown.

The effect of oxidized esters in causing mild or chronic intoxication was investigated. Mature rats were fed diets containing five per cent oxidized esters, not daily but at frequent intervals.

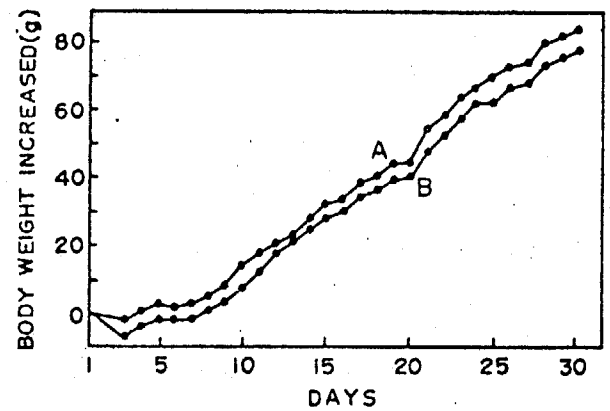

FIG. 72. THE CURVE OF AVERAGE BODY WEIGHT INCREASE

A (Control): Average body weight increment of the rats fed on the diet containing five per cent of ethyl oleate. B: That of rats fed on the diet containing five per cent of the ethyl ester of highly unsaturated fatty acids.

One of the typical examples is illustrated in Fig. 76. The appearance of the animal was quite peculiar. Fur on the face was depilated, the mouth and rear legs were swollen, the remaining fur changed into brown color, and hemorrhages were seen here and there on the skin.

Kaunitz *et al.* (1952) observed that rats developed a flavin-deficiency when rancid lard was given, but it seems that the toxic symptoms in the present case are too acute to be ascribed solely to riboflavin deficiency.

TABLE 68

FEEDING RECORD OF RATS FED UNSATURATED FATTY ESTERS

		Increase of Body Weight, Gm.					
		10th Day		20th Day		30th Day	
Kind of Ester Fed	Sex		Average		Average		Average
Ethyl oleate	♀	+11.0	+13.1	+34.0	+43.3	+ 61.0	+82.1
	♂	+14.0		+51.2		+113.5	
	♀	+15.0		+40.5		+ 70.8	
	♀	+12.5		+47.5		+ 83.0	
Ethyl ester of unsaturated fatty acid	♂	+ 7.7	+ 6.3	+41.0	+38.1	+ 85.0	+75.8
	♀	+ 7.0		+38.5		+ 77.0	
	♂	+ 7.0		+44.5		+ 75.0	
	♀	+ 3.2		+28.5		+ 66.0	
Oxidized form of the above ester	♀	− 3.8 (died on 6th day)					
	♀	− 5.0 (died on 7th day)					
	♀	− 5.0 (died on 8th day)					
	♂	− 8.5 (died on 3rd day)					

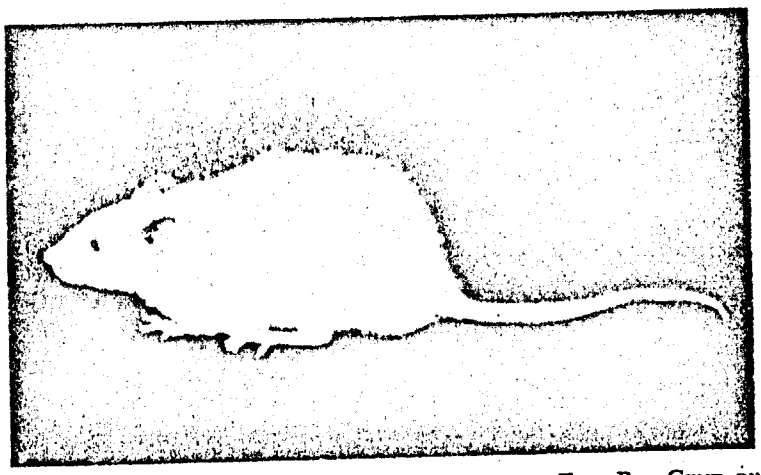

FIG. 73. THE RAT FED ON THE DIET CONTAINING FIVE PER CENT OF ETHYL OLEATE

Control, on the 27th day after the start of the experiment.

FIG. 74. THE RAT FED ON THE DIET CONTAINING FIVE PER CENT OF THE ETHYL ESTER OF HIGHLY UNSATURATED ACID

On the 27th day after the start of the experiment.

Peroxide Formation and Its Relation to Toxicity

Since it semed most likely that the toxicity is related to the formation of peroxide by the autoxidation of unsaturated fatty acid, the amount of peroxide was determined.

The determination of peroxide was carried out following the Wheeler's method as improved by Nakamura (1937): A suitable amount of the sample, usually 0.1 to 0.5 gm., was weighed accurately into a conical flask

FIG. 75. THE RAT FED ON THE DIET CONTAINING FIVE PER CENT OF THE OXIDIZED ETHYL ESTER OF HIGHLY UNSATURATED ACID

On the 4th day after the start of the experiment.

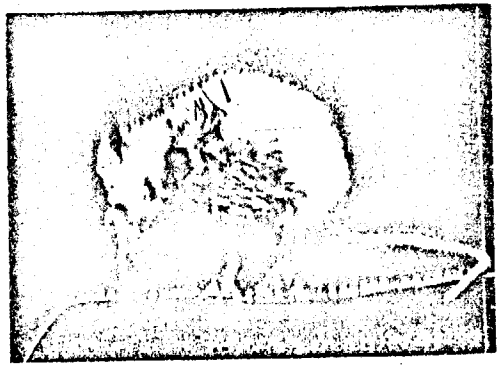

FIG. 76. TYPICAL EXAMPLE OF EFFECT OF FEEDING OXIDIZED ESTERS TO MATURE RATS

Same animal in both photographs.

of 300-ml. capacity; mixed with 20 ml. of carbon tetrachloride, 30 ml. of glacial acetic acid, and 1 ml. of saturated potassium iodide solution. After shaking for one minute, 100 ml. of distilled water were added and the free iodine liberated in the solution was titrated with 0.01N thiosulfate sol

599

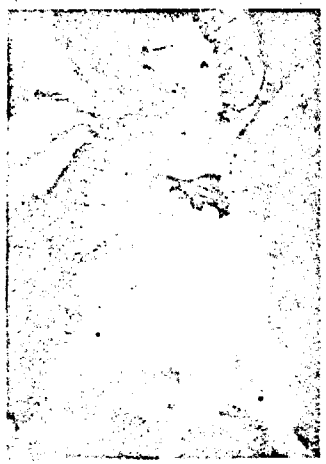

Fig. 6

Fig. 7

as human nutrient when it is prepared and preserved avoiding spontaneous oxidation. Or it may be more safe to hydrogenate fish oil to certain extent to get rid of double bonds.

Besides such important problems of fish oil as fat resources in nutrition, the essential feature of toxic effect of the oxidised unsaturated fatty acid from fish oil is interesting to investigate. In this line the work of Kaunitz and Johnson (*5*) is noteworthy. They observed that rat fell into flavin-shortage when rancid lard was given. But it seems to the author that the toxicity is too acute to ascribe it solely to riboflavin shortage.

SUMMARY

1. When the diet containing 5 per cent ethyl esters of highly unsaturated fatty acids obtained from cuttle fish oil was given to rat, it showed quite the same good nutritive effect as the diet containing oleic acid ester.

2. To the contrary the oxidised form of the above unsaturated fatty acid esters showed an extremely toxic effect to rat.

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[Tokushima Journal of Experimental Medicine, 8, 96-100; 1961]

STUDIES ON THE TOXICITY OF FISH OIL(V)*

TOXICITY OF OXIDIZED COMMERCIAL COD-LIVER OIL

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(Received for publication, April 28, 1961)

Fish oils, containing a large amount of glyceride of such highly unsaturated fatty acid as listed in Table I, show severe toxicity when oxidized, as already reported in detail[1,2]. It is supposed, in the light of the results of the experiments so far conducted, that commercial cod-liver oils will naturally show toxicity when they are oxidized, because they contain a considerable amount of glyceride of highly unsaturated fatty acid.

Table I Highly Unsaturated Fatty Acid

Molecular Formula	Name of Fatty Acid	Number of Double Bonds	Where Found
$C_{18}H_{32}O_2$	Moroctic acid	4	Sardine oil
$C_{20}H_{32}O_2$	Arachidonic acid	4	Sardine & whale oil
$C_{22}H_{34}O_2$	Clupanodonic acid	5	Sardine oil
$C_{24}H_{34}O_2$	Scolidonic acid	5	Tuna oil
$C_{24}H_{32}O_2$	Shibinic acid	5	Tuna oil
$C_{24}H_{30}O_2$	Nisinic acid	6	Sardine oil
$C_{26}H_{40}O_2$	Thynnic acid	6	Tuna oil

Experimental and Results

Expeiment was conducted on commercial cod-liver oil ('Megane' brand) obtained from the livers of 'madara' (*Gadus macrocephalus*) and 'sukesodara' (*Theragra chalcogramma*) (kinds of codfish). This is a light yellowish-brown, transparent liquid whose properties are shown in Table II.

Iodine value of the cod-liver oil was 168.9, measured by the Wijs method. The oil contained no peroxide at all. The oil was placed in a petri-dish, and was subjected to autoxidation in the presence of air at room temperature. The properties of oxidized cod-liver oil are shown in Table III. Generally, with the progress of oxidation, the peroxide increases rapidly and linearly in amount after

* A brief account of this paper was reported at the Kanto regional meeting of the Japan Biochemical Society held in November, 1954. The full text written in Japanese appeared in *Seikagaku*, 29, 812 (1958).

* [illegible]

Table II. Properties of Commercial Cod-Liver Oil Used

n_D^{20}	Iodine Value (Wijs)	Acid Value	Sap. Value	Peroxide
1.4741	168.9	0.53	181.0	0

a certain induction period, and reaches the maximum. It then decreases gradually owing to decomposition and polymerization[2]. Since the polymerized oil, as already reported[3], shows toxicity different from that of peroxide, oxidized cod liver was taken as test material from the portion with increasing peroxide value of 775 mg/100 g. Table III shows the property of the oil.

Table III. Properties of Oxidized Cod-Liver Oil

n_D^{20}	Iodine Value (Wijs)	Acid Value	Sap. Value	Peroxide (mg/100 g)
1.4777	117.8	1.12	190.9	775

Animal experiment was conducted with rats each weighing 60 to 80 g and fed on basal diet containing 10 per cent of oxidized cod-liver oil as shown in Table IV. (In this experiment, oxidized cod-liver oil was mixed with basal diet, while in the case of the experiment on oxidized ethyl ester of highly unsaturated fatty acid separated from cuttle-fish oil, it was given by mouth.)

Table IV. Ingredients of Basal Diet (%)

Starch (rice powder)	75
Casein (ether-extracted)	9
McCollum salt mixture	3
Yeast	3
Sample oil	10

Fig. 1 gives curves illustrating the growth of the rats, the ordinate indicating the body weight of rats and the abscissa days passed since the start of the experiment. The rats fed on original cod-liver oil (not oxidized) grew up well, while those administered with oxidized cod-liver oil showed little growth.

In this experiment, which covered a period of 34 days, the body weight of most rats decreased by 10 g or more, and some by nearly 20 g. No particular outward change was noticed on the rats except that the hair looked like the piles of old carpet.

The toxic effect of peroxides was so rapid and intense that it cannot be regarded as a problem concerning vitamins.

The author then separated the highly unsaturated fatty acid contained in commercial cod-liver oils by fractional distillation. Table V shows the process of separating highly unsaturated fatty acid from cod-liver oil by the soda acetone

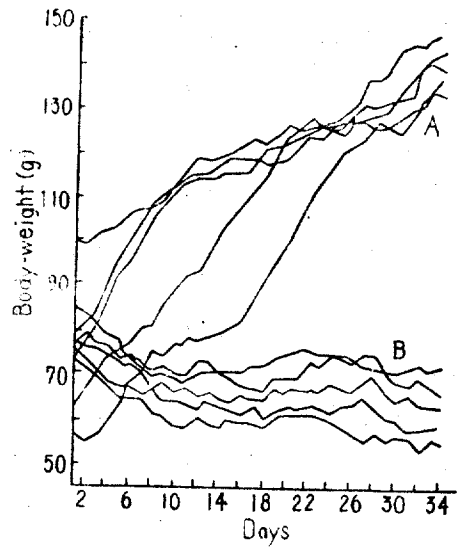

Fig. 1. Feeding record of rats.
A: Original cod-liver oil 10 per cent.
B: Oxidized cod-liver oil 10 per cent.

Table V. Separation of Highly Unsaturated Fatty Acid by Soda-Acetone Method

Commercial Cod-Liver Oil

100% Saponification

Sodium Salt

Acetone, cooling (0——10°C), Filtration

Sodium Salt Insoluble in Acetone

Filtrate

Distillation

Acetone Removed

Sodium Salt of Highly Unsaturated Acid

Decomposition — Dilute Hydrochloric Acid

Washing — Petroleum Ether

Distillation

Ethyl Alcohol

Highly Unsaturated Acid

Ethyl Ester of Highly Unsaturated Fatty Acid

Vacuum Distillation 0.5 mm Hg

~180°C	180-192°C	192-250°C	205-212°C	212-222°C	222-230°C	Residue
4.2%	2.9%	4.7%	3.1%	4.2%	3.0%	0.9%

method[4] employed by Toyama and Tsuchiya.

Cod-liver oil was saponified, mixed with acetone and left overnight at a low temperature. Sodium salt of highly unsaturated fatty acid was then obtained by filtering off sodium salt of not highly unsaturated fatty acid, which was insoluble in acetone. Acetone was evaporated off. The sodium salt obtained in this way was washed in ether in order to remove unsaponified matters and esterified by treating with ethyl alcohol (HCl is used as catalyst).

Then the esters were distilled in vacuum. The percentages of various fractions obtained from commercial cod-liver oil are given in Table V. Table VI shows the properties of each fraction.

Table VI. Properties of Fractions of Highly Unsaturated Fatty Acid Separated from Commercial Cod-Liver Oil ('Megane' Brand)

Fraction	Temp. °C/0.5 mm Hg	%	Percentage Against Raw Oil	Iodine Value (Wijs)	n_D^{20}
1	—180	18.3	4.2	172.5	1.4694
2	180—192	12.6	2.9	221.9	1.4722
3	192—205	20.4	4.7	260.4	1.4764
4	205—212	13.5	3.1	304.0	1.4814
5	212—222	18.3	4.2	314.8	1.4867
6	222—230	13.0	3.0	348.9	1.4910
7	Residue*	3.9	0.9	—	—

* Solid (room temperature)

Discussion

The autoxidized product of the ethyl ester of highly unsaturated fatty acid showed severe toxicity, and the toxicity was attributable mainly to the existence of peroxide.

Since cod-liver oil contains glyceride of highly unsaturated fatty acid in large amount, it was anticipated that the cod-liver oil would show toxicity if an appropriate quantity of peroxide is produced by oxidation. This turned out to be true as a result of experiment. Unlike the case of deficiency of vitamin, toxicity appeared very rapidly. Considering that there is such a difference between oxidized liver oil and non-oxidized one, detection of peroxide is absolutely necessary prior to using liver oil.

Summary

1. Cod-liver oil lost its nutritive value due to the formation of peroxide when exposed to the air for a long time.

2. The properties of the highly unsaturated fatty acid contained in cod-liver oil were made clear.

The Journal of Biochemistry, Vol. 41, No. 4, 1954

STUDIES ON THE TOXICITY OF FISH OIL

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(Received for publication, March 23, 1954)

It was first depicted by Sahashi (*1*) and Somekawa (*2*) that some sorts of fish oil including whale oil are toxic to rat. But it remains undecided whether they are equally toxic to man or not. Since in Japan, where resource of fat is extremely short, the use of fish oils as nutrient is highly desirable, the wide experiments on man were carried out in many clinical as well as biochemical laboratories. The result of such cooperative works revealed that fish oil is not so toxic to man in dosage of 50-100 gms. per day. But quite recently Kaneda and Ishii (*3*) made an interesting observation that fish oil per se is not so toxic even to rat, but when it is once oxidised simply by being exposed to air, turns out to be toxic.

In this case it is well assumed that unsaturated fatty acid in fish oil is the substance easily oxidised and at the same time responsible for toxic effect. Using the unsaturated fatty acids obtained from cuttle fish oil, the author carried out the experiment and fully confirmed the results obtained by Kaneda and Ishii (*3*). Further some chemical nature of the toxic substance was studied, the details of which are partly reported here.

EXPERIMENTAL

Preparations of Unsaturated Fatty Acids

The raw cuttle fish oil produced in Hokkaido was used. Its properties are shown in Table I.

TABLE I

The Properties Raw Cuttle Fish Oil

Density (15°)	Iodine value (Wijis)	Acid value	Sap. value	Unsap. matter (%)
0.9305	182.5	2.03	189.3	2.19

The unsaturated fatty acids were separated by the soda-acetone process (4). Namely, raw cuttle fish oil was saponified by ethyl alcoholic solution of NaOH, mixed with acetone, left over night at 0° and filtered. From the filtrate, which contained acetone soluble sodium-salts of highly unsaturated acids, acetone was distilled off, and its residue was decomposed by dil. HCl.

The free acids, dissolved in petroleum-ether (b. p. below 65°) were washed several times with water, and dehydrated by anhydrous sodium-sulphate and then petroleumether was distilled off, giving the highly unsaturated acids as residue.

Since the sample obtained as above, used to contain somewhat of unsaponificable matter which was existing in raw oil, so it was desirable to remove it as much as we could.

Next, the sample was dissolved in twice the volume of absolute ethyl alcohol containing 2.5 per cent HCl, and heated on the water-bath for about three hours. By this process ethyl esters of the acids were prepared. Then, the ethyl esters were fractionated under a vacuum of 2 mm. Hg at various temperatures. The 205~215° fraction was chiefly used for the author's experiment. This fraction has light yellow colour and no odour. Since these esters are liable to be rapidly oxidised when they are in contact with air, special precaution was paid throughout the preparation.

The container of the esters was always filled with CO_2 gas and was kept in dry-ice during the experiments. An aliquote portion of the sample thus prepared were put into a petri dish and borught into contact with air at the room temperature. As the oxidation proceeded the esters developed light yellow colour. At a certain point of oxidation the reaction was stopped and the ester was kept again in dry-ice avoiding further change.

Ethyl ester of oleic acid was used as control. Some chemical properties of the esters and of their oxidised compounds are indicated in Table II together with those of oleic acid ester for comparison.

TABLE II

Analysis of the Samples Used in the Experiments

	d_4^{15}	n_D^{20}	Iodine value	Sap. value	Unsap. matter (%)
Ethyl ethylesters of highly unsaturated fatty acids	—	1.4823	358.70	163.90	Trace
Oxidised esters of highly unsaturated fatty acids	0.9568	—	264.82	201.49	Trace
Ethyl ester of oleic acid	0.8822	—	82.96	187.69	0

RESULTS

Effect on the Growth of the Rat—The rats of nearly same size and body weight

(about 60 g.) were divided into three groups, each comprising 4 rats. To the first group the feed was given containing 5 per cent of the unsaturated fatty acid esters in the basal diet, to the second group that of the oxidised esters and to the third that of oleic acid ester, which considered as control. The composition of the basal diet is given in Table III.

TABLE III

Ingredients of Basal Diet

Starch (rice powder)	80%	Yeast	3%
Casein (ether extracted)	9%	Liver oil	1 drop/day
McCollum salt mixture	3%		

Total amount of diet per day is 10 g. (from 1st day to 5th day), 12.5 g. (from 6th day to 10th day), 15 g. (from 11th day to the 30th day).

The experimental results are recorded in Table IV and also illustrated in the accompanying Fig. 1 and photographs (Figs. 2, 3, and 4). It is apparent that the rats fed on the unsaturated fatty acid ester containing diet showed the same satisfactory growth as those on the oleic acid ester diet. But it is quite remarkable that all rats that fed on the oxidised ester diet not only showed retarded growth but died within few days after the beginning of the experiment. It was also noticed in this group that white hair turned into brown.

TABLE IV

Feeding Records

Kind of ester fed	Sex	Increase of body weight (g.) 10th day	average	20th day	average	30th day	average
Ethyl ester of oleic acid.	♂	+11.0	+13.1	+34.0	+43.3	+ 61.0	+82.1
	♀	+14.0		+51.2		+113.5	
	♂	+15.0		+40.5		+ 70.8	
	♀	+12.5		+47.5		+ 83.0	
Ethyl ester of unsaturated fatty acid.	♂	+ 7.7	+ 6.3	+41.0	+38.1	+ 85.0	+75.8
	♀	+ 7.0		+38.5		+ 77.0	
	♂	+ 7.0		+44.5		+ 75.0	
	♀	+ 3.2		+28.5		+ 66.0	
Oxidised from of the above ester.	♂	−3.8 (died on 6th day)					
	♀	−5.0 (died on 7th day)					
	♂	−5.0 (died on 8th day)					
	♀	−8.5 (died on 3rd day)					

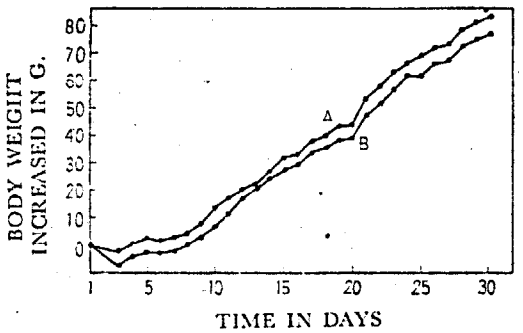

FIG. 1. The curve of average body weight increased. A (Control): average body weight increment of the rats fed on the diet containing 5 per cent of the ethyl ester of oleic acid. B: that of rats fed on the died containing 5 per cent of the ethyl ester of highly unsaturated fatty acids.

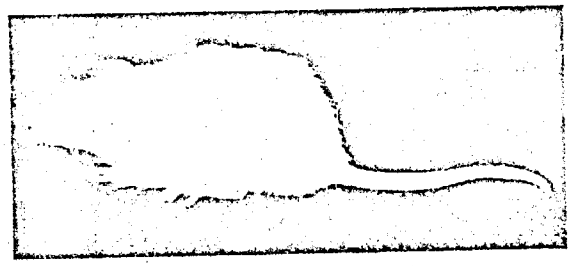

FIG. 2. The rat fed on the diet containing 5 per cent of the ethyl ester of oleic acid. (Control) On the 27th day after the commencement of the experiment.

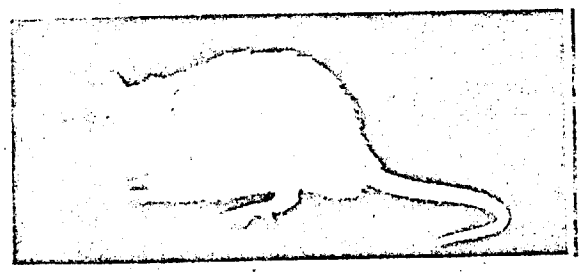

FIG. 3. The rat fed on the diet containing 5 per cent of the ethyl ester of highly unsaturated acid. On the 27th day after the commencement of the experiment.

Effect of More Mild and Chronic Intoxication by the Oxidised Ester—For this purpose the grown-up rats were fed on the diet containing 5 per cent of the oxidised ester not daily but at some intervals.

One of the typical examples is illustrated in Fig. 5 with regard to the change of body weight.

As shown in Fig. 5, the retarted growth of the animal continued during the feeding of the toxic diet, but somewhat recovering was observed when that feeding was stopped. It ended, however, in fatal at the end of 23th day after the commencement

FIG. 4. The rat fed on the diet containing 5 per cent of the oxidised ethyl ester of highly unsaturated acid. On the 4th day after the commencement of the experiment.

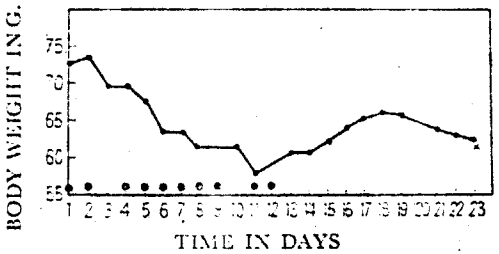

FIG. 5. The curve of body weight changed.
The mark shows the day on which the oxidised ester diet was given.

of the experiment. The appearance of the animal was quite particular. The hair of the face was diplated off, the mouth and rear legs were swollen, the remaining hair changed into brown, and hemorrhages were seen here and there in the skin. (see Figs. 6 and 7).

DISCUSSION

The above results substantially proved the finding first made by Kaneda and Ishii that the highly unsaturated acid from fish oil is not toxic but rather its oxidised form is toxic. As it is usually assumed that unsaturated fatty acid absorbs oxygen forming peroxide at double bonds in the molecule, so it might be plausible that the toxicity of the oxidised acid is caused by peroxide. In this regard the next paper will give full details. At any rate it is promising that fish oil can be utilised

586

EXPERIMENTAL PROCEDURE

A. Purification of lecithins

Tissues were homogenized in chloroform–methanol (1:1, v/v) solution. Lipids were extracted, and fractionated by column chromatography on silicic acid as described previously[1]. The lecithin fractions showed only one component with the thin-layer silicic acid chromatography system of VOGEL *et al.*[10]. The product used in each case contained the bulk of the lecithin fraction, to avoid fractionation with regard to the fatty acid content. Ester[11], phosphorus [12] and plasmogen contents[13] were determined for each lecithin. These, together with the specific optical rotation in chloroform solution, and the ester/phosphorus ratios are set forth in Table I. During the preparation of the fractions trace amounts of hydroquinone were added as an antioxidant, and all samples were stored under N_2.

B. Liberation of fatty acids by phospholipase A (ref. 4)

30 mg of each lecithin were dissolved in peroxide-free diethyl ether to give a final concentration of 20 μmoles/ml. Lyophilized venom of the eastern diamondback rattlesnake (*Crotalus adamanteus*) (Ross Allen's Reptile Institute, Silver Spring, Florida) was dissolved in a solution of 0.1 M Tris buffer, 20 mM $CaCl_2$ and 2 mM EDTA (tetrasodium salt) to give a final concentration of 2.0 μg of venom per μl. The buffered enzyme was adjusted to pH 7.4. To each ml of the etheral solution of lecithin, 10 μl of the enzyme solution was added. The mixture was shaken vigorously for 30 sec and allowed to stand at room temperature for 2 h. 10-μl aliquots were taken at fixed time intervals to check the progress of the reaction by chromatographing the aliquot directly in the thin-layer system mentioned above. The reaction was complete by this test at the end of 2 h. The ether solution was evaporated to dryness under N_2 and the residue dissolved in a minimum of solvent (0.5% methanol in chloroform). The resulting solution was transferred to a 12 mm × 155 mm column containing 5 g of silicic acid equilibrated in the same solvent. The free fatty acids liberated during the reaction were eluted from the silicic acid column by an additional 40 ml of solvent. Elution of unreacted lecithin (trace amounts) was accomplished by the addition of 40 ml of 25% methanol in chloroform to the column. The remaining reaction product, lysolecithin, was eluted from the column with 90 ml of 85% methanol in chloroform. Each of the fractions isolated showed only a single spot corresponding to an authentic sample when subjected to chromatography on the thin-layer silicic acid system. Approx. 90% of the amount used was recovered.

C. Preparation and analysis of methyl esters of fatty acids

Methyl esters of the fatty acids liberated during the reaction were prepared from the first silicic acid chromatography fraction by reaction with 3% (v/v) conc. H_2SO_4 in anhydrous methanol for 2 h at boiling, followed by extraction with light petroleum and washing with water.

The fatty acids remaining esterified to the lysolecithin fraction were methanolized in 5 ml of chloroform–methanol (1:1, v/v) to which was added 1.0 ml of 0.5 N sodium methoxide[14]. The reactants were cooled to 0° before reaction, mixed rapidly, allowed

to stand in the cold for 20 min, then allowed to warm to room temperature, neutralized with 2.5 ml of 3% HCl in anhydrous methanol and evaporated to dryness. The residue was suspended in water and extracted with light petroleum.

Methyl esters from the various fractions were analyzed by gas–liquid chromatography. An Aerograph Hy-Fi (Wilkens Instrument Co., Walnut Creek, Calif.) flame-ionization instrument was used. It was equipped with a 366-cm, 3-mm O.D. glass column which had been treated with dichlorodimethylsilane and packed with 1% EGSS-X on 100–120-mesh hexamethyldisilizane-treated Gas-Chrom P (Applied Science Laboratories, State College, Pa.). The separation was conducted at 190° with N_2 (40 ml/min) as the carrier gas. The instrument was calibrated with authentic standards and National Institute of Health standard mixtures of fatty acid methyl esters.

RESULTS AND DISCUSSION

The data of Table I illustrate the nature of the preparations. Although all lecithin preparations showed only one spot by thin-layer chromatography and corresponded to the expected infrared absorption spectra, the ester/phosphorus ratios are lower for the tuna and menhaden lecithin samples than can be accounted for by their content of plasmalogen. They probably therefore contained small amounts of non-lecithin lipid material. Analyses for the other lecithin preparations gave the expected results within the limits of precision of the methods used. By using the total fraction from the silica-gel column chromatography, it was hoped to avoid the initial fractionation of lecithins noted by RHODES AND LEA[15].

TABLE I

CHEMICAL AND PHYSICAL PROPERTIES OF ISOLATED LECITHINS

Source	*Specific rotation* ($[\alpha]^{25}_{D}$)	*P content* (%)	*Ester content* (*μequiv. per 100 mg*)	*Ester/P ratio*	*Mole % plasmalogen*
Salmon muscle	+4.4	3.60	2.06	1.78	5.5
Tuna muscle	+3.6	3.94	2.22	1.75	8.7
Menhaden muscle	+5.0	3.70	2.28	1.92	5.8
Egg yolk	+6.6	3.76	2.40	2.04	1.5
Rat liver	+6.1	3.59	2.13	1.84	3.8
Beef liver	+6.0	4.00	2.61	2.02	3.8

The specific distribution of fatty acids in the lecithins examined is presented in Table II and summarized in Table III. It is apparent from Table III that, regardless of the origin of the lecithin, the β position is predominantly unsaturated and that the distribution of saturated and unsaturated fatty acids in the β position is very nearly the same in all samples while the mole per cent of saturated fatty acids in the α' position varies considerably. Egg-yolk, menhaden-muscle and rat-liver lecithins were similar in the degree of saturation of the α' position (80%) while beef liver was of an intermediary value (66%) and salmon and tuna muscle of the lowest degree of saturation (35%). Palmitic acid was the predominant saturated fatty acid in the α' position. Indeed, palmitic and stearic acids represented the highest positional enrich-

TABLE II

SPECIFIC DISTRIBUTION OF THE PRINCIPLE FATTY ACID FROM THE ISOLATED LECITHINS

Identification is based on relative retention times of authentic standards of the fatty acid methyl ester or on the relative retention times of homologs followed by hydrogenation and rechromatography. The notation in the first column includes the number of carbon atoms followed by the number of double bonds. "tr" denotes trace amounts; *e.g.*, < 0.1%.

Tentative identification of fatty acids	*Weight per cent of specific fatty acid isolated from*																	
	Salmon			*Menhaden*			*Tuna*			*Egg*			*Rat liver*			*Beef liver*		
	Total	*α'*	*β*	*Total*	*α'*	*β*	*Total*	*α'*	*β*	*Total*	*α'*	*β*	*Total*	*α'*	*β*	*Total*	*α'*	*β*
14:0	0.7	1.9	0.6	0.5	tr	1.3	0.7	tr	1.0	tr	2.3	0	0.1	0	0.4	0.2	0.7	0.3
14:1				tr	tr	tr		tr	1.8	tr	0	tr						
16:0	22	37	10	45	78	6.2	19	36	5.0	34	61	4.8	16	32	2.1	11	19	1.2
16:1	8.0	1.4	3.8	tr	0	tr	2.1	tr	2.3	2.2	0	1.7	0.6	tr	1.1	1.7	2.9	1.7
18:0	2.0	2.7	tr	3.2	5.9	0.9	5.0	7.9	1.0	14	25	2.4	27	50	0.9	23	46	tr
18:1	6.7	8.2	23	7.4	2.3	13	7.6	6.4	16	35	9.5	59	5.6	3.5	6.6	18	19	17
18:2	tr	tr	tr	1.0	tr	2.4	0.7	tr	2.2	13	2.4	26	12	5.2	24	8.9	3.1	16
18:3	tr	tr	tr	tr	tr	tr	0.8	tr	1.1	tr	0	tr	0.3	tr	0.7	1.1	0.5	1.8
20:4	1.0	0.6	0.3	2.2	tr	0.9	2.7	1.3	5.0	2.0	0	6.3	26	4.4	45	8.0	2.0	18
20:5	12	14	17	13	1.7	29	7.1	8.4	15	0	0	0	1.5	4.2	2.6	0.8	tr	2.3
22:1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	7.5	2.3	15
22:4	tr	tr	tr	tr	tr	tr	tr	0	0.5	0	0	0	tr	tr	tr	2.5	1.0	6.9
22:5	tr	tr	tr	tr	tr	tr	tr	tr	tr	0	0	0	tr	tr	1.8	6.1	2.5	14
22:6	43	33	46	28	12.5	42	55	39	48	0	0	0	11	tr	14	2.1	tr	5.6

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.

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BBA 55017

Biochim. Biophys. Acta, 84, 133-139 1964

POSITIONAL DISTRIBUTION OF FATTY ACIDS IN FISH AND OTHER ANIMAL LECITHINS

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(Received October 29th, 1963)

SUMMARY

Phosphatidyl choline (lecithin) fractions were prepared from tuna, salmon, and menhaden muscle, from egg yolk, and from rat and beef liver. Hydrolysis by snake-venom phospholipase A (phosphatide acyl-hydrolase, EC 3.1.1.4) was used to determine the positional distribution of the fatty acids. The fatty acid composition of the hydrolyzed fatty acids (β position) and those remaining with the phosphatidyl moiety (α' position) were separately determined by gas-liquid chromatography of the methyl esters. 36–86 mole % of the fatty acids esterified in the α' position were saturated; 91–99 mole % of the fatty acids in the β position were unsaturated. Palmitic and stearic acids were the predominant saturated acids in the α' position. Some similarity was noted in the β-position distribution of linoleic, arachidonic, eicosapentaenoic, docosaenoic, docosapentaenoic, and docosahexaenoic acids. This observation tends to support the hypothesis of the interchangeability of linoleic and arachidonic acids with "non-essential" polyenoic acids.

INTRODUCTION

This work was initiated as an extension of studies on the nature of the phospholipids of tuna[1] and menhaden[2]. It is known that phospholipase A (phosphatide acyl-hydrolase, EC 3.1.1.4) of snake venom specifically hydrolyzes the β position of both synthetic and naturally occurring phospholipids[3–8], and that these fatty acids are primarily unsaturated[4]. The fish lecithins are characterized by large amounts of highly unsaturated fatty acids[1,2], hence it appeared of interest to determine the positional distribution of these fatty acids in them.

During the course of this investigation BROCKERHOFF *et al.*[9] described the specific distribution of fatty acids in the phospholipids and triglycerides of several marine organisms: cod (*Gadus callarias*), scallop (*Platopectin magellariccus*) and lobster (*Homarus americanus*). The present paper contains our results obtained by similar means on lecithins isolated from albacore tuna (*Thunnus alalunga*), menhaden (*Brevoortia tyrannus*), and salmon (*Oncorhynchus tshawytscha*) muscle, and from beef liver, rat (Long–Evans) liver, and chicken egg yolk.

UNITED STATES PATENT OFFICE

2,627,469

SOYA BEAN OIL COMPOSITIONS

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No Drawing. Application January 29, 1952, Serial No. 268,935

29 Claims. (Cl. 99—144)

1

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.

2

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 0.02% 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	20
3	Cottonseed	75	113				18	
	Soya Bean	25	118	0.4	0.014	0.00	15	21
4	Sesame	60	112				20	
	Soya Bean	40	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	15	92				0.25	12
	Soya Bean	25	112	0.9	0.018	0.001	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 25° F.	At 15° F.
		Percent		*Percent*	*Percent*	*Percent*	*Hours*	*Hours*	*Hours*
1	Cottonseed	100	113				16	10	5
2	Corn	100	127				60	More than 168	64
3	Soya Bean	100	105	4.7	0.12	0.01	0.2	68	30
4	do	100	115	2.2	0.04	0.005	1.5	168	64
5	do	100	106	0.00	0.009	0.00	1	140	50
6	do	100	117	0.00	0.00	0.00	18	More than 168	116
7	Cottonseed	50	114						
	Soya Bean	50	105	4.7	0.12	0.01	-1	44	20
8	Cottonseed	50	114						
	Soya Bean	50	115	2.2	0.04	0.005	7	100	40
9	Cottonseed	50	112						
	Soya Bean	50	106	0.00	0.009	0.00	2	90	35
10	Cottonseed	50	113						
	Soya Bean	50	117	0.00	0.00	0.00	36	More than 168	110
11	Cottonseed	50	114						
	Soya Bean	50	111	0.00	0.00	0.00	14	145	60
12	Cottonseed	25	113						
	Soya Bean	75	115	2.2	0.04	0.005	3	120	55
13	Cottonseed	75	113						
	Soya Bean	25	115	2.2	0.04	0.005	10	60	26
14	Sesame	60	112						
	Soya Bean	40	112	0.6	0.012	0.001	22	160	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)		Overall Acceptability[2]		Relative Flavor Acceptability[3]
				at 95° F.	at 75° F.	at 95° F.	at 75° F.	
		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	35
3	Soya Bean	100	105	4	5	175	400	56
4	Soya Bean	100	115	4	5	165	375	55
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	12	380	825	112
	Soya Bean	37.5	116					
16	Cottonseed	25	115					
	Sesame	35	113					
	Peanut	15	92	6	12	350	710	105
	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	679	17	2.5
B	Corn	100	125–128	351	177	50.5
C	Soya Bean	100	100–120	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	2.977	.01.
			4.140	.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	6.5	.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 56 flavor scorings on each sample by the eight judges at the bi-weekly observation periods during the over-all 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. (1949).

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.

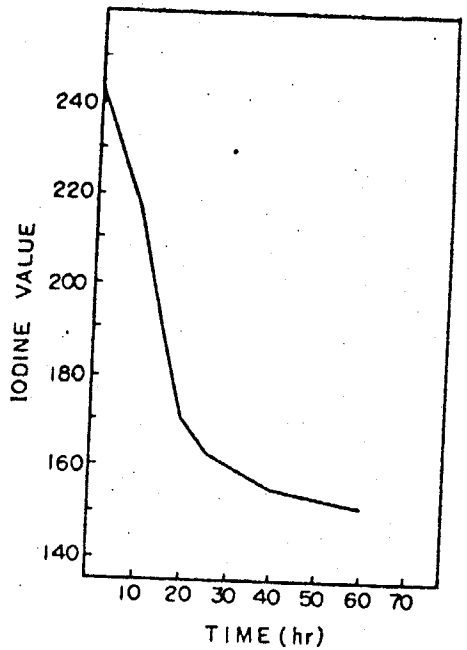


FIG. 93. CHANGE IN IODINE VALUE OF ETHYL LINOLEATE DUE TO THERMAL POLYMERIZATION

respectively. By vacuum distillation, the latter was separated into a distillate (153 to 175°C./1.8–2.0 mm. Hg) and a residue. The distillate is transparent, light yellow, and its yield was 24.7 per cent of the total cyclic ester. The residue is somewhat viscous, transparent, reddish brown, and its yield was 70.8 per cent.

Animal Experiments with the Various Esters.—Rats, each weighing about 60 gm. were fed the basal diet (Table 74) containing, respectively, 20 per cent of each of the sample esters shown in Table 78.

TABLE 78

PROPERTIES OF ESTERS

	n_D^{20}	Iodine Value, Wijs	Saponification Value	Acid Value	Molecular Weight, Rast
Ethyl linolenate	1.4688	242.5	183.1	1.0	302
Polymerized ethyl linolenate	1.4838	157.5	170.1	6.5	
Straight-chain ester	1.4692	201.5	177.4	0.4	
Cyclic ester	1.4891	142.5	168.6	7.3	
Distilled ester	1.4751	159.4	171.0	7.4	293
Residual ester	1.4948	135.8	168.5	8.2	

Ethyl Linolenate.—The rats fed a diet containing ethyl linolenate showed healthy growth as indicated in Fig. 94.

Polymerized Ethyl Linolenate.—As shown in Fig. 95, the rats became gradually thinner and died in about 15 days.

Distilled and Residual Cyclic Esters.—When rats were fed a basal diet containing 20 per cent of distilled cyclic ester, extremely acute toxicity

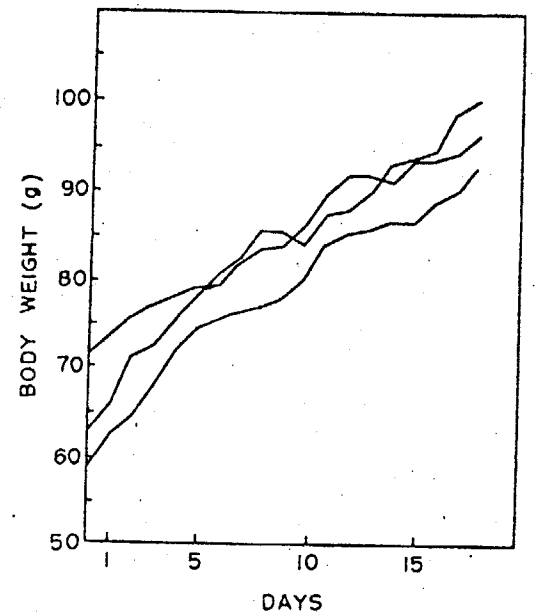


FIG. 94. GROWTH OF RATS FED ON BASAL DIET CONTAINING 20 PER CENT OF ETHYL LINOLENATE

appeared and all rats died in three or four days. However, no particular change was noticed in the appearance of the rats. In the case of feeding a basal diet containing 20 per cent of residual cyclic ester, toxicity was considerably less and there was some increase in the weight of the rats, although they had diarrhea (Fig. 96).

From these observations, it was apparent that distilled cyclic ester was severely toxic.

Properties of Various Esters.—The properties of the esters which were fed are shown in Table 78.

From the iodine value (242.5) of ethyl linolenate (theoretical value: 247.4), it is assumed that the linolenate contained about six per cent of ethyl linoleate, if the contaminant was only ethyl linoleate. The saponification value of this ethyl linolenate was 183.1 (theoretical value: 183.(

and its molecular weight, 302 (theoretical value: 306). The molecular weight of the distilled cyclic ester was 293. From its boiling point, the main component of this cyclic ester is considered to be a cyclic monomer. Since its iodine value was 159.4 it seems that the original linolenate with three double bonds lost one as a result of cyclization. Iodine value of ethyl linoleate with two double bonds is 166.0.

Infrared Absorption Spectra of Ethyl Linolenate and Distilled Cyclic Esters.—The infrared absorption spectra of ethyl linolenate and the distilled cyclic ester, as measured with the Baird B-type spectrophotometer, are shown in Fig. 97.

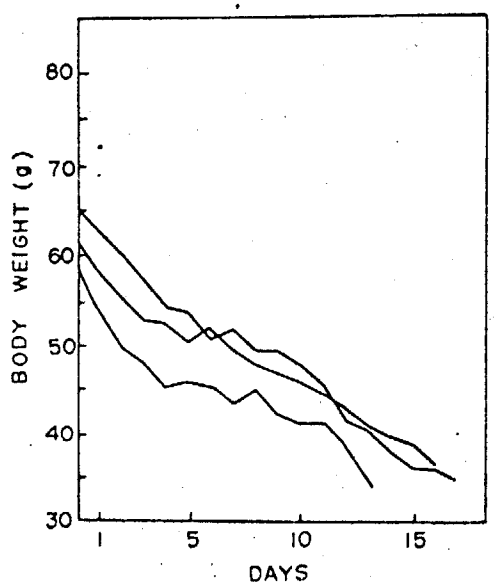


FIG. 95. GROWTH OF RATS FED ON BASAL DIET CONTAINING 20 PER CENT OF POLYMERIZED ETHYL LINOLENATE

All rats died at the ends of the curves

The absorption band at about 14 μ for ethyl linolenate is considerably intense and attributable to cis double bonds.

Linolenic acid has naturally cis double bonds. When it is separated by the bromination method, however, isomerization takes place to some extent and an absorption band of 10.3 μ (970 cm.$^{-1}$) attributable to trans double bonds appears. When the absorption band near 6 μ was examined with increased concentration of the sample, a small absorption was noticed. This absorption is also considered to be attributable to cis double bonds.

When the infrared absorption spectrum (Fig 97—II) of the distilled cyclic ester is compared with that (Fig. 97—I) of ethyl linolenate, it is found that the absorption attributed to cis double bonds is considerably weaker. This seems to be due to the disappearance of some double bonds. A new absorption band was observed at 15.2 μ (660 cm.$^{-1}$). Such absorption was noticed also in the cyclic monomer separated from the prod-

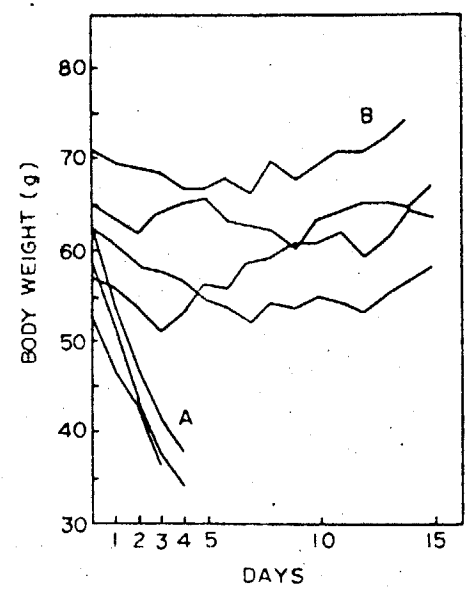


FIG. 96. GROWTH OF RATS FED ON BASAL DIET CONTAINING 20 PER CENT OF DISTILLED CYCLIC ESTER (A) AND RESIDUAL ESTER (B)

All rats died at the end of the A curve.

uct obtained by thermal polymerization of the highly unsaturated fatty acid from cuttlefish oil (Matsuo 1960A).

Cyclohexene shows a band at 670 cm.$^{-1}$ (14.97 μ) (MacDonald 1956). The bands at 663 and 671 cm.$^{-1}$ (15.1 and 14.9 μ) are characteristic of Δ^2 - and Δ^3 -steroids respectively (Jones and Herling 1954), and cis α -ionone (Buchi and Yang 1955) also absorbs in this region. Therefore, this absorption band is considered to be attributable to the formation of a cyclohexene ring resulting from cyclization. This absorption band was also ascertained by MacDonald (1956) as the monomeric, non-urea-adduct-forming material from the ethanolysis of heated linseed oil.

Absorption due to trans double bonds became somewhat greater, but no significant change was observed. Examination of the absorption spe-

599

tra near 6 μ observed with the higher concentration of the sample showed the presence of two small bands. It seems that the new absorption band at about 6.1 μ (1650 cm.$^{-1}$) originates from the cyclic compound.

In the experiment on highly unsaturated fatty acid separated from fish oil, cyclic compounds also showed toxicity, and the cyclic monomer, separated in vacuum distillation, was the main source of toxicity.

