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Hydrogenated oils and polyunsaturated fatty-acid isomers in nutrition

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Hydrogenation is used to change normally liquid oils containing polyenoic fatty acids into semi-solid fats suitable for dietary and manufacturing purposes, e.g. oleomargarine and baking products. Vegetable and marine oils are most commonly used for partial hydrogenations. Vegetable oils contain polyenoic acids of no greater

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unsaturation than the trienoic acid, linolenic acid, which together with linoleic acid are the major polyunsaturated acids in this type of oil. Although the unravelling of the polyunsaturated fatty-acid pattern of marine oils has barely begun it is known that these oils contain only small amounts of essential fatty acids (EFA), but they do contain large quantities of highly unsaturated fatty acids, many of which have carbon chains with more than eighteen carbon atoms.

During recent years new analytical techniques have made possible a better understanding of the chemical reactions underlying the hydrogenation process. The products obtained by partial hydrogenation of an oil vary greatly, depending on the type of oil and no less upon the hydrogenation conditions used, i.e. type of catalyst, its concentration, hydrogen pressure, temperature, and agitation of the mixture during the reaction. Partial hydrogenation of an oil results not only in saturation of some of the double bonds but also in formation of a large number of isomers, geometrical and positional, of the polyenoic fatty acids. Geometrical isomerization and migration of double bonds during partial hydrogenation of oleate was described by Moore (1919) and by Hilditch & Vidyarthi (1929). However, not until recently has renewed interest in this field created a background for the understanding of the reaction mechanisms behind the isomerization processes. Space does not permit an extensive review of this literature.

Hydrogenation is a heterogenous catalysis whereby the double bonds of unsaturated fatty acids are brought into contact with the surface of a catalyst where the reactions take place. Studies by Boelhouwer, Gerckens, Lie & Waterman (1953), Allen & Kiess (1955, 1956*a*, 1956*b*) and Allen (1956) on isomerization by hydrogenation of monoenoic acids, linoleic acid, linolenic acid, and of conjugated dienoic and trienoic acids resulted in a theory of partial hydrogenation–dehydrogenation as an explanation of the simultaneous formation of both positional and geometrical isomers. Further, Emmet (1959) suggested that at least one of the reactants is chemically adsorbed to the catalyst. Thus, when an unsaturated fatty acid is adsorbed onto the surface of the catalyst, such as nickel powder, chemical bonds are established between the fatty acid and the catalyst, i.e. a double bond is 'opened'. The two free bonds created are attached to two of the nickel atoms. The fatty-acid-catalyst complex then reacts with a hydrogen atom and a semi-hydrogenated product is formed in which the fatty acid is still linked with the catalyst by one bond. At this stage several possibilities are open. If the complex reacts with one more hydrogen atom the original double bond is saturated. Another possibility is that the first adsorbed hydrogen atom can be released whereby the original double bond is re-established. However, desorption of a hydrogen atom can also occur at the carbon atom on the other side of the fatty-acid-catalyst link, then a positional isomer is formed because the double bond is shifted one place relative to its original location. This then explains the formation of conjugated and of isolated double-bond systems. Finally, the semi-hydrogenated fatty-acid–catalyst complex is a system with free rotation around the carbon–carbon link. This situation implies that subseque removal of a hydrogen atom and desorption from the catalyst will form the dou bond at the original location but either in the *cis* or the *trans* form. As will be se

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this theory apparently explains the formation of positional as well as of geometrical isomers as a result of partial hydrogenation of unsaturated fatty acids. Similar reaction patterns may possibly be followed in enzymically induced transformations of unsaturated fatty acids in vivo.

It is possible to influence the balance between isomerization and hydrogenation to some extent. Hydrogenation of commercial oils is usually carried out under highly selective conditions in which the more unsaturated fatty acids are hydrogenated first. The main factor determining the selectivity and isomer formation in the hydrogenation reaction appears to be the hydrogen concentration in or on the catalyst. Hydrogenation selectivity is low if the catalyst maintains a high concentration of hydrogen which to some extent can be regulated at will by high hydrogen pressure, thereby increasing the speed of the reaction and keeping the formation of *trans* fatty acids low. A similar effect is obtained by low concentration of the catalyst, fast agitation of the mixture and low reaction temperature. Conversely, low hydrogen concentration on the catalyst due to low hydrogen pressure, slow agitation of the mixture, high reaction temperature, and high catalyst concentration will keep the hydrogenation selectivity high, but unfortunately the rate of formation of isomers is also kept high. The type of catalyst is also of importance for this selectivity. Thus, platinum and palladium catalysts cause more isomerization than a nickel catalyst (Feuge & Cousins, 1960). Other factors of great importance are catalyst poisons which reduce the effectiveness of the catalyst and consequently change the selectivity and the rate of the reaction. Catalyst poisons are, e.g., sulphur compounds, carbon monoxide or ammonia in the hydrogen gas, or soaps, water, phosphatides and mineral acids in the oils. As an example of the importance of hydrogenation selectivity it can be mentioned that under conditions of high selectivity, linoleic acid reacts about fifty times faster than oleic acid, whereas it reacts only about four times faster in non-selective hydrogenation.

Triglycerides would be expected to behave on hydrogenation like methyl esters, but Feuge, Cousins, Fore, DuPre & O'Conner (1953), and Feuge, Pepper, O'Conner & Field (1951) found that under similar operating conditions triglycerides apparently hydrogenated less rapidly than methyl esters, and the amount of *trans* isomers formed was considerably less. Hydrogenation studies with linoleate (Allen & Kiess, 1956*a*; Cousins, Guice & Feuge, 1959) showed that not only are some double bonds saturated but others are transformed from *cis* to *trans* configuration, the divinylmethane arrangement of the double bonds is changed to conjugated systems, and some of the double bonds are moved along the carbon chain. The effect of operating conditions on migration of double bonds during hydrogenation of cottonseed oil was studied by Chahine, Cousins & Feuge (1958). They found double bonds in the positions 6–14 inclusive of the mono-unsaturated fatty acids resulting from the hydrogenation. They also noticed that even mild conditions of saponification cause some shifting of the double bonds of linoleic acid. Scholfield, Jones, Nowakowska, Selke, Sreenivasan & Dutton (1960) hydrogenated linolenate and found about 45% of *trans* acids to be present in both the monoene and diene fractions. Considerable

migration of double bonds from the original 9, 12 and 15 positions occurred, and *cis,cis* dienes were formed which could not be conjugated by alkali.

It is apparent that partial hydrogenation of an oil or of an unsaturated fatty-acid preparation results in a very complex mixture of fatty acids. Thus, an ordinary pentadiene compound like linoleic acid can form *cis* and *trans* forms of no less than nine positional isomeric monoenes. Formation of *trans* isomers tends to give products that are hard at low temperatures but soft at higher temperatures. Positional isomerization results in increasing melting point by conjugation of the double bonds, whereas a progressive decrease in melting point is observed as the distance between a *cis* double bond and the carboxyl group increases. With the *trans* forms no such variations are found.

In general the nutritive value of vegetable oils is high. Thomasson (1953*a*, 1955, 1956*a*) tested the biological value of a large number of fats and oils. He found differences in growth of rats due to a difference in food intake, and a significant correlation between rate of absorption and growth-promoting action of the oils. The decreased rate of absorbability of various oils and fats may perhaps be explained by differences in their fatty-acid composition. The high content of erucic acid, a C_{22} monoenoic acid, in rapeseed oil may thus account for the slow digestion of this fat.

Marine oils, such as whale, herring, seal, menhaden and tuna oil, support good growth in rats (Thomasson, 1953*a*,*b*; Aaes-Jorgensen, 1954; Thomasson & Gottenbos, 1957; Privett, Aaes-Jorgensen, Holman & Lundberg, 1959; Ahrens, Insull, Hirsch, Stoffel, Peterson, Farquhar, Miller & Thomasson, 1959; Privett, Pusch, Holman & Lundberg, 1960). Regarding the content of EFA in these oils, Privett *et al.* (1960) concluded that tuna, menhaden and herring oils contain about 2% of acids belonging to the linoleate family. It is in this connexion interesting to note that Privett *et al.* (1959) found no relief of the dermal symptoms in EFA-deficient rats by supplementation with the polyunsaturated acid esters (600 mg/animal week) from tuna oil, whereas the C_{18}, C_{20} and C_{22} polyenoic-acid esters stimulated growth. The latter result agrees well with the fact that data from many laboratories (e.g. Thomasson, 1956*b*; Klenk, 1958; Klenk & Debuch, 1959) indicate that the major part of the polyenoic acids from marine oils belong to the linolenic-acid family. Acids of this type are known to support growth but do not relieve or prevent the characteristic dermal signs of EFA deficiency.

It is obvious that partial hydrogenation of marine oils will further reduce the original low content of EFA. Therefore, hydrogenated marine oils should only be incorporated into dietary products mixed with oils or fats of a high EFA content, or EFA should be otherwise provided. Partial hydrogenation of vegetable oils is usually less risky from this point of view because of their larger initial content of EFA. An example of the presence of polyenoic-acid isomers in dietary fat was furnished by the examination of typical American margarines and shortenings by Mabrouk & Brown (1956). They found a mean content of *trans* acids of about 30%, besides a large number of positional isomers, especially as conjugated fatty acids. The nutritive value of hydrogenated oils has been studied in several different laboratories, of with rather conflicting results. A discussion in detail of this matter is not poss

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here, but a few more general considerations can be made. Unsaturated fatty acids occur in triglycerides, phosphatides, phosphatidic acids, lipoproteins, proteolipids, esters with steroids and the like, they are present in biological membranes, enzyme systems, and interphases, and take part in vital physiological processes. With this background, a possible relationship has been suggested between the presence of isomers in partially hydrogenated oils and the accentuation of EFA deficiency observed when such oils are given as the sole dietary fat to experimental animals (Aaes-Jørgensen, 1954; Aaes-Jørgensen, Funch, Engel & Dam, 1956; Funch, Aaes-Jørgensen & Dam, 1957). It may be in favour of such a theory that hydrogenated marine oils accentuate EFA deficiency more than hydrogenated vegetable oils (Aaes-Jørgensen, 1954). However, Burr (1942), Deuel, Greenberg, Anisfeld & Melnick (1951), and later Peifer & Holman (1959) suggested that the growth-depressing effect of dietary hydrogenated oils could be explained by an increased demand for EFA with increasing amounts of saturated fat in the diet. Thomasson & Gottenbos (1957) concluded that the cause of the changes in growth effect due to hardening of oils is related to the increase in content of saturated fatty acids and to the presence of fatty acids with twenty or more carbon atoms. A somewhat similar explanation was suggested by Funch *et al.* (1957) from experiments with trilaurin or hydrogenated arachis oil as dietary fat.

It should be emphasized that, in animals on diets containing hydrogenated oils, supplementation with adequate amounts of EFA overcomes the growth retardation and prevents the development of the dermal symptoms (Funch *et al.* 1957; Funch, Jart & Dam, 1960; Thomasson & Gottenbos, 1957). The last authors obtained an even better growth response with such supplementation than with the original oils.

Another way of studying the nutritive effect of unsaturated fatty-acid isomers is by incorporation, in otherwise fat-free diets, of oils rich in one or more of these isomers, or of isolated isomers. Miller & Burr (1937) gave tung oil to mature rats and found that elaeostearic acid was converted into dienoic acid. Reiser (1951) fed laying hens on a diet with conjugated trilinolein or tung oil. Conjugated linoleate appeared in the egg, primarily in the phospholipids, whereas α-elaeostearin appeared most prominently in the neutral fat along with large quantities of newly synthesized dienoic acid. Christensen, Dam & Engel (1957) fed young rats on a diet with 28% hydrogenated arachis oil containing less than 0·1% of conjugated dienoic acids. The content of conjugated dienoic acid in the subcutaneous tissue increased to a maximum of about 2%. This maximum coincided with growth retardation of the rats. They suggested that this retardation may have been due to an unfavourable effect of the conjugated acids as such, or of a high dietary content of fat without EFA. Recently, Kaufmann & Dransfeld (1960) studied the absorption and distribution in the tissues of the seed oil of *Impatiens glanduligera* which contains parinaric acid (a C_{18} conjugated tetraenoic acid). The oil was found to be non-toxic to rats and rabbits. When the oil was administered as a single dose, parinaric acid appeared first in the mesenteric, then in the perirenal and last in the subcutaneous and perigenital fatty tissues. The order of appearance of parinaric acid was reversed, as was the turn-over rate, when the oil was given for longer periods of time,

The nutritive effect of purified conjugated linoleate and of the *trans* isomers, linolelaidate and elaidolinolenate, on EFA-deficient rats was studied by Holman (1951). Conjugated linoleic acid intensified the deficiency signs. The *trans* isomers were inactive as EFA. The primary product of linoleate oxidation is found to be a hydroperoxide with conjugated double bonds (Bolland & Koch, 1945; Privett, Lundberg, Khan, Tolberg & Wheeler, 1953). Holman & Greenberg (1954) gave linoleate peroxide, reduced linoleate peroxide, decomposed linoleate peroxide, and conjugated linoleate to EFA-deficient rats. None of these compounds stimulated arachidonate synthesis, the dermal signs were accentuated, growth rate was decreased, and water consumption remained high. They concluded that these compounds containing conjugated double-bond systems exhibit a toxic effect. Upon feeding weanling rats with conjugated isomers or dienoic and trienoic acids for 17 weeks, Aaes-Jørgensen (1958) found no significant adverse effects on growth rate or on the development of dermal signs. Examination of the polyenoic fatty-acid pattern in various tissues revealed that in general only the content of conjugated dienoic acids was affected markedly by the dietary supplementation. They occurred in large amounts in the depot fat, and conjugated *cis, trans*-linoleate and to a lesser degree ethyl-α-elaeostearate increased the content of conjugated dienes in the lipids of the heart. Apparently very little, if any, of the conjugated compounds was deposited in the lipids of testes or brain. It was concluded that the small amounts of conjugated polyenoic acids usually present in hydrogenated oils do not explain the dietary effects observed in experiments with such oils as the sole dietary fat.

Allen, Kiess, Johnston & Kummerow (1958) fed rats for 16 days on a diet with synthetic triglycerides containing acids with the *trans* bond in the 9 or 8 position. They found the *trans* acids to be efficiently metabolized. This finding was further supported by Johnston, Johnson & Kummerow (1958) who found *trans* acids to be deposited in the tissues only when they were present in the diet. The largest amounts were found in the carcass fat, smaller amounts in the liver, and only small quantities were excreted in the faeces; the major portion of the ingested *trans* acids was metabolized. Removal of the *trans* acids from the diet resulted in gradually decreased amounts in the tissues. Dietary *trans* fatty acids did not inhibit growth when added to a diet which contained 2% of soya-bean oil as an EFA source. As mentioned by Johnston *et al.* (1958), the discrepancy between their results and those of Holman & Aaes-Jørgensen (1956) may very well be due to the fact that the latter authors were carrying out a curative experiment by supplementing the diet of severely EFA-deficient rats.

In studies by Privett, Pusch & Holman (1955) the *cis*-9,*trans*-12-isomer of linoleate was found not to have EFA activity and no 'sparking' effect on the utilization of linolenate. *Trans* double bonds derived from the isomer were retained by the animals and deposited in the tissue. Recently Mattson (1960) depleted rats of EFA and then conducted curative experiments by supplementing their diet with 50 or 100 mg/day of linoleate, *cis,trans*- or *trans,trans*-linoleate, oleate or elaidate, respectively, for 4 weeks. Dermal signs were not reported. On the basis of gain in body weight during this rather short period of supplementation it was concluded that th

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various isomers did not interfere with the utilization of *cis,cis*-linoleate or increase the requirement for EFA. Further, spermatogenesis was normal in the EFA-deficient animals. This latter statement is not surprising since interference with spermatogenesis occurs only after long periods of EFA depletion. Further, even very small amounts of EFA can delay or abolish this impairment (Aaes-Jørgensen *et al.* 1956; Funch *et al.* 1957).

Several experiments seem to indicate that monoenoic *trans* acids apparently are treated in the organism as ordinary fatty acids (Alfin-Slater, Aftergood, Bingemann, Kryder & Deuel, 1957; Thomasson & Gottenbos, 1957), which may not be surprising as it is known that the organism can synthesize various monoenoic acids. Further, *trans* isomerization by intestinal bacteria occurs in ruminants as well as in non-ruminants (Hartman, Shorland & Cleverley, 1958). However, it has been shown in rats that *trans* fatty acids behave differently in at least one respect, namely *trans* fatty acids are not transferred from mother to foetus, but the amount of *trans* acids increases markedly when the young are allowed to suckle mothers on a diet rich in this type of acid (Johnston, Johnson & Kummerow, 1957). A similar picture was seen in human beings (Johnston, Kummerow & Walton, 1958).

It is apparent that several questions regarding the nutritive value of hydrogenated oils are still open for discussion. Some confusion has occurred because occasionally experiments have been compared without enough attention being paid to the fact that some were done with normal and others with EFA-depleted animals. Further, so many isomers are involved that the specific character of those used in the experiments to be compared should be kept in mind. As mentioned above, several reports indicate that hydrogenated oils given as the sole dietary fat have a much poorer nutritive effect than the same oils ordinarily refined, and even markedly poorer than a fat-free diet. A suggested explanation, besides EFA deficiency, is that the increased content of saturated fatty acids formed by hydrogenation in turn may further increase the demand for EFA. However, a biochemical basis for this suggestion is still lacking.

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Fish Oils as a Source of Essential Fatty Acids

INTRODUCTION

The fatty acid spectrum of fish oils is very complex as discussed in Chapter 1. Narrowing consideration of composition to the variation in unsaturated fatty acids, the fish oils still leave us with a high degree of complexity, especially because of the presence of large amounts of tetra-, penta-, and hexaenoic acids mainly of chain lengths C_{18} to C_{22}. A further limitation of the field to survey unsaturated fatty acids with respect to their action as essential fatty acids (EFA) requires consideration not only of the huge variety of unsaturated fatty acids in fish oils, but also to the location of and the configuration at the double bonds. A thorough screening of these many acids was never possible until the development of modern analytical procedures such as gas chromatography, thin-layer chromatography, and mass-spectrometry.

Fish oils used in experimental diets result in a very complicated fatty acid picture in the animal tissues, where *in vivo* conversions are known to take place, i.e., chain elongation and desaturation especially of C_{16}-, C_{18}-, and C_{20}-mono-, di-, and trienoic acids. Furthermore, refined and deodorized fish oils are often partially hydrogenated for commercial purposes, e.g., for production of margarine. Partial hydrogenation of highly unsaturated oils results in a most complex mixture of unsaturated fatty acids including positional and geometrical isomers.

It is the purpose of this chapter to give some of the main trends in the literature on fish oils as a source for information on essential fatty acids.

The criteria for a polyenoic acid belonging to the group of essential fatty acids are: a long chain with *cis*-double bonds in divinyl methane arrangement, and the first double bond in position 6 counted from the methyl end of the molecule (ω-6-position)[1]. This type of fatty acids is now described as belonging to the "linoleic acid family", Thomasson (1953), Klenk (1953); examples are linoleic acid (ω-*cis*-6, *cis*-9-octadecadienoic acid), γ-linolenic acid (ω-*cis*-6, *cis* 9, *cis* 12-octadecatrienoic acid), and arachidonic acid (ω-*cis* 6, *cis* 9, *cis* 12, *cis* 15-eicosatetraenoic acid).

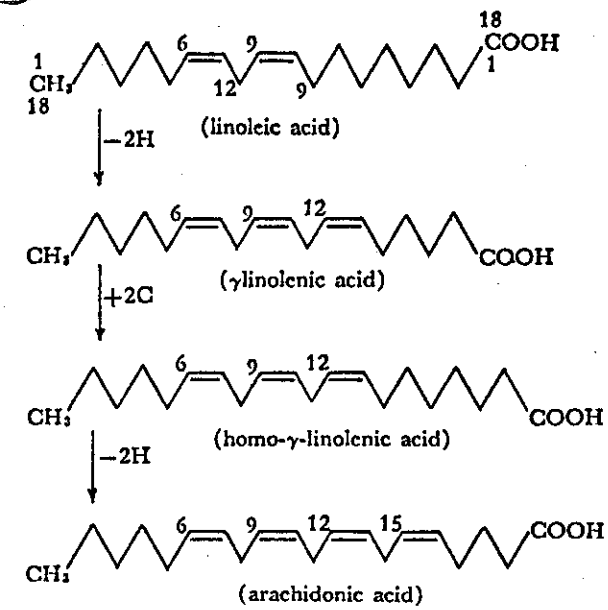
[1] In the text the sign Δ in connection with an unsaturated fatty acid, e.g., Δ 10,-13-nonadecadienoic acid, indicates numbering of the carbon atoms from the carboxyl group.

Only polyenoic acids belonging to the linoleic acid family are considered here as essential fatty acids, i.e., they can prevent or cure the symptoms comprised by the term: the EFA syndrome (Burr and Burr 1929, 1930; Aaes-Jørgensen 1961A). Thus, EFA restore growth and cure the characteristic dermal symptoms in EFA deficient animals; they prevent damage of kidney and reproductive tissues, and prevent a piling up of eicosatrienoic acids in tissues of experimental animals fed on EFA-free or EFA-deficient diet.

Other unsaturated fatty acids of biochemical interest in this connection are the acids belonging in a similar manner to the "oleic acid family," i.e., ω-*cis* 9-octadecenoic acids, to the "linolenic acid family," i.e., ω-*cis* 3, *cis* 6, *cis* 9-octadecatrienoic acids, and to the "palmitoleic acid family," i.e., ω-*cis* 7-hexadecenoic acid. None of the fatty acids of these latter families have EFA activity in the sense that they can cure EFA deficiency. However, acids of the linolenic acid family (ω-3-acids) can promote growth in EFA deficient animals. The ω-9,12,15- and ω-7,10,13-eicosatrienoic acids of the oleic and palmitoleic acid families, respectively, are characteristic for and occur in tissues of EFA deficient animals (Klenk and Oette 1960; Klenk and Pflüger 1963). These acids together with arachidonic acid and ω-7,10,13,16 eicosatetraenoic acid (palmitoleic acid family) can be considered endogenous in animals reared on fat-free diets. Therefore, the ratio of trienoic and tetraenoic C_{20}-acids has been suggested as a chemical indicator of EFA deficiency, Holman (1960), Aaes-Jørgensen (1961). In tissues of normal animals the content of eicosatrienoic acids is very low.

The background for the convenience of using the term "families" in connection with these unsaturated fatty acids is related to enzymatic processes by which the various types of unsaturated fatty acids are further desaturated and/or have their carbon chains extended (Stoffel 1963; Klenk 1965).

In vivo desaturation of the unsaturated fatty acid chain takes place only in the proximal part of the molecule, i.e., between the carboxyl group and the nearest double bond. A new double bond can be introduced only where there are five or more carbon atoms between the carboxyl group and the nearest double bond. All double bonds are methylene interrupted. The introduced double bonds all have *cis*-configuration. Further, chain elongation is also limited to the proximal end of the molecule only. These reactions can be illustrated by the scheme for the *in vivo* conversion of linoleic to arachidonic acid (Mead and Howton 1957).



FISH OILS AS A SOURCE OF EFA

Many nutritional experiments have been carried out with marine oils as the major dietary fat component. However, we shall limit ourselves to discuss the fish oils. In general, fish oils may be described as flesh oil, liver oils, or as oils of the whole fish. A description of the fatty acid composition of the various types of fish oils can be found, e.g., in Hilditch (1956), Hilditch and Williams (1964), Lovern (1938, 1942), Black and Schwartz (1950), Deuel (1951), Swain (1958), Notevarp (1961) and Tsuchiya (1961), and are also considered in detail in Chapter 1. These reviews preferentially list the fatty acids according to unsaturation, i.e., as dienes, trienes, etc. as obtained by alkali-isomerization analyses. Data of this type indicate that fish oils may, or may not, contain EFA. This uncertainty is related to the fact that small amounts of e.g., linoleic acid in the presence of large amounts of highly unsaturated fatty acids are difficult to determine by means of the alkali-isomerization technique. This procedure was introduced by Mitchell, Kraybill, and Zscheile (1943), described in detail by Holman (1957), and further developed by White and Quackenbush (1959). The method is based on conjugation of the double bonds in divinyl methane arrangement by means of heat and alkali. The content of conjugated dienoic, trienoic acids, etc. can be determined by spectrophotometric measurements at their respective absorption maxima in the ultraviolet region. However, data obtained by this procedure give no information concerning the "familial" relationship amongst the various unsaturated fatty acids. Such analyses of fish oils indic[illegible]at linoleic acid was not present, or in very small amounts; also linolenic acid occurs in small amounts only. These data were in agreement with nutritional experiments with fish oils.

In 1953, Thomasson described a biological test for EFA potency of various oils based on growth rate under conditions of restricted water intake. The activity of 10 mg. linoleic acid was defined as a unit of EFA activity. Thomasson found the activity of herring and menhaden oils to be 7.9 and 4.4 units/g, respectively. Peanut oil had 31.1 units/g. Fish oils are thus not entirely devoid of EFA.

Similar results were obtained in our laboratory, Aaes-Jørgensen (1954). Herring oil as the sole dietary fat (7% or 28%) added to a fat-free basal diet to rats gave a growth rate similar to that obtained with diets containing 7% or 28% lard instead of herring oil. Neither were any EFA deficiency symptoms observed during the experimental period of 14 weeks.