From the result of the experiment on ethyl linolenate, it can be concluded that the source of toxicity is a cyclic monomer and that the main

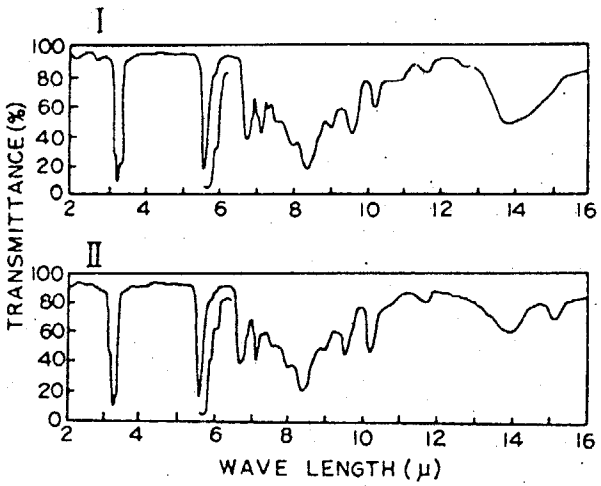


FIG. 97. INFRARED ABSORPTION SPECTRA OF ETHYL LINOLENATE (I) AND DISTILLED CYCLIC ESTER (II)

component of this cyclic monomer is a compound having a cyclohexene ring.

Toxicity of Cyclic Compound of β-Eleostearic Acid

In order to investigate this point, a compound having a cyclohexene ring in the molecule was prepared and its toxicity was examined (Matsuo 1960B).

Preparation of Pure β-Eleostearic Acid.—Tung oil indicated in Table 79–i, was isomerized by the method of Hoffman *et al.* (1957). About 500 gm. of tung oil was mixed with 1 ml. of saturated potassium iodide solution, the mixture was thoroughly stirred, kept air-tight, and exposed to the sunlight to accelerate isomerization. Then the oil was saponified with alkali as usual, and fatty acid was then obtained. This was washed in distilled water, dissolved in ethanol, and allowed to stand overnight at −15°C. The precipitated β-eleostearic acid was collected by filtration and dried in vacuum. The melting point of the acid was 70° to 71°C.

and its infrared absorption spectrum, as shown in Fig. 98-a, was exactly the same as that reported by Hoffman *et al.* (1957).

The ethyl ester of β-eleostearic acid was prepared as usual and distilled in vacuum. The fraction collected at 193° to 196°C./2 mm. Hg showed the properties indicated in Table 79–ii, which were almost comparable to the theoretical values. The infrared absorption spectrum of this ester is shown in Fig. 98-b.

TABLE 79

PROPERTIES OF TUNG OIL AND DERIVATIVES

No.	Sample	n_D^{40}	Iodine Value, Wijs	Saponification Value	Acid Value	Molecular Weight, Rast
i	Tung oil	1.5063	173.7	194.2	5.4	—
ii	Ethyl β-eleostearate	1.4892	165.5 (166.0)	182.2 (183.0)	2.0	301 (306)
iii	Ethyl β-eleostearate acrolein adduct	1.4847	139.8 (140.3)	153.6 (154.7)	1.6	367 (362)

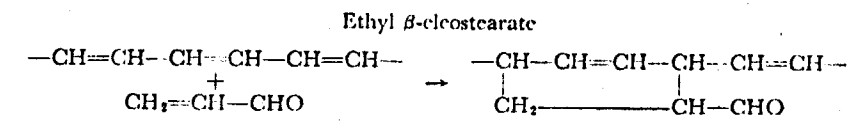
( ) theoretical value.

Preparation of Adduct of Ethyl β-Eleostearate with Acrolein.—Preparation was made by the method of Miyoshi and Kurata (1942) as shown in Fig. 99.

Ethyl β-eleostearate and acrolein were mixed in a sealed tube in the ratio of 4:1 by weight and heated at 130° to 145°C. for 3.5 hours. The reaction product was distilled in vacuum. The distillation was repeated three times and the final distillate at 187.5° to 192°C./1.8 mm. Hg was collected (yield, 7.6 per cent).

This fraction was transparent, light yellowish oil and contained no acrolein when tested by reaction with sodium nitroprusside and piperidine (Feigl 1956). The properties of the adduct are shown in Table 79–iii, and found to be comparable to the theoretical value. Its infrared absorption spectrum is shown in Fig. 98–c.

Ethyl β-eleostearate has three conjugated double bonds, whose configurations are all in the trans form. Cyclohexene ring, as shown in the following equation is considered to be formed by the addition of acrolein through the Diels-Alder reaction.



When the infrared absorption spectrum of ethyl β-eleostearate-acrolein adduct is compared with that of ethyl β-eleostearate, the following

ferences are observed: The strong absorption at 10.1 μ (990 cm.$^{-1}$) due to the conjugated trans-trans double bond, which was found in ethyl β-eleostearate, almost disappeared as a result of the addition of acrolein, and an absorption at 10.3 μ (970 cm.$^{-1}$) due to trans double appeared.

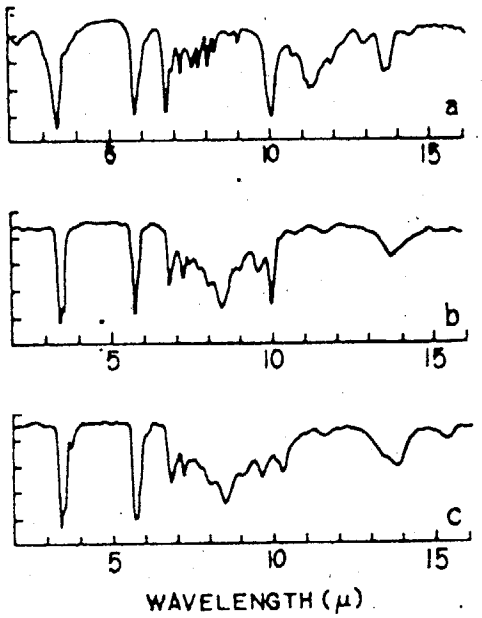


FIG. 98. INFRARED ABSORPTION SPECTRA

(a) β-Eleostearic acid, (b) ethyl β-eleostearate, (c) ethyl β-eleostearate-acrolein adduct.

The absorption at 15.2 μ (660 cm.$^{-1}$) was not found in the spectrum of the original ester. This absorption band was also observed by MacDonald (1956). This absorption can be attributed to the cyclohexene ring. Another absorption, due to the aldehyde radical, was observed at 3.7 μ (2700 cm.$^{-1}$).

TABLE 80

COMPOSITION OF BASAL DIET

	Per Cent
Starch (rice powder)	75
Casein (ether extracted)	9
McCollum salt mixture	3
Yeast	3
Liver oil	One drop a day
Sample	10

These differences seem to be caused by the addition of acrolein to ethyl β-eleostearate as shown in the above formula.

From the properties and infrared absorption spectrum, the product seems to be the adduct of one molecule of acrolein with one molecule of ethyl β-eleostearate.

Toxicity of Ethyl β-Eleostearate-Acrolein Adduct to Rats.—Rats, each weighing 60 to 70 gm., then fed a basal diet (Table 80) containing ten per cent of ethyl β-eleostearate, grew normally.

Rats, each weighing about 60 gm., were fed the same basal diet (Table 80) but containing ten per cent of the adduct of ethyl β-eleostearate and acrolein. In this experiment, as shown in the growth curves given in Fig.

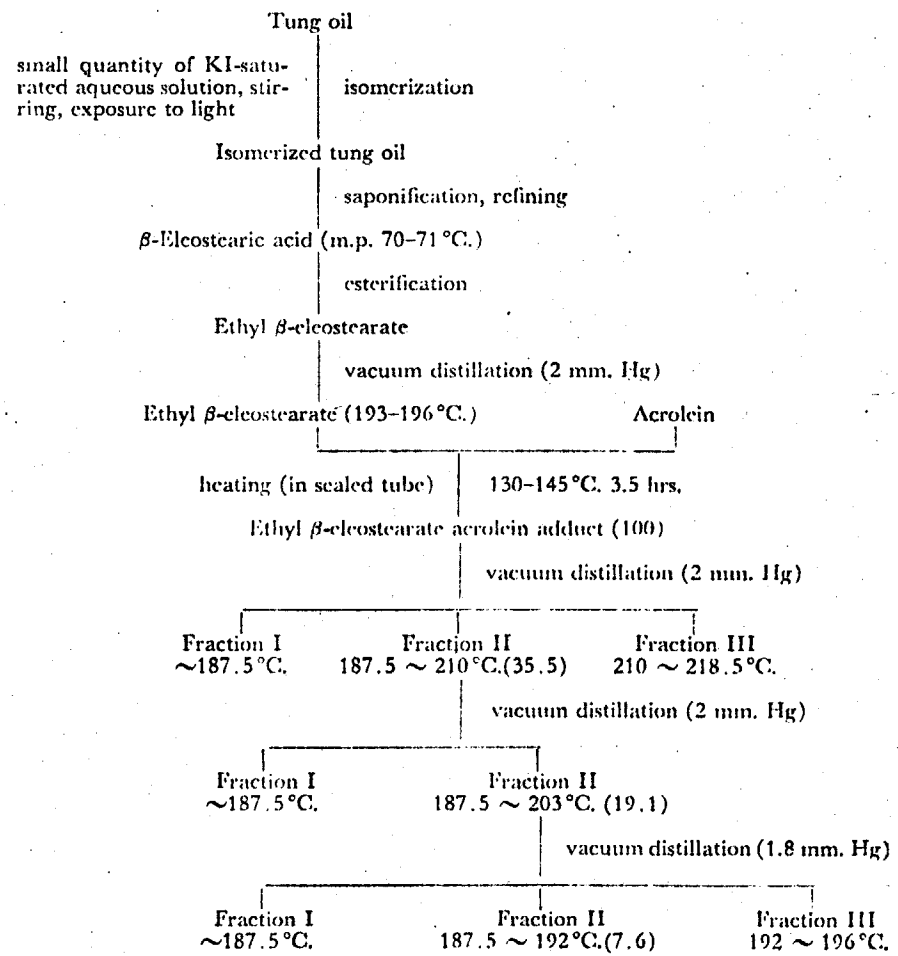


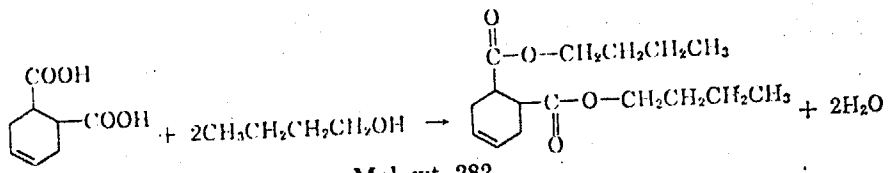
FIG. 99. PREPARATION OF ETHYL β-ELEOSTEARATE-ACROLEIN ADDUCT

599

100, all rats died in 4 to 6 days after showing an abrupt decrease in body weight.

Toxicity of the Cyclic Compound

To obtain further evidence, an experiment was conducted on a compound synthesized from cyclohexene dicarboxylic acid and butanol (Matsuo 1959B). 4-Cyclohexene-1,2-dicarboxylic acid and butanol were reacted by the usual method to form an ester as follows:



Mol. wt. 282

The ester thus obtained was distilled in vacuum twice after being washed thoroughly in water, and finally the distillate at 160 to 165°C./3 mm. Hg was collected. It is a colorless, transparent liquid, and showed the properties listed in Table 81, which are very close to the theoretical values. This ester resembled the cyclic monomer obtained from ethyl linolenate.

TABLE 81

PROPERTIES OF DIBUTYL 4-CYCLOHEXENE-1,2-DICARBOXYLATE

n_D^{20}	Iodine Value, Wijs	Saponification Value	Acid Value	Peroxide Value, Mg./100 Gm.
1.4593	90.1 90.1[1]	398.7 398.0[1]	1.2	0

[1] Theoretical value.

In an experiment in which rats, each weighing about 60 gm., were fed a basal diet containing 20 per cent of the ester, the rats lost weight quickly as shown in Fig. 101, and all died within eleven days.

From these experiments, it is recognized that the cyclic monomer produced by heat treatment of unsaturated fatty acid is a substance responsible for toxicity to animals.

Rats were fed for more than a year on a basal diet containing five to ten per cent of fish oil (cuttlefish oil) thermally polymerized in a stream of carbon dioxide gas at 250°C. for ten hours. Pathological studies on these rats are being made by the present author with the cooperation of Professor Ogata of the Department of Pathology, Faculty of Medicine, Tokushima University.

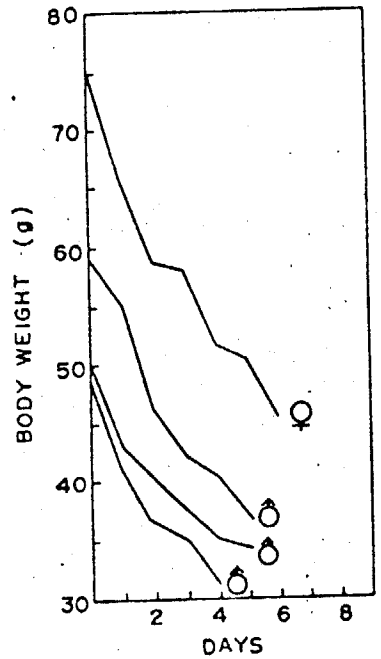


FIG. 100. GROWTH CURVES OF RATS FED ON BASAL DIET CONTAINING TEN PER CENT OF ADDUCT OF ETHYL β-ELEOSTEARATE AND ACROLEIN

All rats died at the end of the curves.

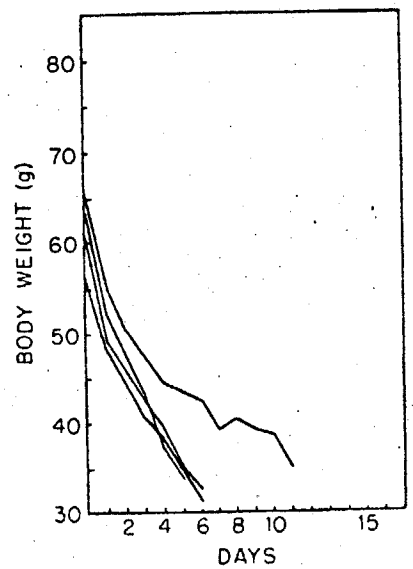


FIG. 101. RATS FED ON 20 PER CENT OF DIBUTYL CYCLOHEXENE DICARBOXYLATE

All rats died at the end of the curves.

SEBORRHEA

The author would like to add a few words with regard to seborrhea. When rats are fed with a large amount of sperm oil, seborrhea is developed and an oily secretion comes out of their skin, making the rats look as if taken out of an oil bath. This fact was discovered by Sahashi

TABLE 82

PROPERTIES OF OLEYL OLEATE AND SPERM OIL

	n_D^{20}	Iodine Value, Wijs	Saponification Value	Acid Value
Oleyl oleate	1.4612	88.6 (95.5)	113.8 (105.3)	4.9
Sperm oil	1.4640	86.7	126.4	0.5

599

(1933) some time ago and was further studied by Somekawa (1933, 1938) and Kaneda (1954C, 1957).

Fig. 102 shows an example of rats with seborrhea (Matsuo 1957D). When rabbits were fed with 15 per cent sperm oil or oleyl oleate having the properties as shown in Table 82, it was also clear that severe seborrhea

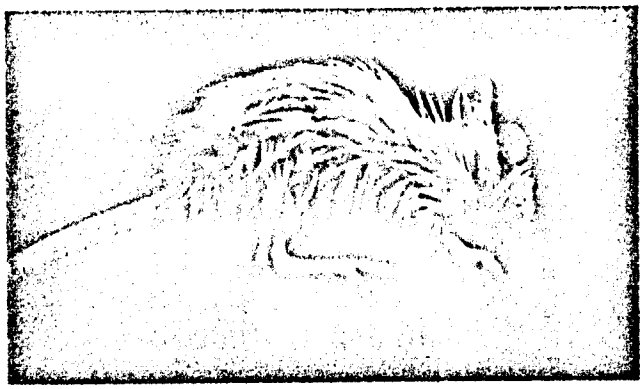


FIG. 102. EXAMPLE OF RAT'S SEBORRHEA

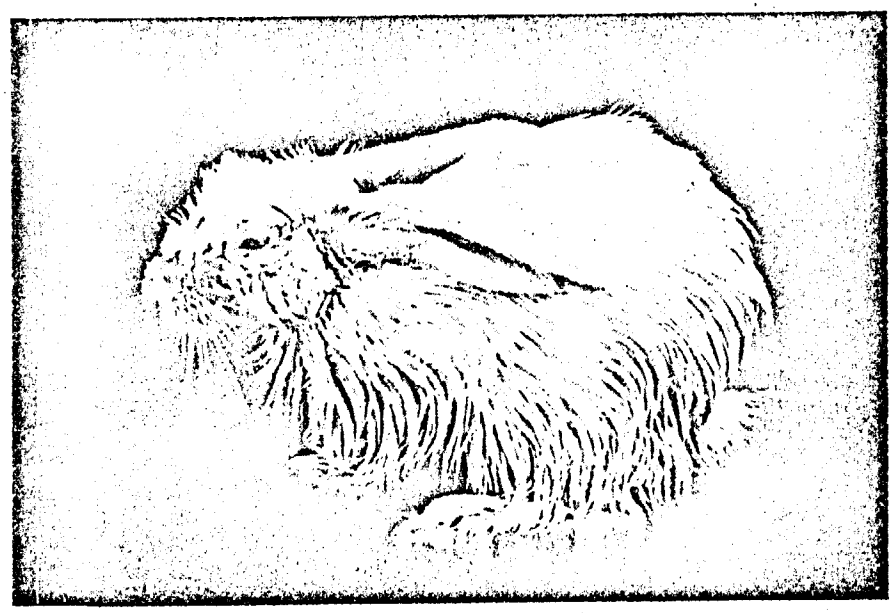


FIG. 103. RABBIT PRESENTING SYMPTOMS OF SEBORRHEA DUE TO FEEDING WITH 15 PER CENT OLEYL OLEATE

27th day after start of experiment.

developed and they died in about a month. Figs. 103 and 104 show rabbits with seborrhea (Matsuo 1960C).

The saturated monohydroxy alcohol esters of oleic acid were prepared (Table 83), and fed to rats at a level of 15 per cent of the feed for the purpose of clarifying the relationship between the number of carbon atoms

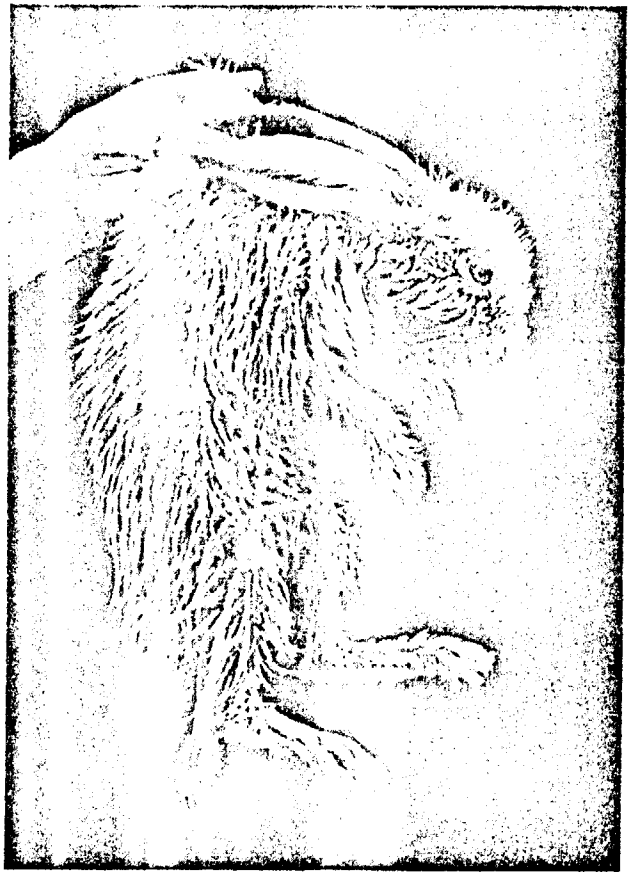


FIG. 104. RABBIT PRESENTING SYMPTOMS OF SEBORRHEA DUE TO FEEDING WITH 15 PER CENT SPERM OIL.

27th day after start of experiment.

and development of seborrhea. Up to the carbon number of 28, there were no symptoms of seborrhea. When dodecyl oleate ($C_{17}H_{33}COOC_{12}H_{25}$) and tetradecyl oleate ($C_{17}H_{33}COOC_{14}H_{29}$) were used, insignificant development of seborrhea was observed.

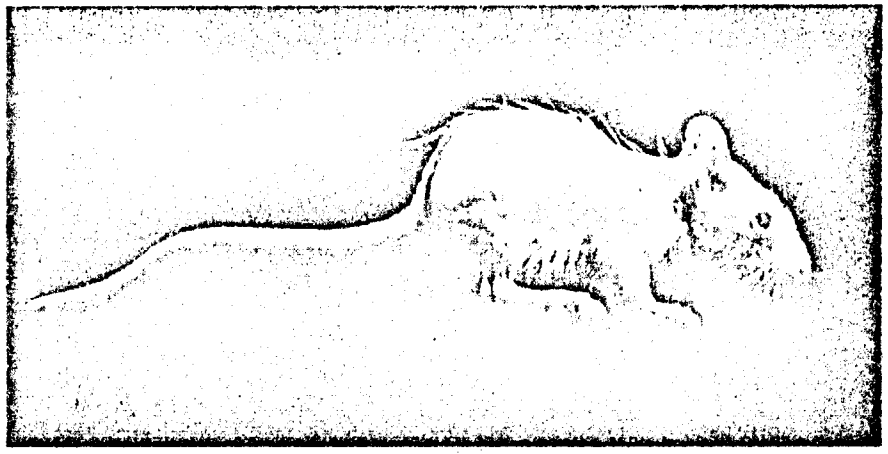


FIG. 105. RAT PRESENTING SYMPTOMS OF SEBORRHEA DUE TO FEEDING WITH 15 PER CENT 4-ETHYL-2-METHYL-4-UNDECYCLOLEATE

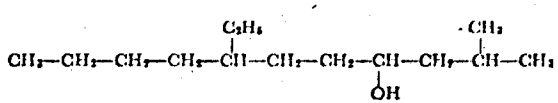
28th day after start of experiment.

TABLE 83

PROPERTIES OF OLEIC ACID ESTERS

Ester	Formula	n_D^{20}	Iodine Value	Saponification Value	Acid Value	Melting Point
Ethyl oleate	$C_{17}H_{33}COO—C_2H_5$	1.4546	84.8 (81.9)[1]	180.9	3.9	-
Amyl oleate	—C_5H_{11}	1.4545	71.6 (72.0)	161.0 (159.1)	4.1	-
Octyl oleate	—C_8H_{17}	1.4529	63.4 (64.3)	142.9 (142.1)	1.5	-
Decyl oleate	—$C_{10}H_{21}$	1.4525	60.2 (60.0)	136.8 (132.7)	2.0	-
Dodecyl oleate	—$C_{12}H_{25}$	1.4550	55.3 (56.3)	126.0 (124.4)	0.6	-
Tetradecyl oleate	—$C_{14}H_{29}$	1.4540[3]	53.6 (53.0)	120.9 (117.2)	4.8	20.0 ~22.0
Cetyl oleate	—$C_{16}H_{33}$	1.4540[3]	50.0 (52.1)	111.8 (110.7)	3.1	31.0 ~32.5
Octadecyl oleate	—$C_{18}H_{37}$	1.4540[3]	47.5 (47.6)	105.9 (104.9)	3.9	32.0 ~33.5
Oleyl oleate	—$C_{18}H_{35}$	1.4664	91.4 (95.5)	106.9 (105.3)	2.3	31.0 ~32.5
Tetradecyl oleate	—$C_{14}H_{29}$[2]	1.4539	54.2 (53.0)	120.4 (117.2)	6.2	-

[1] Numbers in parentheses are theoretical values.
[2] $C_{14}H_{29}OH$: 7-ethyl-2-methyl-4-undecanol.
[3] n_D^{40}.



When cetyl oleate ($C_{17}H_{33}COOC_{16}H_{33}$) or other compounds of larger carbon number were fed, there was a marked development of seborrhea. The average rate of increase in the weight of rats generally decreased with an increase in carbon number (Table 84). For instance, when rats were fed 15 per cent of the ester of 7-ethyl-2-methyl-4-undecyl oleate, which is a saturated monohydric alcohol with a side chain of oleic acid, a particularly remarkable development of seborrhea was noticed.

TABLE 84

OCCURRENCE OF SEBORRHEA

Ester	Formula	Occurrence of Seborrhea	Average Body Weight Increased, Gm./22 Days
Ethyl oleate	$C_{17}H_{33}COO—C_2H_5$	○ ○ ○ ○ ○	60.1
Amyl oleate	—C_5H_{11}	○ ○ ○ ○ ○	60.3
Octyl oleate	—C_8H_{17}	○ ○ ○ ○ ○ ○ ○ ○	53.8
Decyl oleate	—$C_{10}H_{21}$	○ ○ ○ ○ ○ ○ ○ ○ ○	50.4
Dodecyl oleate	—$C_{12}H_{25}$	◐ ◐ ○ ◐ ◐ ○ ○ ○ ○ ◐ ○ ◐ ○	37.4
Tetradecyl oleate	—$C_{14}H_{29}$	◐ ◐ ◐ ◐ ◐	20.6
Cetyl oleate	—$C_{16}H_{33}$	● ● ● ● ●	9.0
Octadecyl oleate	$C_{18}H_{37}$	● ● ● ● ● x	15.0
Oleyl oleate	—$C_{18}H_{35}$	● ● ● ● ● x x	− 6.0
Tetradecyl oleate	$C_{14}H_{29}$[1]	● ● ● ● ● ● ● ● x x x	−17.5

○ Seborrhea not produced.
◐ Seborrhea slightly produced.
● Seborrhea produced.
[1] $C_{14}H_{29}OH$: 7-ethyl-2-methyl-4-undecanol.
x Died.

Fig. 105 shows the appearance of a rat with acute symptoms after feeding 4-ethyl-2-methyl-4-undecyl oleate (Matsuo 1960C).

DISCUSSION

M. E. Stansby.—At the very beginning of your paper in the second sentence, you state "Research conducted recently made it clear that autoxidized or thermally polymerized fish oil shows acute toxicity as compared to other oils and fats." My question is, particularly with reference to the autoxidized part of it, is it not true that other oils and fats if oxidized to the same peroxide value, as you used in your experiments, would be expected to show similar results.

N. Matsuo.—Regarding fish oils, because they have a greater content of unsaturated fatty acids compared with the other oils, also show greater toxicity after autoxidation, I agree that if there is autoxidation in the other fat it will also show the toxicity, but this was not studied.

J. E. Halver.—Have you conducted any longer term feeding studies with lower levels of these autoxidized oils?

N. Matsuo.—Chronic feeding trials have been under way for the period of one year, but the data hasn't been analyzed yet. Chronic studies of three months' duration were carried out.

A. L. Tappel.—In Japan, I understand, you have many fish products that, for example, are dried under rather severe conditions and so you do get thermal polymerization and peroxidation. Is there a human dietary problem because of the consumption of these products?

N. Matsuo.—Most dried fish is heated or cooked before being consumed and the peroxides would therefore be decomposed.

H. Kaunitz.—There has been repeated reports from Spain and particularly from Japan, that the lipid polymerization of fish oil does not render it unpalatable. There was one report I think by Osaki, but I may be mistaken, that when he took the oil of a fish called the plaice and strongly polymerized it and then fed it to rats, that there was no difference in growth of those rats compared to fresh oils. Do you have any experience with this?

N. Matsuo.—I have not seen this literature.

L. J. Machlin.—This is more in the nature of a comment than a question. I noticed that in most of the diets only nine per cent casein was used, and as Dr. Kummerow pointed out that as you increase the protein level you get some protection. Kaunitz used 30 per cent casein. I would suspect that the results are exaggerated in these studies.

N. Matsuo.—I used twelve per cent casein and it gave similar results.

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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,[1] 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 fell 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°.[1]

Before entering upon the problem of self-poisoning, some remarks should be made of impurities occurring in the original oils. Those 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 confimed 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, 22 (1959).
16) O. Toyama: The Review of Phys. Chem. Japan, 14, 88 (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. Scheer, R. Kelley: J. Phys. Chem., 68, 598 (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.

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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[Chem. Pharm. Bull. 13(7) 843~858 (1965)]

UDC 542.941.7 : 541.12

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

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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 Langumuir-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.,

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*1 Presented at the Annual Meeting of Chem. Soc. Japan, April 6, 1951.

*2 Chiba, Japan (三宅良一, 島村芳三, 矢島 尚).

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,*3 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 $-dc/dt=k\,P/V$,[6] 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.[1] 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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*3 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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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 9.3% saturated acids (by Twitchell's lead salt alcohol method). The oil with the above characteristics should contain 87% 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 110 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.[1,11]

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

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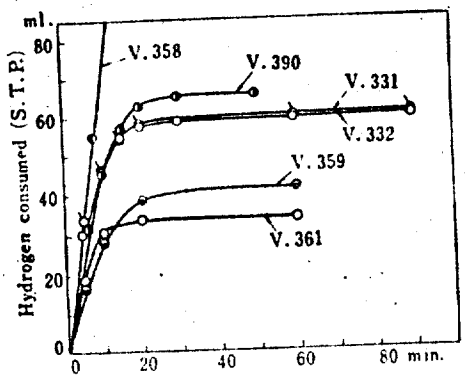

Fig. 1.

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

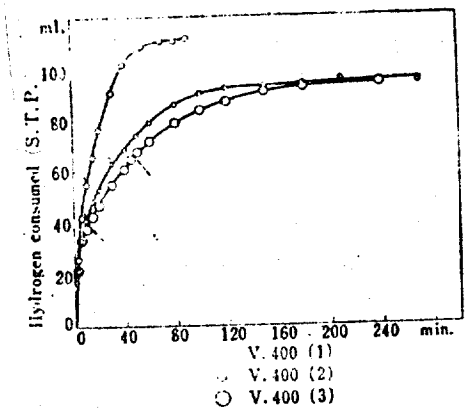

Fig. 2.

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.

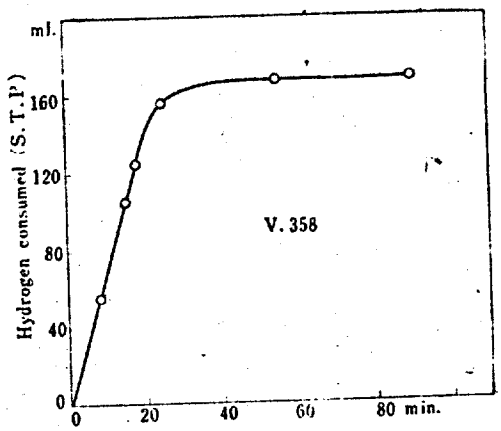

Fig. 3.

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.

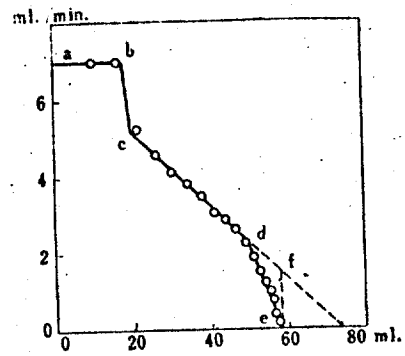

Fig. 4.

dx/dt-x relation for run V. 332. An expression of self-poisoning of catalyst.[1] 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 0.00504 min^{-1} respectively. Formally the whole course may be expressed by the first order formulation: $2.303 \log (60.20-x) = -0.1448t+4.1086$.

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	1.910
V. 359	0.0556	0.6026	1.020[a]
V. 361	0.0548	0.4942	
V. 390	0.1082	0.9443	
V. 358	0.2668	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	1.1440
V. 400 (2)		0.9733
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 suppiying 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. 4). 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 volnme 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.

TABLE III

RELATIVE DISTRIBUTION OF SATURATED AND UNSATURATED FATTY ACIDS IN THE α' AND β POSITIONS OF LECITHIN

Source	*Position*	*Mole % saturated fatty acids*	*Mole % unsaturated fatty acids*
Salmon muscle	α'	38	62
	β	9	91
Menhaden muscle	α'	81	19
	β	7	93
Tuna muscle	α'	36	64
	β	4	96
Egg yolk	α'	86	14
	β	6	94
Rat liver	α'	82	18
	β	3	97
Beef liver	α'	66	34
	β	1	99

ment of any of the fatty acids found. In the fish lecithins, docosahexaenoic acid was the other major fatty acid; however, in the egg-yolk, rat- and beef-liver materials, lower unsaturated fatty acids completed the balance of fatty acids.

Only small amounts of arachidonic acid were found in the fish lecithins; oleic, eicosapentaenoic and docosahexaenoic acids were the prevalent fatty acids in β position. Beef-liver lecithin contained some of the fatty acids normally enconntered in marine organisms. Docosamonoenoic acid was tentatively identified only in the beef sample and was primarily esterified to the β position. Trace amounts of odd chain length and branch-chained fatty acids were also detected in the beef sample but were not positively identified. These fatty acids are possibly the products of rumen bacteria.

The fatty acid composition of the lecithin of egg yolk shows good correlation with that reported by other workers; *e.g.*, in the α' position, palmitic acid 61%, stearic acid 25%, oleic acid 10%, compared to palmitic acid 65%; stearic acid 33%, oleic acid 1% (ref. 5), and palmitic acid 55%, stearic acid 36%, and oleic acid 6% (ref. 16); the correlation is also good for the β position. In the more complex lecithins containing a greater proportion of higher polyenoic acids, two difficulties are of importance: first, the quantitative problem of gas-liquid chromatography, and second, contamination with plasmalogen (compare Table I).

The difficulty with quantitative gas chromatography is illustrated by the data in Table II. Particularly in the fatty acid analyses of the fish-lipid fractions, there are discrepancies in the amounts of total compared to the amounts in the α' and β position. Since careful repetition failed to resolve this difficulty, we are inclined to conclude that it represents an inherent weakness of the methods or equipment. Previous investigators more often than not do not include data for total fatty acids so that direct comparisons are not possible. However DE TOMAS *et al.*[17] have recently published an analysis of phosphatidylethanolamine fatty acids from rats fed diets deficient in essential fatty acids in which similar discrepancies appear.

RAPPORT AND FRANZL[18] report that plasmalogens are attacked by phospholipase A and hence the liberated fatty acids will also contain the fatty acids esterified to the contaminating plasmalogen. No scheme is currently available to differentiate between the lecithin β position and that of plasmalogen. In the plasmalogen-containing lecithins this error would be expected to decrease the apparent recovery of fatty acids not associated with the plasmalogen and to increase the apparent recovery of those associated with the plasmalogen. Should the pattern of fatty acids of plasmalogen follow that of lecithin, triglycerides and other phospholipids having a highly unsaturated β composition[18-22], then one would expect to recover a larger amount of unsaturated and a smaller amount of saturated fatty acids than the theoretically calculated amount.

With the above reservation in mind, it is of interest to relate the current data with that of others and with the schemes of biosynthesis proposed for phosphatidyl choline. First, regarding the distribution of the polyenoic acids of the "linolenic acid family" *vs.* those of the "linoleic acid family", the latter being considered "essential fatty acids", it is apparent that the primary representative of the linolenic-group, docosahexaenoic acid, is primarily esterified to the β position of both the marine and terrestrial lecithins in which it occurs and hence follows the classical distribution of arachidonic and linoleic acid. Linolenic, eicosapentaenoic, docosaenoic, docosatetraenoic and docosapentaenoic acids are also similarly distributed. This distribution is then in agreement with the suggestion of KLENK AND BROCKERHOFF[23], RICHARDSON *et al.*[24], and KLENK AND EBERHAGEN[25] that the so-called essential fatty acids are not absolute requirements in the structure and function of cellular membranes, but that other unsaturated fatty acids may substitute. The enzymatic studies of LANDS AND MERKL[26] would also support such a thesis. Secondly, as was mentioned previously, the most marked positional enrichment of a given fatty acid observed was that of palmitic acid for the α' position.

Dietary effects will influence the relative proportion of saturated and unsaturated fatty acids esterified to the α' position. RHODES[27], for example, found an increase in the unsaturation of egg-yolk lecithin, when the laying hens were fed cod-liver oil. Yet even in those lecithins having the highest α'-position unsaturation, the enrichment of palmitic acid for the α' position was of the same order as that of arachidonic and docosahexaenoic acid for the β position (consider salmon and tuna: palmitic acid: ratio of $\alpha'/\beta = 4$–7; arachidonic acid: ratio $\beta/\alpha' = 0.5$–4; docosahexaenoic acid: ratio $\beta/\alpha' =$ about 1.5). BROCKERHOFF *et al.*[9] also found more palmitic acid esterified to the α' position in cod-muscle and cod-liver, scallop-muscle and lobster-liver lecithin than that found in the α' position of the triglycerides isolated from the same tissue.

ACKNOWLEDGEMENTS

We wish to thank J. R. FROINES AND R. D. JENSEN for their technical assistance, and A. GOODBAN of the Western Regional Research Laboratory, U.S.D.A., Albany, Calif., for determining the optical rotation of the samples.

This investigation was supported in part by funds administered by means of a collaborative agreement between the U.S. Fish and Wildlife Service Bureau of Commercial Fisheries and the University of California.

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United States Patent Office

2,783,260

Patented Feb. 26, 1957

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2,783,260

DEODORIZATION OF HYDROGENATED FATTY OILS

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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 constitutents 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 I

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 then raised to 13[illegible] C. and held there for 3 hours. The oil was then cooled to [illegible] C. to a temperature of 60° C., filtered, and steam deodorized at 235° C. for 3 hours. A control sample of the original hardened oil 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 oil 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° C.–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

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No Drawing. Application August 11, 1955
Serial No. 527,859

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. 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 appreciably 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	80.9
Softening point, ° F	81	102	112	126
Wiley, ° F	93	107	115	128
Setting point, ° C	26.4	29.7	31.4	39.7
Melting point, ° F	94	111	122	131
Solid fat index:				
50° F	23.8	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	18.9	23.5
100° F	0.0	3.6	11.9	19.7
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	75.0
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	28.4	35.9
Solid fat index:				
50° F	23.8	34.4	33.0	34.4
70° F	12.2	27.1	18.0	25.6
80° F	6.8	25.0	10.2	23.1
92° F	1.1	17.3	1.8	15.1
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	0.83	.01	0.57
Percent conjugated diene	0.13	0.39	1.1	0.26
Hydrogenation time (minutes)	13	34	21	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	0.008%	0.016%	0.024%
Iodine value	76.3	76.5	78.4	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	29.5	35.3	43.5
Solid fat index:				
50° F	23.8	27.5	28.8	33.8
70° F	12.2	16.4	21.4	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	12.0	18.5
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	78.6
Softening point, ° F	85	115
Wiley, ° F	88	118
Melting point, ° F	90	123
Setting point, ° C	28.4	35.6
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	.01	0.49
Percent conjugated diene	1.1	1.9
Hydrogenation time (minutes)	21	55

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 to 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 do-

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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	24	6.54 ± 0.34	1.79 ± 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	0.42 ± 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 ± 0.19	<0.01	0.15 ± 0.08
0.18	6	7.75 ± 1.33	4.99 ± 1.00	<0.10	0.32 ± 0.12	0.66 ± 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	0.97	<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	2.24	<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	0.34 ± 0.13
0.04	6	7.10 ± 1.08	2.98 ± 0.43	<0.10	0.28 ± 0.04	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 ± 0.34	<0.01	0.44 ± 0.22
0.14	6	6.12 ± 2.55	6.92 ± 0.99	<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	12.71 ± 4.39	<0.10	0.38 ± 0.24	1.90 ± 0.67	<0.01	0.44 ± 0.16
1.08	2	0.61	20.04	<0.10	0.06	2.22	<0.01	0.18
1.39	2	0.42	26.22	<0.10	0.04	2.87	<0.01	0.18
3.75	2	0.23	32.46	<0.10	0.11	2.31	<0.01	0.14
Linolenate								
0.009	6	7.23 ± 1.89	1.97 ± 0.65	0.09 ± 0.02	0.22 ± 0.07	0.27 ± 0.05	<0.01	0.46 ± 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	0.76 ± 0.30
0.04	6	5.46 ± 1.60	1.74 ± 0.68	0.18 ± 0.09	0.14 ± 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 ± 0.14	0.11 ± 0.05	0.16 ± 0.10	0.03 ± 0.05	1.50 ± 0.65
0.18	6	4.10 ± 0.64	1.08 ± 0.23	0.74 ± 0.37	0.06 ± 0.02	0.10 ± 0.07	0.22 ± 0.19	3.02 ± 1.09
0.32	6	3.24 ± 0.66	0.73 ± 0.15	1.20 ± 0.30	0.06 ± 0.01	0.11 ± 0.06	0.25 ± 0.23	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 ± 0.24	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 ± 0.04	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 ± 0.04	0.92 ± 0.24	3.41 ± 0.56
2.56	4	0.48 ± 0.15	0.69 ± 0.24	5.56 ± 1.29	0.07 ± 0.04	0.13 ± 0.07	2.25 ± 1.16	4.96 ± 1.53
3.56	4	0.47 ± 0.09	0.81 ± 0.04	7.55 ± 0.59	0.09 ± 0.03	0.14 ± 0.04	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 ± 0.04	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".[1]

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.[2]

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 0.65% 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 dond 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.

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

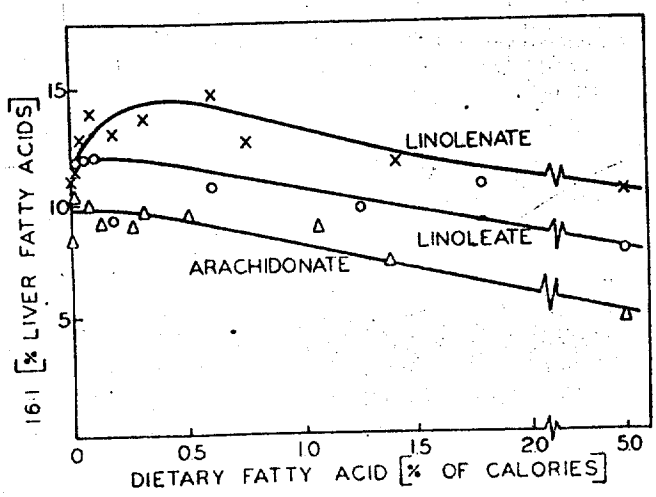

FIG. 3. The effect of varying levels of dietary EFA upon concentration of 16:1 of liver lipids.

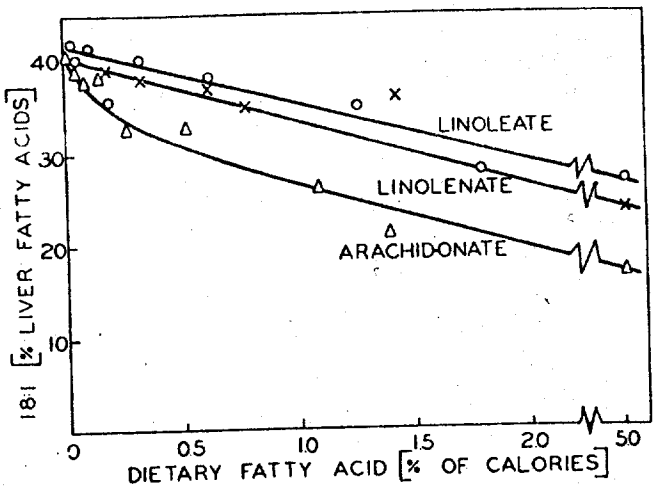

FIG. 4. The effect of varying levels of dietary EFA upon concentration of 18:1 of liver lipids.

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 divinyl-methane 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

[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. Rahm and R. T. Holman.

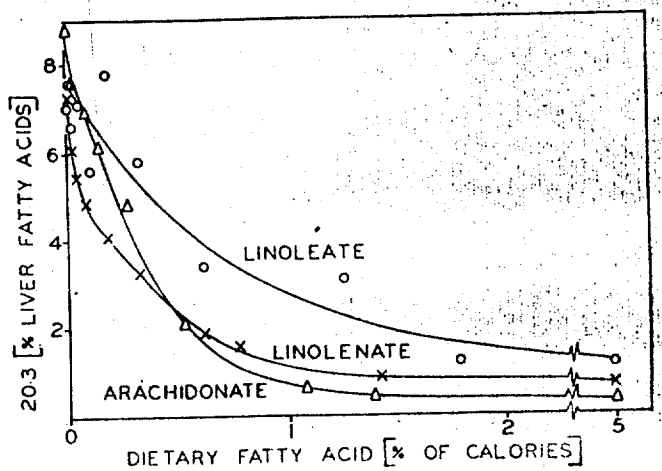

FIG. 5. The effect of varying levels of dietary EFA upon concentration of 20:3 of liver lipids.

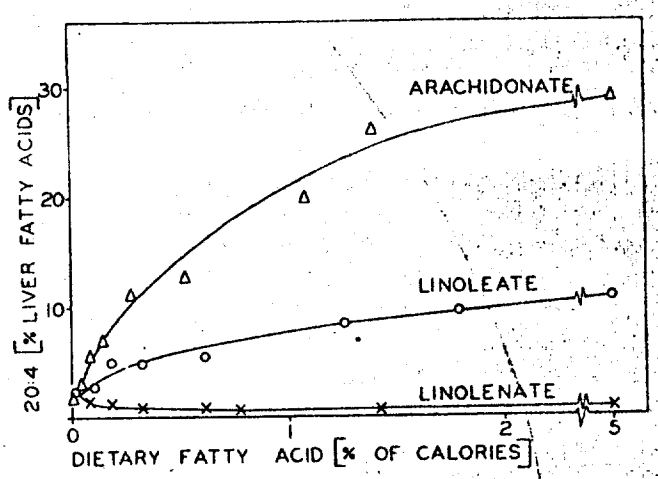

FIG. 6. The effect of varying levels of dietary EFA upon concentration of 20:4 of liver lipids.

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

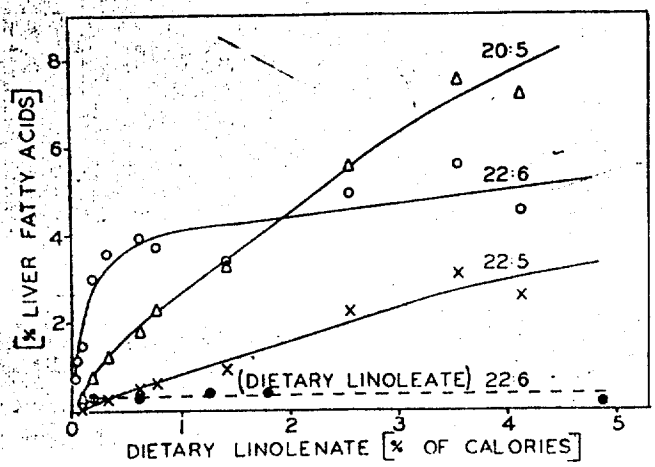

FIG. 7. The effect of varying levels of dietary linolenate upon concentration of 20:5, 22:5ω3, and 22:6 of liver lipids. The dotted line shows the effect of dietary *linoleate* upon the concentration of 22:6.