Based on comparison of the biological value of a large number of fats and oils, Thomasson (1956A) suggested that fatty acids with 20 or more carbon atoms have an unfavorable influence on the growth of young animals. Further, herring oil showed much less growth-promoting effect than did, e.g., summer butter fat. The growth-promoting effect of herring oil decreased with increasing amounts of the oil in the diet. In a following report, Thomasson (1956B) investigated the rate of absorption of several different oils and fats. The studies showed that herring oil was absorbed much more slowly than, e.g., butterfat.

ANALYTICAL DATA OBTAINED BY MODERN TECHNIQUES

Developments during the 1950's in the techniques and methods used in lipid analyses, e.g., GLC, TLC, ozonolysis, infrared spectroscopy, mass-spectrometry, and nuclear resonance spectral analysis, makes it now possible to obtain a much more detailed picture of the fatty acid pattern in fish oils describing not only chain length and degree of unsaturation but also the location of and the configuration at the double bonds within the single fatty acids.

Thus far, thorough analysis of the fatty acid spectrum of a limited number of fish oils have been carried out. The composition e.g., of herring oil has been studied in great detail by Klenk and Brockerhoff (1957, 1958), Klenk and Steinbach (1959), Klenk and Brucker-Voigt (1961). Recently, Lambertsen and Braekkan (1965A) reviewed data from the literature on the fatty acids in herring oil, and reported the acid composition of white muscle, red muscle, liver and roe of herring. Gruger, Nelson, and Stansby (1964) studied the fatty acid pattern of oils from a large number of different fish. Very thorough analyses of menhaden oil have been carried out

by Stoffel and Ahrens (1958, 1960). They found only *cis* double bonds in divinyl methane arrangement, no *trans* or conjugated unsaturated fatty acids. Similar results were obtained by Hashimoto, Nukada, Shiina and Tsuchiya (1963) by means of nuclear magnetic resonance spectral analysis on highly unsaturated fatty acid esters from fish oils. Recently, gas chromatographic data on the unsaturated fatty acids in marine oils were reviewed by Ackman (1964). The fatty acid composition of menhaden oil was examined by Ackman and Jangaard (1963), and of cod flesh by Ackman and Burgher (1964A).

The general trend of the fatty acid composition of fish oils is that acids of the linoleic acid family are present in very small amounts in fish oil, whereas acids of the linolenic acid family are much more abundant. Certain Japanese reports have described unsaturated fatty acids with ethylene interruption of the double bonds. However, such acids seem not to occur in fish oils, Stoffel and Ahrens (1959). Monoenes often dominate in the C_{18}-, C_{20}-, and C_{22}-acids. Tetraenes occur in acids from C_{16} to C_{22}, generally in smaller amounts, penta- and hexaenes are found in the C_{20}- and C_{22}-acids. It should be kept in mind though, that the fatty acid composition of fish oils can vary greatly depending on species, state of feeding, reproduction cycle, season and fishing area, see e.g., Kaufmann and Miyakawa (1958), Jacquot (1961).

RECENT NUTRITIONAL EXPERIMENTS

The uncertainty whether fish oils contain sufficient acids of the linoleic type to meet the needs for EFA led to studies of the *in vivo* activity of concentrates of polyunsaturated acids from tuna oil, Privett, *et al.* (1959). The acids were separated according to chain length, 16, 18, 20, and 22 carbon atoms, and fed as methyl esters (600 mg. per animal/week) to EFA-deficient rats for six weeks. With exception of the C_{16}-fraction, all stimulated growth. But none of the esters of any of the fractions relieved the EFA deficiency symptoms. The results indicate that most of the polyunsaturated acids of tuna oil belong to the linolenic acid family. Further, that inadequate amounts of acids of the linoleic acid family were supplied by the given amounts of these fatty acid fractions.

In a following study, Privett *et al.* (1960) fed EFA-deficient rats on diets containing ten per cent of tuna, menhaden, or herring oil, respectively, for 12 weeks. The results showed that all three oils cured the dermal symptoms and restored growth. These experiments also comprise groups of weanling rats fed on a fat-free basal diet. Some of these latter animals were supplemented daily with 200 mg./rat of the ethyl esters of the same three oils for 14 weeks. The dermal symptoms of EFA deficiency developed equally as fast, and as severely, in the animals receiving the fish oil

esters as in the animals fed the fat-free diet. But the g[illegible]n rates of rats on the fish oil esters compared favorably with that of animals receiving an equivalent amount of ethyl linoleate. After 14 weeks of experiment the oral supplementation of the various esters was discontinued, and they were then administered as ten per cent of the basal diet for an additional 14 weeks. These much larger amounts per animal/day cured the EFA-deficiency in the order of decreasing effectiveness by tuna, menhaden, and herring oils. The authors estimated the content of acids of the linoleic acid family to be approximately two per cent of these oils.

From their analyses of menhaden oil, Stoffel and Ahrens (1959) found a total of 2.6% ω-6-acids, a figure in line with the bioassay value of four units/g found by Thomasson (1962).

Edwards and Marion (1963) studied the effect of feeding menhaden oil to EFA-deficient chicks. Addition of four per cent menhaden oil to the essentially fat-free basal diets improved the growth rates significantly compared to that of chicks on the basal diet alone. Analysis of the liver fatty acids showed no increase of the octadecadienoic and eicosatetraenoic acids when the diets were supplemented with menhaden oil, but the high content of eicosatrienoic acid in the EFA-deficient chicks was reduced to zero. Further, significant amounts of eicosapentaenoic and docosahexaenoic acids were found in the liver only when menhaden was added to the diet. In one experiment, 22 weeks old laying hens were fed diets with 5% of coconut, corn, or menhaden oil, or 2.5% corn oil plus 2.5% menhaden oil, or no fat for 14 weeks. Eicosatrienoic acid was found in liver and heart tissues of hens receiving the basal and the coconut oil diets. This acid was not detected in eggs, serum, heart, or liver tissues of hens fed corn oil or menhaden oil. The level of eicosatrienoic acid in tissues was found to be a valid indicator of linoleic acid deficiency, except when the long chain fatty acids of menhaden oil were included in the diet. These results suggested that the latter acids inhibit the biosynthesis of eicosatrienoic acid from oleic acid.

Klenk *et al.* (1961) gave Δ 9,12-hexadecadienoic and Δ 6,9,12-hexadecatrienoic acids isolated from herring oil to EFA-deficient rats. These acids had no EFA activity, and interestingly enough, were not converted *in vivo* to long chain, further unsaturated fatty acids. The explanation of the latter fact appears to be that a double bond located equivalent to the Δ 9,10-position in a C_{18}-acid (i.e. Δ 7,8-position in a C_{16}-acid) is a prerequisite for desaturation and chain elongation *in vivo*. This conclusion is further substantiated by similar experiments with a number of positional isomers of C_{16}- and C_{18}-acids, Klenk (1965), and for odd-numbered unsaturated fatty acids by Schlenk, Sen, and Sand (1963); Sen and Schlenk (1964).

A number of recent reports have shown that the presence of relatively large amounts of polyenoic acids of one unsaturated fatty acid family depresses the *in vivo* conversion, i.e., desaturation and/or chain elongation, of unsaturated fatty acids of other fatty acid families. Thus, the conversion of linoleate to arachidonate is depressed by linolenate and to a smaller extent by oleate, Machlin (1962), Mohrhauer and Holman (1963A, 1963B), Bozian and Coniglio (1962, 1963).

As mentioned previously, in the EFA-deficient animals oleic acid is converted to Δ 5,8,11-eicosatrienoic acid, and palmitoleic acid to Δ 7,10, 13-eicosatrienoic and Δ 4,7,10,13-eicosatetraenoic acid. These conversions are depressed by supplementation of the diet with linoleic or arachidonic acid. A similar effect is observed with geometric isomers of linoleate, Privett and Blank (1964), and of long chain, highly unsaturated fatty acids as discussed above. These topics are now being studied in several laboratories. The presence in fish oils of large amounts of monoenoic acids and not least of polyenoic acids gives good reasons for speculations about the regulation mechanisms governing the biosynthesis of the highly unsaturated fatty acids in fish oils. Such considerations, of course, also involve the dietary fatty acids of the fish. These problems become not less interesting considering the presence in fish oils of positional isomers and of odd-numbered unsaturated fatty acids.

Many types of fish oils and polyenoic acids as such have been studied not only in connection with their EFA activity but also in relation, e.g., to blood cholesterol. This matter, however, is not linked exclusively to the essential fatty acids, e.g., Ahrens *et al.* (1959).

FISH OIL ODD-NUMBERED FATTY ACIDS AS EFA SOURCE

Straight-chain odd-numbered fatty acids are now known to occur as normal constituents of most natural lipids, although generally in small amounts. Isolation and structure determination have only been carried out for a limited number of these acids as yet. Further, the EFA activity of these acids has only been studied occasionally. However, considering the facts that certain fish oils are very rich in odd-numbered fatty acids, and that the previous discussion of the biological importance of the location of the double bonds in the molecule, an improved knowledge of these fatty acids certainly is most desirable.

Karrer and Koenig (1943) found no EFA activity of Δ 10, 13-nonadecadienoic acid (ω-6,9-C_{19}-acid) when fed to EFA-deficient rats. They gave 50 mg. fatty acid/animal/day for 21 days.

By means of his bioassay procedure, Thomasson (1953) tested a number of different polyenoic acids for EFA activity. The Δ 10,13-nonadecadienoic acid and Δ 7,10,13-nonadecatrienoic acid (i.e., an ω-6,9,12 acid) both showed a slight growth promoting effect on E[illegible]eficient rats. Schlenk, Sen, and Sand (1963) gave methyl linoleate or Δ 10,13-nonadecadienoate (1 gm. per animal/day) for five days to fat-deficient rats on a restricted daily ration. Then the fatty acid pattern of heart, liver, kidney, and spleen was studied. Feeding of linoleate resulted in a high content of arachidonic acid, whereas neither a C_{19}-trienoic nor a C_{21}-tetraenoic acid was detected in the animals given the C_{19}-dienoic acid. On the other hand, appreciable amounts of the most likely degradation product, Δ 8,11-heptadecadienoic acid was found. This proves that the nonadecadienoic acid was absorbed and metabolized. The authors suggest that an explanation of the non-conversion of the Δ 10,13-nonadecadienoic acid into higher unsaturated acids by the fat-deficient rats might be an unfavorable distance between the carboxyl group and the nearest double bond. Rahm and Holman (1965) gave weanling rats a fat-free diet supplemented with synthetic methyl Δ 10,13-nonadecadienoate (about 0.8% of the calories) for 59 days. The C_{19}-dienoic acid showed no EFA-activity as judged by chemical and biological symptoms of fat deficiency.

Gruger, *et al.* (1960) showed mullet body oil to be very rich in odd-numbered polyenoic acids. The amounts and the structure of the odd-numbered fatty acids of this oil was studied in detail by Sen and Schlenk (1964). They found 25% of the fatty acids to be straight-chain, odd-numbered acids, half of which were unsaturated. Regarding the position of the double bonds in the odd-numbered polyenoic acids the vinyl-methane arrangement prevailed, and numerous homologes were found having their double bonds in identical positions relative to the carboxyl group. Apparently the Δ-9 position in the odd-numbered unsaturated fatty acids is a prerequisite for a following *in vivo* elongation and/or desaturation. Thus, the Δ 9-C_{15}-acid can be further desaturated but no chain elongation takes place. Whereas the Δ 9-C_{17} acid is suitable for desaturation and elongation in a similar manner as the Δ 9-C_{18} acid. By far the majority in number and amounts of the polyenoic acids in mullet oil can be derived from the Δ 9-C_{15} and Δ 9-C_{17} acids. A Δ 9-C_{19} monoenoic acid was not found, but a corresponding Δ 9,12-C_{19} dienoic acid was present. The C_{21}-unsaturated fatty acids have not been investigated yet. These data indicate that the C_{15}- to C_{18}- unsaturated fatty acids of mullet oil possess the same proximal structure but different terminal structures. Further, the proximal end of these acids is important regarding whether chain elongation and desaturation takes place or not. A classification referring to the carboxyl group of the odd- and even-numbered acids of mullet oil apparently specifies the 9-10 position as crucial for interconversion which may involve plant or vertebrate desaturation, and it distinguishes the chain lengths of 9-monoenes in regard to their ability to elongate in the course of

further desaturation. The authors emphasize that it is yet uncertain to what extent such classification of the unsaturated fatty acids will lead to positive predictions about the physiological importance of these acids for higher animals. This different concept, however, may possibly contribute to a better understanding of the earlier definition of structures prerequisite for essentiallity of the unsaturated fatty acids.

FISH-LIVER OILS AS A SOURCE OF EFA

Fish-liver oils have mainly been studied for other purposes than their EFA content. However, after the discovery of the essential fatty acids, many different oils have been tested for their ability to cure the skin lesions of EFA deficiency. Amongst these oils were also cod-liver oil.

Sinclair (1929–1930) found that supplementation of a fat-free diet with ten per cent cod-liver oil did not cure the skin signs. Similar results were obtained by Graham and Griffith (1930–1931) by a daily dose of 9–12 drops of cod-liver oil. But Burr *et al.* (1930–1931) noticed that 2–5 drops of cod-liver oil fed daily to EFA-deficient rats renewed growth though the scaly tail was not cured. Also McAmis *et al.* (1929) had observed weight increase of fat-deprived rats by adding 0.02 cod-liver oil to the daily ration. These results indicated that linoleic acid was not present in cod-liver oil. Further, the growth promoting effect of this oil on EFA-deficient animals could probably be ascribed to the presence of linolenic acid. Hume *et al.* (1938) tested the methyl ester of a docosahexaenoic acid isolated from cod-liver oil on EFA-deficient rats. It was found to be potent in promoting weight increase but to have little or no action in curing skin lesions.

Since then the pattern of the unsaturated fatty acids in cod-liver oils has been studied in several laboratories. Modern analytical techniques have also in this case greatly improved the possibilities for obtaining detailed information about the fatty acid composition of these oils. Data of this type are obtained in a number of different laboratories, Klenk and Eberhagen (1957), Klenk and Brockerhoff (1958), Klenk and Eberhagen (1962), Kaufmann and Khoe (1964), Ackman and Burgher (1964A), Gruger, Nelson, and Stansby (1964). Ackman and Burgher (1964B) studied cod-liver oil by gas chromatographic analysis and found a number of acids which are not listed in recent comparable studies. They also examined the fatty acid spectrum of cod roe, Ackman and Burgher (1964C). The component fatty acids of cod roe resembled the flesh lipid fatty acids to a considerable degree.

Lambertsen and Braekkan (1965B) made extensive analyses of the fatty acid composition of cod-liver oil and compared their data with those from nine other laboratories. The octadecadienoic and octadecatrienoic acids

amounted each to 1 to 2%, while the eicosatetraenoic acid made up about 1.5% of the total fatty acids. The authors also listed the polyenoic acids according to families. They found the content of ω-6-acids, i.e., acids structurally related to linoleic acid, in cod-liver oil to be of the order of 1.4.% Those related in a similar way to linolenic acids amounted to about 25% of the total fatty acids.

Seasonal variations in the unsaturated fatty acid pattern of cod-liver oil was studied by DeWitt (1963). He found maximal iodine values during the winter season, and an abrupt fall in March when the cod spawns. These changes corresponded to complementary changes in the total unsaturated fatty acids, while the saturated fatty acids of the oil remained virtually constant throughout the cycle.

The improved knowledge on the composition of fish-liver oils has renewed the interest for biological evaluation of, e.g., the EFA activity of these oils.

By means of his biological assay, Thomasson (1953) found 3.9 biological activity units/gm. in cod-liver oil.

Ershoff (1960) gave immature rats a purified low-fat diet supplemented with ten per cent of cod-liver oil, crude or refined tuna oil, crude sardine oil, or menhaden oil for 14 to 25 days. He found a highly significant growth retardation accompanied by diarrhea in all experimental groups. These effects were largely counteracted by concurrent administration of cottonseed oil at a ten per cent level in the diet. Methyl linoleate when fed at levels of 1, 5, or 10% of the diet, was without protective effect, suggesting thereby that the beneficial effect of the vegetable oil was caused by some factor or factors other than its EFA content. α -Tocopherol at levels of 0.025, 0.05, or 0.1% in the diet had a significant protective effect which probably is related to its antioxidant properties. On the other hand, Ward and Amos (1962) found no growth retardation or diarrhea in rats fed on a basal low-fat diet to which was added ten per cent of cod-liver oil or of cod-liver oil with an initial peroxide value of 20 for 29 days. Dietary supplementation with antioxidants did not influence the growth rate. In the present studies Wistar albino rats were used, while Ershoff used rats of the Holtzman strain. However, in both studies the experimental period was very short.

Recently, Reed (1964) studied the EFA and hypercholesterolaemic activity of cod-liver oil in rats. The diet of EFA-deficient and hypercholesterolaemic rats (5 females and 4 males) was supplemented with 2% of cod-liver oil for 13 weeks. According to gas chromatographic analyses the cod-liver oil contained 1.8% octadecadienoic, 0.8% octadecatrienoic, and 1% eicosatetraenoic acid. Cod-liver oil restored growth and improved the EFA deficiency score of the fur. In the females, dermal symptoms on tail

and hind legs were not cured, but conception was possible. The litters produced were born dead or died shortly after birth or were eaten by the mothers. In the males, scaliness of the tail and hind legs persisted throughout the experiment, the weight of the testes was normal, and the animals were fertile. It is interesting that the $C_{20:3}/C_{20:4}$ ratio was not raised in the rats supplemented with cod-liver oil. However, identification of the fatty acid in these two groups of polyenoic acids probably is necessary for a decisive discussion of this ratio in relation to EFA deficiency because of the complexity of the polyenoic acids of the cod-liver oil.

The experimental diet with two per cent cod-liver oil resulted in a decrease of serum cholesterol from 220 mg.% to 125 mg.% after 12 weeks on the diet. In a parallel group, 2% of maize oil was supplemented instead of two per cent cod-liver oil. Maize oil reduced the serum cholesterol level to 151 mg.%. These results are interesting from the point of view that the content of polyenoic fatty acids in cod-liver oil is only 58% of that in maize oil, whereas the degree of unsaturation is higher in the cod-liver oil than in the maize oil. Apparently the more polyunsaturated fatty acids (especially eicosapentaenoic acid and docosahexaenoic acid) of cod-liver oil are more efficient in reducing serum cholesterol than are the dienoic acids of maize oil. These experiments were followed up by experiments with hypercholesterolaemic (339 mg.% serum cholesterol) female rats supplemented with 40 mg./animal for 20 days of cod-liver oil or of cod-liver oil ethyl esters. The effectiveness of these various fractions regarding reduction of the serum cholesterol level appeared to be approximately proportional to the number of methylene interrupted double bonds of diene unsaturation and upwards, present in the fatty acids, irrespective of whether the fatty acids were of the linoleic or linolenic types.

These latter data are in agreement with previously reported data with cod-liver oil on hypercholesterolaemic rats by De Groot and Reed (1959), Nicolaysen and Ragård (1961), and in chicks by Dam *et al.* (1959). Kingsbury *et al.* (1961) found cod-liver oil more efficient than maize oil in reducing serum cholesterol levels in humans.

FISH OIL FATTY ALCOHOLS AS PRECURSORS FOR EFA

It is known that a number of fatty alcohols *in vivo* can be oxidized to the corresponding fatty acids. EFA activity of linoleyl alcohol was shown by Turpeinen (1938). Supplementation of EFA-deficient rats with 600 mg. linoleyl alcohol or linolenyl alcohol/animal/week stimulated growth and induced changes in the polyenoic fatty acid content of heart and testis tissue similar to those induced by the corresponding acids. Linoleyl alcohol reduced the skin symptoms markedly, whereas linolenyl alcohol did not.

Aaes-Jørgensen *et al.* (1959). Thus, fatty alcohols are a[illegible]d, metabolized and apparently function as the respective fatty acids *in vivo*.

Fatty alcohols are known to occur in the unsaponifiable fraction of marine oils. The oil of sperm whale contains considerable amounts of alcohols, e.g., cetyl-, stearyl-, and oleyl alcohols. Also, certain fish (e.g., Rucovettus preticosus Cocco) contain large amounts of such alcohols. However in most fish oils aliphatic alcohols appear in only minor amounts, Tsuchiya (1961), Toyama and Kaneda (1962). From an EFA, as well as from a nutritional point of view it would be of interest to know more details about the unsaturated fatty alcohols in fish oils, e.g., chain length, degree of unsaturation, and the location of the double bonds in the molecule.

FISH-OIL HYDROCARBONS AS A POSSIBLE SOURCE OF EFA

Hydrocarbons are among the compounds found in the unsaponifiables of fish oils. So far the interest has mainly centered on branch-chain hydrocarbons like squalene ($C_{30}H_{50}$), pristane ($C_{18}H_{38}$) and zamene ($C_{18}H_{36}$). From cod-liver oil can also be isolated another hydrocarbon, gadusene ($C_{18}H_{32}$), Deuel (1951), and Tsuchiya (1961). The hydrocarbons have been studied to a very limited extent only by means of modern analytical techniques. Lambertsen and Holman (1963) gave a partial characterization of the hydrocarbons of herring oil, which contained 0.05% hydrocarbons, about 5% of the unsaponifiable matter.

The hydrocarbons of primary interest for the present discussion, of course, are the unsaturated, non-branched chain hydrocarbons, which by means of ω-oxidation possibly can be converted to the respective unsaturated acids.

If hydrocarbons undergo ω-oxidation, it should be expected that octadecadiene and octadecatriene with the double bonds located in a similar way as in linoleic and linolenic acids could be converted to these acids, respectively, depending on which end of the molecule undergoes ω-oxidation.

In order to test this possibility, EFA-deficient rats were given orally doses (600 mg. per animal per week) of ω-6,9-octadecadiene or ω-3,6,9-octadecatriene for six weeks. Unfortunately, these hydrocarbons acted as very severe skin irritants (e.g., around the mouth) and thus obscured a possible growth-stimulating effect. Analysis of heart and testis lipids of the animals showed changes in the polyenoic fatty acid pattern compared to that observed in EFA deficiency. However, no relief was found for the accumulated trienoic acids characteristic of EFA deficiency. The data indicated that the hydrocarbons were absorbed and metabolized to some extent. It may be that any acids formed from the hydrocarbons are partially metabolized further for energy and that little appear in the tissues as

polyunsaturated acids. Autopsy revealed fatty livers in the rats fed the hydrocarbons, Aaes-Jørgensen *et al.* (1959).

The metabolism of straight-chain saturated hydrocarbons was studied recently by McCharty (1964). In goats rumen perfusion experiments, labeled [1-^{14}C]-octadecane was added to the normal rumen contents. A substantial amount of label was found in the blood, but only as fatty acids. Analysis of the rumen content showed the fatty acids present to be non-radioactive, thus ruling out conversion by the microflora. Intravenous injection of labeled octadecane to goats was rapidly cleared from the blood and apparently taken up by various tissues, e.g., the liver, and oxidized to fatty acids. These acids occurred almost entirely in the phospholipids.

Oral administration of the [1- ^{14}C]-octadecane or [1- ^{14}C]-hexadecane to rats showed that rats are also capable of absorbing and converting the hydrocarbons to the respective fatty acids. A similar administration of the labeled octadecane to laying hens showed that of the isolated labeled fatty acids from the lipids of subsequently laid eggs, 71% of the label was found in the 18-carbon chain fatty acids. Since both of the initial substrates in these various experiments were symmetrical molecules with a methyl group on either end, the reaction must have been ω-oxidation.

For nutritionists, hydrocarbons represent an area where very little is yet known. However, as indicated above, the topic comprises a number of interesting problems.

DIACYL GLYCERYL ETHERS AS SOURCE OF EFA

In fish oils are found varying amounts of diacyl glyceryl ethers together with the triglycerides, Lovern (1937, 1951). In the diacyl glyceryl ethers the hydroxyl groups at the 1- and 2-positions of the glycerol are esterified with fatty acids; the third hydroxyl group is linked by an ether bond to a fatty alcohol. The glyceryl ethers as such occur in the unsaponifiable fraction of the oils. These latter compounds are found in large amounts, especially in liver oils but also in body oils of a number of elasmobranch fish and other marine animals (Karnovsky, Rapson, and Black (1946), Karnovsky, Rapson, and Schwartz (1948)). Recently the composition of glyceryl ethers and of their diacyl derivatives from fish and fish-liver oil have been studied in detail by means of gas chromatography and mass spectroscopy.

Hallgren and Larsson (1959, 1962) studied the glyceryl ethers from liver oils of elasmobranch fish. Besides chimyl-($C_{16:0}$)[2], batyl- ($C_{18:0}$), and selachyl-($_{18:1}$)-alcohols compounds with saturated C_{14} and monounsaturated C_{16}, C_{20}, and C_{22} chains were found. The double bond in a C_{16}

[2] $C_{16:0}$ refers to the long-chain component of the glyceryl ether molecule.

chain was located in position 9, 10. In the C_{20}- and C_{22}-chains, the double bond occurred in position 13, 14. Small amounts of a glyceryl ether with a C_{18} chain with two double bonds were also present. The positions of the double bonds were not established. Guyer, *et al.* (1963) studied the composition of glyceryl ethers in fish-liver oils by gas- and thin-layer chromatography. Besides the generally observed even-numbered side chains in glyceryl ethers they also found minor amounts of C_{15}-, C_{17}-, C_{19}-, and possibly C_{21}-chains in *Squalus acanthias* and *Chimera monstrosa* liver oils.