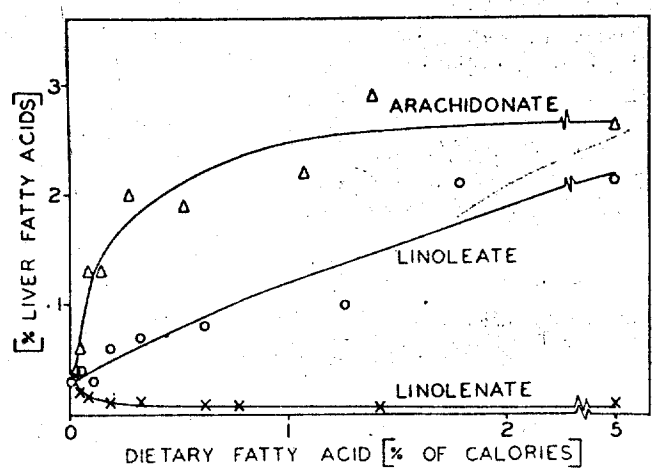

FIG. 8. The effect of varying levels of dietary EFA upon concentration of 22:5ω6 in liver lipids.

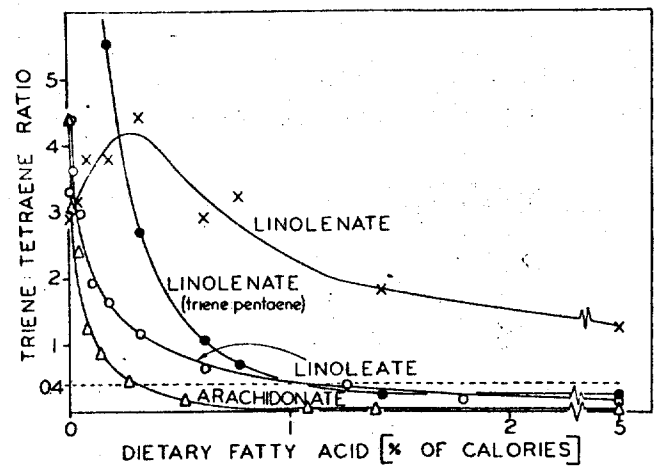

FIG. 9. The effect of varying levels of dietary EFA upon the ratio of 20:3 and 20:4 of liver lipids. Solid circles show the effect of dietary linolenate upon the ratio of 20:3 and 20:5. The dotted line represents a level of the triene/tetraene ratio below which the metabolism of EFA is considered to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

We wish to thank Joseph Seufert and Dennis Sukkestad for their excellent technical assistance and Gary Hansen and John Bonnes for the dependable care of the experimental animals.

We are very grateful for the supply of ethyl arachidonate furnished by Hoffmann La Roche, Nutley, New Jersey.

The authors also wish to express their appreciation to W. O. Caster and Phillip Ahn of the Department of Physiological Chemistry of the University of Minnesota for the discussions of this experiment as well as for the calculation of the standard deviations by computer methods. Helmut K. Mangold and W. O. Lundberg of this institute were very helpful in editing this publication.

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THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 242, No. 19, Issue of October 10, pp. 4507-4514, 1967
Printed in U.S.A.

Chain Elongation of Linoleic Acid and Its Inhibition by Other Fatty Acids *in Vitro**

J. Biol. Chem. 242(19): 4507-4514, 1967

(Received for publication, May 15, 1967)

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SUMMARY

Linoleic acid-1-^{14}C was incubated with subcellular fractions of rat liver to study the mechanism of polyunsaturated fatty acid synthesis. The most effective system for the chain elongation of linoleic acid to eicosadienoic acid was liver microsomes plus malonyl coenzyme A with cofactors, NADPH, and ATP. Under anaerobic conditions, the chain elongation reaction could be separated quantitatively from the dehydrogenation reaction which otherwise required the same system. The chain elongation of linoleic acid was inhibited by saturated fatty acids of which myristic and pentadecanoic acids were most effective. Mono-unsaturated and polyunsaturated fatty acids also act as inhibitors. From consideration of reaction kinetics and inhibition by other fatty acids, the conversion of linoleic acid to γ-linolenic acid seems preferred over the conversion of linoleic acid to homolinoleic acid.

The metabolism of polyunsaturated fatty acids has been studied mainly by feeding experiments with rats, and thus the over-all reactions are well known. Linoleic acid, 18:2ω6,[1] for example, is converted to 20:4ω6 either via 18:3ω6 and 20:3ω6 (1) or via 20:2ω6 and 20:3ω6 (2). Factors governing the preference between pathways in the tissue remain to be determined.

Several attempts have been made to find the location of the enzymes catalyzing chain elongation and dehydrogenation; it is possible that they may be studied separately.

Wakil (3) reported a system located in mitochondria of rat liver which catalyzed the chain elongation of polyunsaturated fatty acids and which was activated by pyridoxamine phosphate. Stoffel (4) described a system which elongated linoleic acid to eicosadienoic acid and which required acetate and bicarbonate. The system was located in the cytoplasm of rat liver, whereas the dehydrogenation of linoleic acid occurred mainly in a microsomal fraction. Later Stoffel and Ach (5) found that the supernatant of the microsome preparation had merely catalyzed the formation of malonyl coenzyme A from acetate and bicarbonate, and that the chain elongation was actually activated by microsomes. Nugteren (6, 7) confirmed that the chain elongation enzyme is mainly present in the microsomes. The microsomal system not only elongated but also dehydrogenated polyunsaturated fatty acids; ^{14}C-labeled linoleic acid thus yielded 18:3ω6, 20:2ω6, 20:3ω6, and 20:4ω6.

In the present report we describe a system in which the enzymatic activity responsible for the lengthening of the chain of linoleic acid and other polyunsaturated fatty acids is retained but in which the dehydrogenation activity is suppressed. Experiments are described which compare the activities of several cell constituents and which specify the optimum reaction conditions. We also describe the inhibition of the reaction 18:2ω6 → 20:2ω6 by many common fatty acids, which shows at the subcellular level inhibitions in the metabolism of polyunsaturated fatty acids which have been predicted in experiments with whole animals (8–10).

EXPERIMENTAL PROCEDURE

Preparation of Subcellular Fractions from Rat Liver—Weanling male rats of the Sprague-Dawley strain were kept on a fat-free diet as described previously (11), except that the protein content of the diet was increased from 18 to 30%, replacing part of the sucrose. Rats kept on this regime develop liver lipids which are poor in polyunsaturated acids. Rats, at 3 to 5 months of age, were killed by drawing the blood from the heart during ether anesthesia. By this method the liver obtained was pale and yielded blood-free subcellular fractions. The livers were quickly removed and cooled in 0.05 M potassium phosphate buffer, pH 7, which contained 0.25 M sucrose (isotonic), 0.15 M KCl, 1 mM EDTA, 5 mM $MgCl_2$, and 1.5 mM glutathione. The livers were then sliced and homogenized in the same solution (1:4, w/v) with the use of a Potter-Elvehjem homogenizer at 0° (cold room). The fractional centrifugation was carried out according to the procedures described by Sickevitz (12) for microsomes (105,000 × *g*, Spinco ultracentrifuge) and Hogeboom

* This investigation was supported in part by United States Public Health Service Research Grant HE 10189 from the National Institutes of Health, Public Health Service.

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[1] The number preceding the colon indicates number of carbon atoms; the number following it denotes number of double bonds; and the number following the ω indicates the position of the last double bond counting from the terminal methyl group.

(13) for mitochondria (17,000 × *g*). The microsomal and mitochondrial pellets were resuspended in a solution containing 0.25 M sucrose, 0.15 M KCl, and 0.1 mM EDTA. The protein contents of the suspensions were determined by the biuret reaction (14). Fatty acids were incubated with aliquots of these suspensions. Subcellular fractions were prepared in the morning, and the incubations were carried out in the afternoon of the same day.

Electron Micrographs of Microsome Preparations[2]—Electron micrographs were prepared to judge the uniformity and morphology of the microsomal preparation obtained by fractional centrifugation. A sample of the microsomal pellet was suspended in a freshly filtered solution of 0.2% ammonium acetate in quartz-distilled water and sedimented at 105,000 × *g* for 60 min. The pellet was then resuspended in the same solution and diluted 1:10,000 and 1:50,000. The particles were sedimented on Formvar-covered copper grids (400 mesh) in a Sorvall RC2 centrifuge with the SV rotor at 30,000 × *g* for 20 min. The specimens were examined in a Siemens Elmscopy I electron microscope and photographed on Kodak projection slides, 6.5 × 9.0 cm.

Preparation and Assay of Malonyl-CoA—Malonyl-CoA was synthesized by transacylation of *S*-malonyl-*N*-decanoyl-cysteamine with reduced coenzyme A by a modification (14) of the method described by Eggerer and Lynen (15). Batches of 12 to 20 μmoles of malonyl-CoA were prepared and stored frozen at pH 5. The solutions were stable for several weeks. However, for most of the experiments a fresh preparation was used. The malonyl-CoA concentration was determined by the delayed nitroprusside reaction as described by Lynen (16) and by the hydroxamic acid test as described by Stadtman (17).

Linoleic Acid-1-^{14}C—This compound, with a specific activity of 37.8 mC per mmole, was purchased from Nuclear-Chicago. Its radiopurity was determined after dilution (1:27, w/w) with cold linoleic acid (Lipid Preparations Laboratory of the Hormel Institute). Gas-liquid chromatography (18, 19) of an esterified sample showed that all of the radioactivity was associated with linoleic acid. Thin layer chromatography of the free fatty acid and autoradiography of the plate showed that only traces of radioactivity, probably due to autoxidation products, remained at the starting point. The content of *trans* double bonds of the fatty acid was approximately 17%, as determined by infrared spectrometry. The content of *trans* double bonds was not taken into consideration for computation of concentrations and yields, because the metabolic conversions of *cis-trans*, and *trans-trans* 18:2ω 6 acids have not been elucidated fully.

Medium chain and long chain fatty acids were purchased from the Lipid Preparations Laboratory of the Hormel Institute. γ-Linolenic acid was synthesized via the triynoic acid in this laboratory.[3] The acids 18:3ω6, 20:4ω6, 20:5ω3, and 22:6ω3 were purified shortly before use by thin layer chromatography on Silica Gel G impregnated with silver nitrate. Gas-liquid chromatography of the respective methyl esters showed that the purified acids were at least 99% pure.

Incubation—Microsomal protein, 10 mg, and 0.3 to 0.6 μmole of malonyl-CoA were placed in Warburg flasks with 120 μmoles of potassium phosphate buffer at pH 7.0, 0.1 μmole of reduced CoA, 0.2 μmole of NADPH, 10 μmoles of ATP, and 15 μmoles of $MgCl_2$ (5, 20). In each case the malonyl-CoA was added in at least a 10% M excess over the fatty acid to be incubated. The oxidizing capacity of the system was suppressed by 3 μmoles of KCN and 125 μmoles of KF (21). The volume of the incubation mixture was adjusted to 3 ml with a solution of 0.25 M sucrose, 0.15 M KCl, and 0.1 mM EDTA.

The substrate and inhibitor fatty acids (1 mg) were dispersed in twice their molar amount of 0.1 N NH_4OH and made up to 1 ml with 1% Triton WR 1339 (Ruger Chemical Company, Irvington on Hudson, New York). Aliquots of this micellar solution were inserted into the side arms of Warburg flasks.

In anaerobic incubations, air was evacuated and the flasks were flushed with argon (99.995%) at least three times. After gassing with argon, the flasks were tipped and the side arms were rinsed with the incubation mixture. The flasks were shaken in a Dubnoff metabolic shaking incubator at 37°. The reaction was stopped by pouring the content of each flask into a precooled (−70°) test tube fitted with a ground joint. The contents were quickly frozen and lyophylized overnight. The dry residue was refluxed with 15 ml of a 5% solution of HCl in methanol for 2 hours in the same tube. The methyl esters were extracted with three portions of light petroleum hydrocarbon which were combined, washed free of acid, and dried over anhydrous Na_2SO_4. This procedure yields a more complete extraction of fatty acids from the incubation mixture than conventional saponification and re-esterification. Therefore it was not necessary to dilute the ester mixture with cold carrier for purposes of handling.

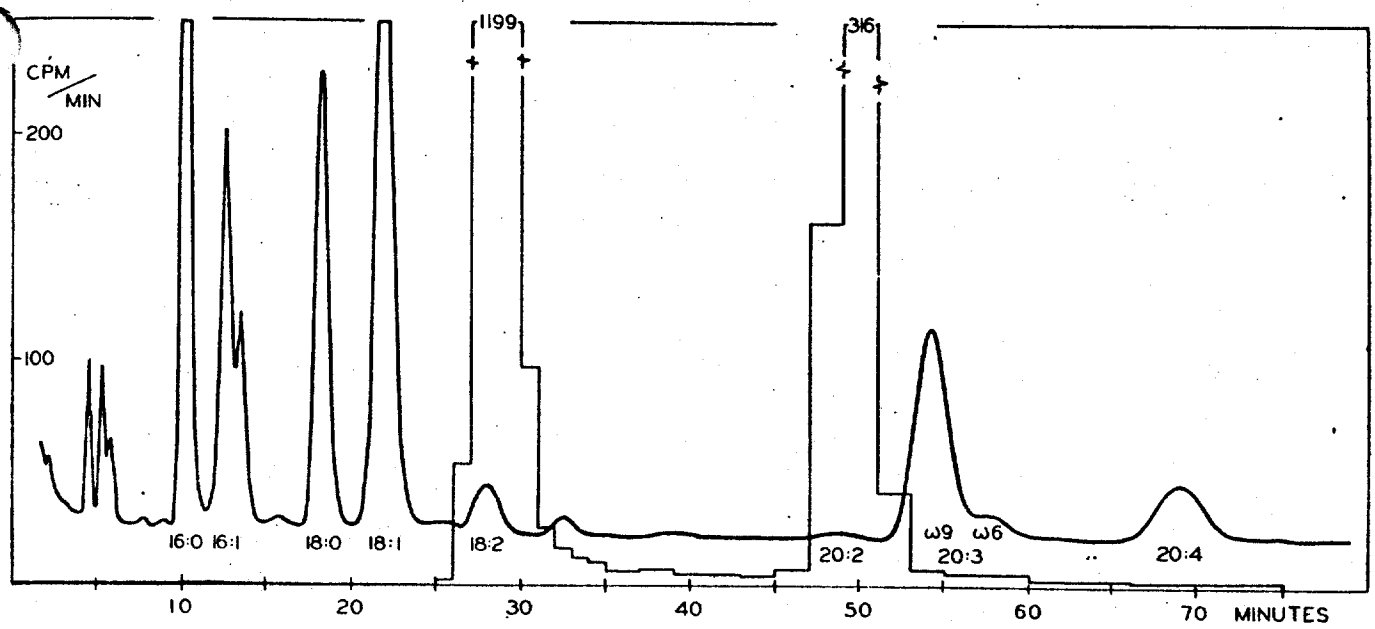
Analysis of Fatty Acid Mixtures—The chain elongation of ^{14}C-labeled linoleic acid to C_{20} acids was observed qualitatively and quantitatively. The mixture of methyl esters obtained from the incubation slurry was analyzed by radio-gas-liquid chromatography by means of a gas chromatograph with radiation ionization detector. The columns employed were aluminum tubing, 300 × 0.6 cm, packed with 20% ethylene glycol succinate plus 2% phosphoric acid on Gaschrom P (Applied Science Laboratories). Temperatures were 180° at the column and 230° at the inlet, in the detector cell, and at the outlet system. Argon flow was 60 to 80 ml per min. The apparatus was equipped with an outlet that allowed collection of the effluent directly into a scintillation counter vial, as described previously (18, 19). In each initial experiment, fractions of the effluent were collected in intervals of 1 to 5 min in order to coordinate the radioactivity with peaks on the chromatographic chart (see Fig. 1). For routine analyses the relevant peak was collected in one vial. Radioactivity was determined in a Packard dual channel scintillation counter. The scintillation solution contained 5 g of 2,5-diphenyloxazole and 0.5 g of 1,4-bis[2-(4-methyl-5-phenyl oxazolyl)]-benzene (Packard Instrument Company) in 1 liter of redistilled toluene.

The rate of conversion of linoleic to C_{20} acids was calculated from the ratio of the radioactivity in the C_{20} acid to the total radioactivity collected from a gas-liquid chromatography run. This method of scanning radioactivity has a distinct advantage over "radio-gas chromatographs" which employ a flow through cell with ratemeter. It is more sensitive because the radioactivity can be counted independently of the flow rate. Therefore the specific activity of the substrates used in biological

[2] We appreciate the help of Dr. H. Jenkin of this laboratory in the preparation of the electron micrographs and of Miss Donna Riki, Department of Microbiology, University of Minnesota.

[3] H. Mohrbacher, K. Christiansen, and M. Deubig, manuscript in preparation.

[4] Y. Marcel and R. T. Holman, manuscript in preparation.



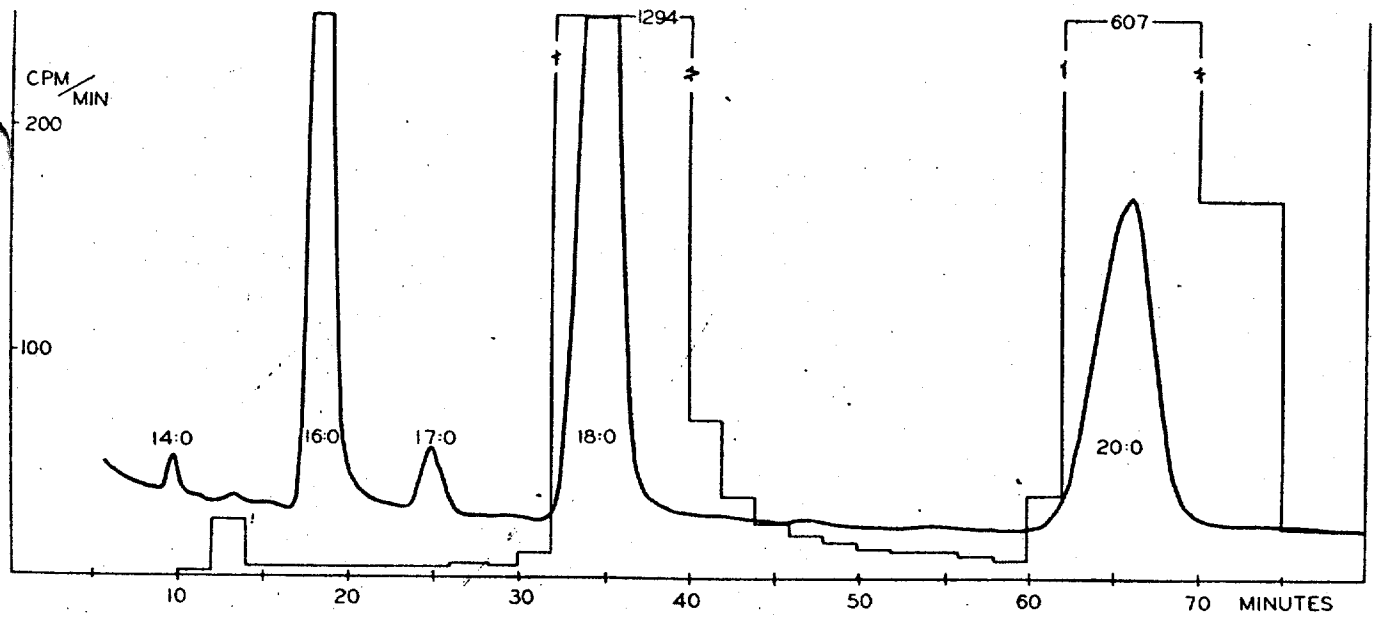


FIG. 1. (*upper*), gas-liquid chromatogram of the fatty acid mixture obtained from an anaerobic incubation of 1-^{14}C-18:2ω6 with liver microsomes (10 mg) from fat-deficient rats for 20 min at 37° with the cofactors described under "Experimental Procedure." Only the portion of the chromatogram is given that is of interest for the chain elongation of 18:2ω6 to 20:2ω6. The *step diagram* depicts the radioactivity in counts per minute that is associated with the gas-liquid chromatographic effluent collected in 1 min. (*lower*), gas-liquid chromatogram of a hydrogenated fatty acid mixture obtained under the same conditions as described above.

experiments can be much lower than that required for ratemeter counting.

Identification of Fatty Acids—Tentative identification of the fatty acids was made via the equivalent chain lengths (22) determined on authentic fatty acid esters. Authentic 20:2ω6, which was synthesized from linoleyl methanesulfonate (23) and sodium malonate (24), confirm the chain length of the metabolic product; the ester mixture was hydrogenated, and the saturated fatty acids were separated by gas-liquid chromatography and identified by internal standards (Fig. 1). Final proof of identity of the principal product, 20:2ω6, was furnished by ozonizing the mixture of C_{20} acids obtained by gas-liquid chromatography of esters from the incubation mixture, and reducing the ozonides with triphenylphosphine (Eastman Kodak) followed by scanning of radioactivity of the resulting mixture of aldehydes and aldehyde esters separated by gas-liquid chromatography (25) under the same conditions as for methyl esters. The peak of radioactivity coincided with the retention time of the authentic C_{11}-aldehyde ester produced by ozonization and reduction of Δ-11-eicosenoic acid, 20:1ω9 (Lipid Preparation Laboratory of The Hormel Institute).

Incorporation of Substrate into Lipids—To investigate the incorporation of the substrate fatty acid and its chain-elongated product into various lipids, the incubation was carried out as

described above. The lyophilized residue was extracted three times with 10 ml of chloroform-methanol (2:1) by shaking in a separatory funnel. The extract was washed with 0.9% NaCl solution and dried over Na_2SO_4. Aliquots of the lipid extract were analyzed on thin layer plates with the solvent systems, petroleum ether-diethyl ether-acetic acid (90:10:1) and chloroform-methanol-water (65:25:4). The lipid classes were identified by the use of standards of phosphatidyl choline and ethanolamine and the thin layer chromatography standard no. 2 from the Lipid Preparations Laboratory of The Hormel Institute. Autoradiograms of the plates were produced on Kodak No-Screen medical x-ray film. The distribution of radioactivity was measured by densitometry of the autoradiograms and by scraping off the spots from the plates and counting of these fractions in suspension according to Snyder and Stephens (26).

Assay for Inhibition of Chain Elongation—The same basic incubation procedure was applied as described above, but varying amounts of the competing fatty acids in concentrations similar to that of 18:2ω6 were incubated together with linoleic acid. The saturated acids tested included even and odd fatty acids from 8:0 to 22:0. The unsaturated acids were 18:1ω9, 18:3ω6, 18:3ω3, 20:4ω6, 20:5ω3, and 22:6ω3. The inhibitory effect of these fatty acids was measured by determining the decrease in yield of 20:2ω6. To measure initial reaction velocity, the incubation was interrupted after 6 min, before the substrate concentration had changed appreciably. From these data Lineweaver-Burk plots were constructed and Michaelis-Menten constants were calculated.

RESULTS AND DISCUSSION

Chain Elongation of Linoleic Acid—A chromatogram obtained from incubation of liver microsomes of fat-deficient rats with ^{14}C-linoleic acid is shown in Fig. 1 (*upper half*). Appreciable amounts of radioactivity are associated only with the substrate fatty acid, 18:2ω6, and with the first product of chain elongation, 20:2ω6. Confirmation of its chain length was provided by hydrogenation of the total fatty acid mixture, and by radio

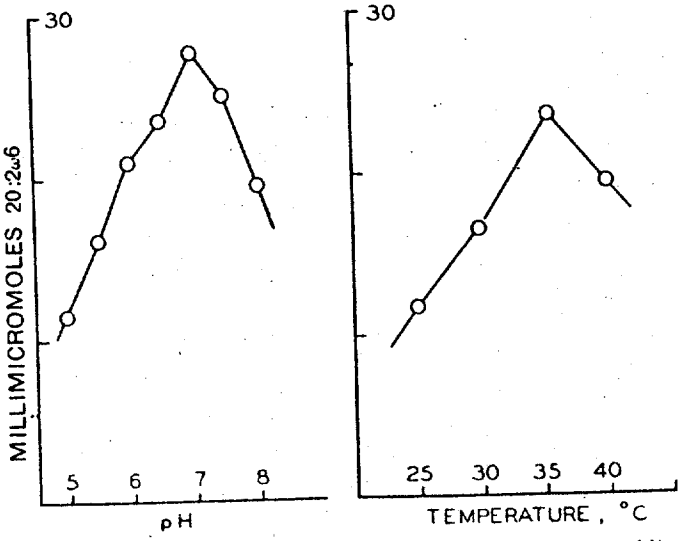


FIG. 2: pH and temperature optima for the conversion of linoleic to eicosadienoic acid by liver microsomes from fat-deficient rats. At varying pH, temperature was 36°; at varying temperatures, pH was 6.5; 450 mμmoles of the radioactive acid were incubated with 10 mg of protein and 0.6 μmole of malonyl-CoA. Other cofactors were as described under "Experimental Procedure."

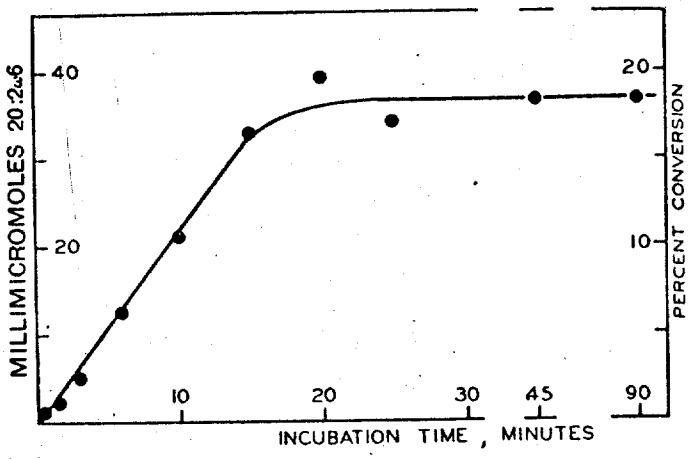


FIG. 3. Time dependence and yield of the chain elongation of linoleic (18:2ω6) to eicosadienoic acid (20:2ω6) by liver microsomes from fat-deficient rats. 1-^{14}C-18:2ω6, 200 mμmoles, was incubated at 37° with 10 mg of microsomal protein, 0.6 μmole of malonyl-CoA, and the other cofactors as described under "Experimental Procedure."

gas-liquid chromatographic analysis. The chromatogram of this preparation is shown in Fig. 1 (*lower half*). The only peaks which were radioactive were C_{18} and C_{20}. Thus the conversion of a C_{18} acid to a C_{20} acid is demonstrated. The location of the first double bond at position 11 in the 20:2 was shown by ozonolysis and reductive cleavage of the ozonides. Radioactivity was associated only with a C_{11}-aldehyde ester.

The optimum conditions for the chain elongation of linoleic acid were found to be near pH 7 and 35° (Fig. 2), and all subsequent incubations were carried out under these conditions. The yields of 20:2ω6 were proportional to time up to 20 min, after which they were constant (Fig. 3).

In the presence of oxygen, liver microsomes catalyze the dehydrogenation of linoleic acid to γ-linolenic acid (20, 27). In the presence of oxygen and malonyl-CoA the reactions 18:2ω6 → 20:2ω6, 18:2ω6 → 18:3ω6, and 20:2ω6 → 20:3ω6 → 20:4ω6 all took place. Chain elongation of 18:3ω6 to 20:3ω6 acid may occur also. In our experiments under aerobic conditions, the chain elongation reaction constituted about 50% of the over-all conversions (Table I). The rigid exclusion of oxygen increased the yield of longer chain products when malonyl-CoA was present. In many experiments we succeeded in completely eliminating the dehydrogenation reaction, so that the only reaction observed was chain elongation (Table I, "Anaerobic 2").

The substances necessary for the chain elongation of linoleic acid are listed in Table II. There is minimal conversion of linoleic acid in the absence of malonyl-CoA, NADPH, or ATP. All other components of the system used in this study served to prevent side reactions, or helped to preserve the structural integrity of the subcellular particles employed. Whereas malonyl-CoA and NADPH are directly involved in the chain elongation cycle (28), ATP is required for the formation of the CoA ester of linoleic acid, and need not be present if the CoA ester of linoleic acid is used as a substrate (5, 20). Only catalytic amounts of CoA are required for the formation of the fatty acid CoA esters, since the CoA is set free at the end of the elongation reaction and may return to the cycle. These small amounts are introduced with the malonyl-CoA and the liver microsomes. Boiling the microsomal preparation for 5 min destroyed most

TABLE I

Separation of chain elongation and dehydrogenation reactions of linoleic acid (18:2ω6) by microsomal protein

1-^{14}C-Linoleic acid was incubated in the amounts indicated with 10 mg of protein, 0.3 μmole of malonyl-CoA, 0.1 μmole of reduced CoA, 2 μmoles of NADPH; 10 μmoles of ATP, 15 μmoles of $MgCl_2$, 4.5 μmoles of GSH, and 125 μmoles of potassium phosphate buffer (pH 7) in 3 ml of a solution containing 10^{-3} M EDTA, 0.15 M KCl, and 0.25 M sucrose at 37°.

	Substrate 18:2ω6	Incubation time	Product of conversion				Chain elongation[a]
			18:3ω6	20:2ω6	20:3ω6	20:4ω6	
	mμmoles	min	mμmoles				% conversion
Aerobic	200	10	6.0	17.4	8.6	8.4	52
	200	20	8.0	20.4	7.6	11.6	51
Anaerobic[b] 1	200	10	1.2	30.0	5.8	2.0	77
	200	20	1.2	38.0	9.0	2.0	78
Anaerobic[b] 2	50	20	<0.1	10.7	<0.1	<0.1	96
	100	20	<0.1	22.1	<0.1	<0.1	98

[a] 20:3 represents one elongation plus one dehydrogenation, and 20:4 represents one elongation plus two dehydrogenations. Therefore, elongation as percentage of total conversions = 100 [(20:2 + 20:3 + 20:4)/(18:3 + 20:2 + 2(20:3) + 3(20:4))].

[b] The amount of dehydrogenation products varied from incubation to incubation, probably in relation to differences in microsomal preparation and efficiency in oxygen removal.

of its activity, an indication that the chain elongation reaction is catalyzed by an enzyme system.

Mitochondria effected a low rate of conversion which did not change after their further purification (Table III). Therefore, a catalytic action of this fraction cannot be ruled out. However, it is definitely not the major site of the enzymes for the chain elongation, as previously suggested (3). The same is true for the supernatant of the microsomal fraction. Even after a very rigorous sedimentation at high speed for a long time, some activity could be observed in the cytoplasmic fraction, but it cannot be considered the primary site of chain elongation. This is contrary to a previous report (4). The postmicrosomal pellet which most likely consists of some microsomes and ribosomes has minimal activity. We agree with Nugteren (6, 7) that the endoplasmic reticulum (microsomes) must be regarded as the major site for the chain elongation of polyunsaturated fatty acids in rat liver. Purification of the microsomal fraction by resuspension and resedimentation did not change the rate of conversion of 18:2ω6 to 20:2ω6. Thus the addition of supernatant to the microsomal fraction represented no advantage. The uniformity of the microsome fraction was checked by preparing electron micrographs. Only particles of similar size could be observed. Mitochondria were not present. Some of the microsomal fragments aggregated, forming lumps of four to eight of the normal pieces, which could not be separated by ultrasonic vibration.

Incorporation of Substrate and Chain-elongated Fatty Acids into Lipids of Liver Microsomes—The substrate and the product of the chain elongation system were incorporated into the lipids of liver microsomes. The lipid classes were separated by thin layer chromatography, and the radioactivity of the fractions was determined after the bands were scraped off into vials containing scintillation solution and Cab-O-Sil (26). The highest radioactivity was found in the phosphatidyl cholines (37%), followed by 32% in the triglycerides. Only 15% was found in the phosphatidyl ethanolamines, 10% in the free fatty acids, and 6% in other polar lipids. In comparable studies of dehydrogenation, Brenner and Peluffo (20) found almost no radioactivity in the triglycerides, only a small amount in the free fatty acids, and the rest of the radioactivity in the polar lipids. Within the phospholipids, the phosphatidyl ethanolamines were found to contain slightly more of the labeled acids than the phosphatidyl cholines.

Inhibition of Chain Elongation of Linoleic Acid by Other Fatty Acids—The inhibition of the conversion of linoleic acid to arachidonic acid by linolenic acid was first observed in nutritional experiments with chickens and rats (9, 29), and this effect has been studied by a number of investigators (10, 30, 31). Recently Brenner and Peluffo (20) have shown that inhibition occurs at the dehydrogenation step of the conversion of linoleic to arachidonic acids by liver microsomes of fat-deficient rats.

TABLE II

Cofactor dependence of chain elongation of 1-^{14}C-linoleic acid (18:2ω6) to eicosadienoic acid (20:2ω6) by microsomal protein

Cofactors	Product of conversion		
	18:3ω6	20:2ω6	20:3ω6
	mμmoles		
Complete system[a]	1.0	21.9	0.9
Boiled 5 min	5.6	0.7	0.6
Minus malonyl-CoA	2.0	0.9	0.4
Minus NADPH	2.7	2.2	0.6
Minus ATP	0.7	0.1	0.3
Minus reduced CoA	1.0	28.6	0.6

[a] The complete system contained: 10 mg of microsomal protein; 200 mμmoles of 18:2ω6; 0.3 μmole of malonyl-CoA; 0.1 μmole of reduced CoA; 2 μmoles of NADPH; 10 μmoles of ATP; 15 μmoles of $MgCl_2$; 4.5 μmoles of GSH; 125 μmoles of potassium phosphate buffer; EDTA, 10^{-3} M; KCl, 0.15 M; sucrose, 0.25 M; pH 7; volume, 3 ml; incubation time, 20 min; temperature, 37°; anaerobic.

TABLE III

Chain elongation of linoleic acid (18:2ω6) by various subcellular fractions of liver from fat-deficient rats

Protein, 10 mg, was incubated with 100 mμmoles of 1-^{14}C-18:2ω6, 0.3 μmole of malonyl-CoA, and the other cofactors as described under "Experimental procedure" at pH 7, temperature 37°, for 20 min. Total volume was 3 ml.

Subcellular fraction	Centrifugal force	20:2ω6 Produced	
		Experiment 1	Experiment 2
		mμmoles	
Mitochondria			
Crude	17,000 × g	3.2	3.6
Purified		4.7	5.3
Microsomes			
Crude	105,000 × g	20.4	19.7
Purified		20.8	23.6
Postmicrosomal pellet	165,000 × g	4.9	4.9
Supernatant		3.2	3.3

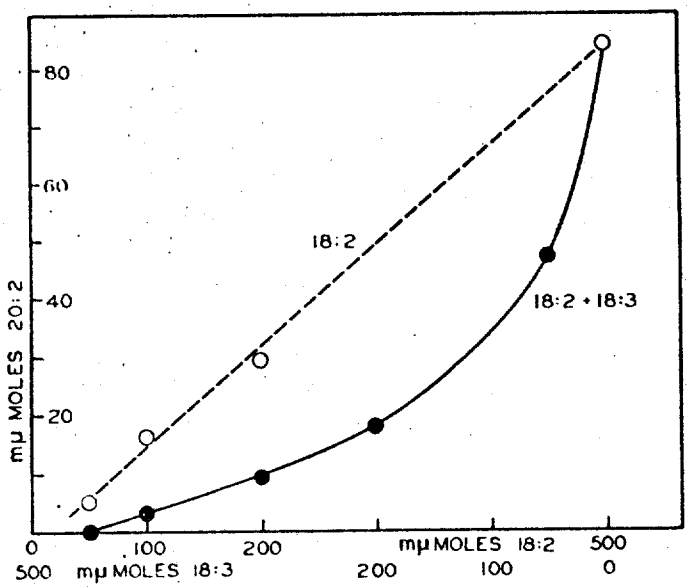


FIG. 4. Biosynthesis *in vitro* of eicosadienoic acid (20:2ω6) in response to increasing amounts of linoleic acid (1-^{14}C-18:2ω6) as substrate and inhibition of this conversion by linolenic acid (18:3ω3). Each incubation contains 10 mg of microsomal protein and 0.6 μmole of malonyl-CoA. The other cofactors are described under "Experimental Procedure." Incubation time, 90 min at 35°. The *dotted line* shows the amount of 20:2ω6 synthesized when 1-^{14}C-18:2ω6 is the only substrate. The *solid curve* shows the suppressed synthesis of 20:2ω6 when 1-^{14}C-18:2ω6 is incubated simultaneously with 18:3ω3.

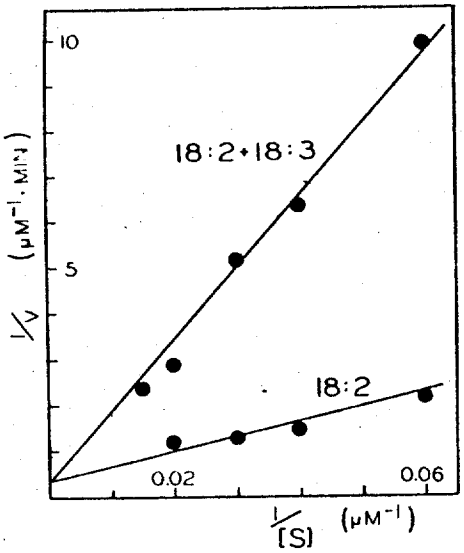


FIG. 5. Lineweaver-Burk plot for the chain elongation of linoleic (18:2ω6) to eicosadienoic acid (20:2ω6) by rat liver microsomes (10 mg). 1-^{14}C-Linoleic acid (50 to 200 mμmoles) was incubated simultaneously with and without 150 mμmoles of linolenic acid for 6 min at 37°, pH 7.0, anaerobically. Cofactors are as described under "Experimental Procedure."

We have shown that the chain elongation step of the conversion of linoleic to arachidonic acids is also inhibited by linolenic acid (Fig. 4). The sum of the amounts of linoleic and linolenic acids was constant in this experiment, and was less than the capacity of the microsomal protein, as shown by the linearity of yield and substrate concentration (Fig. 4, *dotted line*). The yield of 20:2ω6 was smaller in all cases in which 18:3ω3 was added than when linoleate was the only substrate. This inhibition has been verified in several experiments.

The Lineweaver-Burk plots (Fig. 5) of the conversion, 18:2ω6 → 20:2ω6, with and without linolenic acid as inhibitor, were derived from experiments interrupted after only 6 min of incubation time. As in the case of the dehydrogenation reaction (20), the *straight lines* for both the uninhibited and the inhibited reactions intersect the *vertical axis* at about the same point. The Michaelis-Menten constants for the reactions were K_m = 3.3 × 10^{-6} and K_i = 12.1 × 10^{-6}. K_i is about 4-fold higher than the constant for the dehydrogenation reaction derived by Brenner and Peluffo (20). It should be kept in mind, however, that computing a reaction constant for a multienzyme reaction is not very meaningful. Therefore, in our further investigations of substrate inhibition we did not emphasize kinetic considerations.

The chain elongation of linoleic acid is not only inhibited by linolenic acid, but by a large number of saturated and unsaturated fatty acids of different chain lengths. Table IV lists the series of saturated fatty acids from C_8 to C_{22} and all major unsaturated fatty acids occurring in rat liver lipids which have been incubated together with radioactive linoleic acid. In all cases the test level of inhibitor was twice the concentration of 18:2ω6. The conversion 18:2ω6 → 20:2ω6 was measured by the usual procedure. Since the yields were somewhat different in the several experiments needed to study all of these

TABLE IV

Inhibition of chain elongation of linoleic (18:2ω6) to eicosadienoic acid (20:2ω6) by fatty acids

1-^{14}C-18:2ω6, 100 mμmoles, was incubated simultaneously with 200 mμmoles of the nonradioactive inhibitor fatty acid, 10 mg of microsomal protein, 0.4 mμmole of malonyl-CoA, and other cofactors as described under Experimental Procedure at pH 7, 37°, for 20 min. Total volume, 3 ml.

Inhibitor	Product 20:2ω6[a]		% Inhibition
	mμmoles		
Octanoic acid	8:0	27.9	7
Nonanoic acid	9:0	25.2	16
Decanoic acid	10:0	20.4	32
Dodecanoic acid	12:0	14.4	52
Tridecanoic acid	13:0	14.4	52
Tetradecanoic acid	14:0	11.1	63
Pentadecanoic acid	15:0	9.9	67
Hexadecanoic acid	16:0	13.5	55
Heptadecanoic acid	17:0	19.8	34
Octadecanoic acid	18:0	25.2	16
Nonadecanoic acid	19:0	28.8	4
Eicosanoic acid	20:0	27.3	9
Heneicosanoic acid	21:0	30.0	0
Docosanoic acid	22:0	30.0	0
Octadecenoic acid	18:1ω9	15.9	47
Octadecatrienoic acid	18:3ω3	8.4	72
Octadecatrienoic acid	18:3ω6	10.2	66
Eicosatetraenoic acid	20:4ω6	22.2	26
Eicosapentaenoic acid	20:5ω3	13.5	55
Docosahexaenoic acid	22:6ω3	17.0	43

[a] The results are taken from several experiments with different conversion rates. Therefore, the amount of 20:2ω6 has been computed based on a conversion rate of 30% for control experiments without inhibitor.

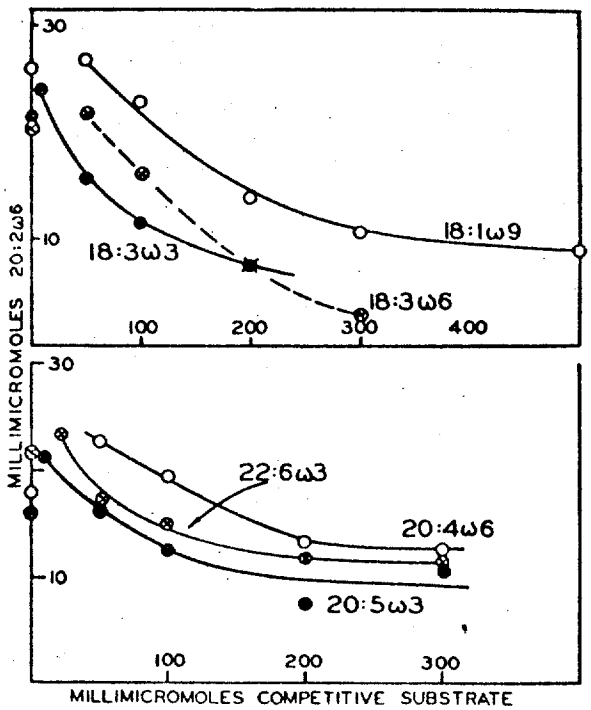


FIG. 6. Inhibition of the conversion by microsomal protein of linoleic to eicosadienoic acid by increasing amounts of other unsaturated fatty acids. 1-^{14}C-18:2ω6 (100 mμ moles) was incubated simultaneously with the nonradioactive inhibitor, 10 mg of microsomal protein, and the cofactors described under "Experimental Procedure," at pH 7, anaerobically for 20 min. Competitive substrates (*upper*): ●, linolenic acid (18:3ω3); ○, oleic acid 18:1ω9); ⊗, γ-linolenic acid (18:3ω6). Competitive substrates (*lower*): ○, arachidonic acid (20:4ω6); ●, eicosapentaenoic acid (20:5ω6); ⊗, docosahexaenoic acid (22:6ω3).

acids, the results were adjusted by computing the amount of 20:2ω6 formed, assuming 30% conversion in the uninhibited reaction.

The saturated fatty acids all inhibit the chain elongation of linoleic acid, and C_{14} and C_{15} acids are the best inhibitors. Expressed in terms of competitive inhibition, they themselves may be elongated. From C_{14} to C_{22} the degree of inhibition decreases, C_{21} and C_{22} acids being ineffective. Thus the most effective inhibitors are those acids which, after chain elongation, produce the most common and abundant saturated fatty acids of animal tissue, C_{14}, C_{16}, and C_{18}.

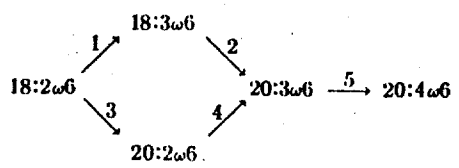
Among the unsaturated fatty acids tested, 18:3ω6 and 18:3ω3 are by far the best inhibitors. The relatively high efficiencies of 22:6ω3 as inhibitor may be product inhibition, because 22:6ω3 is the last product in the sequence of chain lengthening and dehydrogenation in its family of acids.

The inhibition curves obtained with the unsaturated fatty acids are shown in Fig. 6. The yield of 20:2ω6 decreases with increasing amounts of the inhibitor fatty acids in a similar manner for all acids tested. The most dramatic decrease was observed for 18:3ω6, which belongs to the linoleic acid family, and which has the same chain length as the substrate, but has one more double bond.

The conversion rates for 18:2ω6 → 20:2ω6 in these experiments were slightly higher in those samples which contained a small amount of the inhibitor fatty acid than they were for the blanks which contained linoleic acid only (*points* on the *vertical axis* in Fig. 6). It therefore appears that the inhibitors have a stimulating effect at low concentrations. Brenner and Peluffo (20) observed a similar slight increase in yield of the dehydrogenation products when polyunsaturated fatty acids were added to the reaction mixture.

Quite different mechanisms seem to be functioning in the inhibition of the chain elongation reaction and in the dehydrogenation reaction. All 20 fatty acids tested, except 21:0 and 22:0, inhibited the chain lengthening of linoleic acid to some extent. In the case of the desaturation reaction, only 18:1ω9, 18:3ω3, and 22:6ω3 inhibited the conversion of 18:2ω6 to 18:3ω6 (20, 22). Therefore, the enzyme systems for the dehydrogenation must be much more substrate-specific than are those for chain elongation. The latter enzyme system elongates both saturated and unsaturated fatty acids (5).

The metabolic conversions of linoleic acid are shown in the following scheme.



The abilities of various fatty acids to inhibit the dehydrogenation of 18:2ω6 (Reaction 1) and its chain lengthening (Reaction 3) are not equal. For instance, the concentration of 18:3ω3 required to inhibit Reaction 1 by 50% is about 10 times the concentration of the substrate (20), whereas the concentration required to inhibit Reaction 3 by 50% is about equal to the substrate concentration. This suggests that in the presence of fatty acids, such as occur in a living system, the pathway 1-2-5 may be preferred over the pathway 3-4-5.

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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	48.06	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	37.06	0.39	1.26
7	0.34	0.05	2.06	26.17	18.46	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	0.62	< 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	0.62	0.49
12	0.30	1.80	1.61	36.01	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	18.06	1.95	48.05	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	1.26	0.63
18	0.67	1.92	2.70	31.24	20.01	1.77	40.46	1.76	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

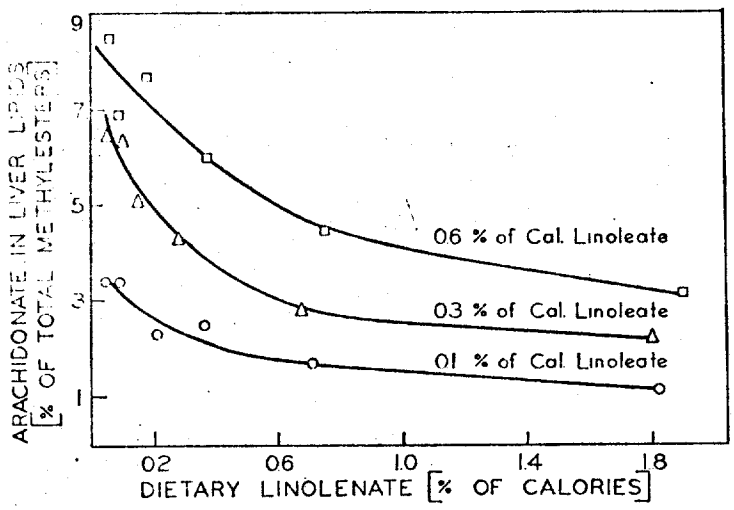

Fig. 1 The effect of varying levels of dietary linolenate upon the biosynthesis of arachidonate in liver lipids.