Regarding the diacyl glyceryl ethers, Hallgren and Larsson (1962) found that the fatty acid diesters of glyceryl ethers constituted about 30% of the liver oil in the Greenland shark and grey dogfish. In the ratfish, the liver fat was composed almost completely of fatty acid diesters of glyceryl ethers with only traces of triglycerides. The fatty acids included relatively large amounts of Δ 11,12-eicosaenoic and a mixture of Δ 13,14- and Δ 11,12-docosaenoic acids. Small amounts of eicosapentaenoic and docosahexaenoic acids were also present.

Malins *et al.* (1965) studied the composition of the diacyl glyceryl ethers and triglycerides of the flesh and liver of the dogfish. Thirty-five per cent of the diacyl glyceryl ethers was found in the flesh oil and 45% in the liver oil. Few significant differences were found between the ether portions of the diacyl glyceryl ethers of the flesh and liver, but the fatty acids were quite different in composition. Those of the flesh diacyl glyceryl ethers contained high percentages of the C_{20} and C_{22} polyenoic acids that are characteristic of most fish lipids. Those of the liver contained little polyenoic acid but high concentrations of C_{20} and of C_{22} monoenoic acids. The contents of C_{18} dienoic and C_{18} trienoic as well as C_{20} tetraenoic acid were very low in the diacyl glyceryl ethers from both sources.

It is interesting that enzymatic cleavage of the ether linkage in glyceryl ethers has been shown to occur in the intestinal mucosa of human, Blomstrand and Ahrens (1959), Bergström and Blomstrand (1957) and in the rainbow trout, Malins, Wekell, and Houle (1964). This cleavage of the ether bond results in formation of the corresponding fatty alcohol which can be further oxidized to the fatty acid which, in turn, is then deposited or metabolized.

The diacyl glyceryl ethers and the glyceryl ethers from fish oils comprise a very interesting field. However, the possibility of EFA activity of these compounds appears to be small, if any at all.

PARTIALLY HYDROGENATED FISH OILS AS A SOURCE OF EFA

Large amounts of fish oils are partially hydrogenated for commercial purposes, e.g., for use in margarine manufacturing. Considering the relatively low contents in fish oils of polyenoic acids of the linoleic acid fami-

ly, it could be expected that partial hydrogenation might be very damaging to the EFA activity of these oils. Further, partial hydrogenation of polyunsaturated fatty acids is known to result in a very complex mixture of positional and geometric isomers of unsaturated acids. Several nutritional experiments have been carried out with different types of partially hydrogenerated oils as dietary fat source, Aaes-Jørgensen (1961A, 1961B, 1965, 1966). However, only very few experiments have been made so far with partially hydrogenated fish oils as dietary fat.

In a study on the effect of partially hydrogenated marine oils as the sole dietary fat to rats, hydrogenated oil was used in an amount of 7% and 28% of an otherwise fat-free diet to weanling rats for 14 weeks, Aaes-Jørgensen (1954). The higher percentage of hydrogenated herring oil (28%) decreased the growth rate significantly as compared with the 7% level. The growth rate of the latter animals was significantly poorer than that of animals on 7% or 28% non-hydrogenated herring oil. Further, on the diet with 28% partially hydrogenated herring oil the animals ceased gaining weight after six weeks on the diet and began to lose weight after nine weeks. After 14 weeks on this diet the rats were moribund. The dermal symptoms indicating EFA deficiency were very pronounced in all the animals reared on diets with 7% or 28% partially hydrogenated herring oil. Hematuria developed in 50% of the animals fed on 7%, and in 100% of the animals fed on 28% hydrogenated herring oil. At the time when these experiments were carried out, the alkali isomerization procedure was the only available technique for determination of the unsaturated fatty acids. Thus, rather limited information only could be obtained concerning the fatty acids in the dietary fats. However, it was beyond doubt that the contents of polyenoic acid, normal as well as conjugated, were low.

Beare (1961) fed diets with two per cent partially hydrogenated herring oil to male, weanling Wistar rats for six weeks. Gas chromatographic analysis of the oil indicated a low content of linoleic acid, the presence of odd-numbered fatty acids, and of several series of C_{20} acids. The rats fed on the hydrogenated herring oil consumed more food and gained more weight than did the animals fed on diets with similar amounts of corn oil, rapeseed oil, or margarine (possessing some marine oil). However, when all weight gains were adjusted for food consumption by covariance analysis, the diet containing corn oil appeared to be the most efficiently utilized, and the diet containing margarine the least; rapeseed and hydrogenated herring oil were intermediate.

The livers of the rats fed partially hydrogenated herring oil were significantly heavier than those of the other rats. Despite the small quantity of dietary linoleic acid available to these rats, the level of octadecadienoic acid in liver and carcass fat was similar to that of rats fed on rapeseed oil or margarine. The author suggests a preferential retention of the sparsely available linoleic acid. In some livers there appeared to be an isomer of the common linoleic acid. The weight of the 14–15 days old litters of mothers fed on hydrogenated herring oil was significantly smaller than those of mothers supplied with corn oil or margarine. The rat milk fatty acids generally reflected more closely the dietary pattern of fatty acids than did those of the tissues.

The nutritive effects of partially hydrogenated fish oils are only vaguely known and present a most difficult area to explore because of the complexity of the unsaturated fatty acid pattern of such oils. However, further studies in this area are needed and no doubt will be undertaken as the analytical techniques become more refined.

FISH OILS, EFA, AND AUTOXIDATION

Problems concerning polyenoic acids and antioxidants are discussed in chapter II. However, it appears reasonable here to stress the fact that because of the relatively low content of EFA in fish oils, and on the other hand, the high contents of PUFA, it is of utmost importance for the dietary value of these oils that there is an appropriate balance between the content of polyenoic acids and antioxidants. It has been estimated that in man the requirement is 0.6 mg. of d-α-tocopherol per gm. of polyenoic acid, Harris and Embree (1963).

In the literature can be found several studies showing decreased nutritive effect of fish oils. These results can probably in some cases be referred, at least in part, to autoxidized oils. Without going into details of this topic, reference to two recent reports may be relevant. Oldfield *et al.* (1963) found that both feed intake and weight gains in swine were progressively decreased as the dietary menhaden oil was more highly oxidized. As oxidation increased, intensity of "yellow fat" increased, and hemoglobin and hematocrite levels were progressively decreased. This situation was abolished by α-tocopherol and ethoxyquin. Similar results were obtained in experiments with rats fed on menhaden oil, Rasheed *et al.* (1963). There is no doubt that these matters are of importance in a discussion of fish oils as dietary fat and thus also as a source of EFA.

CONCLUDING REMARKS

Analysis of the fatty acid pattern of fish oils has revealed a most complex picture. Especially the unsaturated fatty acids appear to be a very fascinating subject for further investigations because of the great variations in amounts and types of acids present. There still is a long way to go before a complete understanding of this matter is reached. However, it appears safe to conclude that polyenoic acids with essential fatty acid ac-

tivity occur in fish oils in minor amounts only. Acids belonging to the linolenic acid family are generally dominating. It should be kept in mind that polyenoic acids, besides being of interest as EFA, are of importance in connection with several other aspects of the nutritive effects of fish oils. Some of these matters have been briefly touched upon in this chapter, others are dealt with in other chapters of this book. One of the most fascinating aspects of the biological effects of the polyenoic acids is their *in vivo* ana- and catabolism. These reactions also comprise the regulatory effects of acids of one family on the *in vivo* interconversions of acids of other families, e.g., the effect of acids of the ω-3-acids (linolenic acid family) on the essential fatty acids, the ω-6-family (linoleic acid family). A more detailed knowledge of these reactions will also be of importance for a better understanding of the functions of polyenoic acids as such in the organism.

There are several indirect proofs that a certain level of highly unsaturated fatty acids are of great importance in the organism. In EFA deficiency, the content of polyenoic acids of the oleic and palmitoleic acid families are significantly increased, because oleic and palmitoleic acids can be biosynthesized, while linoleic and linolenic acids cannot. Several tissues, e.g., testis, brain, liver, adrenals contain remarkable amounts of highly unsaturated fatty acids. Unsaturated acids are important constituents, especially of the phospholipids. The understanding of the biological and physiological importance of these compounds is the subject for studies in many laboratories today. Further, polyenoic acids are involved in the regulation of plasma cholesterol level and, therefore, of importance in relation to hypercholesterolemia and atherosclerosis.

Thus, fish oils as dietary fat are of great general interest. They are not only a source of calories and to some extent of certain fat soluble vitamins, but are also interesting components because of their large content of many different types of polyenoic acids including minor quantities of EFA. The presence of highly unsaturated fatty acids in fish oils means that they autoxidize very easily, e.g., when used in experimental diets. Therefore, the relationship between the content of polyenoic acids and adequate amounts of antioxidants, e.g., tocopherols, should also be considered when fish oils are used as nutrients. Further experiments dealing with fish oils as a dietary source of unsaturated fatty acids, not only from the point of view of their EFA-content, must be awaited with great interest.

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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-

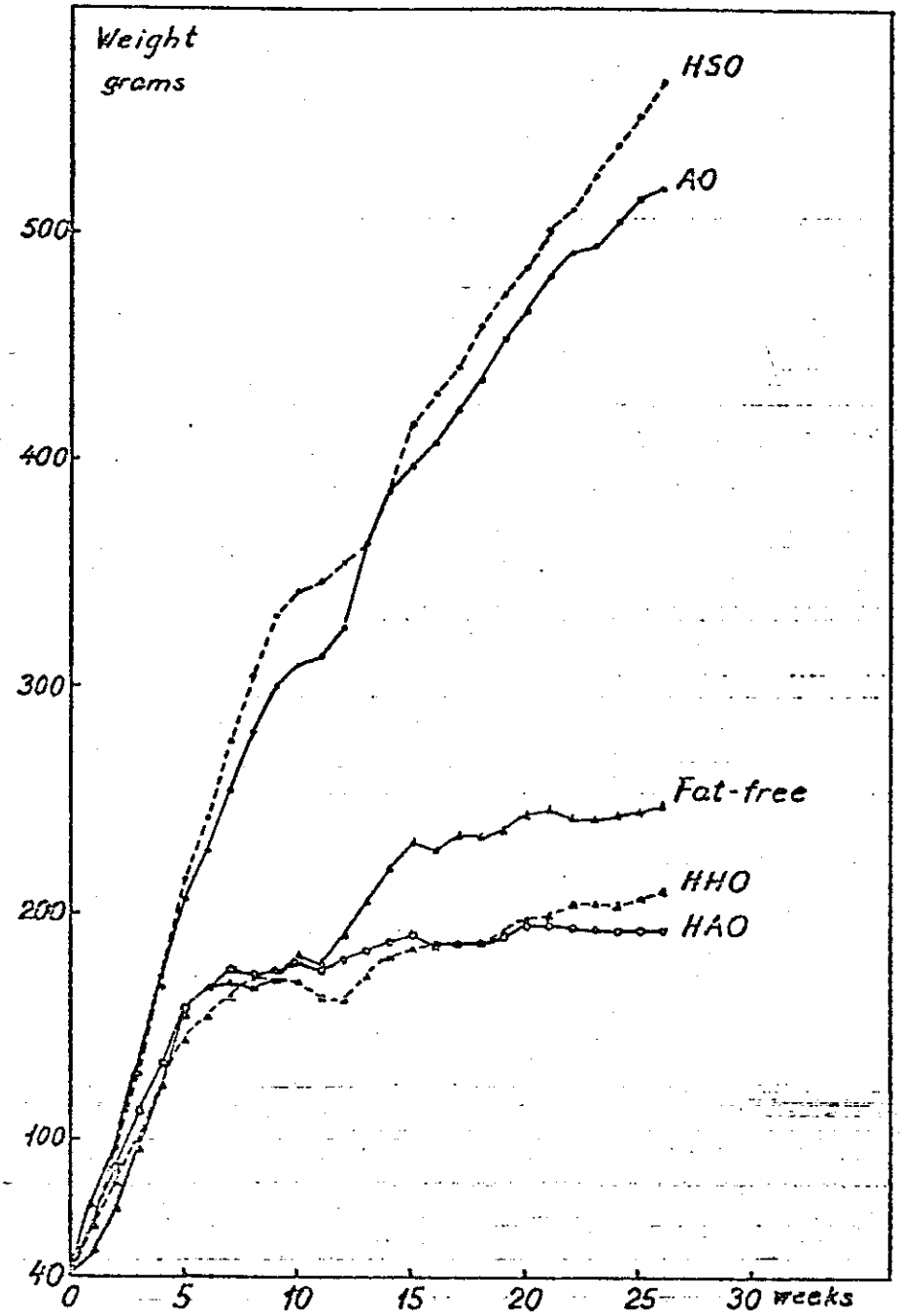

FIG. 1. Average growth rate of male rats fed on a diet with 28% partially hydrogenated soybean oil (HSO), 28% partially hydrogenated herring oil (HHO), 28% partially hydrogenated arachis oil (HAO), 28% arachis oil (AO), or a fat-free diet (Fat-free).

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).

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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		5 weeks	15 weeks	26 weeks
		absolute	relat.[b]	absolute	relat.[b]	absolute	relat.[b]			
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.