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
0.08	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	0.68	0.69	1.44
0.30	1.80	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	1.92	1.46	2.76

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

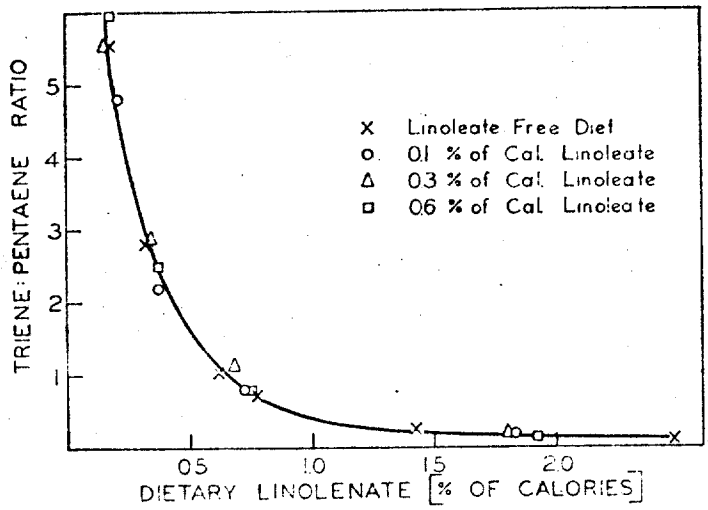

Fig. 2. The effect of increasing amounts of dietary linoleate upon the triene-penetaene ratio at different levels of linoleate intake.

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.

ACKNOWLEDGMENTS

We want to thank Joseph Scufert, Dennis Sukkestad, Gerald Miller and Daryl Williamson for their technical assistance, Gary Hansen and John Bonnes for the dependable care of the experimental animals, and Dr. William O. Caster for help with the calculation of standard deviations by computer methods.

We also wish to thank Prof. F. Klenk of the University of Cologne for his interest in this experiment and his helpful discussions.

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J. Lipid Research, April 1963
Volume 4, Number 2

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

J. Lipid Res.
4(2): 151-159, 1963

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[Manuscript received August 29, 1962; accepted December 14, 1962.]

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-

* Supported in part by grants from the National Institutes of Health (A-4524), USPHS; the Life Insurance Medical Research Fund; the Wesson Foundation; and the National Dairy Council.

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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 impurites 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, 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.

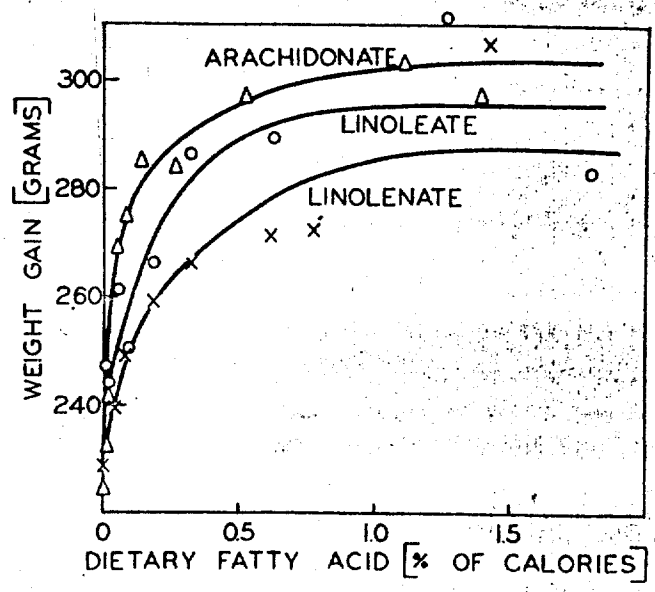

FIG. 1. The effect of varying levels of dietary EFA upon weight gain of rats during the 100-day period of the experiment.

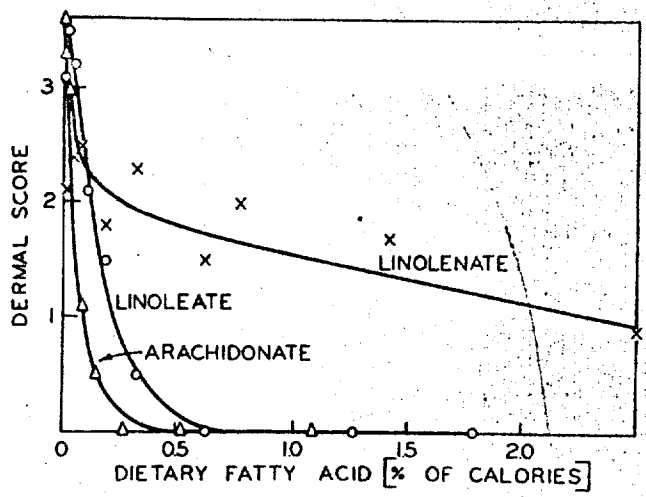

FIG. 2. The effect of varying levels of dietary EFA upon dermal score (the degree of fat-deficiency symptoms on feet and tail of the rats [10]).

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 ± 0.29	40.30 ± 0.58	1.07 ± 0.08	<0.01
Linoleate								
0.009	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 ± 0.22	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	34.50	3.02	<0.01
1.79	2	1.38	27.81	10.83	12.14	27.95	4.28	<0.01
4.87	2	1.00	25.46	7.95	13.60	27.54	5.70	<0.01
Arachidonate								
0.007	6	0.78 ± 0.30	24.14 ± 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 ± 2.49	0.88 ± 0.38	<0.01
0.04	6	1.27 ± 0.49	26.24 ± 1.95	10.57 ± 0.84	10.23 ± 0.75	39.07 ± 2.11	1.40 ± 0.24	<0.01
0.08	6	0.86 ± 0.21	24.24 ± 1.52	10.21 ± 1.00	11.19 ± 1.10	38.01 ± 1.70	1.08 ± 0.22	<0.01
0.14	6	0.88 ± 0.58	23.43 ± 1.96	9.44 ± 2.32	11.61 ± 2.99	38.37 ± 3.99	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	9.04	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	5.06	13.34	16.97	0.54	<0.01
Linolenate								
0.009	6	0.62 ± 0.18	24.98 ± 0.86	11.05 ± 1.83	10.88 ± 1.89	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	40.69 ± 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 ± 4.24	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 ± 1.39	13.93 ± 1.54	12.23 ± 1.61	38.44 ± 1.84	0.41 ± 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 ± 1.05	36.36 ± 1.40	0.50 ± 0.20	1.03 ± 0.19
2.56	4	0.79 ± 0.18	29.40 ± 3.26	12.26 ± 1.04	12.33 ± 1.07	28.64 ± 2.29	0.39 ± 0.08	1.57 ± 0.56
3.56	4	0.69 ± 0.04	29.55 ± 0.52	10.53 ± 1.29	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 ± 2.94	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

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.

The authors wish to thank the Scientific Research Grant of the Ministry of Education for having defrayed part of the expense for this research and Dr. H. Fujii, the director of the Research Institute of Nippon Suisan Co., for use of his facilities for carrying out the experimental work.

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.

(Received March 23, 1965)

J. Nutr. 81:67-74, 1963

Effect of Linolenic Acid Upon the Metabolism of Linoleic Acid[1]

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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

Received for publication February 15, 1963.
[1] Supported in part by grants from the National Institutes of Health (AM04521-03), the Life Insurance Medical Research Fund, and the National Dairy Council.
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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	0.08	0.05	0.79 ± 0.22[2]	21.76 ± 3.09	9.19 ± 1.30	13.75 ± 1.49	37.98 ± 2.68	1.33 ± 0.31
2	0.07	0.09	0.77 ± 0.32	22.43 ± 2.72	10.44 ± 3.18	12.57 ± 3.06	38.05 ± 3.54	1.53 ± 0.33
3	0.09	0.21	0.99 ± 0.27	25.22 ± 2.05	11.09 ± 2.47	11.15 ± 3.12	37.75 ± 4.52	1.34 ± 0.20
4	0.09	0.37	0.70 ± 0.16	21.28 ± 0.66	9.81 ± 0.99	14.42 ± 0.96	33.53 ± 2.35	1.53 ± 0.20
5	0.07	0.72	0.79 ± 0.39	22.78 ± 1.75	10.51 ± 2.46	12.47 ± 2.17	35.63 ± 4.22	1.38 ± 0.32
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	26.83 ± 2.77	8.12 ± 0.98	14.00 ± 2.04	32.68 ± 2.80	2.64 ± 0.81
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	24.64 ± 2.14	10.01 ± 1.26	13.80 ± 1.22	32.82 ± 1.62	2.04 ± 0.41
10	0.28	0.33	0.71 ± 0.09	25.01 ± 2.32	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 ± 2.04	10.68 ± 1.35	13.41 ± 0.89	31.97 ± 0.79	1.95 ± 0.27
12	0.30	1.80	0.84 ± 0.15	21.81 ± 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	31.68 ± 3.39	3.21 ± 0.34
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	2.64 ± 0.81
15	0.59	0.18	0.82 ± 0.10	22.58 ± 2.01	8.42 ± 1.02	14.50 ± 1.13	31.59 ± 1.46	3.40 ± 1.05
16	0.62	0.37	0.74 ± 0.08	24.79 ± 3.39	9.38 ± 0.66	13.56 ± 1.57	30.47 ± 2.37	3.05 ± 0.67
17	0.61	0.75	0.72 ± 0.18	23.81 ± 1.46	9.38 ± 1.71	13.22 ± 1.57	30.52 ± 2.05	3.69 ± 0.81
18	0.67	1.92	0.87 ± 0.21	21.91 ± 1.34	9.86 ± 1.29	13.56 ± 1.87	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] Rahm, 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
0.60 ± 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
0.37 ± 0.06	5.51 ± 2.17	0.48 ± 0.09	3.38 ± 1.26	0.54 ± 0.16	0.46 ± 0.17	0.24 ± 0.15	3.26 ± 1.30
0.35 ± 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
0.66 ± 0.09	4.26 ± 0.90	0.74 ± 0.15	2.49 ± 0.15	1.88 ± 0.44	0.20 ± 0.03	0.68 ± 0.26	7.85 ± 1.16
0.83 ± 0.40	1.83 ± 0.71	0.28 ± 0.15	1.71 ± 0.46	2.43 ± 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
0.35 ± 0.05	5.13 ± 1.14	1.30 ± 0.36	6.33 ± 0.78	0.51 ± 0.07	0.88 ± 0.15	0.27 ± 0.13	3.25 ± 0.75
0.40 ± 0.05	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
0.44 ± 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	6.91 ± 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	8.18 ± 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
0.44 ± 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
0.66 ± 0.20	1.51 ± 0.44	0.89 ± 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	18.08	6.80	18.95	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	0.26	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	4.62	18.01	29.96	9.53	< 0.1	1.92	0.53	12.01	< 0.1	0.59	0.26	2.54
16	0.62	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	18.93	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.

This series of events suggest the dissociation of hydrogen molecules occurring on the catalyst surface, but the resulting hydrogen atoms seem rather unfavorable to the hydrogenation.

Although questionable, the existence of perfectly dissociated atomic hydrogen must be presumed, at least a production of the half dissociated state of hydrogen molecule during hydrogenation, in view of the occurring transformation as follows, exchange reactions of ethylene and butylene with deuterium,[23] isomerization from *cis*-double bond to *trans*-double bond involving migration of double bond,[18] and in deuterogenation on platinum methyl oleate was converted to light methyl elaidate.[24]

This hydrogen molecule featured as radical-like state will promote most probably a formation of a radical olefine, from which polymerization supposedly will be further induced in addition to the above mentioned transformations.

The extension of polymerization in this way will possibly be enhanced by the stabilization of this radically featured or radical olefine, and negative function of carboxlic group linked with the olefines are expected to stabilize its radical state, in the similar way as nitro- or carbonyl compounds.

It is interesting to indicate that the grave problem in determining the kinetic order with respect to the substrates are always confronted with those having electronegative groups in their constitutions.

Actually a deep red polymeric oil was produced in the reduction of 2-nitro-1-butene in glacial acid over Adams' platinum catalyst besides saturated amine,[25] and this polymer is seemingly inhibitory, as reduction stopped at only about 10 per cent of theoretical saturation.

While in simple olefines, cyclic or non-cyclic, and aromatic compounds, zero order kinetics are immediately established by a linear manner of hydrogenation.[6,26~29]

This is only a rough trend, and linearities with those having negative groups have also been reported at the same time.[30,31] For instance, with *p*-nitrophenol it was reported[7] as behaving miscellaneous kinetics completely similar to fat hydrogenation, but the independency of rate was confirmed on dilution with its hydrogenated product, as was done in fat hydrogenation[32].

One will recognize from the references that hydrogenation using much catalyst unexceptionally follows a linear process, even with electronegative groups involving compounds in which the excess catalyst should be regarded as an adsorbent of poison. Indeed, charcoal, and active clay were found on their addition recovering the lowered rate to some degree.[32]

The intermediate type are frequently reported, linearites in the first half being followed by declining rate in the second half.[26~31] On closely tracing the hydrogenation, Tsubaki oil (Fig. 4) shows itself of this type, detailed formulation of which will be opened in a serial paper.

23) J.N. Wilson, J.W. Otvos, D.P. Stevenson, A.D. Wagner: Ind. Eng. Chem., 45, 1480 (1953).
24) J.H. Baxendale, E. Warhurst: Trans. Faraday Soc., 36, 1186 (1940).
25) H.A. Smith, W.C. Bedoit Jr.: J. Phys. Colloid Chem., 55, 1089 (1951).
26) R.H. Price, D.B. Schiewetz: Ind. Eng. Chem., 49, 807 (1957).
27) S.V. Lebedev, G.G. Koblianskii, A.C. Yakubchik: J. Chem. Soc., 1925, 418.
28) G. Gilman, G. Cohn: "Advances in Catalysis" (X). loc. cit., 733.
29) H.A. Smith, H.T. Meriwether: J. Am. Chem. Soc., 71, 413 (1949).
30) S.S. Scholnik, J.R. Reasenberg, E. Lieber, G.B.L. Smith: *Ibid.*, 63, 1192 (1941).
31) H.A. Smith: "Catalysis" Vol. 3 edited by P.H. Emmett, 149 (1955), Reinhold Publishing Corporation, New York.
32) R. Miyake, H. Yajima: Yakugaku Zasshi, 85, 618 (1965).

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 ot 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 lined 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 selecctivity.[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 delation 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 produce corresponding saturated alcohol, where preferential reduction of carboxylic sadical to corresponding olefinic 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.

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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, R. Miyake: Yakugaku Zasshi, 84, 516 (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 catayst 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[29] and rhodium,[28] 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'danskii, S. Yu. Elovich: J. Phys. Chem. (U. S. S. R.), 20, 1085 (1946), C. A., 41, 2973 (1947).

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

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

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.

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

Since the surface is in contact with liquid phase, a further modification is allowed to exercise such that $k_E(E) \gg 1$ 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,[39] 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. 358 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.[34] 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).

39) 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 madé 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 hyd rogenation 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,[33] 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.[20]

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 risng 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 riso to hydrogenation these two quasi compounds must be brought into a close connection, namely it is to construct a pertinent transtional 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." 142 (1944). Zoshindo, Osaka, Japan. H. J. Taufen, 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 shifiting 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 approxinately two, the degree of selective hydrogenation shown by Bailey as 38,[41] 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 hydrogenation 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,[42] 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,[43] 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,

41) A. E. Bailey: J. Am. Oil Chem. Soc., 26, 644 (1949).
42) K. E. Kavanagh, F. F. Nord: J. Am. Chem. Soc., 65, 2121 (1943).
43) H. Yajima: unpublished.

Nutritive Value of Marine Oils. II. Effects of *in Vivo* Antioxidants in Feeding Menhaden Oil to Swine[1,2,3]

J. Am Oil Chem. Soc. 40: 357-360

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Abstract

Five pair-groups of swine were fed diets composed of crude feed materials to which 10% of clay-bleached, light cold-pressed menhaden oil was added. The oil was allowed to oxidize under controlled conditions and aliquots of fresh and oxidized oil were removed at peroxide values (PV) of 2.6, 15.5, and 61—each of which was fed to one group of animals. Two additional animal groups received diets containing the highly oxidized oil (PV=61) plus either alpha-tocopherol acetate or ethoxyquin. All oils were stored frozen and were mixed with the diets daily. Lard was fed at a similar 10% level to a control group of pigs. Both feed intakes and weight gains were progressively reduced as the diet oil was more highly oxidized. As oxidation of the oil increased, intensity of "yellow fat" increased and hemoglobin and hematocrit levels were progressively decreased. Both alpha-tocopherol acetate and ethoxyquin acted *in vivo* to improve feed intake, rate of gain, and blood condition, and eliminated the incidence of steatitis.

Introduction

CERTAIN paradoxical effects have been associated with the inclusion of unsaturated lipids and particularly those of marine origin, in diets for various animal species and man. On the positive side, such lipids are obviously useful sources of the essential fatty acids known to be dietary requirements of rats (6) and of chickens (5,20) not only for maintenance of dermal integrity but also for proper utilization of saturated fat (24). Their contribution to dietary supplies of vitamins A and D has long been recognized (18). It has been further demonstrated that a positive relationship exists between unsaturation in dietary fatty acids and depression of levels of serum cholesterol (33). Holman has pointed out (14), however, that this may not be a simple binary relationship. In addition, there is the basic consideration that all fats, regardless of degree of saturation, contribute significantly to the dietary energy supply. That fish oils may serve in this capacity has been demonstrated experimentally with swine (24).

On the other hand, considerable evidence has been accumulated suggesting that undesirable changes may take place in unsaturated dietary lipids, which lead to profound effects in animals ingesting them. Among resultant symptoms have been listed retarded growth and diarrhea in rats (10,22), depressed growth in chicks (21), and in pigs (4); seborrhea in rats and rabbits (22) and deposition of a yellowish-brown pigment ("steatitis") in depot fat of rats (13), swine (9), cats (7), and mink (16). In most if not all of the above-mentioned instances, data suggest that the dietary lipids involved had been abused in some way prior to feeding. Two types of damage were most frequent: oxidative deterioration and thermal polymerization. Many examples of each of these situations have been documented, but perhaps the data of Andrews et al. (3) for the former and Crampton and associates (8) for the latter are typical. Andrews' studies showed growth depression in rats to be proportional to extent of oxidation of diet fats from peroxide numbers of 100–1200, while Crampton's work indicated a similar proportionality to exist for length of heat treatment of oils held under carbon dioxide (27).

Since most oils in their processing are somewhere subjected to conditions conducive to oxidation, the study reported herein was initiated with the twofold purpose of: 1) examining animal response to dietary lipids oxidized to various known levels, and 2) investigating the effects of added antioxidants on such changes. Menhaden oil, which was specially prepared in a very fresh condition, was chosen for the study. The antioxidants tested were alpha-tocopherol acetate and ethoxyquin. Both of these are known to be effective biological antioxidants, and they gave the opportunity of comparing a natural and a synthetic material. Swine were used as test animals because of their known susceptibility to changes in dietary fat (11).

Methods and Materials

Six pair of Berkshire swine averaging 76 lb in weight and 10 weeks in age were penned individually and fed a basal diet composed of crude natural ingredients with modifications as listed in Table I.

The choice of levels of antioxidants was made after reference to the literature, where successful applications of from 2.4–40 mg alpha-tocopherol per 100 g diet are reported (32,28,19), and 12.5 mg ethoxyquin per 100 g diet appeared to be generally accepted. Residual antioxidant activity in the crude diet ingredients was probably present, but at unknown levels.

Controlled oxidation of portions of the menhaden oil for use in the diets of lots 3–6 was accomplished at room temperature in a closed glass system, to retain volatiles, using a slight positive oxygen pressure. It was assumed, following the work of Privett, et al. (14), that this temperature was satisfactory for for-

TABLE I

Diet Treatments

Lot No.	Diet description[a]
1	Lard diet (lard PV 0.0, TBA number 1)[b]
2	Menhaden oil diet (fresh oil P.V. 2.6, TBA number 35.3)
3	Menhaden oil diet (medium oxidized oil PV 15.5, TBA number 48.8)
4	Menhaden oil diet (highly oxidized oil PV 61.0, TBA number 162)
5	Lot 4 diet + 4.375 mg alpha-tocopherol acetate/100 g diet
6	Lot 4 diet + 12.5 mg ethoxyquin/100 g diet

[a] The basal diet composition was ground barley 60%, lard or menhaden oil 10%, soybean oil meal 20%, meat meal 9%, ground limestone 0.5%, and iodized salt 0.5%. Supplementary vitamins A, D, riboflavin, pantothenic acid and B_{12} and lysine and methionine were provided to meet NRC recommendations (23).

[b] PV = peroxide value; TBA number = Thiobarbituric acid number = mg Malonaldehyde/Kg lipid.

[1] Paper I in this series appeared in the Journal of Nutrition 79, 323–332 (1963).

[2] Carried out under Contract No. 14-17-0001-352, U.S.D.I. Bureau of Commercial Fisheries.

[3] Technical Paper 1615, Oregon Agricultural Experiment Station.

[4] Dept. of Animal Science.

[5] Dept. of Food Science and Technology.

TABLE II
Growth and Feed Data

Lot No.	Treatment	Avg daily gain, lb	Avg. daily feed intake, lb	Avg efficiency lb feed/lb gain
1	Lard diet	1.66	5.83	3.51
2	Fresh menhaden oil diet	1.27	4.32	3.40
3	Med. oxid. menhaden oil diet	1.19	3.89	3.27
4	High oxid. menhaden oil diet	0.82	3.33	4.07
5	High oxid. menhaden oil diet + alpha tocopherol acetate	1.45	5.02	3.45
6	High oxid. menhaden oil diet + ethoxyquin	1.42	4.90	3.45

mation of stable peroxides. In order to maintain diet lipids at the desired state of oxidation, aliquots of lard or menhaden oil were weighed into No. 1 cans, vacuum sealed and quick-frozen at −18F, after which they were stored at 0F. These portions were then mixed with appropriate amounts of dry ration ingredients daily, immediately before feeding. The antioxidants were added to the oil-fed lots 5 and 6 just prior to mixing with the dry feed. Any feed residues remaining were removed and the feed container cleaned prior to addition of new feed.

Feeding of the test diets was continued over a 100-day period, during which time the animals were weighed weekly. At the start and finish of the test, blood samples were drawn from each animal and subjected to determinations for hemoglobin using the Spencer hemoglobinometer and hematocrit using a microhematocrit centrifuge. Plasma cholesterol levels were determined by the method of Zlatkis, et al. (35). At the conclusion of the test the animals were slaughtered, their carcasses examined for evidence of steatitis and other abnormalities, and samples of various body fats were obtained. Peroxide values and iodine numbers (1) and thiobarbituric acid numbers (30) were used as criteria of lipid quality.

Results

Gross evidence of the general state of health of the animals is provided through data relating to gains in body weight, feed intake, and efficiency of conversion of feed to animal weight gains. Such data are summarized in Table II.

Statistical analysis indicates that the lard-fed animals made faster gains than those fed menhaden oil, irrespective of condition of oil or presence of antioxidant under conditions of this experiment. As oils of higher oxidation levels were fed, feed intakes and weight gains were apparently reduced, such reduction becoming statistically significant ($P<0.01$) in the comparison of "medium" and "high" oxidation oils (Lots 3 and 4). Addition of the antioxidants to the highly oxidized oil resulted in significantly improved animal performance over that on the medium and highly oxidized, but unprotected, oil diets, equalling that made on the fresh oil diet.

Since the composition of the blood is widely used as a criterion of nutritional status, examination of data in Table III yields further information on the response of the animals to the various dietary lipid treatments.

It is considered normal for growing pigs to show increases in blood hemoglobin levels and hematocrits with increasing age, due in part at least to the low iron reserves of the milk-fed weanlings. Evaluation of effects of the diets in this experiment on blood characteristics is perhaps best made therefore by comparisons of initial and final values. On this basis, there would appear to be a lowering effect of menhaden oil on both hemoglobin and hematocrit levels when compared with the lard diet with aggravation of the effect when oils of higher oxidation levels were used. Differences between final and initial hemoglobin values during the trial were as follows for lots 1–6, respectively: +2.5, +1.3, +1.2, −1.2, +3.9, and +2.6 g %. Similar changes in hematocrit values were: +10.9, +4.7, +2.4, −2.5, +9.0, and +5.0% in the same order. Addition of the antioxidants to the highly-oxidized oil resulted in hemoglobin and hematocrit values equal or superior to those noted on the fresh oil. The significance of the blood cholesterol changes is somewhat masked by the variation in these values at the start of the trial. It is evident however that while feeding lard resulted in an increased blood cholesterol figure, feeding menhaden oil generally resulted in decreased values. The only exception to this trend was where the highly oxidized oil was fed, unprotected (Lot 4). Use of the *in vivo* antioxidants reversed this oxidation effect.

A lowering of ether-extractable materials in both epinephric and subcutaneous adipose tissue occurred when unprotected fish oil was fed; however there was not a direct correlation with the degree of oxidation of the oil. Inclusion of the two antioxidants in the diets resulted in considerably increased deposition of fat (or ether-extractable materials) to approximately the levels reached in the lard-fed control animals (Lot 1). In addition to the quantitative differences noted, qualitative changes in depot fat were observable. That in animals fed unprotected fish oil was markedly yellow in color—the depth of color being roughly proportional to extent of oxidation of the oil. The fat of the animals fed antioxidant-treated oil was white.

The weights of livers, hearts, spleens, and both kidneys were recorded at slaughter and are presented in Table IV. Although there was an apparent trend towards hypertrophy of some of the organs from animals fed fish oil, differences in the weights of hearts, spleens, and kidneys among the various treatment groups lacked statistical significance. On the other hand, livers from the group fed highly oxidized oil were significantly heavier than those from the groups fed lard, fresh oil, or highly oxidized oil protected with alpha-tocopherol acetate or ethoxyquin. Significantly heavier livers were also shown for the animals

TABLE III
Blood Data

Lot No.	Treatment	Hemoglobin, g %		Hematocrit, %		Plasma cholesterol, mg %	
		Start	Finish	Start	Finish	Start	Finish
1	Lard diet	11.3	13.8	35.3	46.2	114	126
2	Fresh menhaden oil diet	10.6	11.9	34.6	39.3	164	132
3	Medium oxidized menhaden oil diet	9.8	11.0	31.6	34.0	156	137
4	High oxidized menhaden oil diet	10.6	9.4	33.2	30.7	129	138
5	High oxidized menhaden oil diet + alpha-tocopherol acetate	11.3	15.2	36.0	45.0	128	100
6	High oxidized menhaden oil diet + ethoxyquin	12.0	14.6	37.3	42.3	153	101

fed medium oxidized oil in comparison with those from animals fed highly oxidized oil protected with alpha-tocopherol acetate.

Discussion

An interesting feature of the results of this experiment is the degree of protection afforded the animals by addition of antioxidants to the menhaden oil portion of the diet. There seems little doubt that this antioxidant activity took place *in vivo*, rather than in the feed mix prior to consumption. The antioxidants were added immediately before the daily rations were mixed and fed; however it is possible that delay in consumption of all the ration by the animals might have resulted in contact of antioxidant and diet lipid *in vitro* for several hours. More important evidence for *in vivo* activity, however, is the fact that alpha-tocopherol acetate was used, and this would not become biologically active until the acetate moiety was split off, in the digestive tract (2). It should be borne in mind that the oil used in the antioxidant-treated diets was already in an advanced state of oxidation as evidenced by its peroxide value of 61 and TBA no. of 162. These levels are, incidentally, considerably greater than those encountered in most fish oil.

Improvement of feed intakes in the cases of the antioxidant-treated diets was an interesting phenomenon. One would expect *in vitro* antioxidant activity, by depressing oxidative changes in diet fats prior to consumption, to exert a positive effect on feed intake. It is more difficult to reconcile such an effect with *in vivo* activity taking place after considerable preliminary oxidation. One may speculate that such increased feed intakes are rather a reflection of general health and well-being of the animals concerned than an indication of "palatability" per se.

The blood changes, although not extreme, were generally illustrative of a tendency toward anemia as oils of higher oxidation states were fed. Reports exist (31) of occurrence of severe anemia in mink fed raw hake *(Merluccius productus)*, which is, however, a fish of rather low oil content, and of a milder anemia in Eskimos who habitually consume large amounts of dried or raw, frozen fish (29); but in neither of these instances was oxidation level of the dietary lipid implicated. It is significant that deterioration of the blood picture in this experiment was prevented by the use of the two antioxidants. The cholesterol data appear of limited interest, except for the observation that fish oils in the diet generally caused some reduction during the course of the trial. The only exception to this was in the case of the highly oxidized oil, where it may be argued that the points of unsaturation were largely modified. It must be remembered that these feeding treatments were not superimposed on feeding of a hypercholesteremic diet as has been the case in other studies (26,34); therefore the opportunity for plasma cholesterol reduction was slight.

TABLE IV
Organ Weights

Lot No.	Treatment	Liver, g	Heart, g	Spleen, g	Both kidneys, g
1	Lard diet	1721.0	290.0	158.0	348.5
2	Fresh menhaden oil diet	1920.0	376.0	179.5	449.0
3	Med. oxid. menhaden oil diet	2016.5	361.0	198.0	459.0
4	High oxid. menhaden oil diet	2263.5	324.5	177.5	407.0
5	High oxid. menhaden oil diet + alpha-tocopherol acetate	1632.0	308.5	151.0	314.5
6	High oxid. menhaden oil diet + ethoxyquin	1916.5	329.0	140.5	405.5

TABLE V
Diet and Body Fat Characteristics

Lot No.	Iodine numbers			TBA numbers[a]		Ether extractable material, %	
	Diet fat	Back fat	Kidney fat	Inner	Outer	Back fat	Kidney fat[b]
1	61	68.2	58.8	1.7	1.0	86.9	93.5
2	184	91.6	90.3	40.8	10.0	44.1	75.2
3	183	84.8	87.1	36.2	11.0	56.1	64.1
4	181	82.1	74.1	38.5	18.2	54.0	66.7
5	181	95.9	79.5	3.8	16.2	92.2	89.2
6	181	95.7	86.2	12.5	19.1	81.3	89.0

[a] TBA numbers taken on inner and outer back fat.
[b] The designations "Back fat" and "Kidney fat" refer to adipose tissue from these regions.

Differences in depot fat were perhaps most marked by differences in extent of fattening of animals in the various treatment groups, which is a measure of the overall rate of growth. Iodine numbers of body depot fat tended to reflect iodine numbers of dietary lipids; however it is evident from the data in Table V that considerable depot fat must have been synthesized from carbohydrate or some source other than the fish oil. Differences in iodine numbers were not large, but this observation is supported by evidence in the literature that a relatively advanced state of deterioration must be reached before changes in iodine numbers are detectable (17). Generally speaking, iodine numbers decreased with increased oxidation of the diet fat, and this was particularly noticeable in the case of the kidney fat. Antioxidant activity was in the direction of promoting more unsaturated animal depot fat, presumably by protecting double bond sites in the dietary lipids during digestion. It may be of interest that inner backfat appeared to be protected more effectively than outer backfat, as evidenced by TBA values, through the addition of the antioxidants. Garton (12) has previously indicated greater unsaturation in outer than in inner backfat of a pig fed whale oil.

The biological significance of the increased liver weights where menhaden oil was fed, and the heightening of this effect as the oil became more highly oxidized is not explained by the data available. The extent of the liver increases is accentuated when one considers that total body size was simultaneously decreasing (cf. Table II). *In vivo* activity of the alpha-tocopherol apparently reversed the trend toward heavier livers with oxidized dietary lipid.

One may conclude from these data that certain of the adverse effects of oxidized fish oils in diets of swine may be related to the level of oxidation existing in the oils during their metabolism by the animals. Such effects may largely be prevented by inclusion in the diet of such *in vivo* antioxidants as alpha-tocopherol acetate or ethoxyquin at appropriate levels. The two antioxidants apparently performed equally well at the levels used.

ACKNOWLEDGMENTS

Technical assistance and advice in the conduct of these studies by Anita Huang, L. M. Larsen, T. C. Yu, M. E. Stansby and Neva Karrick. Ethoxyquin (6-ethoxy, 1,2 dihydro, 2,2,4 trimethyl quinoline) provided by Monsanto Chemical Co.

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[Received November 26, 1962—Accepted April 24, 1963]

ACTA PHYSIOLOGICA POLONICA
Vol. XVIII, No. 4, 1967

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EFFECT OF EXTREMELY HYDROGENATED FAT ON THE LIPID* METABOLISM

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PALUSZAK J.: *Effect of Extremely Hydrogenated Fat on the Lipid Metabolism.* Acta Physiol. Polon. 18 (4) : 639—649, 1967. — The aim of study was to investigate the effect of fully hydrogenated fats on fat metabolism and to explain the phenomenon of depot fat atrophy in rats fed with extremely hydrogenated vegetable oils. Experiments were performed in [illegible] adult rats, determining, e. a. the body weight, skin changes, quantity of food, digestibility coefficient, blood ketone substances and biotin level of *coecum*. Feeding diets containing 50 percent extremely hydrogenated vegetable oil induces the block of lipogenesis and disappearance of depot fat in rats, leading to the essential fatty acids as well as to biotin deficiency. The biotin deficiency was the result of inhibition of the intestinal synthesis of biotin by bacterial flora in absence of essential fatty acids. Deficiency of essential fatty acids is known to increase cellular metabolism and utilization of biotin due to uncoupling of oxidative phosphorylation. Deficiency of biotin is leading to the block of fatty acid synthesis because of deficiency in the coenzyme of carboxylation of acetyl-CoA to malonyl-CoA. The nonmetabolized acetyl-CoA is responsible for the development of ketonemia.

Recent years brought about a renewed interest in the effect of dietary fat 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 [24].

Partial or complete atrophy of the adipose tissue in experimental animals has been achieved by diets which contained pure palmitic acid [8], trielaidin [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.

wards [illegible]ne systems of the living cell [39]. Trans isomers of unsaturated fatty acids have displayed growth inhibiting properties [4, 20]. These fatty acids are believed to block the synthesis of endogenous fats. It is also known that diets of a high content of the long chain unsaturated fatty acids inhibit the rate of growth of animals [40]. This is possible due to the resulting deficiency of polyunsaturated fatty acids as well as to other aforementioned factors.

The atrophy of the adipose tissue can be brought about also by diets which contain large amounts of raw egg white protein [7]. Such a diet results in biotin deficiency. Biotin is inactivated by the avidin of egg white.

When general causes which might produce the disappearance of the depot fat are analysed, following mechanisms of an impaired synthesis of endogenous fats should be taken into account:

1 — lack of substrate for the synthesis, as for example in starvation, lack of appetite in hypothalamus disturbances as well as in other disturbances of the central nervous system,

2 — deficiency of vitamins and coenzymes as cofactors of the fat metabolism,

3 — presence of inhibitors as for example of substances which bind and inactivate enzymes and cofactors of lipogenesis,

4 — deficiency of enzymes catalyzing the lipogenesis due to the impaired synthesis of enzymatic proteins.

A statisfactory balanced diet excludes the first three mechanisms. An impaired biosynthesis of the enzymes which catalyse the production of endogenous fats can result from a deficiency of dietary exogenic factors or from the errors or lacking of genetical information.

Two principal systems of the biosynthesis of fatty acids in both animals and plant cells are present [15, 31, 41]: mitochondrial and extramitochondrial system. Extramitochondrial system is responsible for the bulk of fats produced in the living body and is biotin dependent.

The aim of the present study was to investigate the effect of extremely hydrogenated fats on the fat metabolism and to explain the phenomenon of depot fat atrophy in rats fed with extremely hydrogenated vegetable oils.

METHODS

Studies were performed in 80 adult male and female rats which weighed between 210—230 g. The animals were separated into groups according to the type of diet they have obtained. In order to elucidate the role of cholesterol in the fully hydrogenated fat diet, some animals were put on the diet which was cholesterol free. A pharmaceutical preparation of vitamin B (vit. B forte „Polfa") was the only source of the

group B vitamins in the diet. This preparation does not comprise biotin. The types of diet employed in the study were as follows:

Group I — basic (control) diet according to *Horst et al.* [22, 23].

Group II — basic diet enriched with biotin 0·2 mg/100 g of the diet.

Group III — high-fat diet which comprised: 100 g of the basic diet, 100 g of hydrogenated rape oil (iodine number = 5) and 1 g of pharmaceutical cholesterol per 100 g of diet.

Group IV — the diet as of Group III but without cholesterol and enriched with biotin 0·2 mg/100 g 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 the diet.

Each group comprised 10 animals. The animals of Group IV were fed with the high-fat diet for 11 weeks and then for additional three weeks with the same diet fortified with unsaturated fatty acids (200 mg daily per rat).

Full hydrogenation of rape oil was achieved at 140—200°C in the presence of nickel catalyst. The hydrogenated fat displayed iodine number 5 and melting point 59°C. Linoleic, linolenic and arachidonic fatty acids were absent.

The animals were weighed and their skin changes evaluated on a weekly schedule. The diets were provided *ad libitum* and consuption was recorded weekly.

Digestibility coefficient was determined by the method of *Van de Kamer* [43]. Ketone substances were determined in 6 rats of each group according to *Hansen* [18] and *Reid* [35]. The determinations were carried out after 5 weeks since placing the rats on experimental diets. After 11 weeks, total fat was determined in three animals of each group [24, 37]. Biotin level of *coecum* content was measured in two rats of Group I and 6 rats of group III by the microbiological technique of *Barton-Wright* [3].

The results were analysed statistically by Student's „*t*" test. Differences were considered significant at $p < 0{\cdot}05$ 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 rate 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 stable while that of rats of Group III was further decreased. At the end of the experiments these rats displayed 25 percent loss of weight. In the rats of Group V and VI (cholesterol-free diet) loss of weight was slower and less extensive (Fig. 1).

The appearance of the rats of Group I and II was not changed during the experiments. Their fur was smooth, fluffy and shiny. Rats of Group

III displayed hair loss and bristly fur already after two weeks of the die. Change of pigmentation was evident in the skin of the tail (brown patches and scales) which resembled horse-tail in appearance. Brown patches were also visible in the skin of ears as well as comb-like shredding of the ear margin. The animals became drowsy and weakly responsive towards ex-

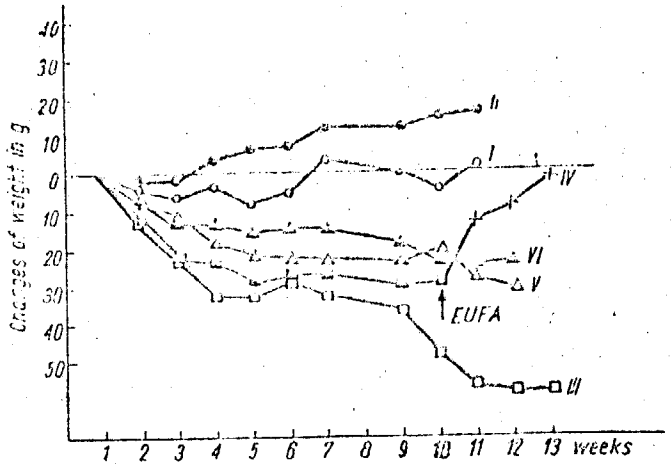

Fig. 1. Weight gain curves in rats. Groups of diets: I. Basal stock diet. II. Basal stock diet + biotin. III. Basal stock diet + 50 percent of maximally hydrogenated oil + 1 percent cholesterol. IV. Basal stock diet + 50 percent maximally hydrogenated oil + 1 percent cholesterol + 1 percent biotin. V. 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).

ternal stimulation. The movements were spastic and the spine humped (kangoroo posture).

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 ± 8·6 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	NS		VI
		NS	NS	NS			

NS — Nonsignificant

b) **Ketone bodies.** The concentration of ketone bodies in blo 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-flod 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 ($p < 0{\cdot}001$), ($17{\cdot}6 \pm 2{\cdot}2$ and $3{\cdot}6 \pm 2{\cdot}4$ 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 c) Biotin like other B vitamins is synthetized by bacterial flora of coecum [18]. 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 mmg/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 *coecum* content of the control animals was five times greater than in the *coecum* 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 weakly 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-

pling of oxydative phosphorylation as well as a possible deficiency 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 intomalonyl 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 [23, 34].

The author is expressing his gratitude to Professor *A. Horst*, M.D. and Professor *L. Działoszyński*, M.D., for their valuable inspiration and instructions, as well as to Docent *D. Rożynek* for her kind help in the interpretation of the results.

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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 P. KHYM.

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The following references are of record in the file of this patent:

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Fish Oils H. S. Olcott (Ed.) 1967

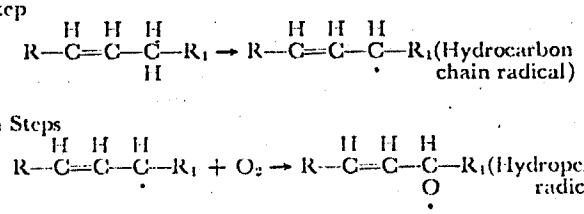
H. S. Olcott

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:

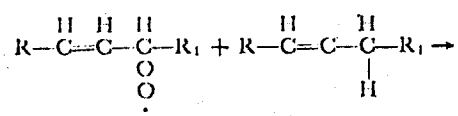
Initiation Step



(1)

Propagation Steps

(2)



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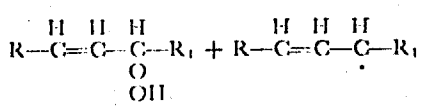
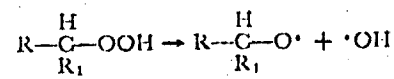


Propagation of the chain involves both hydrocarbon and hydroperoxy free radicals, and a non-radical hydroperoxide is an end-product of the

reaction. Decomposition of the hydroperoxide forms a[illegible]onal radicals (Eq. 4)



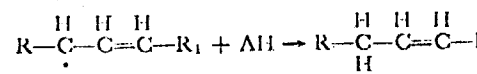
(4)

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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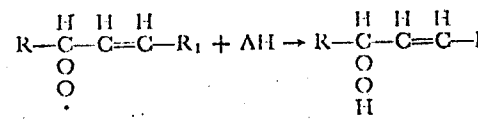

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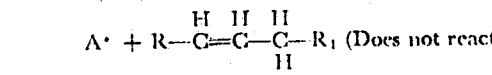

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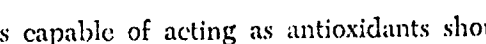


(8)

Substances capable of acting as antioxidants should have readily removable protons and the remaining free radicals should be incapable of

676

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 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.

TABLE 12

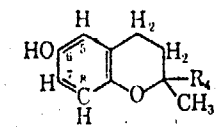
PARTIAL LIST OF FOOD ADDITIVES PERMITTED IN THE UNITED STATES[1]

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
Gum guaiac	Lecithin[4]
	Phosphoric acid

[1] Usually at levels not more than 0.02%.
[2] BHA, BHT, and PG account for 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 a synergist, such as citric acid.
[4] See Table 4.

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 vitamin 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_4 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] R_4 chain of tocol is $-CH_2(CH_2-CH_2-\overset{CH_3}{\overset{|}{C}H}-CH_2)_3H$. R_4 side chain of tocotrienol is $-CH_2(CH_2-\overset{CH_3}{\overset{|}{C}H}=CH-CH_2)_2H$.

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] Einset, E., Olcott, H. S., and Stansby, 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.

Fish Oils, M. E. Stansby (Ed.), 1967

CHAPTER 3

M. E. Stansby

General Analytical Methods

INTRODUCTION

In this chapter it is not the intention to present a description of analytical procedures for analyzing fish oils for the various oil constants and other common determinations. These analyses are made in the same manner as for oils in general and ample references abound in the literature. Rather, this chapter has been prepared to serve as a guide to those aspects of analysis of fish oils where differences exist from methods used for other oils. Thus more emphasis will be placed on sampling procedures and methods of extraction of oils from fish and fishery products than upon description of analytical techniques. Methods for evaluation of the general quality, including odor and flavor, will also be discussed with emphasis on sensory examination procedures.

HANDLING AND SAMPLING FISH OILS

Handling

Fish oils, because of their content of highly polyunsaturated fatty acids are far more unstable, subject to very rapid oxidation, than are most other oils. This makes necessary a very careful attention by the analyst to storage methods suitable to prevent oxidation or other changes from occurring either during storage prior to analysis or even during the analysis itself. Oxidation can occur not only from very small traces of oxygen or air present in any headspace above the oil but even from air dissolved in the oil itself. Fish oil which is to be stored before analysis should, therefore, be provided with an inert atmosphere. Nitrogen is most commonly used for providing such an inert atmosphere which is best applied by bubbling this gas through the oil in the container in which it is to be stored. In order to displace air dissolved in the oil, bubbling should be at such a rate that some circulation of the oil in the container takes place. For prolonged storage of the oil, bubbling should continue for at least one hour.

It is, of course, important that the nitrogen used for this purpose be as free as possible of any small traces of oxygen. While it is possible to purchase specially purified nitrogen containing only about one one-hundredth per cent oxygen, it is usually better to use a regular commercial grade of nitrogen and to pass it over heated copper (Wiberg 1960) before use. In

laboratories doing considerable work with fish oils it is desirable to pipe nitrogen, passed first over heated copper, to outlets at work benches.

Since exceedingly minute traces of oxygen are sufficient to cause alteration in fish oils, in providing an inert atmosphere not only must oxygen-free inert gas be used and bubbled through the oil to displace dissolved oxygen, but also great care must be taken in sealing or stoppering of the container to be sure that minute amounts of air are not admitted at the point where the container is sealed or stoppered. Carbon dioxide offers some advantage over nitrogen at this stage because with a density greater than air it is less likely that air will diffuse into the container at the moment of closing.

Sampling

Fish oil manufacturers require that homogeneous samples be taken of oil from very large lots such as in storage tanks usually ranging from 50,000 to 750,000 gallons or in barge shipments of 200,000 gallons or more. In order for the sample to be representative of such large lots, considerable care is needed to get uniformity. Samples are most frequently taken using a Bacon bomb sampler thief which is attached to a drop cord and lowered down through the tank at a uniform rate. This type of thief consists of a cylindrical body of about 12 inches (305 mm.) long and $2^3/_4$ inches (66 mm.) outside diameter. An opening at the bottom admits the oil through an attached center tube as the bomb sinks to the bottom of the tank. An adjustable needle valve at the top controls the filling speed by throttling the escape of air. The thief has a 16 oz. capacity. It gives a sample representative of a cross-section from top to bottom of the tank.

It is important that in passing down to the bottom of the tank, the bomb should not quite be filled with oil; otherwise it might become completely filled before reaching the bottom and thereby not give a true cross-section sample.

A problem in sampling fish oil, especially at low temperatures, is caused by the presence of stearine which exists as a solid or semi-solid under such conditions. Under severe sampling conditions this problem is overcome by stirring the oil for several hours by means of a side-entry agitator or rarely by heating the oil.