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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-washed Florisil impregnated with silver nitrate (6). The solvents were essentially those reported earlier where silica gel-silver nitrate columns were employed (7,8). The esters of saturated fatty acids were almost completely eluted with 75 ml of *n*-hexane. The *trans* and *cis* monoethylenic esters were eluted in sequence with virtually no overlap by successive applications of 75 ml each of 2,4 and 8% diethyl ether-*n*-hexane mixtures. The respective *cis* and *trans* fractions were further separated by preparative scale GLC with recovery of four chain lengths (C_{16}, C_{18}, C_{20}, C_{22}). Ozonolysis of each specific fraction was carried out in methanol with oxidative work-up, the product acids being identified by GLC after in situ esterification with 2,2-dimethoxypropane (9).

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

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

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

RESULTS AND DISCUSSION

The results of study of this sample of commercially prepared, partially hydrogenated herring oil would indicate the use of a catalyst and conditions favoring selective hydrogenation of the polyunsaturated acids to monounsaturated acids. Only a total of 4% of a variety of artifact polyunsaturated acids remained. The proportions of 22:0, normally absent in herring oil, and 20:0, about 0.1-0.5% in most herring oils, indicate that as little as 8% of the original or newly formed monounsaturated acids may have been hydrogenaged to saturated acids. Total saturated acids were 30% and total monounsaturated acids 66%. Unfortunately the raw herring oil was not available for analysis and the fatty acid composition of herring oil varies with season, age of fish, and catch location. Average figures for 12 Atlantic 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 this sample is not remarkable for a marine oil. Were it not for the presence of 1.64% 20:0, and 2.04% 22:0, the composition could well be that of the saturated acids of any of

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).

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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

	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.*

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 *Spheciospongia 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 [II]	$C_{16}H_{26}O_2$	250.37	.....	.....	.....	.....

[II] An elaidinized α- acid.

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: *Chupanodon 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

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	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

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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.

General References

[1] Deuel, H. J., Jr. 1951. The Lipids; Their Chemistry and Biochemistry. Interscience, New York. v. 1.

[2] Hilditch, T. P., and P. N. Williams. 1964. The Chemical Constitution of Natural Fats. Ed. 4. J. Wiley, New York.

[3] Swern, D., ed. 1964. Bailey's Industrial Oil and Fat Products. Ed. 3. Interscience, New York.

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

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

	Component (Synonym)	Concentration g/100 g total fatty acids
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

	Component (Synonym)	Concentration g/100 g total fatty acids 1/
	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.

General References

[1] Deuel, H. J., Jr. 1951. The Lipids; Their Chemistry and Biochemistry. Interscience, New York. v. 1.

[2] Hilditch, T. P., and P. N. Williams. 1964. The Chemical Constitution of Natural Fats. Ed. 4. J. Wiley, New York.

[3] Swern, D., ed. 1964. Bailey's Industrial Oil and Fat Products. Ed. 3. Interscience, New York.

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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Toxicity of Air-Oxidized Soybean Oil[1,2]

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Although it has long been recognized that overheated or oxidized fat causes toxic manifestations in the rat, neither the exact nature of materials causing these symptoms nor the specific mechanisms by which they are caused have been understood with any certainty. Work prior to 1940 established that highly oxidized fat accelerates the destruction of a large variety of essential nutrients (Burr and Barnes, '43). Subsequent work, however, has indicated that certain oxidized oils contain materials which are toxic to the rat even though apparently adequate steps are taken to protect the easily-oxidizable foodstuffs in the diet.

The identity of the toxic materials in oxidized or overheated fat has been investigated by two routes, differing in the manner by which the oxidized fat was obtained. Several groups of investigators, including Crampton et al. ('51a, '51b, '51c, '53, '56), Common et al. ('57), Granados et al. ('49), and Raju and Rajagopalan ('55) have changed the chemical nature of oils primarily by means of heat treatment, often with complete exclusion of air. Other groups of investigators (Matsuo, '54; Kaneda et al., '55; Kaunitz et al., '55; Andrews et al., '56) oxidized various oils by aeration with little or no elevation in temperature during the oxidation process. Grossly, the results obtained are similar when either type of oxidized oil is fed, namely, rough fur and unkempt appearance and decreased weight gain often followed by death. When, however, those groups feeding heat-treated oil measured the peroxide content of their product, the peroxide concentration in the toxic oil was found to be very low compared with values obtained with aerated oil. On the other hand, in those cases in which oils were oxidized by aeration and some attempt was made to measure the toxicity due to polymers, the polymer content appeared completely innocuous, the toxicity apparently correlating best with peroxide concentration.

In the experiments to be reported in this paper, an attempt was made to reconcile these apparently contradictory results and, further, to identify the toxic mechanism or mechanisms which so frequently lead to death in the rat. There are apparently few or no definitive data on the latter point although it would seem to be of considerable importance in many areas of the world in which cooking customs involve intermittent high-temperature and open air heating of highly unsaturated oils (Raju and Rajagopalan, '55). Consequently, in a preliminary experiment, various levels of peroxidized fat were fed to determine the levels of toxicity and toxic symptoms. Second, the peroxidized oil was fractionated in such a way as to separate peroxide and polymeric material and the fractions were fed. Third, the absorption of peroxidized fat was studied, and fourth, an attempt was made to ascertain the site of the damage.

METHODS AND MATERIALS

Growth experiments. Unless otherwise noted, weanling rats[4] were fed diets containing air-oxidized soybean oil. The animals were individually housed in sus-

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pended cages and, where pertinent, individual diet consumption records were kept. Each rat was weighed twice weekly.

Two diets were utilized in the growth experiments. The percentage composition of diet A was as follows: casein, 25; sucrose, 46; salt mixture, 4; brewers' yeast, 5; fat, 20. The composition of diet B is presented in table 1, as well as the composition of the vitamin and salt mixtures.

Semiweekly administration to each rat of 175 U.S.P. units of vitamin A, 35 units of vitamin D and 1.75 mg of α-tocopherol acetate dissolved in 0.05 ml of sesame oil was utilized to satisfy the fat-soluble vitamin requirement. Initially, this supplement was administered by intramuscular injection in order to avoid the possibility of oxidation by peroxide in the gut. Subsequent experimentation, however, established that similar growth could be obtained in rats given this supplement orally at noon on a semiweekly basis (most of the diet was consumed during the night), and this method of supplementation was used in later experiments.

Preparation of oxidized soybean oil. The raw oil was oxidized by aeration in a water bath at 60°C for approximately one week with the addition of 2 mg/kg each of $CuCl_2$ and $FeCl_2$. Toward the end of the oxidation period the peroxide number (PN), milliequivalents of peroxide oxygen per kilogram of oil, was determined at intervals according to the method of Polister and Mead ('53) so that oxidation might be halted at maximum peroxide concentration inasmuch as the PN decreases rapidly after the maximum is reached. The oxidized oil was then stored at −20°C under nitrogen.

Fractionation of oxidized soybean oil. Two kilograms of raw oil were oxidized in the usual manner and one-half of the oxidized oil was stored at −20°C (fraction C). The remaining oil was dissolved in 1.7 l of petroleum ether (b.p. 30 to 60°) saturated with methanol and was then extracted with 4 l of methanol saturated with petroleum ether. The oil contained in the petroleum ether layer was separated on a silicic acid column into two fractions. This fractionation was accomplished by adding 25-gm quantities of oil in petroleum ether to 8 by 14-cm silicic acid columns and eluting with 500-ml portions of 5% ethyl ether in petroleum ether. Each fraction was collected separately and evaporated to dryness under nitrogen in a warm-water bath. All fractions weighing more than 1 gm were tested for peroxide, as previously described, and those fractions having a peroxide number of less than 20 were pooled. The resulting colorless oil (fraction A) had a peroxide value of 13.2 and contained little or no polymeric material as shown by complete distillation of a methylated sample. After removal of the low-peroxide material from the columns, the remaining oil was eluted with three- to 4-column volumes of methanol. The methanol was evaporated under reduced pressure at a temperature under 40°C. The last traces of solvent were removed under high vacuum at room temperature. The combined sample from all

TABLE 1

Composition of diet B

Dietary component main mixture		Salt mixture		Vitamin mixture	
		Salt	Amount	Vitamin	Amount
	gm/100 gm		*gm*		*mg/gm*
Oxidized oil	15.0	$CaCO_3$	300.0	Choline	120,000
Casein	18.0	$CaHPO_4$	75.0	Thiamine	200
Sucrose	60.8	K_2HPO_4	322.5	Riboflavin	200
Glycerol	1.0	NaCl	167.4	Niacin	400
l-Cystine	0.2	$MgSO_4$	49.8	Calcium pantothenate	800
Salt mixture	4.0	$MnSO_4 \cdot 2H_2O$	0.5	Folic acid	8
Vitamin mixture	1.0	Zn acetate	0.402	Biotin	4
		Fe citrate	27.4	Pyridoxine	100
		KI	0.8	Vitamin B_{12}	0.[illegible]
		$CuSO_4 \cdot 5H_2O$	0.3	*i*-Inositol	4.[illegible]
				Sucrose	273,500

of the columns was an amber, moderately viscous oil with a peroxide number of 927 (fraction B). The original methanol fraction was extracted with petroleum ether at 0°C for 6 hours in a liquid-liquid extractor. The sample, in methanol, was stored under nitrogen at −20°C until shortly before use. The methanol was then removed under reduced pressure in an ice bath with a slow stream of nitrogen. High vacuum was utilized to remove the last traces of the solvent. The sample (fraction D) was colorless, extremely viscous and had a peroxide number of 3,185. Peroxide numbers and spectral properties of the fractions are listed in table 2.

Absorption studies. The animals used in these investigations were mature rats[4] which were subjected to thoracic duct cannulation according to the technique of Bollman et al. ('48). Preliminary investigation revealed that pre-operative administration of 2 ml of oxidized soybean oil (PN = 1200) was frequently fatal to the subject. A similar amount of peanut oil was employed, therefore, in the pre-operative procedure. Lymph was then collected for a 20- to 24-hour period at room temperature and was stored at −20°C. These samples served as the source of control lymph fat. After the initial period of lymph collection, a dose of air-oxidized soybean oil was administered to the cannulated rat. Lymph collection was then continued for periods extending up to 12 hours. These samples were also stored at −20°C. Similar volumes of both control and experimental samples were then extracted with a 3:1 mixture of alcohol and ether, the protein removed by centrifugation, and the alcohol-ether layer evaporated in a tared flask under nitrogen on a warm-water bath, for determination of peroxide number. Other lymph fat samples were examined for conjugated diene by dissolving the fat isolated from 2 ml of lymph in 50 ml of ethanol and examining solution at 232 μ with a Carey recording spectrophotometer.

TABLE 2

Peroxide number and conjugated diene concentration in the various fractions of oxidized soybean oil

Fraction	Peroxide number	Meq. conj. diene/kg oil[1]
A	13	0
B	927	1190
C (unseparated)	1156	780
D	3185	1602

[1] The fact that the peroxide numbers and conjugated diene concentrations do not change in parallel fashion probably indicates that the unsaturated centers of many molecules have suffered further attack after the initial peroxide formation.

Xanthine oxidase assay. The intestinal xanthine oxidase of rats was assayed as follows: the animals were decapitated and the upper third of the small intestine excised; intestinal contents were flushed out with approximately 10 ml of saline and 0.5 gm of the upper end of the washed intestinal section placed on dry ice until homogenized; homogenization was conducted at 5°C in 10 ml of an 0.015 M sodium pyrophosphate buffer contained in an all glass homogenizer. The general method of enzyme assay was that of Dhungat and Sreenivasan ('54). Milk xanthine oxidase was purified according to the method of Ball ('39).

RESULTS

Rat growth experiments. In the initial growth study, oxidized oil (1200 PN) was diluted with fresh oil to give peroxide numbers of 800, 400 and 100. Four variations of diet A were prepared: three with each of the diluted oils and the 4th with the original 1200 PN oil. A 5th diet containing fresh oil served as the control. Extraction of the fat in these diets showed that the peroxide number did not change on mixing, after three weeks' storage at −20°C, or on standing at room temperature for three days.

After consuming the control diet for 6 days, 50 weanling rats were divided into 5 groups of 10 each, equally divided between males and females. One of these groups was assigned to each of the diets previously described, and the animals were weighed individually twice a week for the ensuing 70 days. The average growth curves of the female rats in each group are presented in figure 1. The diets containing 20% of fat with peroxide numbers

[4] Sprague-Dawley strain.