A sample of one pint is generally taken as being representative from tanks or lots up to 10,000 gallons. For larger lots, the sample size is increased, and for a 200,000-gallon barge load of fish oil, four quarts is considered adequate.

For cross-section sampling the "average" type Bacon bomb thief is employed. Where samples representative of the oil at different levels in the tank are desired, a special type thief is used.

GENERAL ANALYSES

Commercial fish oils are usually analyzed for iodine number, free fatty acid, moisture, impurities, and color as Gardner number. Is some cases bleach color (Lovibond) and occasionally the hot bleach color at 288° to 305°C. are measured. Rarely, unsaponifiables and saponification numbers are run.

AOAC (1960) methods are employed for running those analyses for which such methods are specified. The Wijjs method is used for iodine number except that some fish-oil plants employ for routine analysis a small hand-type refractometer. American Oil Chemists' Society methods (Mehlenbacher, Hopper, and Sallee 1965) are employed for some procedures such as the fat stability test sometimes determined on fish oils to be used in animal feed.

Cold-and-heat-bleach tests are occasionally used on fish oils to determine the color after treatment with specified bleaching earths, either with or without heat. The AOCS official method—Bleaching Test—C 8 b—52 (Mehlenbacher *et al.* 1965) designed primarily for use with soybean oil is commonly applied to fish oils as a cold bleach test. For a hot bleach test, the ASTM (1965) tentative heat-bleach method is sometimes used, especially where the fish oil is to be used in manufacturing alkyd vehicles. The test aids in predicting the final color of the alkyd.

ANALYTICAL EXTRACTION OF OILS

So far, we have been considering analyses of oils already extracted such as industrially manufactured fish oils. Often it is desired to analyze a sample of fish or of a fishery product which contains fish oil. Oils may be extracted from various fishery products in order to determine the oil content, or, in some cases, for the purpose of making a further examination as to some property of the extracted oil. Problems concerned with such procedures will be considered first with respect to extraction from fish flesh, then from fish meal.

Extraction of Oil from Fish Flesh

A sample is prepared for oil extraction by comminution in some sort of cutting-type grinder. Although it is possible to use equipment similar to the common kitchen meat-grinder in which the material, fed by a spiral shaft, is forced through a plate with holes, better grinding leading to a more homogeneous sample results from use of a sausage-type grinder in which the flesh is cut by a rotating, circular knife. Ordinarily fish samples for oil content determinations consist of bone-free material such as fillets. If bone is present, the above-mentioned types of grinders are unsuitable unless the fish can be cooked under pressure which will soften the

bones. If raw fish containing bone must be ground, more powerful mills such as of the hammer type may be necessary.

Moisture is rapidly lost by evaporation from fish, especially after the flesh has been minced. In any quantitative work, therefore, it is important to keep the minced sample in tightly closed containers until the sample has been weighed for analysis.

In some cases, especially when dealing with fish which has been frozen, the flesh releases moisture slowly after mincing, and fluid collects gradually. It is, therefore, important to stir the flesh thoroughly immediately before sampling in order to incorporate any separated fluid uniformly throughout the flesh.

For quantitative determination of the oil content of fish flesh the method of the AOAC (1960), involving acid hydrolysis followed by extraction in the cold with ethyl and petroleum ethers and using Mojonnier equipment, is quite generally employed.

Where the extracted oil is to be used for some subsequent analysis a more gentle extraction procedure is usually indicated. In order to obtain the fish oil with a minimum of change during extraction, the minced flesh can be dried in a vacuum desiccator over concentrated sulfuric acid after mixing with sand as is done in the AOAC (1960) method for moisture in grain and stock feeds, followed by extraction in the cold by appropriate fat solvent. A more generally applied technique is that of Bligh and Dyer (1959) in which the undried, minced fish flesh is extracted by blending with methanol and chloroform. The sample size is adjusted to give a constant amount of moisture, regardless of moisture content of the sample. A ratio of chloroform, methanol, and water is obtained which results in a layer of chloroform containing the lipid. As has been shown by Folsch *et al.* (1951) all lipid is extracted (at least from brain tissue) except any diphosphoinositide which is tightly bound by protein and can be released only by acid hydrolysis.

Extraction of Oil from Fish Meal

Conventional methods for determining the oil content of stock feeds AOAC (1960) involving continuous extraction of a dried meal sample, with ether, often gives extremely low values when applied to fish meals which have been stored for considerable periods of time after manufacture. This situation is worse, the higher the degree of unsaturation of the oil in the fish meal. When antioxidant is added to meal which is commonly done, the decrease in solubility of oil in ether occurs at a slower rate but nevertheless does take place. Ordinarily the problem is not severe for analysis of meals with oil of relatively low iodine number like herring meals, and even menhaden meals, unless stored for extremely long periods

of time, do not present any great problem. With fish meals such as pilchard or anchovy, very misleading results can be obtained if the usual methods are applied to meals which have been stored. Methods such as that of Bligh and Dyer give better results but for extreme cases other techniques may be necessary.

Samples of pilchard meal which when freshly prepared have an oil content of 15% may, if analyzed by conventional ether extraction methods, show values after storage of two years of as little as two per cent oil. The situation is improved markedly if acetone is substituted for ether but all of the lipid cannot be recovered unless the meal is given an acid hydrolysis treatment. The lipid extracted by acetone is, after extraction, then soluble in ether. Apparently the lipid originally present in the freshly prepared fish meal, as a result of oxidation and/or polymerization during storage, becomes tied up in some way with the remainder of the meal so that the lipid is extracted to only a slight extent by ethyl ether.

These facts are the basis for the 2-stage extraction procedure in the AOAC (1960) method for fat in fish meal. Most of the oil is removed by continuous Sohxlet acetone extraction. The residue is then digested with acid and the remaining feed lipid is extracted by acetone.

This procedure, involving two separate extractions, is time-consuming and is, therefore, inconvenient to use. Research is under way to find and standardize upon a simpler procedure.

Lovern (1965) has reviewed some more recently developed methods when applied to fish meal. The simple Bligh and Dyer (1959) technique is shown to give as good or better extraction of lipid from whitefish meal than the AOAC (1960) method. A modification of this method by Hanson and Olley (1963) is recommended. It remains to be demonstrated whether such methods will, without acid hydrolysis, extract all oxidized lipid from meals made from species like pilchard containing oil of very high degree of unsaturation. Even if this is not the case, a combination of the modified Bligh and Dyer (1959) method with an acid hydrolysis step would probably provide a convenient method.

EVALUATION OF FLAVORS AND ODORS

Two general types of procedures are possible in evaluating oxidative or other changes in fish oils leading to development of off-odors and flavors. In one type, accumulation of deterioration products are measured either by sensory or by chemical methods. In the alternate approach the stability of the oil is measured by determining the length of the induction period. A variety of methods and techniques are available for such measurements. Chemical tests for rancidity are discussed in Chapter 10 and will not be considered here.

CHEMICAL TESTS FOR NON-RANCIDITY FLAVORS AND ODORS

Our knowledge of the causes of "fishy" flavors in fish oils, other than those that arise from oxidative deterioration, is so very meager that there are few tests that can be considered. A measurement of trimethylamine or tertiary nitrogen would probably be helpful but unfortunately the quantity present in fish oils is usually so exceedingly small that its quantitative measurement is most difficult. Usually it is sufficient to measure the total nitrogen content of the oil. Even this, with the quantity of nitrogen present being very small, is not easy to do. A micro-Kjeldahl nitrogen digestion followed by a colorimetric determination of liberated ammonia has been used by Chipault (1960) for determining traces of nitrogen in fish oils. The quantity present is sometimes considerably smaller than the amount of nitrogen present as impurities in the Kjeldahl reagent chemicals which may result in larger blanks than in the sample. Use of a Dumas nitrogen determination method avoids this difficulty and often given better results.

SENSORY EXAMINATION METHODS

Importance

A number of chemical methods are available for evaluating flavor of fish oils, especially where flavor results from oxidative rancidity (see Chapter 10). Such objective methods, however, are of practical value only when they are standardized and frequently checked against the actual flavor of the fish oil. As has been pointed out by Stansby (1963), values obtained for rancidity tests in fish oils at identical flavor stages vary depending upon such conditions as storage temperature and presence or absence of antioxidants. Thus unless sensory tests are used in conjunction with scientific tests, the latter alone may give meaningless results.

Sensory Panels

As with sensory judgment of flavor in other food products, appraisal made by a single observer of the flavor or odor of fish oil is not sufficiently consistent to give reproducible results. It is necessary, therefore, to use a panel of several individuals. Panel members for evaluations of this kind should be experienced and familiar with the different types of odors and flavors normally encountered in fish oils. Thus, a consumer preference type of panel is not applicable to such work. Flavors and odors of fish oil are generally made up of a blending of a number of different components. Unless panel members are fully familiar with these individual flavor or odor components, no adequate evaluation is possible.

Judging Flavor of Fish Oils

A panel of at least four persons (preferably more), experienced in flavors of fish oils, is necessary in order to get a satisfactory evaluation. It is important to limit the number of oil samples compared at one time in order to reduce fatigue. As a rule, reproducibility of results falls off rapidly if more than about six samples are compared at one time. Since it is sometimes difficult to completely remove oil, previously examined, from glassware, it is convenient to present fish-oil samples to the panel members in 9-cm. disposable plastic petri dishes as suggested by Stansby and Jellinek (1965). These dishes give a large area for preliminary odor examination and can be discarded after use. Using plastic spoons, samples of oil can be conveniently tasted, a small portion of the oil being taken on the tip of the spoon. This procedure prevents excess oil from adhering to the lips which might interfere with successive tasting of several oil samples. Since fish oil often has persistent after-taste, it is helpful to remove flavor between oil samples in some suitable manner. For this purpose apple juice is useful. It is just tart enough to readily remove the fish-oil flavor without excess acidity such as present in citrus juices which interferes with subsequent sample tasting.

Judging Flavor of Oil in Fishery Products

When comparing flavor samples of fish oil contained in such products as cooked fish, many of the same considerations pointed out in the preceding section on fish oil flavor evaluation apply. In addition, it is important to remember that the oil in fish is not uniformly distributed throughout the flesh and furthermore, oxidative rancidity occurs to a far greater extent in oil in areas of dark flesh than in the light tissue; with some species virtually all the oxidation takes place in the oil in the dark muscle (Brown *et al.* 1957). In tasting samples of such fish it is important that these facts be well recognized. Otherwise, one panel member may be judging primarily light flesh in which the oil has not oxidized to any appreciable extent, and another panel member may be judging exclusively the dark flesh. It is possible to present to the panel a homogenized sample in which light and dark meat are blended together so that each panel member receives essentially identical samples. This procedure, while eliminating much discrepancy in judgments among panel members, also decreases sensitivity in picking up off-flavors. The dark tissue ordinarily occurs in fish in much smaller quantities than the light tissue. Hence the oxidized dark sample containing most of the flavor component is diluted with the light flesh containing largely unaltered oil. It is usually much better to have panel members each separately evaluate the flavor of some of the dark as well as of the light flesh. This same principle applies to chemical rancidity tests

where it is advisable to run peroxide numbers, TBA, etc., on oil samples extracted from light and dark flesh separately rather than on a composited sample. Often minimal or zero values are obtained on oil from the light flesh where the corresponding oil from the dark flesh gives high rancidity values.

Induction Period Measurements

Often fish oils may, at the time they are analyzed, all have good flavor but, upon oxidation, some oils will develop rancid or other flavors more rapidly than others. A measure of the induction period will give an estimation of stability of the oil, and sometimes sensory tests offer the best means of judging when the end of the induction period has been reached.

Ordinarily the oil is stored under some reproducible, accelerated condition to hasten oxidation. This is usually accomplished by aeration, heating to some elevated temperature at which the oil is stored, or a combination of both. Use of elevated storage temperatures usually alters the pattern of oxidation so that if two oils have an induction period ratio of 1:3 at the elevated temperatures, this ratio may be quite different at some lower temperature, at which the oil is apt to be stored. Another method sometimes more suitable for accelerating oxidation rate in induction period measurement is by increasing the surface of oil exposed to air. For example, fish oil spread on filter paper may oxidize one hundred times as fast as when stored in a beaker. Samples stored on filter paper can be tasted at intervals of storage at room or lower temperature until flavor changes have occurred to give a measure of the induction period, or chemical tests can be applied.

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SP-3; CP-211
Draft 3
August 12, 1968

U.S. FISH MEAL AND OIL INDUSTRY

by

Maurice E. Stansby and George M. Knobl, Jr.

ABSTRACT

This article presents a comprehensive review showing past developments, present operations, and future possibilities.

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Oil sample	Location produced	Level endrin found (ppm)[a]			
		Lab 1	Lab 2	Lab 3	Average[b]
SBO-1	Area suspected of contamination	0.61	0.78	0.48	0.62
SBO-2	Area suspected of contamination	0.77	0.51	0.14	0.48
SBO-3	Area suspected of contamination	0.41	0.43	0.36	0.40
SBO-4	Area suspected of contamination	0.35	0.24	0.07	0.22
SBO-5	Area not suspected of contamination	BDL	BDL	0.03	0.01
CSO-1	Area suspected of contamination	BDL	0.03	BDL	0.01
CSO-2	Area suspected of contamination	BDL	0.19	BDL	0.06
CSO-3	Area suspected of contamination	BDL	BDL	0.03	0.01
CSO-4	Area suspected of contamination	I	BDL	BDL	BDL
CSO-5	Area not suspected of contamination	BDL	0.05	BDL	0.02

[a]SBO = Soybean oil.
CSO = Cottonseed oil.
BDL = Below detectable limits of analysis.
I = Interference in sample analysis.
[b]Calculated by taking average of values reported by the three laboratories.

substance contained in the biological samples analyzed, it was believed that the lower limit of confident analysis was approximately 0.03 ppm. Based on extensive recovery data, values below 0.03 ppm were not considered analytically significant in this study.

Results and Discussion

Pilot Plant Processing of Oil

Data obtained in the pilot plant processing of the vegetable oils are shown in Table I. The weight of soapstock can be calculated by determining the differences between the crude oil refined and refined oil recovered. The weight of soapstock is dependent upon content of the free fatty acids, phosphatides, etc., in the crude oil and the amount of neutral oil occluded with the soapstock. In the batch refining procedure used in this pilot plant study, more than usual occluded neutral oil occurred in the soapstock because of difficulties in obtaining a clean-cut soapstock-neutral oil separation. Samples of soapstock analyzed for neutral oil showed that approximately 30–35% of the weight of soapstock was neutral oil. Therefore, the refining loss was high when compared to commercial refining where the normal range is 15–22% occluded neutral oil in soapstock.

During deodorization, approximately 9 lb. of stripping steam was passed through the oil. The stripping steam and the vaporized materials were pulled from the closed vessel and were partially condensed in a water condensate trap. The amounts of condensate recovered per batch varied from none to 4 lb. Freezing out more condensate was attempted and found impractical.

Analytical Laboratory Results

Table II shows data on endrin contamination of the four test soybean and four test cottonseed oils that were randomly sampled from two crude oil mills in the area suspected of producing crude oils with pesticide residue contamination. One crude soybean oil and one crude cottonseed oil were chosen from an area which uses little or no endrin; the oils were analyzed and found free of pesticide contamination prior to processing. This table shows that the negative control soybean and cottonseed oils were at or below the sensitivity of the analytical methods for endrin. Chlorinated pesticide contamination of the crude cottonseed oils used in this study was doubtful.

Table III illustrates a comparison of endrin analytical values for four soybean oils in stages of processing. Considerable variation was observed between the endrin content of the oil lots. Within the precision of this experiment, endrin levels were equivalent in the crude, refined and bleached oils; none was detected at the sensitivity level of the analytical method in deodorized oil.

The average analytical values obtained when two fortified cottonseed oils were processed and analyzed by three laboratories are presented in Table IV. Processing had a similar effect on all chlorinated pesticides studied: the refined and bleached oils had similar amounts of pesticides present, while pesticide contamination could not be established in the deodorized oils. Trace levels of DDT and endrin were reported by one laboratory; however, the other two laboratories could not confirm the presence of detectable levels of chlorinated residues.

Table V gives calculated amounts of endrin in each of the processing fractions. Since essentially all the endrin can be accounted for during refining and bleaching, it would appear that these processes would be of minimal importance in reducing the pesticide level of contaminated oils.

Vacuum deodorization removed endrin and other chlorinated pesticides studied. This was not surprising considering the similarity in the physical and chemical properties of the related chlorinated pesticides and the previous research reports on the removal of certain pesticides from oil by deodorization (1,2,11). These reports should eliminate possible concern over edible oils being produced from crude oils containing significant amounts of pesticides since all edible vegetable oils are deodorized in processing.

Two soybean oils were hydrogenated prior to deodorization and the level of endrin was reduced to near or below the lower detectable limits for the analytical method. These results confirm observations by Gooding (1) and Shell (2).

Since it has been established that hydrogenation or deodorization, or both, will eliminate endrin from the resulting edible oil, the question arises as to the fate of these pesticides during these processes.

TABLE III

Effect of Processing on Endrin Residue Levels in Four Soybean Oils Initially Suspected of Pesticide Contamination[a]

Oil sample	Crude oil	Refined oil	Bleached oil	Deodorized oil
		ppm		
SBO-1	0.62	0.59	0.51	BDL[b]
SBO-2	0.48	0.30	0.35	BDL
SBO-3	0.40	0.64	0.48	BDL
SBO-4	0.22	0.30	0.29	BDL
Mean	0.43	0.46	0.41	BDL

[a]Individual crude, bleached and deodorized oil values represent mean analyses of the three laboratories. Two analyses compose the refined oil values.
[b]BDL = Below detectable limits of analysis.

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	12.5	[c]
DDE	0.92	0.83	BDL[d]
Aldrin	0.55	0.65	BDL
Dieldrin	0.72	0.64	BDL
Heptachlor	0.58	0.69	BDL
Heptachlor epoxide	0.77	0.86	BDL

[a] Average pesticide value of two oils with three independent laboratory analytical values per oil.
[b] Values (CSO-1, 0.07; CSO-2, 0.08) 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 a 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 (530 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.

ACKNOWLEDGMENT

The authors express gratitude to Chester Gooding (Corn Products Co., Bayonne, N.J.), James Thigpen (USDA), Garlon Harper (National Cotton Products Association), and C. McMichael (Votator Div., Louisville, Ky.) for their recommendations and assistance.

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[Received April 8, 1968]

Fish Oils: ME Stansby (Ed.) 1964

CHAPTER 20

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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

(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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ments allowed as great a deposition of total polyunsaturates in heart tissue as did an equal dosage of linoleate. The polyunsaturated acids present in the 4 fractions of tuna oil esters were not the metabolic equivalent of linoleate. With the exception of the C_{16} fraction, all stimulated growth as do both linoleate and linolenate. The lack of growth stimulus, and the high proportion of trienoic acid deposited in the hearts of rats fed the C_{16} fraction suggest that this fraction has a nutritional and metabolic activity less than or different from the other groups of unsaturated acids tested.

No evidence was found in this investigation that any portion of esters of unsaturated acids from tuna oil contained essential fatty acids (linoleic acid family) in detectable amounts. Marked growth-promoting activity was present in the polyunsaturated acids of the 18-, 20- and 22-carbon fractions, suggesting that their acids were of the linolenic acid family.

SUMMARY

Highly unsaturated acids from tuna oil were concentrated and their esters were separated according to chain length by fractional distillation. Each chain-length fraction was fed as a supplement to fat-deficient rats and the effects were compared with those induced by ethyl linoleate.

None of the fractions containing fatty acids of 16, 18, 20 or 22 carbon atoms in length relieved the dermal symptoms of EFA deficiency. However, all the fractions except the 16-carbon fraction showed marked stimulation of growth. Only the 16-carbon fraction allowed the deposition of abnormally high amounts of trienoic acid in heart and testis tissue.

ACKNOWLEDGMENT

The authors wish to acknowledge the technical assistance of F. J. Pusch, P. A. Rusley and Arlen E. Viste.

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Survival Time of Hypertensive Rats Receiving Fish-oil Extracts

The demonstration by A. Grollman (*J. Pharm. exp. Therap.*, 1945, 84, 128) that the blood pressure of animals with experimentally induced hypertension can be reduced by the oral administration of various extracts derived from oxidized oils raises the question as to the beneficence of this form of therapy. It is obviously important to know if the decline in blood pressure is accompanied by any deleterious effects such as are noted following the injection of many noxious agents which are also capable of reducing the blood pressure. We have therefore determined the effect of administering an oxidized crude fish oil on the survival of rats rendered hypertensive by the procedure of Grollman (*Proc. Soc. exp. Biol. Med.*, 1944, 57, 102).

The animals used for the experiment were a piebald strain reared in the laboratory and weighing 150 to 400 grams. A week following the operation on the right kidneys, the animals were divided into two groups, alternately, according to the level of their blood pressure. One group received the normal laboratory diet; the other, the same diet plus the addition of 10 grams of oxidized fish oil daily for the group. Two weeks following the first operation, the left kidneys in both groups were removed. Blood pressures were determined twice a week.

Observations were continued for two months following the nephrectomies. At the end of this period 21 of the control group of 44 animals had died, while only 11 of the 42 rats in the treated group had died. The average blood pressure of the control group was 134; that of the treated group, 129. The observed drop in blood pressure was, thus, apparently insignificant, due to the relatively small amount of activity present in the doses of oil used, as well as to the fact that the average blood

pressure of the animals was not greatly elevated above normal.

A more convincing experiment was carried out on a group of 24 mice, the right kidneys of which were constricted and the left extirpated. Half of this group was treated with a more potent extract than was available in the case of the experiment on rats, which probably accounts for the better response observed. Of the 12 control animals, 10 had died at the end of a six-week period of observation, compared to only 2 in the group of 12 treated animals.

The experiments cited suggest that the administration of extracts of oxidized fish oil prolongs the life of animals with chronic hypertension.

The work described was supported by grants from the John and Mary R. Markle Foundation. I am indebted to Dr. Grollman for supplying the oils used in these experiments and for aid in performing the operations.

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Logic and the Science Curricula

J. Am. Chem. Soc 45: 866-869, 1968

Removal of Chlorinated Pesticides From Crude Vegetable Oils by Simulated Commercial Processing Procedures

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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 these 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.

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.

Pilot Plant and Oil Processing

Before processing each individual oil sample, all pilot plant equipment was thoroughly washed with hot (180 F) tap water, rinsed with commercial grade hexane followed by a 20° Be sodium hydroxide solution and finally flushed with distilled water. Extensive care was taken in each processing step to prevent external contamination of the samples.

Crude oil lots were thoroughly mixed and then emptied into a 50-gal stainless steel refining kettle. The crude oil was rapidly propeller-agitated (550 rpm) and the caustic solution was slowly added according to AOCS specifications (9). The agitation was continued for 20 min at ambient temperatures (80–90 F). Agitation was then reduced to 10–15 rpm (paddle-type-agitator) and the oil was heated (SBO, 160 F; CSO, 140 F) by circulating steam in the jacket of the refining kettle. When the desired temperature was reached, steam and agitation were turned off and the oil was allowed to settle until a soap break was observed. The soapstock was drained, weighed and sampled.

The refined oil was washed with 15 wt per cent hot water (SBO, 150 F; CSO, 140 F). Under rapid agitation, the temperature of the oil was increased (SBO, 170 F; CSO, 160 F) and held at temperature for 30 min. The oil was then allowed to settle until the break was complete. Wash water was removed and samples of wash water and refined oil were obtained.

The refined oil was transferred to the vacuum bleaching vessel and bleaching agent added (SBO, 1% acid clay; CSO, 1% neutral clay plus 0.2% activated carbon; both oils, 0.5% filter aid). Under vacuum (50 mm Hg abs), the oils were slowly heated (approx ½ hr) to temperature (SBO, 220 F; CSO, 200 F) and held at temperature for 20 min. The oil was then pumped through a horizontal-plate pressure 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 number was obtained (70–80), the oil was cooled under vacuum (50 mm Hg abs to approx 175–185 F). Vacuum was then broken with nitrogen and the hydrogenated oil was filtered through a horizontal-plate pressure filter to remove the used catalyst. The hydrogenated oil was weighed and sampled.

During deodorization, the refined-bleached oils or refined-bleached-hydrogenated oils were heated to temperature (SBO, 485 F; CSO, 460 F) under vacuum (6 mm Hg abs) and held for 1 hr. While the oil was being taken to temperature, 3 lb. (1½ wt per cent) stripping steam was added to agitate the oil. When the oil was at temperature, a total of 6 lb. (3 wt per cent) stripping steam was passed through the vessel to accomplish deodorization. Component weights were recorded and samples were obtained for chemical analysis.

Analytical Procedures

The three participating laboratories employed analytical methods for determination of the chlorinated hydrocarbons which were essentially equivalent to those described in the FDA Pesticide Analytical Manual (10). Small variations in procedures used by the respective laboratories are considered of no significance in this collaborative effort.

In general, the oil material was extracted with hexane, partitioned into acetonitrile and exchanged back into hexane. After washing and drying the hexane solution with sodium sulfate solution, aliquots were cleaned by liquid-solid chromatography through an activated Florisil column. Three separate elutions were collected and subjected to gas chromatographic analysis for chlorinated hydrocarbons.

The utilization of standard GLC methods employing electron-capture and microcoulometric detection systems permitted extreme sensitivity to the chlorinated hydrocarbons. Based on the equipment capability, normal background noise, and the interfering

TABLE I

Results Obtained in Processing the Vegetable Oils Under Pilot Plant Conditions[a]

Process	SBO-1	SBO-2	SBO-3	SBO-4	SBO-5	CSO-1	CSO-2	CSO-3	CSO-4	CSO-5
Refining										
Quantity of oil refined, lb.	222	226	210	222	295	225	212	221	203	295
Free fatty acids in oil, wt. %	0.71	0.72	1.17	0.30	0.30	2.70	2.75	2.60	2.20	0.49
Caustic used, lb.	12.6	19.2	20.4	16.9	22.5	21.6	20.8	21.0	17.9	23.2
Refined oil recovered, lb.	209	204	182	157	260	191	178	182	178	241
Refining loss, wt. %	5.9	9.7	13.3	15.8	11.9	15.1	16.0	18.4	12.3	18.3
Bleaching										
Quantity of oil bleached, lb.	206	201	179	184	255	188	175	179	175	236
Bleached oil recovered, lb.	197	200	176	175	245	187	168	176	162	225
Hydrogenation										
Quantity of oil hydrogenated, lb.		197		172						
Hydrogenated oil recovered, lb.		147		86						
Deodorization										
Quantity of oil deodorized, lb.	194	147	173	200[b]	200	184	165	173	159	200
Deodorized oil recovered, lb.	189	136	170	193	190	172	156	166	144	186
Condensate recovered, lb.	0	0.25	0.25	4.0	4.0	3.75	0.5	0.25	0.25	0.25

[a] SBO = Soybean oil.
CSO = Cottonseed oil.
[b] Composed of 86 lb. hydrogenated SBO-4 and 114 lb. deodorized SBO-2.

THE EFFECT OF CONCENTRATES OF POLYUNSATURATED ACIDS FROM TUNA OIL UPON ESSENTIAL FATTY ACID DEFICIENCY[1]

J. Nutr. 67: 423-432, 1959

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(Received for publication August 23, 1958)

INTRODUCTION

Marine oils are known to contain large proportions of highly unsaturated acids, which are generally regarded to have low potency in the relief of dermal symptoms of essential fatty acid (EFA) deficiency. Recently accumulated evidence suggests that the polyunsaturated fatty acids may be grouped into a few classes depending upon the location of the first double bond from the methyl end of the fatty acid molecule (Thomasson, '53). Thus far, only those acids which have a terminal structure like that of linoleic acid have been found to exhibit all the activities of an essential fatty acid. That is, only linoleic and arachidonic acids, having their first double bond between the 6th and 7th carbon atoms from the methyl group, and a second double bond between the 9th and 10th carbon atoms are able to mantain normal skin and stimulate growth of rats when added to an otherwise fat-free diet. It is not known with certainty whether fish or marine oils contain sufficient acids of the linoleic acid type to meet the needs

[1] Hormel Institute publication no. 181. Supported in part by grants from the Hormel Foundation, Fish and Wild Life Service of the Department of Interior and the Office of Naval Research, contract N8onr66218, 101-344.

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for essential fatty acids. It was therefore of interest to test a typical marine oil for its EFA activity. In order that the information be more precise, the individual chain-length fractions from tuna oil unsaturated methyl esters were tested in rats for their effects upon growth, dermal symptoms and polyunsaturated acid contents of representative tissues.

PREPARATION OF MATERIALS

Tuna oil was saponified and the unsaponifiable matter was removed by extraction of the soaps. The fatty acids were liberated with acid and the free acids were subjected to a

TABLE 1

Characterization of the ester supplements

SAMPLE	UNSATURATED COMPONENTS BY PAPER CHROMATOGRAPHY		IODINE VALUE	MOST UNSATURATED COMPONENT BY ALKALINE ISOMERIZATION
	Major	Minor		
C_{16}	2	2	154.5	tetraenoic acid
C_{18}	2	2	200.5	tetraenoic acid
C_{20}	1 (pentaenoic acid)	2	352	pentaenoic acid
C_{22}	1 (hexaenoic acid)	1	414	hexaenoic acid
Ethyl linoleate	1	0	160.5	dienoic-acid

scheme of low temperature crystallization in light petroleum ether (Skelly F). The fraction which was soluble at —70°C at a 10:1 ratio of solvent to solute was recovered and further fractionated by urea crystallization. Most of the less unsaturated acids formed urea-inclusion compounds at —15°C, and the highly unsaturated acids which comprised the bulk of the sample remained in the filtrate. This fraction was esterified with methanol and then was fractionated according to chain length by distillation through a Podbielniak whirling band column. The chemical characterization of the 4 fractions fed in this study are given in table 1. It should be emphasized that

the fractions fed represent concentrates of the highly unsaturated acids from tuna oil and do not represent the total fatty acids of tuna oil fractionated according to chain length. The C_{22} fraction represents a high concentrate of methyl docosahexaenoate having only one unsaturated component detectable by the paper chromatography (Schlenk et al., '57) although its iodine value, 414, is less than the theoretical 445. The ultraviolet absorption spectra after alkaline isomerization of the 4 fractions indicated that the most highly unsaturated components were tetraene for C_{16}, tetraene for C_{18}, pentaene for C_{20} and hexaene for C_{22}. The hexaenoic acid present in the C_{22} portion was found to be 4, 7, 10, 13, 16, 19-docosahexaenoic acid by the method of von Rudloff ('56).

NUTRITIONAL EXPERIMENT

A fat-free diet was fed ad libitum to 48 male weanling rats of the Sprague-Dawley strain. The diet consisted of 16% vitamin-test casein, 4% α-cellulose, 74% sucrose, 4% Wesson salt mixture ('32), 1% of a mixture of vitamins in casein and 1% of a mixture of choline chloride in casein (Aaes-Jørgensen and Holman, '58). After 11 weeks, the growth rate was rather slow, and distinct dermal symptoms of essential fatty acid deficiency had appeared in all animals. The rats were then divided into 6 equal groups and continued on the same fat-free diet during the period of supplementation. Group 1 received no supplement, group 2 received the C_{16} fraction, group 3 the C_{18} fraction, group 4 the C_{20} fraction, group 5 the C_{22} fraction and group 6 ethyl linoleate. All supplements were administered in doses of 200 mg thrice weekly. The rats were weighed and inspected each week. After 6 weeks of supplementation the rats were killed by ether anaesthesia and the hearts and testes were taken for analysis. Polyunsaturated acid contents were determined using the method of Holman and Hayes ('58).

RESULTS AND DISCUSSION

Biological effects. The effects of the various fatty acid supplements upon the EFA-deficient rats is shown in table 2,

and the condition of the hind feet and tail of representative rats from each group is shown in figure 1. None of the supplements except linoleate was able to cure the dermal scaliness or stimulate the development of the normal yellow-brown skin pigment. The rats from all groups except those fed linoleate had stiff, extremely scaly tails. In figure 1 it will be noticed that the tail from the linoleate animal was relatively straight, whereas the remainder had curled tails which the rats could

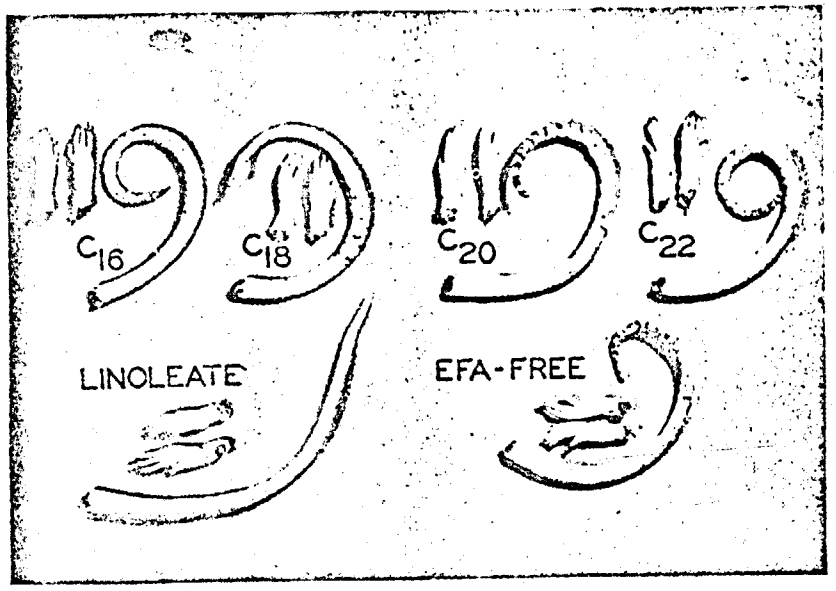

Fig. 1 Tails and feet from representative rats of each group.

not straighten. From these observations it may be concluded that none of the fractions of highly unsaturated fatty acid esters from tuna oil contained sufficient EFA to effect even a partial dermal cure in 6 weeks. Eicosapentaenoic acid and 4,7,10,13,16,19-docosahexaenoic acid have been shown here to stimulate growth but not to cure the dermal symptoms of EFA deficiency. These observations strengthen the concept that unsaturated fatty acids which have identical terminal structures have similar biological activities. Since all the fractions

from tuna oil stimulated growth except the C_{16} fraction, and none affected the dermal symptoms, it appears that the major polyunsaturated acids in tuna oil resemble linolenic acid rather than linoleic acid in their biological activities.

Fatty acids of heart tissue. The hearts of rats chosen at random from each experimental group were analyzed for their contents of polyunsaturated fatty acids. The data, shown in table 3, indicate a general uniformity in the amount of total polyunsaturated acids in the heart tissue of the several groups.

TABLE 2

Effects of supplements upon EFA-deficient rats

SUPPLEMENT	AV. WT.		AV. DERMAL SCORE [1]		SKIN PIGMENT [2]	CAUDAL NECROSIS [3]
	Initial	Final	Initial	Final		
	gm	*gm*				
None	231 ± 6.3 [4]	262 ± 6.6	1.8	2.0	0	1
C_{16}	231 ± 13.9	265 ± 10.7	1.8	1.8	0	1
C_{18}	232 ± 12.9	291 ± 13.6	1.5	1.2	0	3
C_{20}	231 ± 9.4	305 ± 8.9	1.5	1.8	0	1
C_{22}	231 ± 6.1	287 ± 8.0	1.5	2.0	0	2
Linoleate	235 ± 9.5	296 ± 8.2	2.0	0.4	8	0

[1] Average scores for tail, forelegs and hind legs, each evaluated on a scale of 0 to 3.
[2] Number of rats having skin pigment.
[3] Number of rats having necrosis for at least 1 cm of the tail.
[4] Standard error of the mean.

The group fed ethyl linoleate had somewhat more total polyunsaturated acids in the heart tissue than did the others.

As was expected from previous studies (Rieckehoff, Holman and Burr, '49; Aaes-Jørgensen and Holman, '58) the proportion of dienoic acids was low in the fat-free group and high in the linoleate-fed group, whereas trienoic acid was very high in the fat-free group and low in the linoleate-fed group.

Supplementation with the esters of the unsaturated C_{16} acids resulted in higher contents of pentaenoic and hexaenoic acids than found in the rats fed the fat-free diet. However, the dienoic acid contents of hearts of the C_{16} group remained low and the trienoic acid contents remained high, comparable

to the condition found in EFA-deficient rats. Although pentaenoic and hexaenoic acids were synthesized when the C_{16} fraction was fed, neither the supplement nor the metabolites produced were adequate to meet the need for EFA.

TABLE 3

Polyunsaturated fatty acid content of hearts from rats fed various fatty acid esters

SUPPLEMENT	ACID					
	Hexaenoic	Pentaenoic	Tetraenoic	Trienoic	Dienoic	Total
	mg/100 gm	*mg/100 gm*	*mg/100 gm*	*mg/100 gm*	*mg/100 gm*	*mg/100 gm*
None	18	6	46	240	29	339
	19	5	44	224	96	388
	13	7	59	261	2	342
	17	13	101	417	− 14	543
C_{16}	55	23	71	288	4	441
	61	27	96	284	34	502
	18	10	44	121	− 23	170
	44	28	74	190	63	399
C_{18}	132	33	67	58	37	327
	85	40	101	109	69	404
	61	39	136	123	14	373
	57	23	61	126	88	355
C_{20} (largely pentaenoic)	115	63	135	54	17	384
	73	61	115	33	11	293
	110	97	198	51	10	466
	102	79	180	59	5	425
C_{22} (largely hexaenoic)	147	46	53	0	5	251
	153	52	57	38	7	307
	248	69	73	106	− 5	491
	140	61	95	131	142	569
Ethyl linoleate	14	49	249	55	158	525
	14	30	162	76	226	508
	16	43	311	68	149	587
	29	50	307	50	143	570

Supplementation of a fat-free diet with the C_{18} fraction induced increased deposition of pentaenoic and hexaenoic acids in the hearts of the rats. However the contents of dienoic acids remained low, suggesting that the dienoic acid of the sample was not incorporated in the tissue and that reduction

of the more highly unsaturated acids of the sample to dienoic did not take place to any great extent. Trienoic acid appeared in the heart tissues in amounts intermediate to those obtained in deficient rats and rats fed linoleate. The hexaenoic and perhaps the pentaenoic acids of the hearts apparently arose from less unsaturated precursors in the supplement fed.

Feeding the C_{20} fraction, which was largely pentaenoic acids, resulted in a pattern of polyunsaturated acids similar to that found in hearts of rats fed the C_{18} fraction. That is, the dienoic content was low, the trienoic acid content was equivalent to that found in linoleate-fed rats, and the tetraenoic, pentaenoic and hexaenoic acids were present in high proportions. Therefore, it may be concluded that the hexaenoic acid of the heart tissue was synthesized from the pentaenoic acid fed, for the supplement contained no hexaenoic acid.

The C_{22} supplement, which was mostly hexaenoic acid, caused the deposition of a high proportion of hexaenoic acid in the hearts of rats. The pentaenoic acid content of the hearts was one magnitude greater than that found in rats fed fat-free diet. However, the tetraenoic acid content was about the same as in the deficient rats, suggesting that hexaenoic and pentaenoic acids were not reduced to provide tetraenoic acid. The trienoic acid content was found to be low, suggesting that abnormal synthesis of trienoic acid did not take place when the highly unsaturated acids were provided, even though these did not relieve the external symptoms of EFA deficiency.

Fatty acids of testis tissue. The testes of EFA-deficient rats were found to contain more trienoic acid and less dienoic acid than those from the rats fed linoleate (table 4). None of the supplements of esters from tuna oil increased the dienoic acid content of testes significantly. The trienoic acid content of testis tissue was found to be abnormally high only in the group fed the C_{16} fraction. All fractions of unsaturated esters increased the hexaenoic acid content of the testis when fed as a supplement to the fat-free diet, suggesting that all contained significant proportions of acids which are precursors of hexaenoic acid and are thus presumably of the linolenic acid family.

TABLE 4

Polyunsaturated fatty acid contents of testes from rats fed various fatty acid esters

SUPPLEMENT	ACID					
	Hexaenoic	Pentaenoic	Tetraenoic	Trienoic	Dienoic	Total
	mg/100 gm	*mg/100 gm*	*mg/100 gm*	*mg/100 gm*	*mg/100 gm*	*mg/100 gm*
None	5	38	30	152	21	246
	8	63	148	174	10	403
	9	68	143	167	16	403
C_{16}	17	74	108	109	4	312
	15	46	86	101	7	255
	17	45	83	102	4	251
	21	66	90	125	56	358
C_{18}	34	75	108	81	19	317
	48	77	140	113	35	413
	77	41	69	60	5	252
	30	46	87	86	6	255
C_{20}	42	67	90	55	8	262
	36	70	97	65	17	285
	43	102	146	85	12	388
	63	160	193	134	46	596
C_{22}	28	29	51	53	4	165
	55	53	85	91	17	301
	31	35	50	37	5	158
Ethyl linoleate	3	52	49	30	16	150
	15	12	20	101	46	194
	7	128	105	39	20	299
	9	161	144	39	22	375

CONCLUSIONS

From this study it appears that the concentrated highly unsaturated fatty acids of tuna oil do not show complete EFA activity when fed at the level of 600 mg per week. None of the fractions, which were essentially single chain lengths, relieved dermal symptoms of EFA deficiency. None induced the deposition of dienoic acid in heart tissue such as is observed when linoleate is fed. However, only the C_{16} fraction allowed the deposition of abnormally high trienoic acid in heart tissue, such as is observed in EFA deficiency. None of the supple-

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J. Am. Oil Chem. Soc. 44, 397-399, 1967

Standardization of Nuclear Magnetic Resonance Measurement of Solids in Fats and Shortenings

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Abstract

An investigation of factors that influence the measurement of solids in fats and shortenings by nuclear magnetic resonance was directed toward standardization of the method. The aim was to select abbreviated tempering conditions that would yield results comparable with those existing at temperature equilibrium. Conditions were found which reduced the time and gave an accuracy that made the method suitable for both research and control purposes.

Introduction

REPORTS ON THE APPLICATION of nuclear magnetic resonance (NMR) measurements to the determination of solids in fats and shortenings (1–4), have been concerned primarily with showing the solids content of samples at selected temperatures and pointing out the superiority of these measurements over other techniques for evaluating solids content. Although investigators have standardized the testing for their own purposes, little or no information is available on the factors necessary for obtaining comparable results in different laboratories. These factors involve instrument calibration, appropriate chilling temperature and time, and tempering time that will give results comparable with equilibrium conditioning in as short a time as possible. This report will deal with the effect of these various factors on the determination of solids and selection of the conditions which, in a minimum time, give results identical with or close to the equilibrium values.

Measurement by Nuclear Magnetic Resonance

For this purpose a low resolution NMR instrument, Schlumberger NMR Analyzer Model 104,[1] was employed. The first step in the application of an instrument of this type is the establishing of appropriate settings for the various parameters, based upon characteristics of the material to be tested and upon the size of sample to be employed. The conditions employed for the reported data were:

Sample size	4.5 to 5.5 grams
Instrument settings	
Sweep time	1 minute
Sweep constant	1 second
R.F. selector	40 df
Sweep amplitude selector	0.5 gauss
Sensitivity selector	X 500
Modulation amplitude selector	0.1 gauss
Integrator settings	
Read-out multiplier	2
Threshold, mv	0.1
Signal multiplier	5

In preparing a sample for testing, a procedure must be followed which is reproducible. The principal steps are heating the sample to a selected starting temperature, chilling to a point where the solids formed are greater than they will be at the testing temperature, and tempering in a bath with proper temperature control for a period that will give equilibrium or conditions close to those existing at equilibrium.

After appropriate instrument settings and a satisfactory procedure for preparing the sample have been established, the next step is calibration of the instrument.

Instrument Calibration

A calibration curve can be established from a sufficient number of measurements on a series of liquid oils, which will give a reliable average value for the liquid oils, at the desired testing temperature. The calibration curve for the selected temperature is prepared by plotting the average of the series of measurements on the liquid oil as 0% (zero) solids and zero NMR measurement as 100% solids and then drawing a straight line between the two points. Measurements on a solid fat are not necessary as a completely solid fat will measure zero. Samples of vegetable oil hydrogenated to a low iodine value may measure 0 to 1 mv/g at room temperature but, on cooling, will measure zero, indicating the presence of a small amount of liquid at room temperature.

Measurements made on a series of oils at 0, 28, and 60C indicated that soybean, cottonseed, and peanut oil were similar and that safflower oil gave slightly lower NMR values, also that these values vary with temperature. Typical measurements are given in Table I. Thus, in preparing calibration curves, one must take into account both the testing temperature and the type of oil. Since soybean and cottonseed oils are the most typical oils processed, the calibration curves for different temperatures given in Figure 1 are based on the average values for these oils over the range of 0 to 60C. Values below 0C are taken from an extrapolation of the data to −15C. This extrapolation is in line with data for safflower oil, which were carried to −10C without solids appearing in the sample, as evidenced by the NMR measurements and the visual clarity of the sample.

In the past it has been the practice to set up calibration curves which were based on measurements made of known blends of completely hydrogenated oils with liquid oil. These have followed a straight line connecting the NMR value for the liquid oil (0% solids) and zero NMR value (100% solids), within the limits of the method, when the measurements were made in the region (0 to 30C), where the hydrogenated oil has not gone into solution appreciably.

TABLE I

Typical N-M-R Measurements on Liquid Vegetable Oils

Temperature	Safflower oil	Soybean oil	Cottonseed oil	Peanut oil
0C	65.2	65.5	64.6	64.5
28C	60.7	61.5	62.2	61.6
60C	56.5	57.6	57.4	57.6

[1] This model has been replaced by Varian Associates as Model PA-7 Process Analyzer.

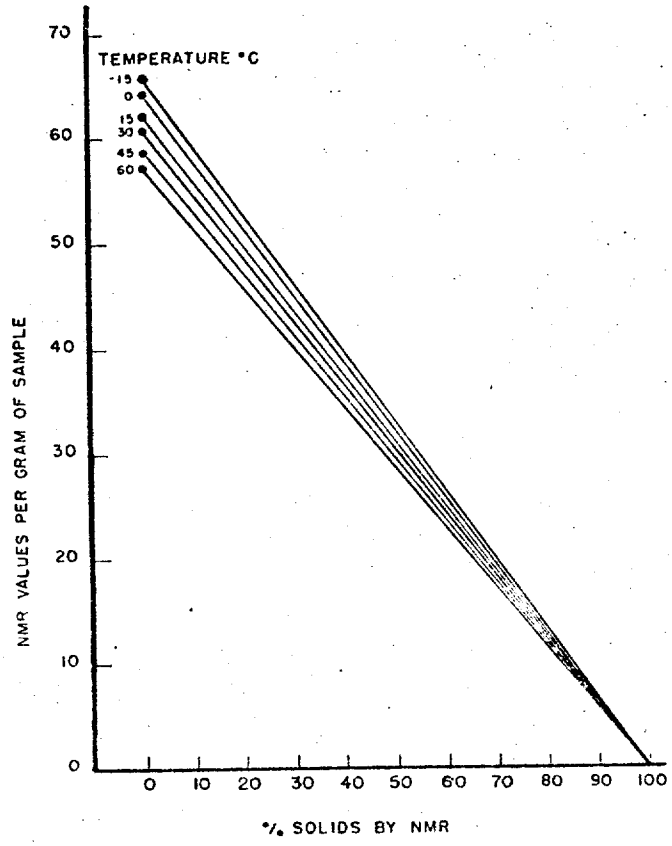

FIG. 1. Calibration graphs showing the effect of temperature.