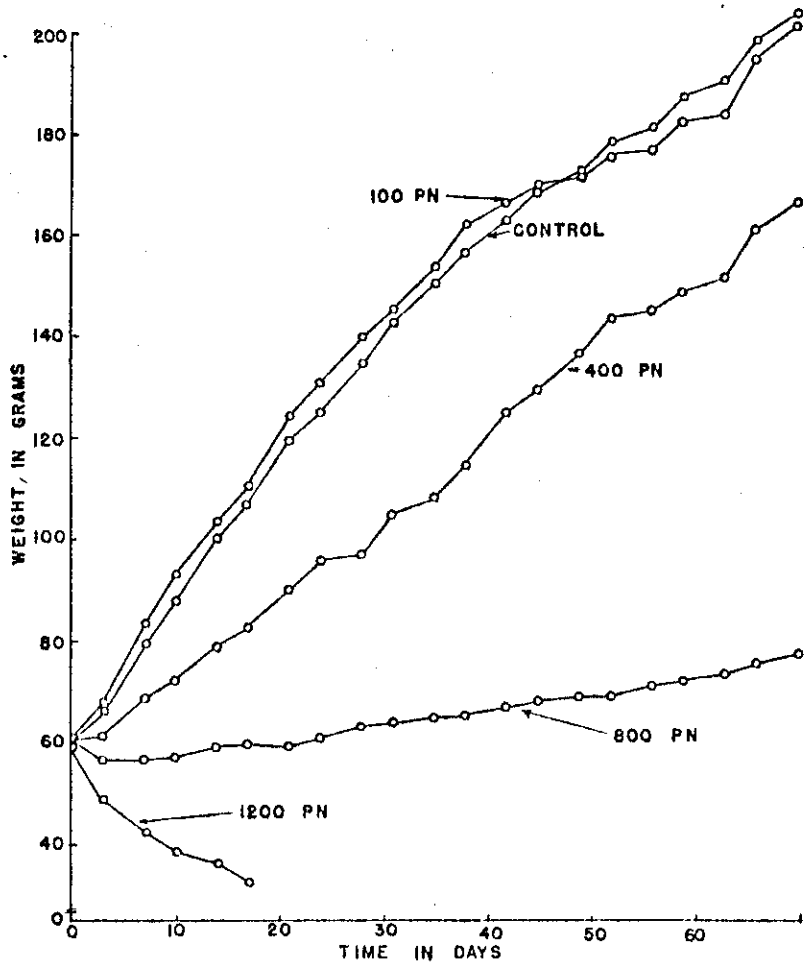

Fig. 1 Growth of female rats receiving various levels of oxidized fat.

of 400 to 1200 caused a reduction in growth rate directly related to the peroxide number. Consumption of the 1200 PN diet caused death within approximately three weeks. The only other symptoms observed in the animals fed this diet were weight loss and severe diarrhea. Animals receiving the 800 PN diet developed moderate to severe diarrhea, but this symptom had subsided somewhat by the 8th week. Similar effects were observed in the growth of male rats fed these diets.

The data obtained in this experiment indicate that the diet containing 100 PN oil has no effect on growth. It seemed conceivable, however, that under conditions of stress a difference between the control and 100 PN diets might become apparent. Accordingly, an experiment was conducted in which 25 rats, 11 males and 14 females, consumed the 100 PN diet for 8 weeks and then were subjected to a whole-body X-irradiation dose of 500 r.[5] A similar group of animals served as concomitant controls. In both the control and 100 PN diets the concentration of brewers' yeast was increased from 5 to 7.5%

[5] Irradiation was carried out using a Picker 250 KV Industrial X-Ray apparatus, with the following factors: 15 ma, 0.28 Cu parabolic, 0.21 Cu inherant and 1 Al; field size 47 cm², FOD 55 cm.

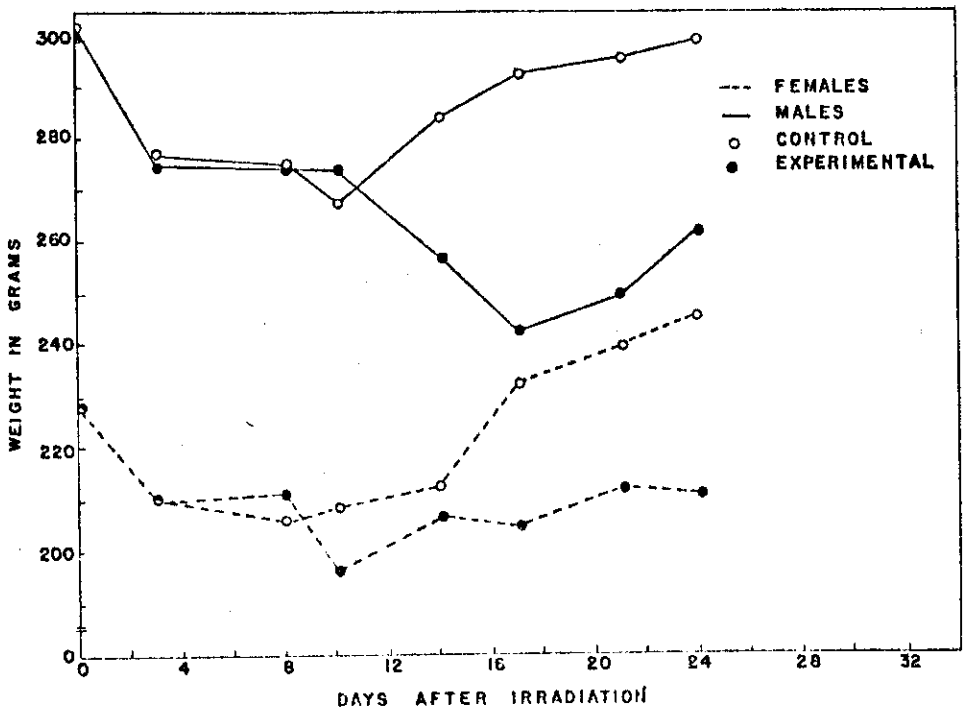

Fig. 2 Effect of diet on recovery from whole body irradiation.

at the expense of sucrose. Figure 2 shows the average group weight changes over the 27-day period following irradiation. The results obtained in this experiment indicate that consumption of the 100 PN diet caused a slower than normal recovery from the stress of irradiation.

In an attempt to compare the effect of the lipid peroxide with that of a simple water-soluble peroxide, and to eliminate diet flavor as a factor, *t*-butyl hydroperoxide, synthesized according to the method of Milas and Surgenor, ('46) was used in both feeding and injection studies. In preliminary injection experiments, 8 mg of this compound in a non-physiological solution was found to be immediately fatal when intravenously administered to rats weighing approximately 400 gm. Intravenous injection of a physiological solution of 1 mg of *t*-butyl hydroperoxide every three or 4 days over a two-week period caused a weight loss and some loss of hair. A growth study was conducted with 4 weanling rats fed control diet A by substituting a solution of 4×10^{-2} M *t*-butyl hydroperoxide for the drinking water. Figure 3 illustrates the average growth of these animals compared with three rats consuming the same diet and drinking tap water. This figure also illustrates the marked loss of weight which occurs when a high-PN diet is substituted for the control diet.

Fractionation experiments. In an attempt to settle the question of whether the toxic principle in oxidized oils is peroxidic or polymeric in nature, the fractions obtained from air-oxidized soybean oil were fed to weanling rats. It was assumed that fraction A contained most of the non-hydroxylated or peroxidized glycerides, while fraction D consisted primarily of glycerides containing fatty acid peroxides. Fraction B was assumed to be a mixture of the two materials. Since incorporation of a highly oxidized oil, namely, fraction D, in the diet makes the food unpalatable, the rats were fed a fat-free diet similar to diet A and the appropriate oil was force-fed. Diet A was modified by increasing the brewers' yeast from 5 to 7.5% and adding

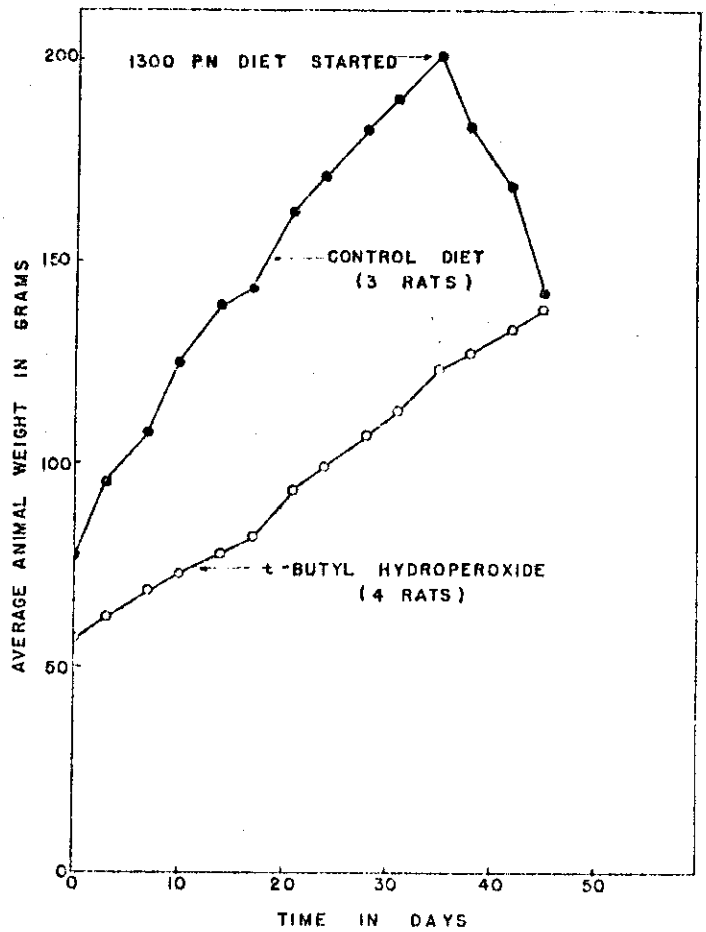

Fig. 3 Growth of rats under several dietary regimes.

2% of guar gum,[6] both at the expense of sucrose. Preliminary considerations indicated that each rat should receive an amount of oil equivalent to 38% of the total amount of calories consumed during the previous 24 hours. Ten grams of the dry diet were weighed into tared glass feed cups for each rat. Two milliliters of water were then mixed with the diet in each cup to make a thick paste which soon hardened and which the animals could eat with a minimum of spillage. An extra cup of diet was prepared each day and used to correct food consumption figures for water evaporation. Fifteen male and 5 female weanling rats ate the fat-free diet for an initial 8-day period. During this period some diarrhea was observed but weight gains were satisfactory and the animals appeared otherwise healthy. At the end of the 8-day period, the male rats were divided into three groups (A, B and C) of 5 each with the female rats comprising a 4th group (D). These group designations correspond to the fraction designations of the oxidized oil. All animals received orally twice the previously described volume of fat-soluble vitamin mix once a week about three hours before any fat was force-fed. If the amount of oil to be administered was more than 0.5 ml, it was given in two equal doses at noon and 4:00

[6] Donated by the Stein-Hall Co., New York.

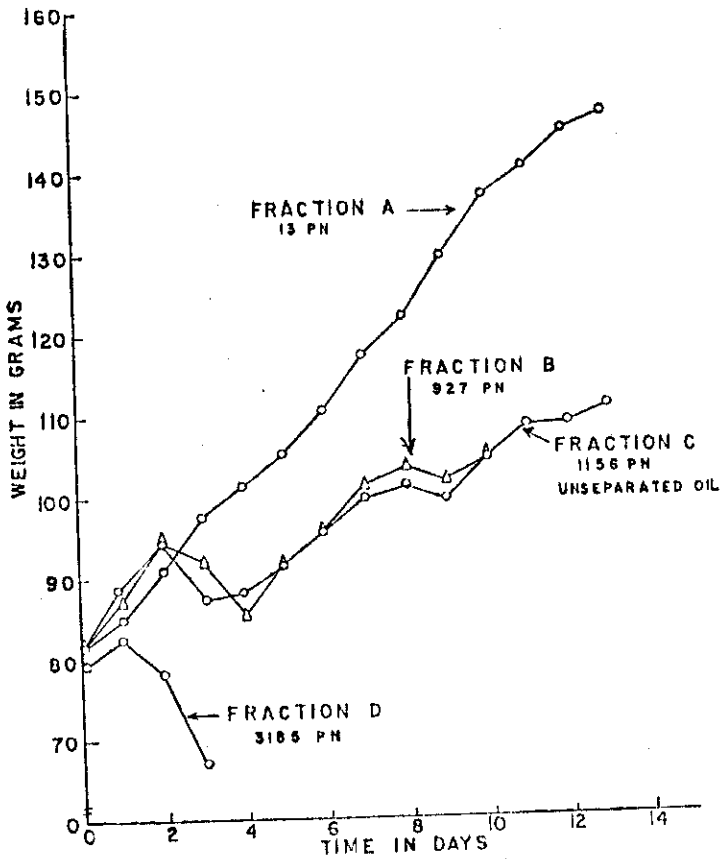

Fig. 4 Growth of rats receiving various fractions of oxidized soybean oil.

P.M. The rats were weighed daily during the experimental period. The average weight gains for the 4 diet groups are presented in figure 4.