This new approach to the preparation of a calibration graph saves time and increases the accuracy of the measurement of solids at a selected temperature.

Chilling and Tempering

In previous reports by the authors, samples were chilled at 0C and for a time were comparable with that used in the Solids Fat Index (SFI) method (3) because subsequent measurements were going to be compared with SFI data. Subsequent work has revealed that chilling at 0C for the short periods selected was not adequate to solidify all the material that would be solid at 10C. This was evidenced by the increase in solid content of certain samples with time, when held in the 10C bath after chilling at 0C.

To attain results in a short time which will be comparable with those at equilibrium, it is necessary to approach the equilbrium conditions from the solid side. To accomplish this the sample must be chilled to a point where more solids are formed than will be present at the tempering temperature. If chilling is to be done rapidly, so that the method is suitable for routine testing, a very low temperature bath must be used. A carbon dioxide-acetone bath maintained at below −60C was the simplest and most convenient means for chilling the samples. Samples heated to 70C and placed in the carbon dioxide-acetone bath were chilled to −30C in 10 min. At −30C all of the glycerides solid at 0C and some or most triglyceride liquid at 0C will be in the solid state. Samples chilled as low as −40C attained the temperature of the tempering bath in 15 min as measured by a thermocouple in the center of the sample. This provided conditions satisfactory for obtaining an excess of solids prior to tempering. The temperature attained in the sample during tempering appeared to offer conditions that would be close to equilibrium after 30 min of tempering.

Tests made to show the effect of 5-, 10-, and 15-min chilling in the −60C bath, followed by tempering at 10C for 20, 30, and 50 min and for 16 hr, are shown graphically in Figure 2 for a lard and a tallow. Previous tests on these two fats after chilling at 0C had indicated inadequate chilling. The results in Figure 2 indicate that 10 min of chilling in the −60C bath, followed by 30 min of tempering, would probably be satisfactory for routine testing and give results comparable with those at equilbrium (16 hr of tempering).

Measurement Tempered at 10 to 37.8C

In routine tests at a variety of temperatures the instrument will be maintained at a constant room temperature. If the samples are colder than room temperature, melting of the solids will occur with time. If the samples are warmer than room temperature, solids will form with time. Under these conditions it may be appropriate to deviate from the normal practice of taking the average of the first two sweep measurements. The operating procedure that will give the best results, based on tests made on 15 shortenings and five hydrogenated vegetable oils in which the samples were heated to 70C, chilled 10 min in the −60C bath, then measured after 30 min and 16 hr of tempering, are summarized in Table II. All measurements were based on the average of the first and second sweep except when indicated otherwise.

Under the conditions illustrated in Table II the precision of the method, as expressed by the standard

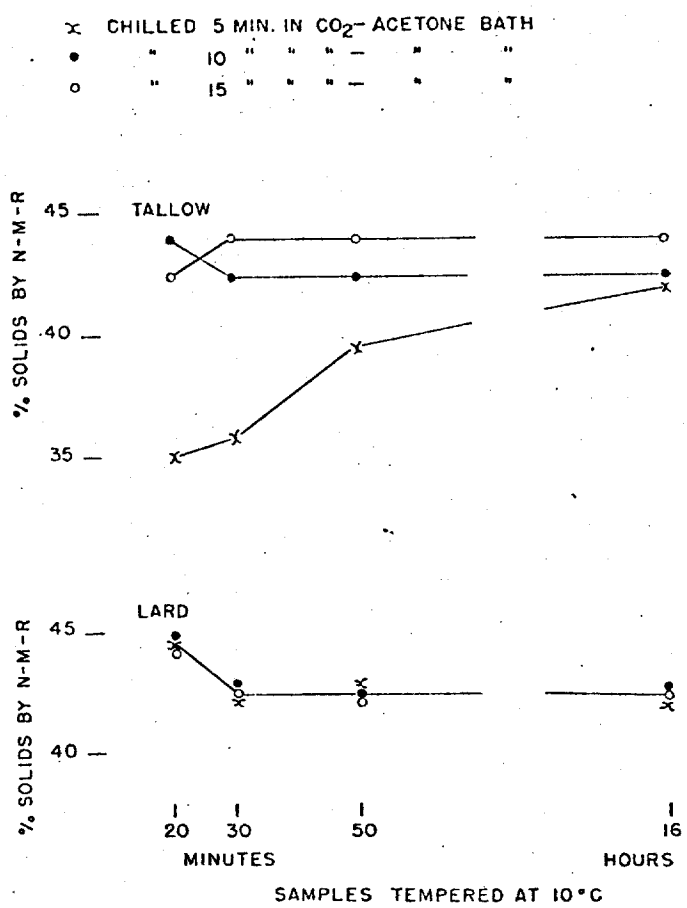

FIG. 2. Effect of chilling and tempering time on solids found when tempered at 10C.

TABLE II

Summary of Data for the Determination of Solids in 15 Shortenings and Five Hydrogenated Vegetable Oils after 30 Minutes and 16 Hours of Tempering

	Tempering Temperature, °C				
	10	21.1	26.7	33.3	37.8
Tempered 30 minutes	50.3[a]	32.4	23.7	11.2	10.2
Tempered 16 hours	50.4	31.1	22.6	11.2	9.2

[a] Average of the second and third sweep used for calculating the solids content.

deviation, is approximately ±1.0%, and the 95% confidence limits for a single value (average of two sweeps) is approximately ±2.0%.

The precision of the method and accuracy of the results obtained after 30 min of tempering are satisfactory for routine analysis, control analysis, and many research studies. Greater accuracy can be attained as required, by appropriate changes in chilling, tempering, and/or increasing the number of determinations on the same sample.

The conditions reported in this paper covering instrument calibration, chilling, tempering, and measurements have simplified the preparation of a calibration graph without sacrificing accuracy and reduced the time required for a test to a minimum while at the same time retaining accuracy. The testing parameters set forth should make it possible to obtain comparable results in different laboratories which would be only slightly more variable than those within a laboratory, for example, than those reported in this paper.

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[Received October 17, 1966]

L. Analysis of meal and solubles

1. Protein content

2. Oil content

3. Ash content

4. Moisture content

5. Pepsin digestibility

6. Special considerations for solubles and homogenized condensed fish

M. Analyses of fish oil

1. Sampling

2. Analytical methods

III. Production from miscellaneous whole fish

A. Herring

B. Trash fish

C. Alewives

D. Pacific hake

IV. Production from trimmings

A. Tuna

B. Mackerel, sardine, and anchovy

C. Fillet waste

D. Crab scrap

V. Use

A. Fish meal

1. Meal produced from fin fish

2. Meal produced from nonfin fish

B. Fish solubles

C. Fish oils

1. Drying oils
2. Lubricants
3. Animal feeds
4. Fatty acids and other chemical derivatives

VI. Statistics and economics

A. Fish meal

1. U.S. production
2. Imports
3. U.S. demand for fish meal
4. Prices
5. Competing feeds

B. Fish solubles

C. Fish oil

VII. Future possibilities

INTRODUCTION

The purpose of this report is to give a comprehensive picture of the fish meal and oil industry. This industry has been subject to marked change. Over the years, types of raw material used and ways in which processing has been carried out have altered greatly. Accordingly, before current operations and future possibilities are discussed, these developments will be traced in order to give a better understanding of the present status and what changes the future may bring.

I. HISTORICAL DEVELOPMENTS

The development of the industry will be considered in relation to five eras: pre-1850, 1850-1890, 1890-1920, 1920-1940, and 1940-1968.

A. Pre-1850 Era

Prior to 1850, the protein of fish was not used in the manufacture of any fishery industrial product. Whole fish were used to a small extent as a fertilizer, but they were never processed. In a few scattered instances, either raw or cooked fish had been fed to animals or poultry, but such use was more of a scientific curiosity than an established practice.

A whale-oil industry existed, and whale oil was in much demand for a variety of uses, the chief of which was as an illuminant for burning in lamps. A small cod-liver-oil industry also existed. Although knowledge of vitamins A and D was completely lacking, it was well known that cod-liver oil had medicinal value. Some cod-liver oil made from old livers also had modest sales as an industrial oil.

An early effort to make menhaden oil got underway at Portsmouth, Rhode Island, in 1811 (Deblois, 1882) when Christopher Barker and John Tallman cooked menhaden in iron pots, skimmed off the oil, and sold it through a firm in Newport, Rhode Island. Their plant was destroyed by gales in 1815 but was rebuilt a few years later. A larger installation was subsequently built in 1841. In addition, another menhaden-oil plant was built on the Merrimac River, Massachusetts, in 1842. All these early efforts toward the manufacture of menhaden oil were on a small, crude basis, and the product was not widely used.

B. 1850-1890 Era

During the years 1850-1890, the menhaden industry developed from a small, purely oil operation to a large industry producing both oil and fertilizer. A beginning was made also in the use of other species of fish for industrial products.

Because the rise of the menhaden industry was centered in Maine and because the earlier, very small operations farther south in Rhode Island and Massachusetts have largely been forgotten,the start of the industry is generally credited to a farmer's wife in Mount Desert, Maine. Noting oil on the surface of water in which she had cooked menhaden for feeding her chickene, she skimmed it off and later started a small commercial operation, selling the oil to brokers in Portland, Maine. In all the operations prior to the American Civil War, only oil was produced; the protein material was discarded. Immediately after the Civil War, in about 1865, several large operations centered in Maine began. Not only menhaden but also herring was processed. About this time, the protein material was manufactured for fertilizer. The industry soon spread southward to Connecticut, New York, and New Jersey. By 1890, a sizable operation was underway with an annual oil production of about 3 million gallons.

C. 1890-1920 Era

During the 30-year period from 1890 to 1920, the menhaden industry grew slowly while several other species of fish became minor sources of raw material for making fish fertilizer and oil. Among these sources, in addition to herring mentioned earlier, were waste from salmon canneries in Alaska and the Pacific Northwest and a small quantity of tuna trimmings. In 1910, the first extensive use of a solvent-extraction process was employed at Eliza Island near Bellingham, Washington. Salmon cannery waste given a preliminary drying in Alaska was shipped to the solvent-extraction plant for extraction of the oil. This operation was carried on for about 10 years. During this period, another new product, fish flour (today called fish or marine protein concentrate) for human consumption, was produced on a small scale in Alaska from salmon cannery trimmings.

Perhaps the greatest innovation was the use of dried fish meal for poultry feed instead of for fertilizer. This use got underway during World War I, although as late as 1920, considerable quantities of fish meal were still used as fertilizer. Though fish meal had been used for animal feed in Europe since the last quarter of the nineteenth century, it was not until about 1918 that any considerable quantity was used in this way in the United States.

D. 1920-1940 Era

Three important events occurred in the industrial fishery products field in the United States during the 2-decade period ending in 1940.

The trend toward using fish meal as poultry feed rather than as fertilizer was completed, so that, by 1930, practically none was used for anything other than animal feed. By this time, the menhaden industry had spread southward with main operations from New York to Texas.

With increased knowledge of the nutritional importance of vitamins A and D, the fish-liver-oil industry expanded greatly. No longer was the production of vitamin oil limited to the use of cod livers. Huge quantities of shark livers (principally dogfish and soupfin) were processed, primarily on the Pacific Coast. Livers of other species, especially halibut and tuna, also became important sources of vitamin oils.

The third important development was the rise of the pilchard or California sardine industry. These fish were taken off the Pacific Coast from British Columbia to Southern California. Starting from practically zero production in 1920, by 1929, the sardine industry had grown larger than the menhaden industry (in 1929, 420 million pounds of sardines were processed compared with 352 million pounds of menhaden). By 1937, the production of sardine exceeded 1 billion pounds, which was more than double the amount then produced by the menhaden industry.

Fish Oils, M.E. Stansby (Ed.), 1967

Appendix

PHYSICAL AND CHEMICAL CONSTANTS OF FISH OILS

Only scattered mention has been made of physical and chemical constants such as refractive index and saponification value for oil made from various species of fish. We know that these values vary over such wide ranges, even for oil made from the same species of fish, that most of the values scattered throughout the literature and usually reported for some small batch of oil have very little meaning. Some ranges of constants for four fish oils of North and South American origin were tabulated in Chapter 16, Table 20. These ranges of values, being based in part upon analyses of large batches of oil, have considerable significance.

In Table 64 are listed some ranges of values for refractive index, iodine

TABLE 64

CONSTANTS OF FISH OILS

Type of Oil	n_D^{65} (= 1.4··)		Iodine Value		Slip Point (°C)		Saponification Value	
	Limits	Mean	Limits	Mean	Limits	Mean	Limits	Mean
Whale	554–579	565	116–132	121	17–25	22	194–198	195
Herring	573–602	586	123–148	134	9–17	14	181–187	184
Menhaden	590–623	608	150–165	159	22–28	24	192–199	196
Pilchard	634–648	641	159–192	180	20–23	22	191–196	193
Cod-liver	615–621	618	159–166	162		<10	185–187	186
Cod-liver, non-destearinated		572		126		20		193
Peruvian fish		659		199		24		192
(another sample)		671		206				

value, slip point, and saponfication value, and these are based upon averages of many analyses of different batches of oils made at the research laboratory of one of the world's largest processors of fish oil. The values are averages of analyses made during the period of 1950–1953 except in the case of the Peruvian fish oil all samples of which were from 1963. The constants were run on crude filtered oil except that in cases where free fatty acid exceeded 5%, the oil was first neutralized. The values in Table 64 (and Table 65 as well) were tabulated by J. Hannewijk and were made available through the courtesy of Unilever Research Laboratory, Vlaardingen, Netherlands.

A relationship exists between iodine value and refractive index such that I.V. $= (n_D^{65} - 1.4429) \times 8560$.

Use of Table 65 makes possible the identification of a number of different fish oils provided their saponification value and refractive index are known.

TABLE 65

IDENTIFICATION OF FISH OILS BY THEIR SAPONIFICATION VALUE AND REFRACTIVE INDEX

Saponification Value	<190		> 190			
Refractive Index n_D^{60} (= 1.4[illegible])	<610	>610	550–580	590–630	630–650	>650
Type of oil	Herring	Cod-liver	Whale or non-destearinated cod liver	Menhaden	Pilchard	Peruvian fish

Buyers usually prefer the meal to have an oil content of about 10 percent. Less than 5 percent of oil is undesirable because the meal tends to be dusty. More than 10 percent of oil is undesirable because it increases the hazard of spontaneous combustion during storage and also gives a dark-colored meal.

3. Ash content.--(AOAC, 22.010, p. 328). The ash content is determined by carefully burning the meal sample. The content of ash is calculated from the difference between the weight of the original sample and the weight of the ash residue.

An abnormally high content of ash would probably adversely affect price because the buyer might fear that the meal had been adulterated with a low-cost mineral constituent. Authentic meals made from small, bony fish, however, may have more than 25 percent ash, though this high amount is unusual.

A simple analysis for ash is not completely informative as to the contribution the meal will make to the animals' nutritional requirements for minerals. More helpful would be analyses for phosphorus, calcium, and other elements needed for growth, such as magnesium, manganese, iron, copper, zinc, tin, and iodine. Again, these analyses are expensive and their exact benefits are difficult to calculate, so they normally are not made.

4. Moisture content.--(AOAC, 22.003, p. 327). The moisture content is determined by evaporating the water from the sample in a drying oven. The decrease in weight upon drying is taken as moisture.

Buyers prefer the moisture content of a meal to be in the range of from 6 to 10 percent. A moisture content of less than 6 percent is contrary to shipping regulations, presumably owing to the potential danger of spontaneous combustion. A moisture content as high as 12 percent places the meal in the danger zone of spoilage, usually by mold organisms. Strangely enough, high moisture may also increase danger of overheating, either through a catalytic effect on oil oxidation or from heat resulting from increased action of spoilage organisms, or both.

5. Pepsin digestibility.--(AOAC, 22.025-22.031). Sometimes the pepsin digestibility is also determined in addition to proximate composition. Recently it was found (Pepsin Digestibility as an Index of Quality in Fish Meal: Parts I and II, Fishing News International, vol. 3, nos. 3 and 4 1964) that using smaller concentrations of pepsin resulted in an improvement of the test as an index for protein quality of fish meals. Essentially the major difference from the AOAC method is that the concentration of pepsin is reduced from 0.2 to 0.002 percent.

6. Special consideration for solubles and homogenized condensed fish.--Solubles and homogenized condensed fish are sold on the basis of solids content. The analysis consists simply in evaporating off the moisture from the sample. The residue is considered to be nonvolatile solids.

Producers try to attain a solids content of 50 percent. If the content of solids rises appreciably above this level, the product may become so viscous as to be difficult to pump from the tank car, and if the content falls below, the consumer receives a product of lesser protein, vitamin, and mineral content, and accordingly demands a lower price.

A difficulty is sometimes encountered in manufacture in which a gel is formed before the level of 50- percent solids is reached. In these instances, solubles with a solids content of from 38 to 45 percent become so viscous that they are impossible to pump. Generally, the addition of acid to a pH of 3.5 to 4.0 is sufficient to break the gel and to allow further evaporation.

M. Analysis of fish oil

1. Sampling--Fish oil which is to be analyzed is commonly stored in tanks or barges in lots of as much as 50,000 to 750,000 gallons. In order to obtain a homogenious sample a special sampling device such as a Bacon bomb sample thief is employed. This device consists of a cylindrical body of about 12 inches in length and about 2 3/4 inches in diameter with two openings one at the bottom to admit oil as the equipment is lowered to the bottom of the tank. The other opening consists of an adjustable needle valve at the top. It controls admittance of air and hence rate of filling

with oil. Such equipment usually has a 16-ounce capacity. When lowered from the top to the bottom of a tank of oil it gives a sample representative of a cross section from top to bottom of the tank.

For tank lots of oil up to 10,000 gallons, a one-pint sample is considered to be representative. With increasing size of lots of oil, the sample size is increased. For a 200,000 gallon lot, for example, a 4-quart sample is considered adequate.

2. Analytical methods--Commercial fish oils are usually analyzed for free fatty acid content, moisture content, impurities and for color. Rarely saponifiable number and unsaponifiable content analyses are also included. For special uses of the oils other determinations may be made such as bleach color or hot bleach color. These might be run if the oil were to be used in manufacture of a paint where the bleach color test will predict the effect of the oil color on the final color of the paint.

Analytical methods found in the Official Methods of Analysis of the Association of Official Agricultural Chemists Tenth Edition 1965 are employed for analysis of fish oils. For methods not described in this book other procedures especially those described in Official and Tentative Methods of the American Oil Chemists Society (Mehlenbacher, Hopper, and Gallee 1965) may be used.

III. PRODUCTION FROM MISCELLANEOUS WHOLE FISH

Operations in which whole fish other than menhaden are rendered take place in several herring plants in Alaska, and several very minor operations occur on Puget Sound, Washington. The supply of herring in Alaska has shown a decided downward trend in the past few years. The result has been a marked decline in the production of herring meal and oil.

A second type of whole fish used for meal and oil are the so-called trash fish--species of bottomfish that are not considered marketable as human food. Fish of this kind are reduced primarily in the New England states, the Gulf of Mexico, and the State of Washington. These fish, a mixture of species, are taken by otter trawl. For the most part, the reduction operation uses batch-type dry-rendering equipment, although one plant in New England uses the Viobin azeotropic ethylene dichloride process (Levin and Finn, 1955).

Other whole fish presently used as a minor source of meal and oil include hake in the Pacific Northwest and alewife in Lake Michigan. These two developments, now at a very early stage, may ultimately become sizable operations. Several plants have been built in Wisconsin to render alewife from Lake Michigan. In 1966, a plant was built at Aberdeen, Washington, to process hake caught in the adjacent area of the Pacific Ocean.

A. Herring

Aside from menhaden, the principal species rendered into meal and oil from whole fish has been herring. This industry is centered in Alaska; until recently, each of three areas in Alaska had four plants. These plants were operated in Southeastern Alaska, Prince William Sound, and on Kodiak Island. Within very recent years, the availability of herring in Alaska has tapered off sharply, causing many plants to close until, in 1966, only two were in operation--one in Southeastern Alaska and one on Kodiak Island.

In the Alaskan herring fishery, the fish are taken mostly from June to October. The fishery is conducted by purse seines, and the fish, which are caught relatively close to the reduction plant, are transported without refrigeration. They are unloaded from the fishing vessels by means of pumps, and the fish are measured by the barrel, a barrel consisting of 250 pounds. The fish are held in tanks until ready for processing. The manufacturing operation follows the typical wet process, the herring being cooked with live steam and pressed through a continuous-screw-type press. The meal is dried in a flame dryer. No attempt is made to recover fish solubles as a separate product.

The meal is not cured prior to being bagged in burlap, and shipment from Alaska to Seattle is made by barge. The herring oil is shipped also by barges, which each carry two 70,000-gallon tanks. Herring meal is of uniformly high protein content, The oil, being of relatively low iodine value, does not oxidize rapidly in the meal. This stability eliminates the need for the addition of antioxidant or for special curing operations. The meal is of high nutritional value. The oil finds markets in the protective-coating field and in the manufacture of chemical intermediates.

B. Trash fish

Considerable quantities of whole fish are rendered into meal and oil. Much of these operations are carried out on a small scale in plants at widely separated parts of the United States. In Puget Sound, Washington, several plants reduce bottomfish into meal and oil. Mixed trash fish containing dogfish, ratfish, and numerous unwanted small species, taken by otter trawl, are processed by the dry method (cooking and drying is carried out in one step following by pressing to remove oil). At New Bedford, Massachusetts, a Viobin solvent-extraction plant produces meal from such trash fish.

E. Pressing

One type of press consists of a cast-steel screw, surrounded by a cylindrical screen, suitably reinforced at intervals along the area of pressure. The screw is machined to fit tightly against the screen, and either the pitch of the flights progressively decreases or the screw is tapered, resulting in compression and development of pressure on the solids as they progress toward the discharge end of the press. The size of the discharge throat may or may not be variable. The sections of the cylindrical screen that form a part of the press may have perforations of about $\frac{3}{64}$ - inch diameter at the inlet end and of about $\frac{1}{32}$ - inch diameter at the discharge end. The press liquor is screened or centrifuged to remove the fine solids and then is sent to centrifuges for the recovery of the oil.

A press that works on a different principle consists of two 4-foot-diameter press-wheels rotating on an angle-hub. The wheels rotate by means of ring and pinion gears, and the face of each wheel is made up of removable steel plates covered with a perforated screen. The hub is set at such an angle that the close point between the press wheels is slightly ahead of the bottom point toward the discharge side, thus giving a uniform, continuous pressing action. The pressed cake is discharged by means of a spade protruding between the plates and is transported to the driers. The liquors are disposed of in the same manner as with the screw-type press.

The solid pressed fish, called presscake, is transported to the driers for further treatment, which will be discussed in the drying section. The press liquor contains two components of value: oil and solubles.

F. Recovery of the oil

Centrifugal separators have been extensively used to replace settling tanks for recovery of the oil from the liquid portion called press liquor, resulting from the pressing operation. Several different companies have designed and manufactured centrifuges, but there are essentially only two types. One, a three-phase machine, called a sludger, is designed to handle liquor containing oil, water, and some suspended solids. The solids, and part of the water, are continuously discharged through the ports in the wall of the separator bowl. Usually the machine is adjusted so that all the oil and a small amount of water are discharged at the oil spout. This adjustment ensures that no oil will be lost but does produce a wet oil or emulsion. This emulsion then is fed, with the addition of a quantity of hot water, to the second type of centrifuge, the oil purifier, where the last traces of solids and water are removed. The sludger machine can be adjusted so that, with careful operation, an oil suitable for direct marketing may be obtained. However, the possibility always exists that oil may be lost into the water discharge in the separation process. Then, too, the processing capacity of the centrifuge is somewhat less if this technique is used. For these reasons, the two types of centrifuges are customarily used in conjunction.

If gravity settling is used, the liquor passes to a series of settling tanks. Use of heat, acids, bacteria, or enzymes may be necessary to break the emulsion of oil and water that normally comes from the presses.

C. Recovery of solubles

In the earlier days of the industry, the liquid portion, called stickwater, remaining after the oil was removed, was allowed to drain off into the tidal waters near the plant. This practice gave rise to foul odors. Pollution regulations in most areas now prevent stickwater from being discarded. Furthermore, as a result of research, the stickwater has been turned to profitable use. In one process, it is pumped into tanks, where acid is added in sufficient amount to stop bacterial and enzymatic action. Any precipitated solids and entrapped oil are removed by centrifugation, and the stickwater is dehydrated to a content of 50 percent solids.

In the menhaden industry, however, the stickwater is often first concentrated. Acid then is added to the finished solubles in an amount sufficient to lower the pH to about 4.5. This acidity acts primarily as a preservative. Both processes are illustrated in Figure 3.

The solubles are stored in large steel tanks and are shipped in tank cars, tank trucks, or in some areas, tank barges.

H. Drying

Drying is generally accomplished by means of one of three types of driers: direct-heat, steam-tube, or air-lift.

1. Direct-heat drier.-- In the direct-heat drier, the drier unit consists of a furnace of the Dutch-oven type as a source of heat, and the horizontal drier shell. For 10-ton-per-hour capacity, the shell is about 6 feet in diameter and 50 feet in length. The drier shell is mounted on trunions; a slow rotation of the shell is accomplished by reducing the speed of the driving motor through a jack-shaft arrangement. From the jack shaft, the power is applied through a gear to the bull ring attached to the shell. Wet meal is introduced by an overhead hopper or an under-feed screw conveyor. The inlet end of the drier shell is mounted to turn in a collar in the rear arch of the furnace. The products of combustion and the additional air drawn through the Dutch-oven heating area pass through the drier parallel to the path of the fish meal. The steel plates or lifters attached to the inside wall of the drier shell cause the meal to be carried up the sides and then to be spilled continuously into the path of the hot gases. A fan draws the spent gases and the suspended fine meal through a cyclone separator, where the meal is recovered and the gases are discharged. The major portion of the dried meal is discharged from the end of the drier and is then sent to the grinder.

2. Steam-tube drier.-- The steam-tube drier differs somewhat from the previously described direct-heat drier in that heat is applied through steam tubes mounted in circles concentric and adjacent to the inside drier wall. As the drier rotates, the meal falls through the spaces between the heated pipes, and a slight inclination of the drier toward the discharge end causes the meal to move in that direction. A fan and a cyclone separator may be used to collect fine particles of meal and to remove the moisture-laden air from the drier.

3. Air-lift drier.-- In the air-lift drier, wet presscake is introduced into the bottom of a funnel-shaped tower about 25 feet high and 7½ feet in diameter. At this point, the upward velocity of air is sufficient to entrain and bring all materials into suspension. As the material dries, it is carried through a blower to a cyclone, which separates the air and the dried product. A coal furnace, steam coils, or a combination gas- and -oil furnace can be used to supply the hot gases needed for drying; by means of a regulated volume of air, the gases can be made to enter the base of the tower at a temperature of from 250° to 300°F. Under these conditions, the meal contains sufficient moisture to prevent it from being scorched.

I. Deodorizing

The moisture-laden gases drawn off after the removal of the fine particles of meal contain undesirable odors. In many localities, the odor of the gases must be decreased before they may be discharged to the outside air. The condensable portion of these gases may be removed in scrubbers; the remaining noncondensable portion may then be burned.

J. Curing

The dried product called scrap is conveyed to storage sheds, where it accumulates in large piles. The residual oil in the scrap now becomes a factor in handling. In general, fish oils are characterized by a high degree of unsaturation--that is, the molecule of the fish-oil fatty acid may contain as many as six double bonds. These double bonds have the property of combining with the oxygen in the air, and this reaction releases heat. To retard the rate of oxidation of the residual oil and thereby lower the rate of heating, many operators add antioxidants, usually to the dried scrap but occasionally to the presscake before it is dried. Thus, depending on the rapidity of accumulation of the scrap, the amount of residual oil present, and whether or not an antioxidant was added, the piles of scrap are turned and aerated perhaps several days to cool. Otherwise, the scrap might undergo spontaneous combustion. The turning of the scrap pile, which presents a problem when large quantities of scrap accumulate rapidly, is accomplished by use of tractors and overhead conveyors. The scrap, after being turned and cooled, is left unground or is ground into meal and is sold either in bulk or in burlap or paper bags.

K. Preventing losses

Processing losses inherent in the wet-reduction method include suspended fine solids to the extent of from 1 to 5 percent of the weight of the raw fish and water-soluble protein material at the rate of from 15 to 20 percent of the protein contained in the raw fish. The condition of the raw fish, its freshness, and the method of operating the equipment are the factors that largely determine whether these losses will be high or low and that therefore must be controlled if the losses are to be minimized. Additional equipment such as rotary or vibrating screens, basket centrifuges, or foot presses may be used to increase the recovery of the solids suspended in the press liquor.

L. Analysis of meal and solubles

In the following subsections, analytical procedure for meal, solubles, and homogenized condensed fish are discussed. Each lot of meal usually is given what is called a proximate analysis for: (1) protein, (2) oil, (3) ash, and (4) moisture content. Ordinarily, the sum of the protein, oil, ash, and moisture contents of the meal will equal very nearly 100 percent, which fact gives a means of estimating the reliability of the combined analyses. The methods used are those found in the Official Methods of Analysis of the Association of Official Agricultural Chemists, Tenth Edition, 1965.

1. Protein content.-- (AOAC, 2.044, p. 16). The amount of total nitrogen is determined by the Kjeldahl method. A weighed sample of the meal is digested with sulfuric acid and a catalyst. This process converts the nitrogenous material to ammonium sulfate. The ammonium sulfate is treated with sodium hydroxide, liberating ammonia. The ammonia is distilled into a measured amount of standard acid. From the amount of acid neutralized, the amount of nitrogen in the original sample can be calculated, and this amount, when multiplied by the factor of 6.25, gives an acceptable approximation of the amount of protein present.

Menhaden meal is usually sold on a 60-percent-minimum-protein basis. It should be emphasized that this is an average figure and that individual lots of meal vary above and below this average by several percentage points.

2. Oil content.--(AOAC, 22.032, 22.033, p. 331). The oil content of the meal is determined by extracting a sample with an oil solvent--such as ethyl ether, petroleum ether, or acetone--which dissolves the oil from the meal. When the solution is filtered off and the solvent is evaporated the oil originally in the meal is left as a residue. From the weight of the original meal and the weight of the residue, the amount of oil in the meal can be calculated.

E. 1940-1968 Era

The period 1940-1968 saw the decline and practical disappearance of both the fish-liver-oil and the California pilchard industries. The former ceased to operate in the United States because of production of low-priced synthetic vitamin A and of importations of low-priced liver oil from Japan. The pilchard industry reached a maximum production of 1.15 billion pounds and thereafter declined to such a low level that none were available for reduction to meal and oil. Other species, such as mackerel, became a minor source of raw material in California; and with the rapid growth of the tuna industry, the production of tuna meal and oil increased. The production of herring meal and oil also became of some significance, especially in Alaska.

Meantime, the production of menhaden expanded considerably, making menhaden by far the major source of meal and oil in the United States. In very recent years, there has been a considerable decline in the volume of menhaden landed.

II. PRODUCTION FROM WHOLE MENHADEN

A. Introduction

Meal and oil is manufactured in the United States exclusively in shore plants to which the raw material, chiefly whole fish, usually menhaden, is brought. A very small part of the raw material is waste cuttings from filleted or canned fish and from shellfish, and production from these materials as well as from whole fish other than menhaden will be discussed in subsequent sections.

By far the most important raw material for meal and oil is menhaden, which is processed whole. The menhaden fishery over the past several years has accounted for almost 80 percent of all fish meal produced in the United States.

Menhaden meal, with other fish meals, is highly esteemed as a source of essential nutrients for use in commercial mixed feeds, primarily for poultry and swine. It contains protein with an excellent balance of amino acids and, many nutritionists believe, unidentified growth factors as well as valuable minerals, including calcium and phosphorus.

The menhaden fishery extends from the Middle Atlantic area southward and around into the Gulf of Mexico area--that is, from New York to Texas. Important locations for menhaden plants on the Atlantic Coast include several points on the New Jersey coast, the coast of Delaware, several points on bays in Virginia,and points in North Carolina. Important plant areas along the Gulf of Mexico shore include an area near Pascagoula, Mississippi, and near the Mississippi delta in Louisiana and nearby in Texas. Whereas in the early days of the menhaden fishery, the center of operations was in Northern New England (Maine), today the most northerly located plant is on Long Island, New York. Although sizable operations are carried out inthe Middle Atlantic area, the largest sustained operations occur in the Gulf area.

As was just indicated, menhaden are found from Southern New England down the Atlantic Coast to Florida, and along the Gulf Coast from Florida to Texas. Ichthyologically speaking, four species are now recognized along the coast of the United States--two in the Atlantic waters and two in the Gulf of Mexico--with Brevoortia tyrannus being the most common species in the Atlantic and the related Brevoortia patronus the most common species in the Gulf. The other two species, distinguished chiefly by their smaller and more randomly distributed scales, are relatively rare.

The Atlantic Coast species, Brevoortia tyrannus, has become less available within the last few years. This trend is apparent when one considers that in 1967, 61 percent of the menhaden meal produced was from the Gulf area. In 1960 the reverse was true, even though the tonnage produced from the Gulf area remained relatively constant.

Menhaden are usually captured by purse seines. To preserve the quality of the fish, the fishermen ordinarily catch them close to the processing plant and bring them to the plant during the same day of capture. A number of menhaden vessels, however, are equipped with refrigeration, which permits fishing at considerable distances from the plant with assurance that menhaden of acceptable quality will be landed.

After the menhaden are unloaded from the vessel, they are processed by the wet-reduction method[1/]. This method involves three main stages of operation: cooking, pressing, and drying. The fish are cooked with live steam under pressure; are pressed to expel most of the oil and some of the water; and, finally, the pressed fish are dried to remove most of the remaining water.

A more detailed description of the overall process (Figures 1, 2, and 3) and the equipment used is presented in the following subsections, which also includes a discussion of certain other steps including deodorizing, curing, the prevention of losses, and methods used in analyzing the products.

[1/] Another method is currently being investigated by the menhaden industry. Details of this process are not available. Interested readers should refer to United States Patent 2,651,647, Process of Dehydration of Fatty Materials: Charles Greenfield, River Edge, New Jersey, September 8, 1953.

Much of the information presented in this section was obtained from the following sources: Butler (1949), Lee (1953), Sanford and Lee (1960, 1961), Stansby (1963) and Stansby (1967).

B. Unloading

Unloading involves removing the menhaden from the hold of the vessel, measuring the quantity unloaded, and placing them in storage until they are needed for cooking, pressing, and drying.

Upon being brought to the dock, the menhaden are removed from the hold of the vessel. In most plants, this unloading is accomplished by partially flooding the hold with fresh water and removing the menhaden by centrifugal pumps of positive displacement. These pumps are large and are capable of emptying a hold of ½ milion fish in less than an hour. The pump water used to unload the fish is held either to reclaim the soluble protein at a later time or for disposal in such a way that it will not pollute the inshore waters.

The menhaden are separated from water by means of a large rotary sieve. From the dewatering sieve, they discharge to a "quarter box," which measures the quantity of menhaden by volume rather than by weight.

In some plants, the fish are drained of water, pumped directly onto huge conveyor belts, and passed over an automatic scale (or weighing conveyor) to determine the weight landed.

C. Holding

From the quarter box or automatic scale, the fish are either carried directly to the cooker or to temporary storage in a "raw box." These raw boxes may be large enough to hold up to several million menhaden and are designed so that the floors slant toward the middle of the box where a screw or flight conveyor can pick up the fish and transport them, as needed, to the cooker.

D. Cooking

Although cookers vary in size (16 to 30 inches in diameter) and in length (30 to 40 feet), for the purposes of this article, we shall describe a continuous steam-cooker screw press of 10-ton-per-hour capacity.

The cooker portion of a 10-ton-per-hour unit consists of a steel shell 24 inches in diameter and about 30 feet in length. The menhaden are admitted through a hopper at one end and are slowly moved through the cooker by a revolving screw. Steam is introduced through a series of jets in the cooker wall from two manifolds paralleling the cooker. As the screw pushes the mass toward the discharge end of the cooker, any oil and water freed in the cooking process is allowed to escape through a 4- to 6-foot section of screen on the bottom half of the cooker shell. At the discharge end, the cooked menhaden drop through a hopper to the press directly below. The conditions of cooking may be altered to suit the varying raw materials by the use of a suitable steam pressure in the cooker (usually 5 to 10 pounds) and by changing the speed of the screw. The cooking process should break down the oil cells and coagulate the protein, but overcooking or undercooking prevents the development of a satisfactory presscake and usually results in an excessively wet and oily cake accompanied by an excessive loss of fines in the press liquor.

CONTENTS

Introduction

I. Historical developments

A. Pre-1850 era

B. 1850-1890 era

C. 1890-1920 era

D. 1920-1940 era

E. 1940-1968 era

II. Production from whole menhaden

A. Introduction

B. Unloading

C. Holding

D. Cooking

E. Pressing

F. Recovery of the oil

G. Recovery of solubles

H. Drying

1. Direct-heat drier

2. Steam-tube drier

3. Air-lift drier

I. Deodorizing

J. Curing

K. Preventing losses

C. Alewives

A relatively recent operation is the use of alewives from Lake Michigan as a source of meal and oil. Two plants are currently operating on Lake Michigan--one at Milwaukee and one at Pensauke, Wisconsin. The plants employ conventional wet-rendering equipment, Although present operations are at a relatively low level (about 15 million pounds per year), they are expected to expand considerably.

D. Pacific hake

In 1966, the first Pacific hake reduction plant of the conventional wet-rendering type got into operation. An exceedingly large stock of hake exists off the coasts of Washington and Oregon. It is estimated to be large enough to support a fishery of several hundred million pounds per year. Several additional plants are in the planning stages. The supply picture has now become complicated, however, by the entry of the Russians into the fishery. They recently conducted an extensive dragging operation beyond the 12-mile limit.

IV. PRODUCTION FROM TRIMMINGS

Trimmings primarily from fish canneries, but in some cases from crab plants and filleting plants, are made into meal and oil. Thus the processing of fish fillet and crab wastes constitute another category of operations. Plants for rendering these materials are all relatively small and are widely scattered about the country in areas where fish is filleted in quantity or where crab is handled.

Menhaden meal and oil, which as we have seen accounts for nearly 80 percent, on a value basis, of the meal and oil produced in the United States, is in first place in the value of industrial products from fish. Of the remaining products, tuna and mackerel meal and oil made from cannery trimmings is in second place in value. In the production of tuna and mackerel meal and oil, the principal plants are located in or near Terminal Island and San Diego, California. Trimmings from the sardine (herring) canneries in Maine also serve as a source of raw material for making meal and oil.

A. Tuna

The most important operation from trimmings, in the United States, is the manufacture of meal, oil, and solubles from waste produced by tuna canneries, primarily in Southern California at Terminal Island and San Diego.

The portion of the fish used for manufacture into these tuna by-products consists of the carcasses of tuna after the light meat has been removed for canned human food and the red meat has been removed for canned pet food. The whole fish have already been pre-cooked so that, unlike the raw material in other meal-and-oil operations, the tuna scrap has already been partially cooked when it enters the byproducts plant. The waste material passes directly from the cannery plant to the byproducts plant, which is usually located in a separate building. When the distance between these facilities is not great, the scrap may be transported by conveyor belts; otherwise, it is transported by truck.

To facilitate the separation of oil in the subsequent pressing operation, some plants for processing tuna have used lime mixed with the scrap. At least one plant still follows this procedure. About one part of lime is added and mixed to each 100 parts of tuna scrap.

Rendering of tuna meal and oil follows conventional procedures using the usual direct steam cookers and screw presses. Most plants use flame driers for drying, but one large plant uses steam-tube driers. Condensed fish solubles are prepared by the usual multieffect vacuum evaporation of the acidified stickwater.

Crude tuna oil is ordinarily of a very dark, almost black, color. Simple alkali refining generally gives a satisfactory color with a Gardner number of about 6. Considerable amounts of tuna oil are heat bodied and then used for the production of alkyd resin.

Because most tuna reduction plants are located in heavily populated areas where not only odors but also smog is a problem, adequate treatment of stack gases is important. Several plants use chlorinated water in a countercurrent system. The water, containing about 20 parts per million chlorine, flows down the stack over firebrick, thereby meeting the rising flue gases. These gases emerge at the top without almost no odor remaining.

B. Mackerel, sardine, and anchovy

In California, the trimmings from several other species of fish that are canned are made into meal, oil, and solubles in a manner essentially the same as that used with tuna. For many years, the cannery waste from Pacific and jack mackerel and from California sardines (Lassen, 1963) have been used in this way; recently a similar operation has been based on waste from anchovy canning.

At one time, as we saw earlier, the California sardine or pilchard provided raw material exceeding a billion pounds per year for reduction purposes. Although this resource has all but disappeared, a very small industry still remains. The trimmings from pilchard canneries are generally rendered in a mixed operation in which the pilchard scrap is rendered together with trimmings from mackerel, tuna, or both.

The sardine (herring) rendering plants of Maine also use cannery trimmings as a source of raw material for meal and oil. The operations resemble closely those in California using tuna and mackerel cannery trimmings. Several small plants operate in such areas as Eastport and Portland. Most of the plants use wet-reduction equipment with either flame or steam-tube driers, although a few batch-type dry-rendering installations are also operated. One plant uses the Carver-Greenfield process in which the raw material is heated in fish oil under high vacuum.

C. Fillet waste

Few reduction plants exist in the United States for the sole purpose of rendering trimmings from filleting operations to meal and oil. In most instances, the quantity of raw material is either so small or is available so irregularly as not to be able to keep a plant operating continuously. Also, competition often exists for this type of raw material for other purposes, such as freezing it for animal feed. This competition further complicates the situation by decreasing the regularity of supply.

When fillet waste is processed into meal and oil, it is therefore usually handled in plants designed to render a wider variety of raw material. These plants may range from those accepting meat and poultry as well as fishery raw material to others that process only fishery material, which might range from cannery trimmings and fillet carcasses to whole trash fish. Plants using fillet trimmings are generally of the batch type involving a combined cooking and drying step. Such plants are more versatile in handling a wide variety of raw materials occurring in irregular supply. Some fillet waste, however, is rendered in multipurpose plants used also for widely diverse raw material, and these may be of the wet-rendering type. At least one such plant uses an air-lift type of drier.

D. Crab scrap

Crab waste is processed from crab operations, mostly on the Atlantic Coast and particularly in Virginia, Maryland, and New Jersey. The processing is in small batch-type dry-rendering equipment.

V. USE

A. Fish meal

1. Meal produced from fin fish.--As we have seen, fish meal was used primarily as a fertilizer prior to the 1920's before research results indicated its true value in animal rations. These results show that fish meal is (1) a concentrated source of protein containing a good distribution of the essential amino acids as well as of many non-essential amino acids; (2) a good source of such vitamins of the B complex as niacin, riboflavin, thiamine, pantothenic acid, choline, and vitamin B_{12}; (3) a good source of certain unidentified growth factors; and (4) a particularly valuable source of trace elements and of available phosphorus, which are essential feed ingredients that are relatively expensive if bought separately.

These properties have produced a demand for fish meal in rations for poultry and swine, the use in poultry feed being by far the most important in terms of sales volume. Fish meal is valuable as a supplement for the vegetable proteins, such as those found in soybean meal, which are deficient in some of the essential amino acids and also are lacking in the unidentified growth factors found in fish. Relatively little fish meal is used for feeding cattle because ruminants can effectively and economically utilize simpler forms of nitrogen--for example, urea--owing to the ability of their microbial flora to synthesize essential amino acids from these simple feed ingredients.

Fish meal is incorporated into the various animal diets at different percentages primarily depending upon the type of productivity required of the species. For instance chicken broiler diets may contain from 0 to 5 percent while breeder and layer diets vary from 0 to 3 percent. Turkey grower diets contain as much as 7.5 percent with a maximum of 5 percent being used in the breeder diets. Swine starter diets may contain as much as 5 percent fish meal. However little if any fish meal is used in growing-finishing diets except under special circumstances.

The practice of incorporation of fish meal into pet foods increased markedly with levels of 2.5 to 10.0 percent being used. Fish meal is also used in fish hatcheries.

2. Meal produced from nonfin fish.--Small amounts of meal are also produced from sources other than fin-fish such as crabs, shrimp, and oysters. Crab scrap meal has a relatively low protein content, about one-half that of regular fish meal. It is used primarily in poultry rations. Shrimp and oyster meals are also used in poultry rations. Interestingly enough, pink shrimp meal has been reported to be valuable for feeding hatchery-reared salmonoids, since this meal imparts a desirable pink color to the flesh of these species.

B. Fish solubles

The solubles, being liquid, require special handling for incorporation into feeds. Ordinarily, the equipment for incorporating molasses is suitable. Some of the smaller feed dealers, however, do not have these facilities. Accordingly, dry solubles would be a more desirable product for these users. The difficulty in the manufacture of dry solubles as a separate product, however, is that it tends to be hygroscopic; that is, it may absorb moisture from the air and become sticky and thus present problems in packaging, handling, storage, and incorporation into rations. Condensed solubles often are added back to the partially dried presscake and re-dried to make "full" fish meal, a process that was developed abroad earlier and that has come into increased application following the sharp break in the market price of solubles in 1959.

Condensed solubles may also be mixed with a carrier of vegetable origin--such as wheat middlings, soya bean oil meal, or cottonseed meal--and dried. In these products the solubles supply certain nutritive factors in which the carrier is deficient, and the mixtures are much less hygroscopic than are straight dry solubles.

The use of fish solubles in animal feeds is confined primarily to chick and turkey growing and breeding diets ranging from 0. to 3.5 percent of the diet. Usage is based principally on the unidentified growth factor or hatchability factor found in a greater concentration in solubles.

C. Fish oils[2/]

Outside the United States, about 75 percent of the fish oil produced is used for edible purposes. Within the United States, where Federal Food and Drug regulations forbid commercial fish oils made by the usual processes to be used in edible margarines, shortenings, and other foods, such fish oil is used only for nonedible purposes. However, a substantial part (over half) of the fish oil produced in the United States is exported to European countries, where it is hydrogenated for edible use.

The menhaden oil (representing about 90 percent of U.S.production) that is consumed domestically finds use in drying oils, lubricants, animal feeds, fatty acid products, and a wide variety of other products. In 1963 (the last year for which complete statistics are available), of the 104 million pounds of menhaden oil consumed domestically, 27 percent was used in lubricants, 23 percent in drying oils, 22 percent in animal feeds, 12 percent in fatty acid products, and 16 percent in all other products. The use in drying oils in 1963 was abnormally low, the usual proportion being close to one-half the domestic use; ordinarily this use is the principal one for fish oils in the United States.

2/ Much of the material for this section is from Chapters 16 (Fineberg and Johnson, 1951) and 19 (Dyer, 1967) of the book, "Fish Oils" by M. E. Stansby (1967).

1. Drying oils.--When fish oils are used for their drying properties, they find use in protective coatings--paints, varnishes, resins, and lacquers--and for caulks, sealants, inks, and putty. Fish oils are in demand for such uses both because of their relatively low price and for the quality of the resulting product. When fish oils are used in protective-coating formulation, their relative high content of saturates represents a disadvantage, which often necessitates a preliminary separation by some process, such as winterizing.

An increasing use for fish oils in the protective-coating field has been for the production of alkyd resin for use in paints and varnishes. Menhaden-oil alkyds are being prepared for use in flat, glossy, and semi-glossy paints, in aluminum paints, enamels, house paints, zone-marking paints, rust-inhibitive primers, and in many other products. These alkyds are formed first by reacting the oil with glycerine to produce a monoglyceride and then by reacting the latter with phthalic anhydride to form an oil-modified alkyd resin. Maleated menhaden oil and fish oil copolymerized with cyclopentadiene are also used as varnish components.

Heat-bodied (polymerized) fish oils, when used in paints, show certain advantages for some applications, including improved alkali resistance and increased gloss. Air-blown fish oils used to some extent in protective coatings add properties such as improved compatibility with some pigments but cause some loss in the resistance of the paint to water.

2. Lubricants.--Hydrogenated menhaden and herring oils, or more frequently the fatty acids made from them, form the basis for making metal soaps (for example, sodium, potassium, and recently also lithium and barium). The relatively high proportion (up to 45 percent) of C_{20} and C_{22} fatty acids present in these fish-oil soaps imparts better solubility of the soaps in mineral oil when the lubricants are formulated. Lithium hydrogenated fish-oil fatty-acid soaps make greases of improved water resistance, greater chemical stability, and high dropping point. Aluminum soaps from herring oil impart to greases improved setting properties at high temperature and also result in a nongrainy texture.

3. Animal feeds.--Fish oils contain fatty acids that, although not classified as being essential, nevertheless stimulate growth. The extent to which they can be included in animal feeds depends upon two circumstances. First, the price of fish oils, in relation to that of competing oils and fats (especially tallow), may place an economic brake upon their wide use in feeds. For example, the past high price of fish oils discouraged their wider use for this purpose. Second, for some uses, the possibility of their imparting a fishy flavor to the resulting food product limits the quantity that can be used in a given diet. For example, when fish oils are fed to chickens, a maximum proportion of 1 percent is considered optimum; otherwise, a fishy flavor may be imparted to the meat.

4. Fatty acids and other chemical derivatives.--Fatty acids prepared from fish oils are sometimes sold and used directly as the acids. For example, they are sometimes used both as ore-flotation collectors in the concentration of ores and as a sizer for hardboard to prevent the absorption of water. More frequently, however, fish-oil fatty acids, especially after being hydrogenated to give saturated ones, are used as chemical intermediates. These, in turn, are converted into such end products as esters, alcohols, and halogen or nitrogen derivatives. Of these, the esters and nitrogen derivatives are currently the most important. Methyl, ethyl, and butyl esters are prepared and used in such applications as alloy metal working, die-casting, plastic manufacture, and textile spinning. Among nitrogen derivatives prepared from fish-oil fatty acids are amides, nitriles, and quaternary ammonium compounds. These derivatives are used in such products as ore-flotation agents, corrosion inhibitors, fungicides, paper coatings, and fuel additives.

VI. STATISTICS AND ECONOMICS

A. Fish meal

This section deals with U.S. production, imports, U.S. demand, prices, and the effect of competing feeds on the sale of fish meal.

1. United States production.--The production of fish meal during 1967 is estimated to be 185.4 thousand short tons. This represents a decrease of 12 percent from the 1966 production and a 24 percent decrease from the 1962-66 average (Tables 1 and 2). Lower menhaden landings (Table 3) were the primary cause of this significant decline in domestic production. Only 119.1 thousand short tons of fish meal were produced from menhaden in 1967 (Table 3). This is a 33 percent decrease from the 1962-66 average. Menhaden landings doubled from 1950-56, but since 1956 have fluctuated widely. Increases in landings were the result of increased fishing effort, intensity, and efficiency due to increased use of larger vessels, airplane spotters, power blocks, and suction pumps. On the other hand, the fishery has suffered from a series of poor year classes since 1958.

Landings along the Gulf Coast have increased, while the Atlantic Coast landings have decreased. Since 1960, the percentage contribution of the two fisheries has been reversed. In 1960 the Atlantic catch was 58 percent of the total menhaden landings. The Gulf Coast claimed 62 percent of the landings in 1965, remaining steady with 61 percent being produced in the Gulf Coast area in 1967. Note, however, that the production of menhaden meal in both the Atlantic and Gulf Coasts has decreased over the past several years, with a 27 percent decrease in 1967 on the Gulf Coast and a 41 percent decrease in 1967 on the Atlantic Coast below the 1962-66 average (Table 4.).

Even with this decrease in the production of menhaden meal, it is still the largest source of fish meal in the United States, representing 64 percent of the total production. The remaining 36 percent was comprised of tuna, mackerel, anchovies, herring, ground fish, and alewives (Table 2).

2. Imports.--Fish meal imports during 1967 totaled 651.5 thousand short tons--up 45 percent from 1966 and up 82 percent from the 1962-66 average.

Peru is the major source of imported fish meal. In 1960 Peru supplied 52 percent of the United States fish meal imports, in 1964, 79 percent but has dropped back to 68 percent in 1967. Norway supplied 17 percent (113.2 thousand short tons) of the fish meal imports to the United States in 1967, an increase of 12 percent from 1966. Chile conversely supplied 20 percent of the imports in 1966, but only 6 percent in 1967 (Table 5).

3. U.S. demand for fish meal.--The U.S. demand for fish meal has been increasing for a number of years, keeping pace with the rapid development of the poultry and swine industries. Broilers use most of the fish meal, consuming about 70-80 percent of all the fish meal available in the United States. This usage in broiler diets is closely related to the price of fish meal and also to the decrease in the ratio between average fish meal price and the average price of major competing feed components, such as soybean meal. Hence, any decrease in the price of fish meal usually results in increased usage in broiler diets.

4. Prices.-- The price of menhaden meal in the United States was $131.60 per short ton F.O.B. at New York City in December 1967. The last previous time menhaden meal had sold at this price was in September 1964 (Table 6). Peruvian meal also is lower than in past years. The average price in December 1967 was $121 per short tone F.O.B. East Coast ports--the lowest price since September 1963. Low prices for both menhaden and Peruvian anchovetta meals are the result of large stocks of fish meal being held in foreign countries, with Peru and Norway the major holders. In addition, fish meal production rates have continued to increase and have added to the problem of oversupply.

5. Competing feeds.--Linear programming is becoming increasingly important in least-cost broiler ration formulation. Most broiler producers are using this tool to formulate least-cost broiler ration which will fulfill the required levels of several amino acids and the required energy level. These amino acids and energy requirements are usually met by a combination of fish meal and corn, or a combination of soybean meal, meat and bone meal, and fat, with the addition of synthetic methionine. The extent to which fish meal is used in broiler rations, therefore, is determined by the competitive price level of the fish meal/corn combination as compared with the soybean meal/meat and bone meal/fat/synthetic methionine combination. During 1967, the relationship between these price levels was favorable for nearly maximum usage of fish meal in broiler rations.

For example, during 1965, least-cost broiler ration formulation for the Delmarva area was conducted under contract for the Bureau of Commercial Fisheries using June 1, 1965 and December 1, 1965 wholesale price levels of the important feed ingredients. The price of one feed ingredient at a time was varied to obtain a price map of the utilization of fish meal. This indicated fish meal use as a proportion of the ration for a range of fish meal prices when all other feed ingredient prices were held constant at the June 1 and December 1 levels.

The use of fish meal in the linear programmed broiler rations decreased rapidly as fish meal prices increased beyond $150 per ton, using June 1 wholesale price levels. With December 1 wholesale price levels of broiler ration ingredients, the use of fish meal began to decrease as fish meal prices increased beyond $140 per ton. The combined effect decreases in the price of corn meal, fat, and soybean meal and an increase in the price of meat and bone meal were contributing factors. At both the June 1 and December 1 price levels, very little fish meal would be used in broiler rations with fish meal prices greater than $165 per ton. Actually, early December fish meal pricers were above $180 per ton.

B. Fish solubles

Production of fish solubles in the United States in 1967 totaled an estimated 73.7 thousand short tons (Table 7). This represents a 12 percent decrease from the 83.4 thousand short tons produced in 1966 and a 24 percent decrease from the average amount produced from 1962-66.

Over past years, the ratio of soluble production to meal production has increased as health authorities and local officials have become more restrictive in the disposal of stickwater. Moreover, the stickwater was found to contain large quantities of dissolved protein, vitamins, and other essential nutrients, and was an important source of revenue when sold in the wet form. Normally, fish solubles contain twice as much water and half as much protein as fish meal and therefore should sell at about half the price of fish meal. Since fish meal prices have averaged more than double solubles prices for the past few years, it has become more profitable to remove the water content of fish solubles to increase the production of fish meal.

Prices of fish solubles dropped in 1967 from $80.85 per short ton in January to $60 per ton in November and December (Table 8).

C. Fish oil

Domestic fish oil production in 1967 was 119.6 million pounds. This represents a 27 percent decrease from the 164 million pounds produced in 1966 and a 39 percent decrease from the 1962-66 average (Table 9). Menhaden oil in 1967 totaled 98.4 million pounds (82 percent of the total oil production--Table 10). Other fish yielding oil in 1967 were tuna, mackerel, and anchovies, which together accounted for 5 percent of the total production, and herring which accounted for 4 percent of the total production. Following the trend of fish meal, menhaden oil is priced lower than it has been for the last 4½ years. In 1967 menhaden oil dropped from 8.9¢ per pound in January to 6¢ per pound in December (Table 11). These prices are quotations from New York City for crude oil in tank cars. Fish oil consumption in the United States has decreased 8 percent from 1966. The United States exported 76.8 million pounds of fish oil in 1967 compared to an average of 146 million pounds from 1962-66. In 1966, 32 percent of the United States fish oil exports went to the Netherlands and 78 percent to the United Kingdom (Table 12). In 1967 the percentage of United States exports to the Netherlands increased to 48 percent, while the percentage to the United Kingdom dropped to 9 percent. Twenty-seven percent of the United States oil exports went to Sweden and 10 percent to West Germany. In 1967, no oil was shipped to Norway, while 15 percent was exported in 1966.

Table 1.--U. S. production of fish meal by month, 1966, 1967, and 1962-66 average[1]

Month	1962-66 average		1966		1967[2]	
	Thousand short tons	Percent	Thousand short tons	Percent	Thousand short tons	Percent
January	2.7	1.1	2.8	1.3	6.5	3.5
February	2.8	1.1	2.3	1.1	3.8	2.0
March	3.9	1.6	5.7	2.7	4.5	2.4
April	9.6	3.9	11.0	5.2	9.0	4.9
May	31.6	13.0	19.8	9.4	15.5	8.4
June	46.1	18.9	36.8	17.4	30.6	16.5
July	45.2	18.5	36.5	17.3	33.2	17.9
August	38.2	15.7	31.3	14.8	31.0	16.7
September	24.7	10.1	23.2	11.0	17.2	9.3
October	17.2	7.1	10.9	5.2	11.0	5.9
November	13.1	5.4	18.9	8.9	14.0	7.6
December	8.5	3.5	12.0	5.7	9.1	4.9
Total[3]	243.7	100.0	211.3	100.0	185.4	100.0

1/ Excludes meal made from shellfish.

2/ Preliminary.

3/ Figures may not add to total because of rounding.

Table 2.--U. S. production of fish meal by raw material source, 1966, 1967, and 1962-66 average

Source	1962-66 average		1966		1967[1]	
	Thousand short tons	Percent	Thousand short tons	Percent	Thousand short tons	Percent
Alewives	1.2	0.5	3.7	1.8	4.5	2.4
Groundfish	3.4	1.4	6.3	3.0	7.9	4.3
Herring	9.3	3.8	11.8	5.6	9.3	5.0
Menhaden[2]	179.0	73.5	135.0	63.8	119.1	64.2
Tuna, mackerel, and anchovies	25.1	10.3	29.8	14.1	32.4	17.5
Unclassified	25.7	10.5	24.7	11.7	12.2	6.6
Total	243.7	100.0	211.3	100.0	185.4	100.0

1/ Preliminary.

2/ Includes a small quantity of other species.

Table 3.--Menhaden landings by area, 1966, 1967, and 1962-66 average

Area	1962-66 average		1966		1967 1/	
	Million pounds	Percent	Million pounds	Percent	Million pounds	Percent
New England	4.1	0.2	-	-	-	-
Middle Atlantic	265.3	15.7	17.0	1.3	38.3	3.4
Chesapeake	283.2	16.7	252.4	19.8	203.0	17.9
South Atlantic	193.3	11.4	214.1	16.8	193.5	17.0
Total Atlantic	745.9	44.0	483.5	37.9	434.7	38.3
Gulf Coast	948.6	56.0	792.1	62.1	700.8	61.7
Total U.S.	1,694.5	100.0	1,275.6	100.0	1,135.5	100.0

1/ Prelimary.

Table 4.--Production of menhaden meal by area and month, 1966, 1967, and 1962-66 average[1]

Month	1962-1966 average		1966		1967[2]	
	Thousand short tons	Percent	Thousand short tons	Percent	Thousand short tons	Percent
			Atlantic Coast			
January	0.2	0.2	0.5	0.9	0.4	0.8
February	-	-	-	-	-	-
March	-	-	-	-	-	-
April	0.7	0.9	0.2	0.4	0.6	1.3
May	8.3	10.4	1.1	2.1	1.9	4.0
June	14.6	18.2	4.6	8.7	6.6	14.1
July	12.6	15.7	6.0	11.3	6.0	12.8
August	11.7	14.6	7.2	13.6	6.8	14.5
September	8.5	10.6	5.8	10.9	3.6	7.7
October	9.3	11.6	5.7	10.8	4.4	9.4
November	9.2	11.5	14.8	27.9	10.4	22.2
December	5.0	6.3	7.1	13.4	6.2	13.2
Total[3]	80.0	100.0	53.0	100.0	46.9	100.0
			Gulf Coast			
January	-	-	-	-	-	-
February	-	-	-	-	-	-
March	-	-	0.1	0.1	-	-
April	3.9	4.0	3.7	4.5	3.7	5.1
May	16.2	16.4	12.9	15.8	8.4	11.7
June	23.5	23.8	22.5	27.5	16.7	23.2
July	22.9	23.1	18.9	23.1	18.6	25.8
August	18.0	18.2	12.4	15.1	16.4	22.7
September	10.9	11.0	11.4	13.9	7.5	10.4
October	3.3	3.3	-	-	0.8	1.1
November	0.2	0.2	-	-	-	-
December	-	-	-	-	-	-
Total[3]	99.0	100.0	81.9	100.0	72.2	100.0
			Total[3]			
January	0.2	0.1	0.5	0.4	0.4	0.3
February	-	-	-	-	-	-
March	-	-	0.1	0.1	-	-
April	4.6	2.6	3.8	2.8	4.3	3.6
May	24.4	13.6	14.0	10.4	10.3	8.7
June	38.1	21.3	27.1	20.1	23.3	19.6
July	35.6	19.9	24.9	18.4	24.6	20.6
August	29.8	16.7	19.7	14.6	23.2	19.5
September	19.4	10.8	17.2	12.8	11.1	9.3
October	12.6	7.0	5.7	4.2	5.3	4.5
November	9.3	5.2	14.8	11.0	10.4	8.7
December	5.0	2.8	7.1	5.2	6.2	5.2
Total[3]	179.0	100.0	135.0	100.0	119.1	100.0

1/ Includes a small amount of meal from other species.
2/ Preliminary.

Table 5.--U. S. imports of fish meal by country of origin, 1966, 1967, and 1962-66 average

Country of origin	1962-66 average		1966		1967	
	Thousand short tons	Percent	Thousand short tons	Percent	Thousand short tons	Percent
Canada	47.2	13.2	43.6	9.7	46.6	7.2
Chile	28.2	7.9	89.4	20.0	40.9	6.3
Norway	4.6	1.3	21.0	4.7	113.2	17.4
Peru	261.2	73.1	276.6	61.8	442.5	67.9
South and South West Africa	10.5	3.0	6.6	1.5	-	-
Other	5.5	1.5	10.5	2.3	8.1	1.2
Total 1/	357.2	100.0	447.8	100.0	651.5	100.0

1/ Figures may not add to total because of rounding.

Table 6.--Average monthly price of menhaden meal[1] f.o.b. east coast ports, 1962-67

Month	1962	1963	1964	1965	1966	1967
	Dollars per short ton					
January	120.40	131.35	132.00	144.15	[2] 179.80	153.10
February	122.00	129.00	130.00	146.25	[2] 172.50	[2] 154.00
March	123.00	126.15	128.25	150.00	[2] 170.60	[2] 154.00
April	124.00	126.50	128.00	153.00	[2] 163.00	135.00
May	125.25	125.00	128.25	156.00	[2] 153.80	135.80
June	123.00	125.00	129.80	160.90	160.35	139.00
July	122.70	125.00	130.90	170.25	164.00	137.40
August	123.25	125.00	131.00	172.10	169.00	137.10
September	123.75	125.00	132.90	175.00	166.20	136.75
October	126.80	127.70	140.20	185.40	160.00	136.00
November	128.25	129.25	140.75	186.00	151.55	[2] 132.00
December	130.50	133.90	140.75	186.00	153.10	[2] 131.60
Average[3]	124.40	127.40	132.75	165.40	163.65	140.15

1/ 60 percent protein, bagged, as quoted at New York City.

2/ Nominal price as presented in the Oil, Paint and Drug Reporter.

3/ Simple average.

Sources: U. S. Department of Interior, Bureau of Commercial Fisheries, and the Oil, Paint and Drug Reporter.

Table 7.--U. S. production of fish solubles by month, 1966, 1967, and 1962-66 average

Month	1962-66 average		1966		1967 1/	
	Thousand short tons	Percent	Thousand short tons	Percent	Thousand short tons	Percent
January	1.5	1.5	1.4	1.7	2.6	3.5
February	1.4	1.4	1.8	2.1	1.0	1.4
March	1.9	2.0	3.6	4.3	1.8	2.4
April	3.7	3.8	3.6	4.3	4.0	5.4
May	12.4	12.8	8.2	9.8	6.1	8.3
June	17.5	18.0	15.1	18.1	13.4	18.2
July	17.3	17.8	13.9	16.7	12.4	16.8
August	16.0	16.5	12.7	15.2	12.1	16.4
September	10.5	10.8	9.8	11.8	6.6	9.0
October	7.6	7.8	5.3	6.4	5.0	6.8
November	4.6	4.7	5.6	6.7	5.3	7.2
December	2.6	2.7	2.4	2.9	3.4	4.6
Total 2/	97.0	100.0	83.4	100.0	73.7	100.0

1/ Preliminary.

2/ Figures may not add to total because of rounding.

Table 8.--Average monthly price of menhaden solubles[1] f.o.b. east coast plants, 1962-67

Month	1962	1963	1964	1965	1966	1967
	Dollars per short ton					
January	58.00	65.00	75.00	58.60	66.25	80.85
February	58.75	66.25	75.00	59.40	66.50	79.00
March	59.60	62.50	62.25	61.25	66.70	70.75
April	59.70	61.25	52.25	56.25	68.75	64.60
May	56.25	61.25	62.25	53.75	68.75	64.00
June	53.10	61.25	62.25	55.75	68.75	64.40
July	53.25	61.25	62.25	56.85	68.75	64.05
August	54.05	61.75	61.95	60.00	68.75	62.85
September	55.40	64.10	61.20	61.25	72.50	61.25
October	58.30	66.55	61.05	63.95	78.75	60.75
November	64.95	75.00	61.25	65.25	79.25	60.00
December	64.15	75.65	61.25	66.25	81.90	60.00
Average[2]	57.95	65.15	64.00	59.90	71.30	66.05

1/ In tank cars; prices quoted at New York City.

2/ Simple average.

Table 9.--U. S. production of fish oil by month, 1966, 1967, and 1962-66 average

Month	1962-66 average		1966		1967 1/	
	Million pounds	Percent	Million pounds	Percent	Million pounds	Percent
January	0.6	0.3	0.4	0.2	2.1	1.8
February	0.3	0.2	0.2	0.1	0.6	0.5
March	0.6	0.3	1.0	0.6	0.8	0.7
April	6.5	3.3	5.4	3.3	3.5	2.9
May	27.8	14.2	19.2	11.7	9.1	7.6
June	40.4	20.6	34.6	21.1	20.1	16.8
July	36.3	18.5	29.6	18.0	21.5	18.0
August	31.3	16.0	22.4	13.6	22.1	18.4
September	22.1	11.3	20.4	12.4	13.0	10.9
October	15.4	7.8	7.7	4.7	9.1	7.6
November	10.2	5.2	16.3	10.0	11.9	9.9
December	4.5	2.3	6.8	4.1	5.9	4.9
Total 2/	196.0	100.0	164.0	100.0	119.6	100.0

1/ Preliminary.

2/ Figures may not add to total because of rounding.

Table 10.--U. S. production of fish oil by raw material source, 1966, 1967, and 1962-66 average

Source	1962-66 average		1966		1967 1/	
	Million pounds	Percent	Million pounds	Percent	Million pounds	Percent
Alewives	0.7	0.4	2/	2/	1.8	1.5
Groundfish	2.1	1.1	0.5	0.3	1.3	1.0
Herring	7.6	3.9	7.9	4.8	5.1	4.3
Menhaden 3/	176.5	90.0	144.2	87.9	98.4	82.3
Tuna, mackerel, and anchovies	4.9	2.5	4.9	3.0	6.2	5.2
Unclassified	4.2	2.1	6.5	4.0	6.8	5.7
Total	196.0	100.0	164.0	100.0	119.6	100.0

1/ Preliminary.

2/ Included in "unclassified."

3/ Includes a small quantity of other species.

Table 11.--Average monthly price of menhaden oil[1] f.o.b. east coast plants, 1962-67

Month	1962	1963	1964	1965	1966	1967
	Cents per pound					
January	4.75	4.70	8.40	9.15	9.50	8.90
February	4.75	5.40	8.35	9.50	9.70	8.90
March	4.75	5.50	8.50	9.50	9.75	8.60
April	4.75	5.75	8.75	9.50	9.75	7.20
May	4.70	5.60	8.50	9.45	9.75	6.90
June	4.40	6.50	8.65	9.15	9.60	6.95
July	4.20	6.75	8.75	8.95	9.40	6.70
August	4.25	7.10	8.80	9.30	9.00	6.00
September	4.05	7.30	9.05	9.50	9.45	5.80
October	3.95	7.60	9.20	9.50	9.45	6.00
November	4.00	7.75	9.40	9.50	9.20	6.00
December	4.00	8.00	9.40	9.50	8.90	6.00
Average[2]	4.40	6.50	8.80	9.35	9.45	7.00

1/ Crude oil in tank cars; prices quoted at New York City.

2/ Simple average.

Table 12.--U. S. exports of fish oil by country of destination, 1966, 1967, and 1962-66 average

Country	1962-66 average		1966		1967 1/	
	Million pounds	Percent	Million pounds	Percent	Million pounds	Percent
Canada	4.4	3.0	1.4	1.8	0.3	0.4
Netherlands	26.8	18.7	24.5	31.7	37.1	48.3
Norway	12.2	8.5	11.4	14.7	-	-
Sweden	41.9	29.2	6.6	8.5	20.4	26.6
United Kingdom	29.7	20.7	14.3	18.5	6.6	8.6
West Germany	20.8	14.5	9.4	12.2	8.1	10.5
Other	7.8	5.4	9.7	12.5	4.3	5.6
Total 2/	143.6	100.0	77.3	100.0	76.8	100.0

1/ Preliminary.

2/ Figures may not add to total because of rounding.

VII. FUTURE POSSIBILITIES

As was seen in Section I, the fish meal and oil industry in its 100-year-old history in the United States has been far from static. As will be recalled, after starting out as an exclusively menhaden-rendering business, the industry diversified during the first third of this century until menhaden represented much less than half of the total meal and oil production. During the next third century, the importance of menhaden increased, however, primarily as a result of the decline in abundance of sardine.

Today, with the menhaden resource indicating at best an unchanging volume and with other presently utilized raw materials representing a tiny fraction of the whole, there is a movement to find new sources of raw material. The first large-scale venture in this direction is occurring on the Pacific Coast, where one new plant to reduce Pacific hake has recently started operation and where others are in the planning stage. Although hake is a relatively lean fish and will yield little oil, plants for handling such fish are expected to diversify their operations. Species such as anchovy, which may yield considerably more oil, will doubtlessly be utilized later. Thus, the production of both meal and oil should increase, assuming no large drop in the production of menhaden such as occurred in the production of sardine.

Other nonutilized species in the Atlantic and Gulf areas, such as thread herring, may be used later. Another new operation involves the reduction of Great Lakes trash fish, particularly alewives from Lake Michigan, to meal and oil. Although initial efforts at three plants have involved at present only a few million pounds of fish per year, possibilities for expansion to as much as 200 million pounds per year appear to be good.

In addition to the possibility of obtaining new species to serve as raw material for direct use in fish meal and oil production, large-scale operations to produce FPC (fish protein concentrate) may result in a new source of oil as a byproduct. When this product is to be used for human consumption, virtually all the oil must be removed in order to prevent flavor reversion. In the existing methods of producing fish meal, several percent of oil always remains in the meal as a residue. Removal of the last traces of oils for FPC results in simultaneous release of pigmented substances, which usually give a much darker-colored oil. This darkening of the oil may result in refining problems, which, until research has developed new techniques, will hinder use of the oil from such an operation. Nevertheless, the development of a large-scale FPC operation in the United States would undoubtedly increase the quantity of fish oil produced.

With efforts both to find new sources of raw material and to develop new processing methods already underway, the chances for increased production of both meal and oil in the United States are good, assuming that the stocks of menhaden do not decline appreciably. In addition, developments toward improved processing methods indicate that higher quality meal and diversified uses for oil may cause an increase in value of these fishery industrial products.

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Pesticide Levels in Fish of the Northeast Pacific

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During the past year, a program was initiated to monitor the pesticide levels of edible fish in the Pacific Northwest. The residues monitored were DDT and its metabolites, DDE and TDE. The species sampled were anchovy (Engraulis mordax), Dungeness crab (Cancer magister), English sole (Parophrys vetulus), hake (Merluccius productus), ocean perch (Sebastodes alutus), starry flounder (Platichthys stellatus), true cod (Gadus macrocephalus), and yellowtail rockfish (Sebastodes flavidus)[1]. Since only minimal information about pesticides in fishery products appears in the literature, our results may prove useful to others in the field.

Experimental Methods

The samples were extracted (1) and analyzed (2) according to standard methods. The sample size was adjusted to keep the fat

[1]Oysters were intentionally omitted from this study because they are under monthly surveillance as part of the National Pesticide Monitoring Program initiated by Philip A. Butler, U.S. Bureau of Commercial Fisheries Biological Laboratory, Gulf Breeze, Florida.

Bulletin of Environmental Contamination & Toxicology,
Vol. 3, No. 4, 1968, published by Springer-Verlag New York Inc.

content below 2 g. Except for individual analyses of anchovies, all fish samples weighed at least 20 g. Although the florisil PR[2] was activated at 145°, part of the endrin still appeared in the 94+6 fraction. Dieldrin, however, was completely recovered in the 85+15 fraction. Since the endrin GLC peak was well separated from the other peaks, its presence in the 94+6 eluate was ignored. A Wilkens 600D gas chromatograph with a tritium detector and a 5'x1/8" glass column containing 2% SE-30 on HMDS treated 80/100 mesh Chromosorb W at 195° was used for quantitation. The identity of peaks was confirmed using p-values (3).

The reported values for DDT include both o,p'- and p,p'-DDT. They were obtained by comparison with technical DDT, in order to better approximate the toxicologically significant amount of organochlorine compounds present.

In general, a standard error of about 15% can be assumed.

Results

The data are tabulated. All samples included in an individual entry are from the same catch of fish. Where male and female samples are listed sequentially, they are from a single catch. The inclusion of anchovy and hake as edible species derived from the current use of meal from them for livestock feeding and the projected production of marine protein concentrates from species

[2]Trade names referred to in this publication do not imply endorsement of commercial products.

TABLE

Pesticide Residues in Various Species of Fish and Shellfish

Species	Source	Sex	Number of fish analyzed[a]	Residues (ppm.) DDE	TDE	DDT
Anchovy	Grays Harbor, Wash.		24	0.074	0.074	
			19	0.074	0.098	
			4[a]	0.058-0.172	0.073-0.244	
Crab	Destruction Island, Wash.	M	1	0.039	0.011	---
	Ilwaco, Wash.	M	4[a]	0.027-0.040	0.017-0.021	trace-0.013
English sole[b]	Blaine, Wash.	F	3[a]	0.009-0.016	0.009-0.016	0.010-0.019
		Immature	1	0.053	0.071	0.058
Hake	Saratoga Passage, Wash.	F	10	0.058	0.047	0.090
	" "	M	12	0.042	0.030	0.043
	Port Susan, Wash.	F	11[a]	0.038-0.083	0.030-0.090	0.065-0.147
	" " "	M[c]	5[a]	0.057-0.111	0.062-0.074	0.101-0.223
	Near Cape Foulweather, Ore.		12	0.074	0.068	0.143
Hake meal	Near Aberdeen, Wash.			0.267	0.027	0.080
Ocean perch	Hecate Strait, B.C.		d	0.012	trace	0.013
Starry flounder[b]	Blaine, Wash.	F	1	0.018	0.026	0.013

TABLE (continued)

Species	Source	Sex	Number of fish analyzed[a]	Residues (ppm.)		
				DDE	TDE	DDT
True cod[b]	Blaine, Wash.	Unknown	2	0.005-0.006	0.006-0.007	0.004
Yellowtail rockfish						
Fillets	Hecate Strait, B.C.	F	8	0.017	Trace	0.004
Remains[e]	" " "	F	8	0.042	0.006	0.021
Fillets	" " "	M	18	0.030	Trace	0.014
Remains[e]	" " "	M	18	0.076	0.009	0.051
Fillets	Ilwaco, Wash.	F	13	0.119	0.022	0.048
Remains[e]	" "	F	13	0.256	0.055	0.104
Fillets	" "	M	14	0.092	0.028	0.036
Remains[e]	" "	M	14	0.416	0.092	0.194

[a]Composites, unless a range of values is given.

[b]Fillets only.

[c]Different catch from preceding entry.

[d]16 fillets.

[e]Fish after removal of fillets.

not currently consumed directly. In comparing the data for hake meal with that of whole fish, the difference in water content must be considered (hake meal 4-6%; whole fish 75-80%).

Analysis of DDT in anchovy is complicated by a GLC peak with a retention time only slightly longer than that of DDT. The interference had a p-value in acetonitrile/hexane of 0.71 compared to DDT 0.38 in this laboratory or 0.35 in the literature (3). The presence of a small amount of DDT could be recognized by a shoulder on the leading side of the interference peak but, since only about 0.010 ppm. appeared to be involved, further analysis was dropped.

Since o,p'-DDT overlapped with p,p'-TDE in the GLC quantitation on SE-30, the presence of large amounts of DDT required correction of the apparent TDE concentration for o,p'-DDT included. Correction was achieved by using the p,p'-DDT concentration and the ratio 21:70 of o,p'- to p,p'-DDT in technical DDT (4) and, therefore, in nature.

The 85+15 chromatographic fraction caused gross contamination of the detector and the facilities for cleanup were not regularly available. Consequently, the 85+15 pesticide extracts from fish were merely examined cursorily. At random about one out of three samples was quantitated. The dieldrin and endrin content were generally insignificant. Occasionally a sample would show up to 0.006 ppm. of one of these compounds. p-Value analysis was not, however, performed so the presence of these compounds is scarcely confirmed.

Discussion

The drainage of pesticides through the Columbia River and Puget Sound provides an obvious source of contamination of significant toxicological consideration to the fishing industry and the consuming public of the Pacific Northwest. Evidence from fish collected in California waters (5) indicates that pesticide levels in that area have not been alarmingly high, but no information about Pacific Northwest specimens has been publicly available. To keep abreast of the current situation a survey of the more common local species has been undertaken.

As the data indicate the level of pesticide residues in marine products is substantially below the currently acceptable FDA tolerance for beef (7 ppm.). As might have been anticipated, fillets contain substantially lower pesticide levels than the remaining carcasses. The pesticide levels in fillets undoubtedly represent an even more accurate estimate of the human ingestion level than do whole-fish data, although the latter are of more direct biological interest.

The most striking results are those for yellowtail rockfish. A distinctly larger amount of pesticides occurs in the fish from Ilwaco, Washington, at the mouth of the Columbia River than in those from Hecate Strait, British Columbia, where no major river is entering the ocean. Obviously the agricultural run-off from Washington and Oregon is reaching the ocean fish nearby.

Acknowledgment

The able technical assistance of Michael Metcalf is acknowledged.

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(133) Studies on the Partial Hydrogenation of Fish Oil, IX

The Hydrogenation of Herring Oils (Ōnishin Oil and Konishin Oil)

By Masakichi Takano and Fukuo Kikuya

(Received May 25, 1938)

The oil obtained from young herring is called in Japan "konishin oil" and that from adult herring "ōnishin oil". Both of these herring oils are used in the manufacture of hardened oil. M. Takano, one of the authors and Y. Kumeno have formerly studied on the hydrogenating course of sardine oil (*This Journal*, 1935, 38, 252 B).

Now, the present paper deals with the investigation of hydrogenating course of the above herring oils in a similar way as was stated in the case of sardine oil. Each of the herring oils which had preliminarily been refined with acid clay, was separately hydrogenated to the various degrees of iodine value at 200~210°C under the atmospheric pressure using Ni-kieselguhr catalyst, and the respective fatty acids were prepared from them in the usual manner. Each of the fatty acids thus obtained was analysed; the ether-insoluble bromide (bromide I), the petroleum-ether-insoluble bromide (bromide II), and their bromine content were determined.

The results are as follows:—(The original refined oils are designated as No. 0 in the following table 1 and 2).

In each case, the iodine value of fatty acids decreases steadily, while the thiocyanogen value lowers comparatively slowly, with the result that the two values gradually approach each other with the progress of hydrogenation. The fatty acids were then treated by Twitchell's Pb-salt alcohol method, whereby they were separated into solid and liquid fatty acids as follows:—

Table 1—Ōnishin oil

Sample No.	Hydrogenated oil: Iodine v. (Wijs)	Hydrogenated oil: n_D^{25}	Hydrogenated oil: M.P. (°C)	Corresponding fatty acid: Neutr. v.	Corresponding fatty acid: Iodine v. (Wijs)	Corresponding fatty acid: Rhodan v.	Bromide I: Yield Br (%)	Bromide I: content (%)	Bromide II: Yield Br (%)	Bromide II: content (%)
No. 0	116.5	1.4743	—	194.5	122.2	89.6	15.25	71.73	20.36	52.90
1	103.0	1.4739	—	193.4	108.1	80.4	5.17	69.20	15.44	47.89
2	64.6	—	38~40	193.0	66.8	54.0	0.73	—	5.75	45.82
3	35.5	—	48~50	191.6	39.5	25.9	—	—	Trace	—
4	12.7	—	51~53	191.5	15.6	12.5	—	—	—	—

Table 2—Konishin oil

Sample No.	Hydrogenated oil: Iodine v. (Wijs)	Hydrogenated oil: n_D^{25}	Hydrogenated oil: M.P. (°C)	Corresponding fatty acid: Neutr. v.	Corresponding fatty acid: Iodine v. (Wijs)	Corresponding fatty acid: Rhodan v.	Bromide I: Yield Br (%)	Bromide I: content (%)	Bromide II: Yield Br (%)	Bromide II: content (%)
No. 0	142.8	1.4788	—	198.8	149.7	90.0	25.46	71.81	44.89	54.14
1	113.2	1.4773	—	198.0	120.9	89.7	8.19	69.62	39.79	52.23
2	96.9	1.4764	—	197.1	103.6	82.5	2.69	69.01	27.15	46.70
3	78.2	—	32.5~34	195.6	84.2	76.9	1.21	68.84	12.77	42.69
4	74.5	—	34 ~35.5	195.3	80.9	74.5	Trace	—	5.36	40.46
5	59.6	—	37.5~40	193.2	58.7	50.4	—	—	Trace	—
6	43.6	—	43 ~45	191.0	47.0	40.0	—	—	—	—
7	28.0	—	48.5~51	189.1	31.5	26.3	—	—	—	—

Table 3—Ōnishin oil

Sample No.	Solid acid: Yield (%)	Solid acid: M.p. (°C)	Solid acid: Iodine v. (Wijs)	Solid acid: Neutr. v.	Liquid acid: Yield (%)	Liquid acid: n_D^{25}	Liquid acid: Iodine v. (Wijs)	Liquid acid: Neutr. v.
No. 0	17.6	43 ~44.5	27.9	214.7	80.1	1.4606	129.2	190.9
1	20.4	42.5~44	29.4	212.4	76.6	1.4565	112.8	190.1
2	50.8	40 ~41.5	42.2	199.3	45.7	1.4560	78.4	188.1
3	72.3	44 ~46	22.4	195.3	23.6	1.4553	70.6	180.2
4	86.9	52 ~54	10.3	189.4	9.1	1.4546	58.8	176.0

Table 4—Konishin oil

Sample No.	Solid acid: Yield (%)	Solid acid: M.p. (°C)	Solid acid: Iodine v. (Wijs)	Solid acid: Neutr. v.	Liquid acid: Yield (%)	Liquid acid: n_D^{25}	Liquid acid: Iodine v. (Wijs)	Liquid acid: Neutr. v.
No. 0	21.1	43.5~45	24.5	217.8	74.7	1.4635	159.2	191.8
1	23.9	43 ~44.5	25.4	215.5	73.3	1.4626	130.1	191.6
2	30.4	42 ~43.5	32.4	205.7	66.6	1.4604	117.5	193.7
3	41.4	41 ~42.5	40.1	197.4	56.1	1.4600	108.0	190.4
4	46.4	40 ~41.5	42.0	197.6	49.6	1.4598	107.1	188.8
5	60.0	40 ~41	44.6	195.2	36.9	1.4592	90.7	186.6
6	65.0	42 ~43	33.7	192.8	31.6	1.4586	80.0	185.0
7	74.7	44 ~45.5	20.4	190.7	21.5	1.4573	69.2	184.3

The amount of so-called isooleic acid may be calculated from the expression as 90 (a=yield of solid acid, s=iodine value of solid acid); in this case, however, the iodine value of the solid unsaturated acid is not necessarily 90. But, on the assumption that the solid unsaturated acid can be calculated from as 90, the amount of the saturated acid may be calculated from the following formula.

Solid acid—Solid unsaturated acid=Saturated acid

Unsaturated acid which gives bromide I=Unsaturated acid which gives bromide II=Unsaturated acid having an unsaturation of linolic series and an unsaturation of higher degree (Unsaturated acid A).

Liquid fatty acid—Unsaturated acid A=Liquid unsaturated acid of oleic series (Unsaturated acid B).

Thus, the following figures were obtained:

In each case (ōnishin oil and konishin oil), the selective hydrogenation referred to the unsaturation of fatty acid, was observed as in the case of hydrogenation of sardine oil previously stated; the

Table 5—Ōnishin oil

Sample No.	Saturated acid (%)	Solid unsaturated acid (%)	Unsaturated acid A (%)	Unsaturated acid B (%)
No. 0	12.1	5.5	13.9	66.2
1	13.7	6.7	9.7	66.9
2	27.0	23.8	3.3	42.4
3	54.3	18.0	Trace	23.6
4	76.9	10.0	—	9.1

Table 6—Konishin oil

Sample No.	Saturated acid (%)	Solid unsaturated acid (%)	Unsaturated acid A (%)	Unsaturated acid B (%)
No. 0	15.4	5.7	27.8	46.9
1	17.1	6.8	21.5	51.8
2	19.4	11.0	15.3	51.3
3	23.0	18.4	7.6	48.5
4	24.7	21.7	5.0	44.6
5	30.3	29.7	Trace	36.4
6	40.5	24.5	—	31.6
7	57.8	16.9	—	21.5

highly unsaturated acid which is originally contained is most quickly hydrogenated and any appreciable amount of saturated acid is not formed until the former has disappeared almost completely, whilst with the complete disappearance of the highly unsaturated acid, the formation of the saturated acid becomes more remarkable.

The iodine value of hydrogenated konishin oil, whose bromide I has just disappeared, is incomparably lower than in the case of sardine oil, and konishin oil falls between the two; in other words, the lower the iodine value of the original oil, the more quickly the bromide I disappears. The solid unsaturated acid increases at first, and then decreases after the maximum was reached. As for the amount of such acid, sardine oil is the greatest, while konishin oil the smallest.

The amount of liquid unsaturated acid of oleic series is somewhat increased at first, but is ultimately decreased in every case. The tendency of such increase is most conspicuous in sardine oil.

Konishin oil has just the intermediate nature with respect to every point stated above.

[HAKODATE FISHERIES COLLEGE]

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(48) On the Cooling Curves of Oils, Fats and Waxes in their Natural States as well as Hardened, and Paraffins. I

The cooling curves of fats and waxes in their solid state and those of similar substances

by Sei-ichi Ueno and Shin-ichi-Nakaguchi

(Received December 5, 1935)

This experiment having the purpose of obtaining cooling curves for various substances: oils, fats, natural waxes, as well as hardened substances (in other words, those saturated by hydrogenation), -- is very interesting both from the theoretical and practical points of view. But it seems that no substantial publication on this subject has appeared up until now.

We want to adopt the cooling curve as an indicator of the nature of each of the above-mentioned substances, since the form of the curve strongly depends on the nature of each of the substances.

We give here the curves for hardened substances (oils, fats and waxes) at the same time as for natural products (fats, solid waxes, paraffins and ceresin) which are in the solid state at ordinary temperatures.

This report is only a preliminary one for our studies, and our intention is to obtain many more cooling curves for oils, fats and waxes which are in the liquid state and to look for relationships

between the curve and the composition of each of these substances.

Experimental Section

Certain quantities of oil, fat and wax were hardened by hydrogenation, which was done during 2 to 4 hours under elevated pressure of 50 atmospheres, at a temperature of 180°C and with nickel as a catalyst. In general, we used as samples, substances whose iodine value is under 3, but for wood and sesame oils, we had to settle for samples whose iodine value is a little higher than 3. As for solid natural wax, since it is difficult to hydrogenate, we used raw samples.

We put into a M. Shukoff bottle (Chem. Ztg. 1901, 25:95) a mass of 30 grams of each of the above mentioned samples, and having first warmed it to a temperature surpassing by more than 10°C the point of solidification, we let it cool, all the while shaking it, until the moment where the temperature reached exactly 10°C above the point of solidification. At this time, we stopped shaking the bottle, and we began reading the temperature every 60 seconds.

The cooling curves which are reproduced below were obtained in this manner, and they are concerned with oils, fats and waxes which were hardened as well as the natural products: fats, solid waxes and paraffins.

Summary

In taking the point of view that the cooling curves for oil, fat, etc. can serve well as indicators of their nature, we obtained these curves for the following substances: 16 kinds of hardened substances, of which 3 are varieties of animal oil, 2 kinds of animal fat, 8 kinds

of vegetable oil, 1 kind of vegetable fat, 2 kinds of liquid wax; and 13 kinds of natural substances of which one is a variety of solid fat, 5 kinds of solid wax, 7 kinds of paraffin.

We have shown that there was in general, a remarkable temperature elevation after surfusion for each kind of hardened oil and hardened fat; only for hardened sardine and herring oils was there only a slight elevation in temperature; nevertheless, we were able to ascertain that there was a rise.

On the other hand, for all kinds of solid wax and for paraffin, no elevation of temperature after surfusion was visible. For hardened wax, a very small elevation was visible, but we may assume that this was caused by the presence of glyceride.

The results of our experiments allowed us to see that there was a distinct difference between the cooling curves for oils and hardened fats and those for hardened wax, and also between the curves for solid fats and those for solid waxes or for paraffins.

Furthermore, among the varieties of oils and hardened fats, we showed that for some the cooling curve had a particular form following the nature of the substances as we had thought.

[Department of Applied Chemistry, Engineering Faculty, Imperial University of Osaka]

(48) Sur les Courbes de Refroidissement des Huiles, des Graisses, des Cires en leur Etat Naturel ainsi que Durci, et des Paraffines. I

Les Courbes de Refroidissement des Graisses, des Cires en leur Etat Solide et des Substances Semblables

Par Sei-ichi Ueno et Shin-ichi Nakaguchi

(Reçu au 5 Décembre 1935)

L'expérience en vue d'obtenir les courbes de refroidissement de ces diverses substances: huiles, graisses, cires naturelles ainsi que celles durcies (c'est-à-dire qui sont saturées par hydrogénation), cette expérience doit être très intéressante au point de vue théorique et pratique. Mais aucune publication concrète sur ce sujet n'a pas été faite jusqu'ici, semble-t-il.

Nous voulons adopter la courbe de refroidissement comme un indicateur du caractère pour chacune des substances susmentionnées, puisque la forme de la courbe dépend bien du caractère de chacune de ces substances.

Nous donnons ici les courbes de ces substances durcies (huiles, graisses et cires) ainsi que de ces produits naturels (graisses, cires solides, paraffines

J. Soc. Chem. Ind. (Jap.) 39: 79B-80B, ~~1936~~ 1936

et cérésine) qui sont à l'état solide à la température ordinaire.

Ce rapport n'est qu'un préliminaire pour nos études et nous avons l'intention d'obtenir encore beaucoup de courbes de refroidissement pour les huiles, les graisses et les cires qui sont à l'état liquide et de chercher les relations entre la courbe et la composition de chacune de ces substances.

Partie expérimentale

Certaines quantitées d'huile, de graisse et de cire ont été durcies par une hydrogénation, qui s'est faite pendant 2 à 4 heures sous une haute pression de 50 atmosphères, à la température de 180°C, et avec du nickel comme catalyseur. Nous avons, en général, adopté comme échantillons des substances dont la valeur d'iode est au-dessous de 3, mais pour l'huile de bois et de sésame, nous nous sommes contentés d'échantillons dont la valeur d'iode est un peu plus haute que 3. Quant à la cire solide naturelle, puisqu'il est difficile de l'hydrogéner, nous avons employé des échantillons brûts.

Nous mettons dans un flacon de M. Shukoff (*Chem. Ztg.*, 1901, **25**, 95) une masse de 30 grammes de chacune des échantillons sus-mentionés et l'ayant d'abord chauffée jusqu'au degré qui dépasse par plus de 10°C le point de solidification, nous la laissons refroidir, tout en l'agitant, jusqu'au moment où sa température atteint 10°C exactement au-dessus du point de solidification. A ce moment nous cessons d'agiter le flacon et nous commençons à lire sa température toutes les 60 secondes.

Les courbes de refroidissement que nous reproduisons ci-dessous ont été obtenues de cette façon et elles concernent huiles, graisses et cires qui sont durcies ainsi que ces produits naturels: graisses, cires solides et paraffines.

Fig. 1—Huile de Sardine durcie

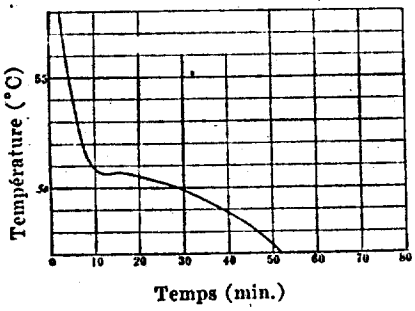


Fig. 2—Huile d'Hareng durcie

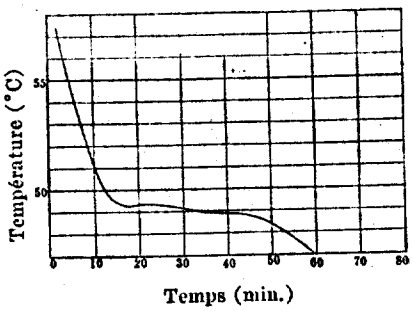


Fig. 3—Huile d'Abura-garei durcie

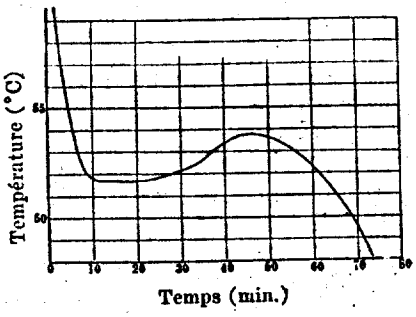


Fig. 4—Graisse de Cheval durcie

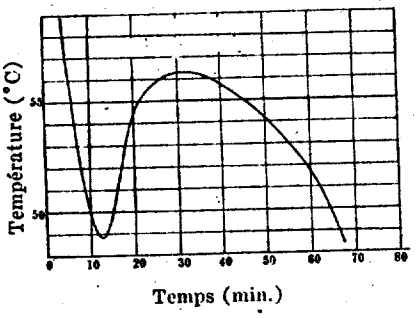


Fig. 5—Suif de Boeuf durcie

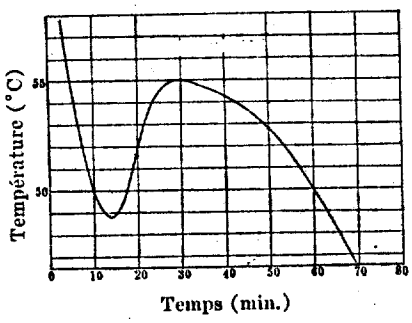


Fig. 6—Huile de Bois du Japon durcie

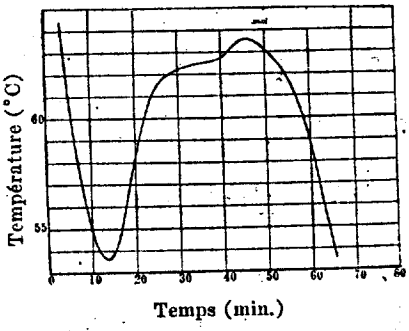


Fig. 7—Huile de Bois de Chine durcie

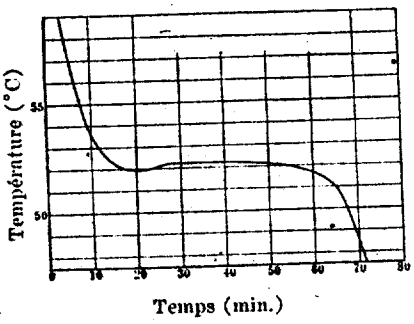


Fig. 8—Huile de Sésame durcie

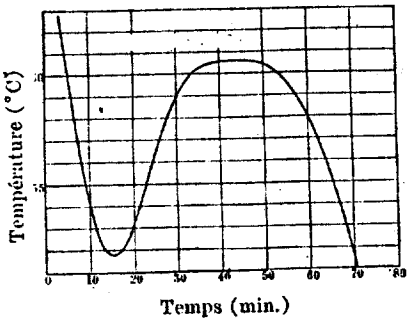


Fig. 9—Huile de Coton durcie

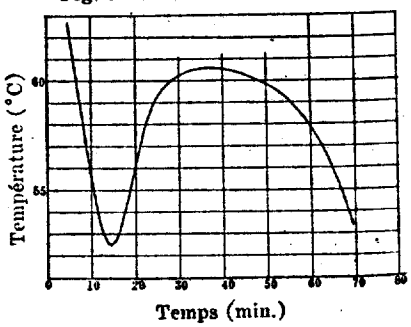


Fig. 10—Huile de Chalmougré durcie

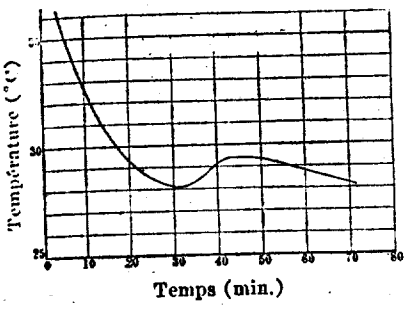


Fig. 11—Huile d'Arachide durcie

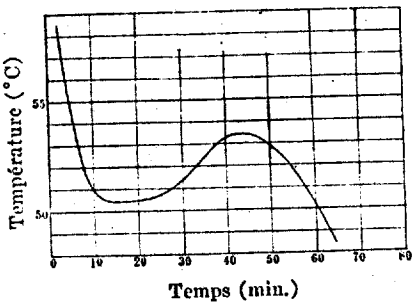


Fig. 12—Huile de Ricin durcie

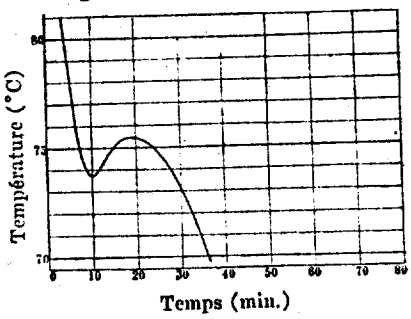


Fig. 13—Huile d'Olive durcie

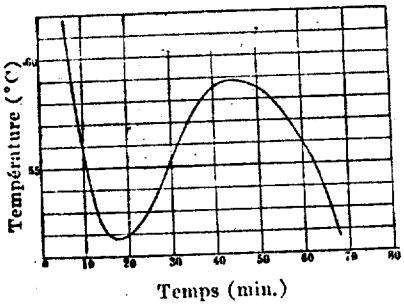


Fig. 14—Cire du Japon durcie

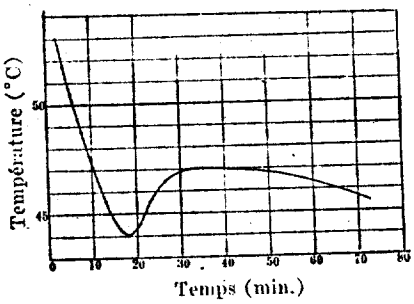


Fig. 15—Cire de Spermacéti durcie

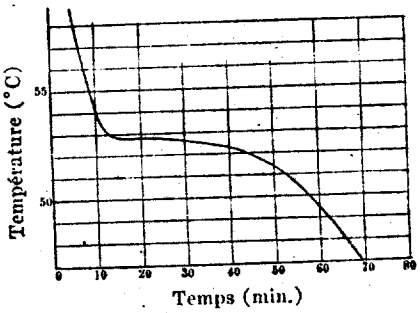


Fig. 16—Cire de Spermacéti arctique durcie

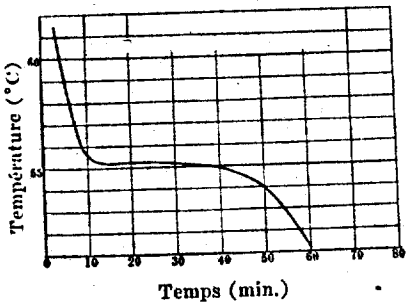


Fig. 17—Cire du Japon

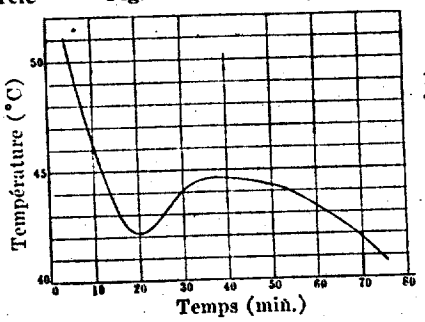


Fig. 18—Cire de Carnauba

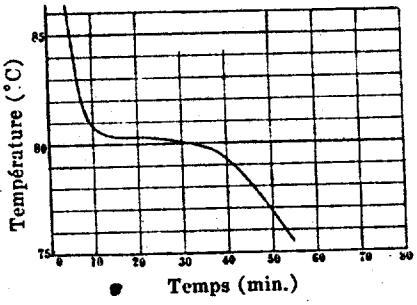


Fig. 19—Cire de Candelilla

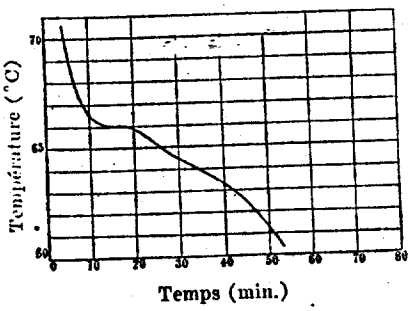


Fig. 20—Cire d'Abeilles

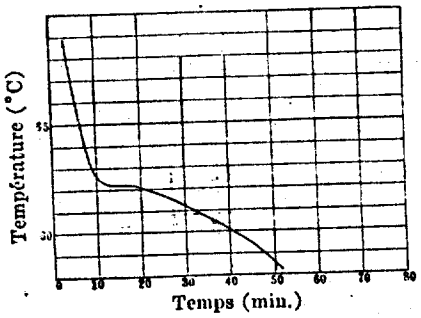


Fig. 21—Cire d'Insectes

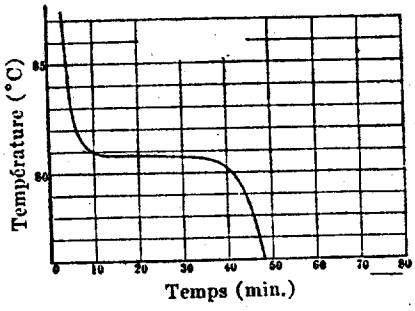


Fig. 22—Cire de lignite

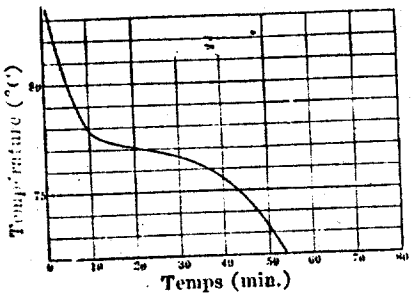


Fig. 23—Cérésine (Kahlbaum)

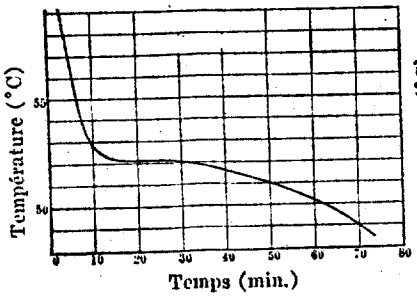


Fig. 24—Paraffine A (Californie)

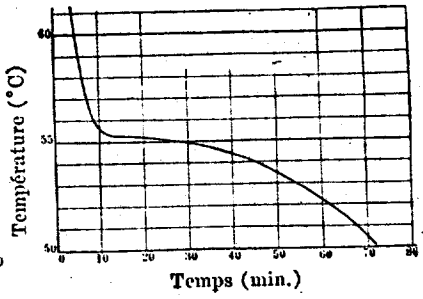


Fig. 25—Paraffine B (Echigo)

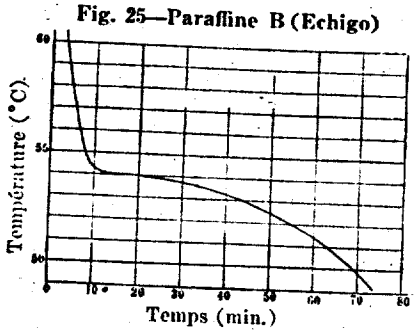


Fig. 26—Paraffine C (d'huile de schiste)

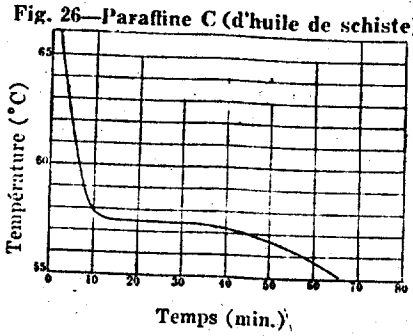


Fig. 27—Paraffine D (Niigata)

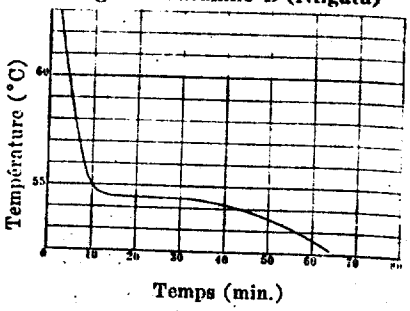


Fig. 28—Paraffine E (Formose)

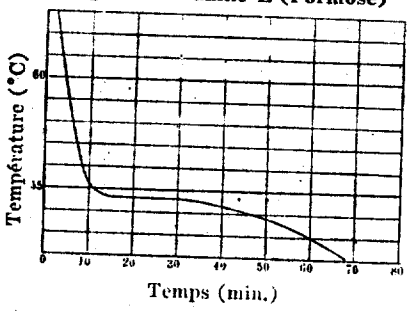
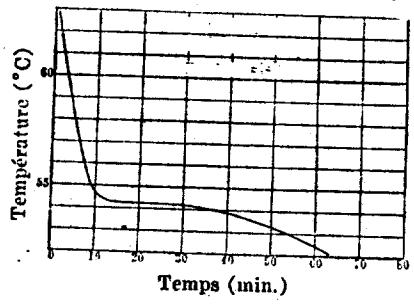


Fig. 29—Paraffine F (Californie)



Sommaire

En nous plaçant sous ce point de vue que les courbes de refroidissement de l'huile, la graisse, etc. peuvent bien servir comme des indicateurs de leur caractère, nous avons obtenu ces courbes pour les substances suivantes: 16 sortes de substances durcies, soit 3 sortes d'huile animale, 2 sortes de graisse animale, 8 sortes d'huile vegétale, 1 sorte de graisse vegétale, 2 sortes de cire liquide; et 13 sortes de substances naturelles, soit 1 sorte de graisse solide, 5 sortes de cire solide, 7 sortes de paraffine.

Nous avons constaté qu'il y avait, en général, une élévation remarquable de température après la surfusion pour chaque sorte d'huile durcie et de graisse durcie; seulement pour l'huile de sardine durcie et celle de hareng durcie, il n'y avait qu'une faible élévation de température; néanmoins nous avons pu apercevoir que cela avait eu lieu.

Au contraire, pour toutes les sortes de cire solide et pour la paraffine, aucune élévation de température après la surfusion n'a été visible. Pour la cire durcie une petite élévation a été visible, mais nous pouvons supposer que cela a été causé par la présence de la glycéride.

Les résultats de nos expériences nous ont permis d'apercevoir qu'il y avait une différence bien nette entre les courbes de refroidissement de l'huile et de la graisse durcies et celles de la cire durcie, et de même entre les courbes des graisses solides et celles des cires solides ou des paraffines.

En outre, parmi ces sortes d'huile et de graisse durcies, nous avons constaté qu'il y en avait dont la courbe de refroidissement avait une forme particulière suivant les espèces comme nous l'avions bien pensé.

[Rayon de Chimie Appliquée, Faculté des Ingénieurs, Université Impériale d'Ôsaka]

J. Soc. Chem. Ind., Japan, 41(6): 200B-202B, 1938

(106) The Relationship between Thiocyanogen Value and Iodine Value in the Course of Oil-hardening

By Sei-ichi Ueno

(Received April 30, 1938)

The author has previously published several reports on the progress of hydrogenation of various oils. In this experiment sardine oil is subjected to hydrogenation under the same conditions as adopted in the practical oil-hardening on a large scale. The catalyst is the reduced nickel supported on kieselguhr, using in the ratio of 0.5% metallic nickel to oil. The procedure of hydrogenation is just the same as described before, employing both the ordinary pressure and a little higher pressure at about 180°C. With the progress of the hydrogenation small portions of reaction products are drawn out from the hydrogenation vessel at intervals of 1 hour, and the iodine value and thiocyanogen value of these samples are examined. The results are given as follows:

Exp. 1—Hydrogenation at 5~7 atm. p.

Lapse of Time hours	Iodine Value	Thiocyanogen Value	Melting Point
(Original Oil)	176.1	95.9	—
1	163.6	92.4	—
2	138.8	91.5	—
3	105.5	74.2	—
4	89.4	72.2	—
5	68.8	54.5	26.8~28.6

Lapse of Time hours	Iodine Value	Thiocyanogen Value	Melting Point
6	42.9	34.9	36.3~37.5
7	22.1	17.5	44.4~45.8
8	9.2	5.8	50.8~51.8
9	7.0	2.7	54.9~55.7
10	6.3	2.5	55.3~56.0
11	6.0	2.4	55.4~56.1
12	5.8	2.3	55.4~56.1
13	5.5	2.2	55.5~56.2

Exp. 2—Hydrogenation at 5~7 atm. p.

(Original Oil)	180.3	89.0	—
1	175.1	85.3	—
2	158.7	84.7	—
3	123.6	82.1	—
4	92.7	74.1	28.4~30.2
5	74.1	52.3	33.2~36.7
6	61.5	44.3	38.9~40.3
7	52.5	41.0	42.3~43.6
8	39.0	25.2	45.6~47.1

Exp. 3—Hydrogenation at ordinary pressure

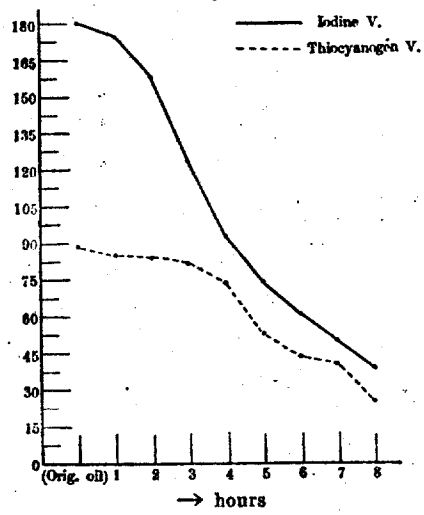
(Original Oil)	176.9	95.8	—
1	169.3	93.5	—
2	156.7	92.9	—
3	140.8	92.0	—
4	129.5	86.6	—
5	118.4	85.4	—
6	107.0	80.4	—
7	99.9	79.0	28.9~30.6
8	95.1	76.2	29.4~31.3
9	79.1	49.7	34.7~36.4
10	67.3	43.7	38.5~39.9
11	63.6	38.9	39.1~40.4

Exp. 4—Hydrogenation at ordinary pressure

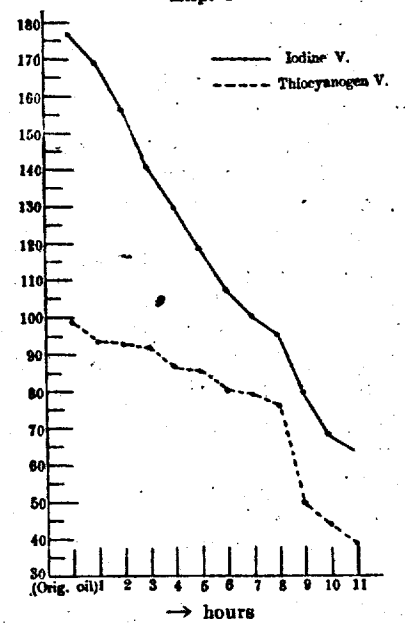
(Original Oil)	176.7	90.2	—
1	167.6	88.9	—
2	140.0	87.8	—
3	120.8	81.6	—
4	103.1	79.2	—
5	95.0	75.2	33.6~35.3

The diagrams in figs. 1~4 constructed from the data of this experiment show the relationship between thiocyanogen value and iodine value during the hydrogenation course of the sardine oil. From

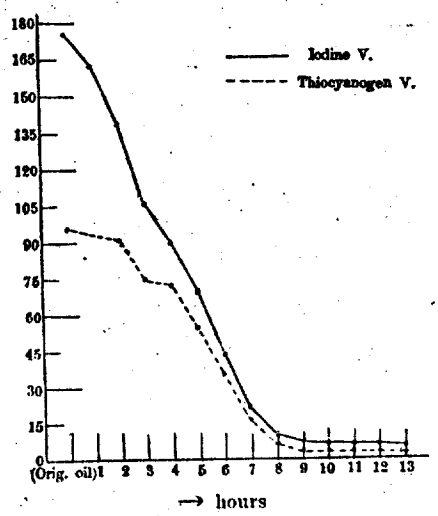
Exp. 2



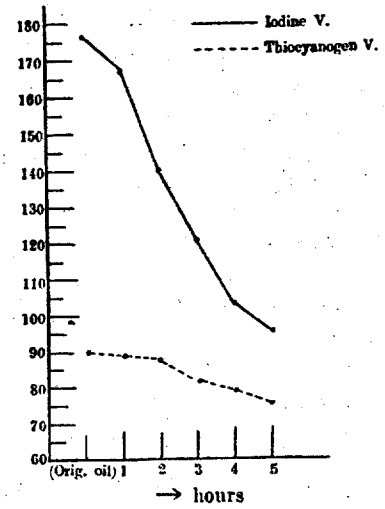
Exp. 3



Exp. 1



Exp. 4



these curves it will be easily seen that the sardine oil shows a rapid decrease in thiocyanogen value at the stage of the hydrogenation process where the iodine value falls to 70~80. This fact is considered to indicate the selective and step-wise character of the hydrogenation process.

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ABSTRACTS FROM THE ORIGINAL COMMUNICATIONS

ON THE NUTRITIVE VALUE OF THE HARDENED OILS. II

By Seiichi Ueno, Matasaku Yamashita and Yasuo Ota

RECEIVED DECEMBER 23, 1927

The present study was begun for the purpose of ascertaining the stability of nutritive value of oils towards hydrogenation.

In this experiment sardine oil, herring oil, finback whale oil and chrysalis oil were used.

Hydrogenation of oils was carried out at a low temperature of 110–115°C in the presence of nickel catalyser with extreme care.

The properties of the oils were given in the following table.

	Sardine oil	Hardened sardine oil	Herring oil	Hardened herring oil
$d_{4^\circ}^{15^\circ}$	0.9299	($d_{4^\circ}^{100^\circ}$) 0.8581	0.9174	($d_{4^\circ}^{100^\circ}$) 0.8555
$n_D^{20^\circ}$	1.4799	($n_D^{40^\circ}$) 1.4621	1.4712	($n_D^{40^\circ}$) 1.4588
M. P.	—	29.6–31.8	—	31.1–34.7
Acid. V.	0.23	0.30	0.24	0.25
Sapn. V.	190.3	190.7	187.1	187.0
I. V. (Hanus)	166.2	64.2	106.6	55.7
R. M. V.	0.4	0.3	0.7	0.4
H. V.	92.4	93.9	93.6	93.8

	Finback whale oil	Hardened finback whale oil	Chrysaris oil	Hardened chrysalis oil
$d_{4^\circ}^{15^\circ}$	0.9205	($d_{4^\circ}^{100^\circ}$) 0.8535	0.9232	($d_{4^\circ}^{100^\circ}$) 0.8524
$n_D^{20^\circ}$	1.4718	($n_D^{40^\circ}$) 1.4589	1.4751	($n_D^{45^\circ}$) 1.4583
M. P.	—	28.1–31.7	—	41.3–42.8
Acid. V.	0.65	0.28	0.39	0.79
Sapn. V.	192.9	189.3	192.8	192.5
I. V. (Hanus)	106.8	64.5	135.9	68.6
R. M. V.	0.5	0.4	0.4	0.4
H. V.	93.8	93.7	93.3	93.8

Animals employed in this experiment were young healthy rats weighing about 40 grs. selected from our carefully bred stock and fed with an artificial ration, the constitution of which being as follows.

J. Soc. Chem. Ind. (Japan) 31: 92-93 1928

Rice starch	70 pts.
Cane sugar	5 ,,
Purified horse flesh	10 ,,
Mc. collum's salts	4.5 ,,
Oryzarine powder	0.5 ,,
Testing oil or fat	10 ,,

The results of the animal test indicated that nutritive values of the hardened fish oils and the hardened chrysalis oil prepared at a low temperature were clearly superior to those of their original oils.

J. Food Sci 28: 305-312, 1962

Autoxidation of Fish Oils. II. Changes in the Carbonyl Distribution of Autoxidizing Salmon Oils[a,b]

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(Manuscript received October 1, 1962)

SUMMARY

The carbonyl compounds of autoxidizing salmon oil were qualitatively and quantitatively analyzed at predetermined levels of oxidation. The 2-thiobarbituric acid (TBA) number, peroxide values, and total carbonyls were determined in addition to the individual volatile and Girard-T-isolable carbonyls. The C_1-C_{12} alkanals, C_3-C_{12} alk-2-enals, and the C_6-C_{10} alk-2,4-dienals composed the major part of the monocarbonyl fractions. The shorter-chain carbonyls were predominant, with methanal and propanal in the highest concentration. It appears that the Girard T reagent, as employed in the investigation, degrades the precursors of the volatile carbonyl fraction.

The hydroperoxides were readily destroyed in unpolymerized oil by the Girard T reaction conditions, but as polymerization proceeded the yield of hydroperoxides increased and that of the Girard-isolable monocarbonyls and malonaldehyde decreased. Polymerization of the salmon oil stabilized a large portion of the measurable hydroperoxides toward the degradative effects of the Girard and TBA reactions.

Carbonyl compounds have been implicated in the flavor deterioration of lipids (Lea and Swobodi, 1953) and in the rusting or browning reaction of fishery products (Nonaka, 1957). Work on the chemical definition of carbonyls in autoxidizing salmon oil has been carried out by Yu *et al.* (1961), and a wealth of information has been accumulated for other lipids in recent years (Day and Lillard, 1960; Lillard and Day, 1961; Gaddis *et al.*, 1960, 1961a; Forss *et al.*, 1960a,b,c).

The carbonyl compounds have been classified as volatile, or free, and nonvolatile, or bound (Gaddis *et al.*, 1960). The volatile fraction has been defined for many lipids, and it is usually less than 3% of the total measurable carbonyls (Lillard and Day, 1961; Gaddis *et al.*, 1960). The volatile monocarbonyls consist largely of alkanals, alk-2-enals, alk-2,4-dienals, and traces of vinyl ketones (Stark and Forss, 1962), and if the lipid is thermally oxidized, methyl ketones may occur (Crossley *et al.*, 1962). The remainder of the volatile carbonyl fraction has been reported as dicarbonyls in salmon oil (Yu *et al.*, 1961) and in lard (Gaddis *et al.*, 1960), but chemical definition of the mixture has not been reported.

Gaddis *et al.* (1960) distinguished between volatile and free carbonyls by finding that a portion of the free carbonyls were not accountable in the volatile fraction. The free carbonyls were measured by the Pool and Klose (1951) procedure. Recent work indicates, however, that their interpretation may be incorrect because of inherent errors in the original Pool and Klose method. Keith and Day (in press) modified the procedure to enable measurement of the major monocarbonyl classes, thus eliminating the quantitative error in the original method. They found that the volatile monocarbonyl fraction in milk fat was comparable to the free monocarbonyl fraction measured by the modified Pool and Klose procedure.

The nonvolatile (bound) carbonyl fraction has not been chemically elucidated, but it may be defined as that portion of the

[a] Technical paper no. 1607, Oregon Agricultural Experiment Station.

[b] This investigation was supported in part by PHS Research Grant EF-182 from the National Institutes of Health, Toxicology Section.

total measurable carbonyls that is not distillable or detectable by the Pool and Klose procedure. The fraction appears to be largely derived from the degradation of precursors by the reagent used in measuring it. Gaddis *et al.* (1960) attempted to define the fraction more clearly by using the Girard T reagent. They employed a procedure that caused degradation of the carbonyl precursors, and the carbonyls were isolated as the water-soluble hydrazones. They found that the volatile portion of the Girard-T-isolable compounds was at least superficially similar to the free volatile carbonyls, but they also found nonvolatile monocarbonyls and dicarbonyls.

Both the volatile and bound carbonyl fractions of salmon oil are of practical significance. The volatile fraction appears to be largely responsible for the rancid odor (Yu *et al.*, 1961), and, from available evidence, it is logical to assume that the "so-called" nonvolatile fraction, which exists as carbonyl precursors, is the origin of the volatile fraction. Both fractions undoubtedly are implicated in the rusting of frozen salmon and the browning of fish meal. Since the extent to which the salmon oil or the oil-containing products are oxidized varies so widely, the work reported herein was designed to study changes in the carbonyl composition of the oil at different stages of oxidation. An attempt was made to extend the qualitative identification of the volatile carbonyl fraction reported on previously by Yu *et al.* (1961) and to characterize the nonvolatile fractions. Attention was also directed to ascertaining the effect of oxidation on both the total and relative quantities of the various carbonyl compounds in the two fractions.

METHODS

Solvents. Hexane, chloroform, benzene, and ethylene chloride were purified by the procedure of Schwartz and Parks (1961). Absolute ethanol was refluxed for 3 hr with 2,4-dinitrophenyl-(DNP)hydrazine and trichloracetic acid, followed by distillation. Equilibrated hexane was prepared by shaking hexane with excess acetonitrile, and the acetonitrile layer removed.

Column-chromatography packing. Analytical-grade Celite was dried 18 hr at 160°C. Celite 545 was dried 24 hr at 160°C. Seasorb 43 (Fisher Scientific) was activated for 48 hr at 400°C. The iodine adsorption number of the Seasorb was 58. The iodine adsorption number of the Seasorb was determined by weighing to the nearest 0.01 g a 4-g sample of Seasorb 43. One hundred ml of 0.0500*N* iodine in carbon tetrachloride was added, and the sample was shaken for 15 min. After settling for 5 min, 25 ml of the clear solution was pipetted into a flask containing 50 ml of 0.03*N* KI in 75% ethanol. The solution was titrated with standard 0.05*N* sodium thiosulfate (detailed procedure available from Food Machinery Corporation, New York, New York). The silicic acid-Celite 545 mixture was prepared by using five parts of 100-mesh silicic acid with one part Celite, and the mixture was dried 12 hr at 200°C.

Oxidation of salmon oil. Fresh salmon oil, edible grade, was oxidized by maintaining it under positive oxygen pressure in a sealed bottle at room temperature. The oil was shaken daily and analyzed periodically for peroxide value and 2-thiobarbituric acid (TBA) number. The fresh oil and four samples taken at predetermined levels of oxidation were subjected to carbonyl analysis.

Peroxide value. Peroxides, determined by the AOCS (1959) method, are expressed as mg of peroxide per kg of oil.

Thiobarbituric acid number. The procedure of Sinnhuber and Yu (1958) was used to measure the mg of malonaldehyde per kg of oil.

Total carbonyl values. Total saturated and unsaturated carbonyls were determined by the method of Henick *et al.* (1954).

Isolation of volatile carbonyl compounds. The volatile carbonyls were isolated by means of the reduced-pressure distillation apparatus of Day and Lillard (1960) except that 100-g samples were used and 100 ml of distillate were collected. The distillate was reacted with an equal volume of 5*N* HCl, saturated with DNP-hydrazine. The DNP-hydrazones of the monocarbonyls were isolated by extraction with hexane. The extracts were combined and the hexane removed under reduced pressure. The bis-DNP-hydrazones of the dicarbonyls were subsequently isolated from the reaction mixture by benzene extraction.

Isolation of water-soluble Girard T carbonyl derivatives. The Girard T reagent was used to isolate the carbonyls from salmon oil. Reaction conditions were selected to give the maximum yield of carbonyl derivatives. The procedure was essentially as outlined by Gaddis *et al.* (1960) except the reflux time was increased to 3 hr. One hundred grams of oil, 10 g of Girard T reagent, 2 g of Dowex 50 cation-exchange resin, and 200 ml of carbonyl-free ethanol were refluxed 3 hr. The mixture was cooled, 2 L of distilled

water were added, and the oil was removed by extraction with petroleum ether. Sodium chloride was added during the extraction step to help break the emulsion and to facilitate removal of final traces of oil. The water-soluble hydrazones were converted to DNP-hydrazones by reacting equal volumes of the aqueous solution and 5*N* HCl saturated with DNP-hydrazine. The reaction was allowed to proceed 12 hr at room temperature, after which the DNP-hydrazones were isolated by extraction with chloroform. Upon removal of the chloroform, the DNP-hydrazones of monocarbonyls were separated from bis-DNP-hydrazones by exhaustive extraction with hexane. The procedure as outlined gave an average yield of 57% of the estimated total carbonyls in unpolymerized salmon oil.

Quantitative analysis of DNP-hydrazones. The DNP-hydrazones were separated into classes with the Seasorb-Celite-ethylene chloride procedure of Schwartz *et al.* (1960). The quantity of DNP-hydrazones represented in each class was determined by measuring the absorbance of chloroform solutions at 358, 373, and 390 mμ for alkanals, alk-2-enals, and alk-2,4-dienals, respectively, and calculating the millimoles of carbonyl using molar absorptivity indices of 22,500, 27,500, and 37,500 for the respective classes.

Each of the DNP-hydrazone mixtures obtained from the class separation was chromatographed by the column-partition method of Corbin *et al.* (1960). The column was modified in that no water was used during preparation. The eluate from the column was collected in 10-ml fractions, and the absorbance of each fraction was measured at the wavelength of maximum absorption for the class of DNP-hydrazones being separated. The data, when plotted in the manner described by Corbin *et al.* (1960), depicted the typical chromatograms in which each peak represented a specific member of a DNP-hydrazone class. The area under each peak was determined with a planimeter, and these values were used to calculate the percentage of each compound in each carbonyl class. Since the total carbonyl concentration of each class had been determined in a previous step, the amount of each carbonyl was easily calculated from the percentage composition data.

Quantitative analysis of bis-DNP-hydrazones. Excess DNP-hydrazine present in the crude bis-DNP-hydrazone mixtures was removed by the cation-exchange procedure of Schwartz *et al.* (1962). The bis-DNP-hydrazones were separated on the silicic acid-Celite column of Wolfrom and Arsenault (1960). All of the chromatographic bands gave positive tests for the bis-DNP-hydrazones of vicinal dicarbonyls. The absorbance of each fraction, in chloroform, was measured at 393 mμ, and the moles of carbonyl were calculated using a molar absorptivity value of 4.3×10^4.

Qualitative analysis of DNP-hydrazones. Evidence to establish the identification of DNP-hydrazones was based on the following criteria. Class separation of the DNP-hydrazones as described by Schwartz *et al.* (1960) and confirmed in our laboratory was effective in separating the various hydrazone classes of the monocarbonyls. The chain length of compounds in each class was ascertained as described by Corbin *et al.* (1960) through preparation of reference chromatograms from authentic DNP-hydrazones. The chromatographically resolved compounds were checked by determining absorption maxima, and chain lengths were confirmed by comparing chromatographic behavior with authentic derivatives on paper chromatograms. Melting-point analyses were conducted on questionable derivatives.

RESULTS AND DISCUSSION

Table 1 shows gross changes in the salmon oil during autoxidation. The oil was checked periodically for the TBA number and peroxide values. Aliquots were taken for detailed analyses at the points indicated in the table.

The quantity of Girard-isolable monocarbonyls reached a maximum during the oxidation period and the subsequent decrease in yield was accompanied by visible evidence of polymerization of the oil. The percent Girard-isolable monocarbonyls of the total estimated carbonyls decrease from 89% in Sampling 3 to less than 4% in Sampling 5. Peroxide values for Samplings 1 and 5, after treatment of the oil by the Girard procedure, were 0 and 153.5, respectively. This means that in advanced stages of oxidation, polymerization appears to stabilize a substantial portion of the hydroperoxides towards the degradative effects of the Girard reaction. The decrease in yield of Girard-isolable monocarbonyls with the concomitant stabilization of hydroperoxides also suggests that hydroperoxide degradation effected by the Girard reaction accounts for a large portion of the carbonyls isolated by this procedure. This is true also for the Girard-isolable dicarbonyls even though the quantity in this fraction increased as oxidation proceeded. The maximum yield (3% of the total carbonyls) was obtained for Sampling 3.

Table 1. Gross changes in salmon oil during oxidation.

	Sample no.				
	1	2	3	4	5
Days' storage	0	16	31	66	163
TBA no.	8.35	350	1102	2024	1472
Peroxide value	4.0	62.6	171	224	358
Total carbonyls [a]	9.5	52.6	110.8	312.3	569.8
Volatile monocarbonyls [a]	0.096	0.176	0.562	1.463	2.057
Girard-isolable [a] monocarbonyls	4.456	3.820	98.670	77.888	19.209
Volatile dicarbonyls [a]	0.039	0.047	0.289	0.308	0.890
Girard-isolable [a] dicarbonyls	0.194	1.114	3.309	5.972	8.250

[a] mM/kg of oil.

The above conclusions may be questioned because the quantity of Girard-isolable monocarbonyls is lower in Sampling 2 than in Sampling 1 while the peroxide value is much higher in Sampling 2. A possible cause for this discrepancy may be the poor recovery of the monocarbonyls at one of the several steps in the isolation procedure. The data in Table 3 show that methanal and propanal are the compounds accounting for the decrease in yield. These compounds, along with ethanal, are the most difficult to recover because of their limited solubility in the solvents employed.

The decrease in TBA number, while not simultaneous with the decrease in Girard-isolable monocarbonyl fraction, also appeared to be related to stabilization of hydroperoxides. Previous investigators (Day, 1960; Day and Lillard, 1960; Sinnhuber *et al.*, 1958; Patton and Kurtz, 1955) have reported that only a small percentage of the total measurable malonaldehyde exists free in oxidizing lipids, and Day (1960) has suggested that the remaining malonaldehyde results from degradation of hydroperoxides during the analysis.

Tarladgis and Watts (1960) observed that malonaldehyde production and the rate of oxygen absorption reached a maximum simultaneously in oxidizing fatty acids and that the rancid-odor intensity decreased concomitantly with the TBA number. In the present study, no attempt was made to evaluate odor intensity quantitatively. However, it appeared to increase throughout the oxidation period. The observed increase in volatile carbonyls (Table 1) and the reported high correlation between flavor intensity of oxidizing fat and volatile monocarbonyls (Lillard and Day, 1961) lends credence to this point.

Table 2 shows qualitative and quantitative composition of the volatile monocarbonyl fractions of the salmon oil. In earlier work, Yu *et al.* (1961) obtained evidence for tentative identification of a number of methyl ketones. The ketones were not found in the present work except as contributed by contaminated solvents. It is believed that the methyl ketones found by Yu *et al.* (1961) came from the same source. Compounds found in this investigation that were not reported by Yu *et al.* (1961) were the C_1, C_{10}, C_{11}, C_{12} alkanals; C_{12} alk-2-enal; C_6, C_8, C_9 alk-2,4-dienals.

Substantial amounts of volatile monocarbonyls were found in the fresh oil, and the total quantities continued to increase during autoxidation. Of possible significance was the observed coincidence of the TBA number maximum and the peak concentration for a large number of the alkanals. In fact, only hexanal continued to increase during oxidation, and if quantitative data were available for the C_1, C_2, C_3, and C_{12} alkanals of Sampling 4 (Table 2) it is probable that the total alkanal value would show a maximum at that point.

The volatile alkanal concentration increased at an early stage whereas no substantial increase was apparent in the unsaturated carbonyls until after the 16th day of oxidation. The large increase in the

Table 2. Volatile monocarbonyl compounds from autoxidizing salmon oil (mM/kg).

	Sample no.				
	1	2	3	4	5
Alkanals					
n-Dodecanal	.0029	.0017	.....	.....	.0126
n-Undecanal	.0023	.0022	.0058	.0709	.0194
n-Decanal	.0022	.0018	.0102	.0705	.0675
n-Nonanal	.....	.0019	.0058	.1292	.0606
n-Octanal	.0015	.0016	.0087	.1212	.0720
n-Heptanal	.0020	.0023	.0109	.0758	.0755
n-Hexanal	.0024	.0043	.0310	.2234	.2549
n-Pentanal	.0025	.0097	.0358	.1794	.0269
n-Butanal	.....	.0036	.0375	.0698	.0274
n-Propanal	.0079	.0421	.1287	.....	.1875
n-Ethanal	.0168	.0062	.0621	.....	.1600
n-Methanal	.0111	.0720	.1335	.....	.0514
Total	.0516	.1494	.4700	.9400	1.0517
Alk-2-enals					
Dodec-2-enal	.0008	.0006	.0010	.0031	.0407
Undec-2-enal	.0011	.0006	.0008	.0041	.0101
Dec-2-enal	.0016	.0015	.0018	.0139	.0275
Non-2-enal	.0010	.0010	.0025	.0291	.0407
Oct-2-enal	.0012	.0013	.0019	.0272	.0473
Hept-2-enal	.0023	.0022	.0025	.0595	.0891
Hex-2-enal	.0024	.0030	.0205	.0724	.2463
Pent-2-enal	.0054	.0034	.0262	.0451	.2386
But-2-enal	.....	.....	.0146	.....	.....
Total	.0159	.0135	.0717	.2543	.7412
Alk-2,4-dienals					
Dec-2,4-dienal	.0022	.0010	.0008	.0198	.0080
Non-2,4-dienal	.0012	.0004	.....	.0120	.0627
Oct-2,4-dienal	.0025	.0011	.0023	.0225	.0458
Hept-2,4-dienal	.0227	.0102	.0143	.1704	.1005
Hex-2,4-dienal	.....	.0009	.0029	.0441	.0471
Total	.0285	.0135	.0204	.2688	.2641

alkanals is accountable primarily by methanal and propanal, both of which could arise from the oxidation of terminal unsaturated fatty acids and the fatty acids with a double bond between the third and fourth carbons from the methyl end of the chain. These acids are very common in menhaden oils (Stoffel and Ahrens, 1960), and it is conceivable they would occur in salmon oil.

Table 3 shows qualitative and quantitative data for the Girard-isolable monocarbonyls. The qualitative composition of the various classes is comparable to that of the fraction isolated by distillation (Table 2). The Girard procedure was not as sensitive or consistent as the distillation procedure in detecting the compounds occurring at lower concentrations in the oil. For some unknown reason, some of the compounds were not detectable at consecutive sampling periods. This is especially noticeable for Sampling 4, in which the longer-chain members of each class were conspicuously absent. A recheck of the sample failed to reveal the missing compounds. To check this point further, a second sample of fresh salmon oil was oxidized to a comparable TBA number (2125) and the analysis of the Girard monocarbonyls again did not reveal the missing compounds.

When the individual monocarbonyls in the volatile fraction (Table 2) are compared with those of the Girard-isolable fraction (Table 3), in practically all cases the rela-

Table 3. Monocarbonyl compounds from autoxidizing salmon oil isolated with the Girard T reagent (mM/kg).

	Sample no.				
	1	2	3	4	5
Alkanals					
n-Dodecanal	.....	.....	.....	.....	.2883
n-Undecanal	.....	.....	.....	.....	.4580
n-Decanal	.....	.....	.3619	.....	1.2045
n-Nonanal	.....	.....	.4891	.....	.8991
n-Octanal	.....	.....	.4206	.....	1.0603
n-Heptanal	.....	.0328	.8706	.4669	1.0008
n-Hexanal	.1253	.1669	3.8345	1.7714	2.7226
n-Pentanal	.1473	.3036	6.5442	1.7714	.7548
n-Butanal	.....	.....	10.7700	11.9812	.6106
n-Propanal	1.8208	1.2328	30.5690	24.8687	2.4767
n-Ethanal	.2598	.7253	11.8360	9.4270	.6785
n-Methanal	2.0433	1.0537	32.1440	18.3666	2.0696
Total	4.3960	3.5200	97.8200	68.6600	14.2240
Alk-2-enals					
Dodec-2-enal	.....	.0065	.0056	.....	.0063
Undec-2-enal	.....	.0045	.0048	.....	.0208
Dec-2-enal	.....	.0076	.0286	.....	.0444
Non-2-enal	.....	.0056	.....	.....	.0679
Oct-2-enal	.....	.0140	.0413	.0755	.1160
Hept-2-enal	.....	.0118	.1255	.3186	.4804
Hex-2-enal	.....	.0101	.3440	1.7440	.4947
Pent-2-enal	.....	.0109	.0564	2.2247	.5808
But-2-enal	.....	.....	.0330	.2953	.0798
Total	.....	.0710	.6391	4.6580	1.8912
Alk-2,4-dienals					
Dec-2,4-dienal	.0101	.0206	.0047	.....	.1234
Non-2,4-dienal	.....	.....	.0152	.1352	.1898
Oct-2,4-dienal	.0101	.0422	.0116	.5407	.1234
Hept-2,4-dienal	.0403	.1669	.1625	2.8585	2.2965
Hex-2,4-dienal	.....	.....	.0174	1.0356	.3606
Total	.0605	.2297	.2113	4.5700	3.0936

tive amounts in the carbonyl classes are comparable for both fractions. Apparently the conditions of the Girard reaction cause degradation of the volatile carbonyl precursors in the oil.

A portion of the ethanal shown in Table 3 may have resulted from dehydrogenation of the ethanol used in the Girard reaction, in the manner described by Gaddis *et al.* (1961b). The amount could not have been significant, however, because the ethanal concentration closely followed the trend of the other compounds in the mixture during oxidation of the oil.

Methanal and propanal formed a large percentage of the alkanal fraction of the Girard carbonyls, and, again, the most feasible origin of these compounds would be as mentioned for the volatile fractions. An additional mechanism that may help account for methanal has been described by Bell *et al.* (1951), in which decomposition of the primary alkoxy radical could give rise to methanal and the alkyl radical:

$$R - CH_2O \cdot \rightarrow R \cdot + CH_2O$$

Relatively large amounts of methanal were also reported in oxidizing milk fats by Lillard and Day (1961). The decrease in methanal and propanal may be associated with the destruction of the fatty acid precursors. Similarly, the drop in the alk-2-enals and alk-2,4-dienals in advanced stages of oxidation is undoubtedly related to oxi-

Table 4. Volatile dicarbonyl compounds from autoxidizing salmon oil (m*M*/kg).

Fraction	Sample no. 1	2	3	4	5
1	.0019	.0150	.0742	.0213	.2211
2	.0345	.0065	.0550	.1455	.1982
3	.0027	.0098	.0631	.0923	.0734
4	.....	.0164	.0966	.0491	.3968

Table 5. Dicarbonyl compounds from autoxidizing salmon oil isolated with Girard T reagent (m*M*/kg).

Fraction	Sample no. 1	2	3	4	5
1	.0542	.4307	.6341	.7991	4.7725
2	.0511	.2193	1.000	1.2045	1.9545
3	.0890	.4636	1.059	1.3229	1.5225
4	.....	.....	.6159	1.7729	.....
5	.....	.....	.....	.8727	.....

dative destruction of the aldehydes and the polyunsaturated fatty acids.

The quantitative data for the bis-DNP-hydrazones are presented in Tables 4 and 5. Positive identification of these fractions is not complete. Recent work in our laboratories has indicated that the Wolfrom and Arsenault (1960) procedure does not give complete separation of mixtures of dicarbonyl derivatives such as isolated from oxidizing fats and oils. The percentage of volatile dicarbonyls in the volatile carbonyl fraction ranged from 17 to 35%. There was not a consistent trend in changes of the relative amounts of individual fractions in either the volatile or Girard-isolable dicarbonyls during oxidation of the salmon oil.

ACKNOWLEDGMENT

The authors thank R. O. Sinnhuber for helpful suggestions, and T. C. Yu for assistance in determination of the peroxide values and TBA numbers.

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