The rats in group A appeared healthy and ate well in contrast to those in groups B, C and D. Those in groups B and C were indistinguishable in appearance but rats in group D began losing weight after the first day of fat administration. On the third day of the experiment two of the rats in group D were found dead and, since the remaining three appeared moribund, they were killed for pathological examination.

Histopathological examination[7] of these three animals revealed no obvious anatomical basis for their condition. The livers of these rats showed slight enlargement of the nuclei of the parenchymal cells and prominent nucleoli. Moreover, the small intestines of these animals had cytoplasmic vacuoles distending the mucosal walls. Examination of all other organs generally gave negative findings.

Absorption experiments. In order to ascertain the actual site of action of the toxic principle in oxidized fat, it was deemed of primary importance to determine whether any of the peroxide-containing material could be absorbed through the intestinal wall. For this study, rats with thoracic duct cannulae were given small doses of oxidized soybean oil, and the lymph was collected for periods varying between two and 24 hours. After isolation, the lymph fat was analyzed for peroxide and conjugated

[7] Histopathology performed under the direction of Dr. D. Moyer of the Dept. of Pathology, UCLA School of Medicine.

TABLE 3

Peroxide numbers of lymph fat obtained from rats fed either 0.5 to 1.0 ml of air-oxidized soybean oil (PN = 1200) or 2 ml of peanut oil

Rat no.	Oil fed	Lymph extracted	Weight of extracted fat	Peroxide no. of fat
		ml	*mg*	
A	Peanut	4.0	213.7	0.9
	Oxidized soybean	4.0	242.9	32.7
B[1]	Peanut	4.0	188.5	13.1
	Oxidized soybean	4.0	135.0	13.9
C[1]	Peanut	3.0	71.1	4.2
	Oxidized soybean	3.0	161.5	4.3

[1] Rats B and C are presented as typical of the 25 rats in which no significant change in the PN of the lymph fat was found.

diene. The results of the peroxide determinations are shown in table 3. The results of the experiment with rat A led to the conclusion that fatty acid peroxides may be absorbed as such. However, in subsequent experiments involving 25 cannulated rats it has been impossible to detect a significant rise in the peroxide number of lymph fat as the result of feeding oxidized soybean oil with a PN of 1200. The results obtained with rat A were, therefore, assumed to be either an artifact or an exceptional case of absorption.

Ultraviolet examination of a 1:50 dilution in ethanol of lymph fat from cannulated rats yielded the results typical of those presented in table 4. It can be seen that there was an increase in light absorption at 232 mμ (the maximum for conjugated diene) in the lymph of those rats which had been fed the oxidized oil. In the case of rat 6, 3 ml of oxidized oil (PN = 1100) were fed by stomach tube before the cannulation. Collection was begun immediately after the operation (two hours after feeding) and at no time did this animal receive further administrations of oxidized oil. It therefore appears that even though the peroxide itself may not appear in the lymph, its reduction products may be absorbed. The results of the absorption experiments suggest that the fatty acid hydroperoxides are destroyed during or before the absorption process and further, that the toxic effect probably takes place primarily in the intestinal cells.

Table 4

Optical densities at 232 mμ observed in lymph fat from rats fed oxidized soybean oil

Rat no.[1]	Amt. ox. oil fed	Length of collection time	232 mμ O.D. at[2]
	ml	*hours*	
9	0.5–1.0	4.5	0.075
	0.5–1.9	4.0	0.024
6	3.0	21.5	0.030
		5.0	0.130
		3.5	0.021
		16.0	0.058
		8.0	0.008
		16.0	0.000

[1] These results are presented as typical of several experiments performed.

[2] The optical density at 232 mμ (the maximal for conjugated diene) is a measure of the absorption of the fatty acid chain containing the unsaturated center prior to feeding the oxidized oil. The lymph fat had no significant absorption at this point under these conditions of dilution.

Intestinal xanthine oxidase assay. Since the absorption studies had led to the hypothesis that the primary site of toxicity was the intestine, and the pathology of these animals was generally negative, an investigation of the effect of oxidized fat on intestinal xanthine oxidase as a representative intestinal enzyme was undertaken. This enzyme was selected because it is fairly well characterized and is sensitive to lack of a dietary component (McQuarrie and Venosa, '45).

Twelve mature male rats were fed a control diet similar to diet B except that unoxidized soybean oil was substituted for the oxidized oil and the level was increased from 15 to 20% at the expense of sucrose. This control diet was continued for 10 days, at the end of which period the animals were paired on the basis of weight.

TABLE 5

Inhibition of intestinal xanthine oxidase by the ingestion of oxidized fat

Days on experiment	Diet	Feed ingested	Enzyme activity[1]	Intestinal protein[2]	Animal weight	
					Initial	Final
		gm/day	*μl O_2*	%	*gm*	
0	Control	0	23.5	73.9	226.5	
	Control				212.5	
2	Oxidized	2.4	8.0	62.4	211.0	207.0
	Control	2.4	28.5	59.4	201.5	—
5	Oxidized	3.0	1.5	64.5	193.0	178.5
	Control	3.0	4.5	63.6	194.0	180.0
7	Oxidized	3.7	0.5	68.7	212.0	189.0
	Control	3.7	10.0	62.1	204.0	194.0
9	Oxidized	4.9	1.0	66.9	203.0	177.5
	Control	4.9	13.0	63.6	204.0	209.0
12	Oxidized	5.9	0.0	62.1	200.5	188.0
	Control	5.9	25.0	56.7	197.0	203.5

[1] Corrected for endogenous activity.
[2] Determined by method of Lowry et al. ('51).

One pair was killed at this time for enzyme assay. One animal in each of the 5 remaining pairs was randomly assigned to an experimental diet containing 20% of air-oxidized soybean oil (PN = 1200). The control diet was pair-fed for the duration of the experiment. Pairs of rats were killed at intervals of 2, 5, 7, 9 and 12 days after initiation of the experiment and the intestinal xanthine oxidase of each animal assayed. The results of these assays are presented in table 5.

The observed inhibition of intestinal xanthine oxidase appears to be specific since it cannot be due to either lower protein intake or intestinal edema. One possible explanation for this enzyme inhibition is oxidation of sulfhydryl groups as suggested by Potter and DuBois ('43) in their study of succinic dehydrogenase. For an investigation of this possibility a group of 12 weanling female rats were paired and subjected to the same dietary regimen as in the previous experiment. Pairs of rats were killed each day for 6 days and the levels of SH in each intestine determined. The intestinal samples were prepared as described above and the method of Grunert and Phillips ('51) was used to determine the levels of SH, reported as glutathione. The results obtained in this experiment are given in table 6.

Apparently, the inhibition of intestinal xanthine oxidase is not directly connected with oxidation of sulfhydryl groups. In order to investigate further the mechanism of this enzyme inhibition, milk xanthine oxidase was purified by the method of Ball ('39) and *t*-butyl hydroperoxide was used as the inhibitory compound. The assay

TABLE 6

Levels of intestinal glutathione in weanling female rats consuming either oxidized or unoxidized soybean oil

Rat no.	Oil	No. of days on experiment	Intestinal glutathione level
			μg/mg fresh tissue
7	Control	1	0.38
8	Oxidized		0.57
9	Control	2	0.51
10	Oxidized		0.75
23	Control	3	0.27
24	Oxidized		0.21
11	Control	4	0.24
12	Oxidized		0.37
19	Control	5	0.19
20	Oxidized		0.22
25	Control	6	0.27
26	Oxidized		0.43

TABLE 7

Warburg assay of the inhibition of milk xanthine oxidase by t-butyl hydroperoxide

Flask components						Activity[4]
Buffer[1]	Enzyme	t-Butyl hydroperoxide 1.1×10^{-4} M	FAD[2,3] 1 mg/ml	Xanthine 1 mg/ml	H_2O	O_2 uptake: 60 min. totals
ml	*ml*	*ml*	*ml*	*ml*	*ml*	*µl*
1.0	0.3				1.7	0.0
1.0	0.3			1.0	0.7	80.3
1.0	0.3	0.1		1.0	0.6	73.9
1.0	0.3	0.1	0.1	1.0	0.5	90.6

[1] 0.039 M K–$NaPO_4$, pH 7.54.
[2] Flavin adenine dinucleotide.
[3] Obtained from California Corporation for Biochemical Research, Los Angeles. Reported absorption ratio at pH 7.0, 264 mµ/450 mµ = 3.22.
[4] Average of duplicate flasks.

TABLE 8

Warburg assay of the inhibition of rat intestinal xanthine oxidase by t-butyl hydroperoxide

Flask components						Activity[1]
Buffer[2]	Enzyme[3]	t-Butyl hydroperoxide 1.1×10^{-4} M	FAD[4,5] 1 mg/ml	Xanthine 1 mg/ml	H_2O	O_2 uptake: 60 min. totals
ml	*ml*	*ml*	*ml*	*ml*	*ml*	*µl*
1.0	0.5				1.5	43.6
1.0	0.5			1.0	0.5	89.1
1.0	0.5	0.1		1.0	0.4	42.4
1.0	0.5	0.1	0.1	1.0	0.3	71.7

[1] Average of duplicate flasks.
[2] 0.039 M K–$NaPO_4$, pH 7.54.
[3] A 5% intestinal homogenate.
[4] Flavin adenine dinucleotide.
[5] Obtained from California Corporation for Biochemical Research, Los Angeles. Reported absorption ratio at pH 7.0, 264 mµ/450 mµ = 3.22.

technique was essentially the same as that previously described except that a 0.039 M phosphate buffer with equal concentrations of sodium and potassium at pH 7.5 gave higher oxygen uptake and was, therefore, substituted for the pyrophosphate buffer. In view of the work of Bernheim, et al. ('52) and Ottolenghi et al. ('55) the effect of flavin adenine dinucleotide (FAD) on the inhibited enzyme was investigated. The data obtained in this experiment are reported in table 7.

The results of this experiment indicate that *t*-butyl hydroperoxide is capable of inhibiting milk xanthine oxidase and further, that this inhibition may be reversed by FAD. The high value for the flask containing FAD probably means that the enzyme preparation is somewhat deficient in FAD. A similar assay using a 5% intestinal homogenate from a stock rat as the enzyme source yielded the results presented in table 8.

This experiment corroborates the similarity of the inhibitory effects of the hydroperoxide and the ability of FAD to reverse these effects although apparently not as completely as with the purer preparation.

DISCUSSION

The investigations described above support earlier work in establishing that the toxicity ascribed to air-oxidized oils correlates well with the fatty acid peroxide content of these materials. The feeding experiment in which *t*-butyl hydroperoxide was utilized substantiates, at least in some degree, the toxicity of fatty acid peroxides, although this substantiation must be qualified because of differences in structure

and probable mode of absorption. Nevertheless, from the data obtained in this experiment it can be calculated that the consumption of peroxide in the form of t-butyl hydroperoxide corresponds to a diet containing 20% of oil with a PN of approximately 600. It is interesting to note that the average growth curve of these animals falls between those for rats receiving diets with oils of 400 and 800 PN. The fractionation of air-oxidized soybean oil showed predominantly polymers of a polar nature, probably polyperoxides, and little or no polymers of the type found in heat-oxidized oil. This study also illustrates the complexity of the reactions occurring in the autoxidation of an oil, since if a simple generation of peroxide groups were the only reaction occurring, equivalence between peroxide oxygen and conjugated diene should be observed. Such is not the case, however (table 2). Although the work reported here demonstrates the toxicity of oxidized oil to be due entirely to peroxide formation, we have not investigated the toxic principle of anaerobically heated oils. Thus, it may be suggested that two separate classes of toxic compounds are possible in autoxidized oil—fatty acid peroxides when the fat is oxidized in air at only slightly elevated temperatures for 1.5 weeks or less and fatty acid polymers when the oil is heated to temperatures in the neighborhood of 250°C with the exclusion of oxygen for periods of one to two days. The conditions of autoxidation for a maximum yield of one class or the other will vary, however, with different oils. In our laboratory we have observed that extended aeration of soybean oil results in an oil of lower than expected peroxide content, and that the application of high temperatures at any point during the autoxidation process will have the same effect. It is probable that either of these modifications tends to decrease the peroxide content of the oil by formation of degradation products and polymers.

The primary site of toxicity, as demonstrated in the cannulated rats, appears to be the intestine. The gross observations and the limited pathology data available appear to support this conclusion. Even the irradiation experiment lends some indirect support to this hypothesis since it is known that the intestine is one of the most sensitive organs to whole-body irradiation, and extended recovery periods were observed in those animals receiving a dietary fat containing peroxide.

The mechanism or mechanisms by which fatty acid peroxides cause the observed toxic effects still need clarification and amplification. In the absence of observable gross changes in any of the organs examined, it seems likely that the injury is of a more subtle nature. The inhibition of intestinal xanthine oxidase, however, points to the possibility that choline oxidase and amine oxidase may be inhibited in a similar manner and that succinoxidase and cytochrome oxidase may also be inhibited but in a manner as yet unknown (Bernheim et al., '52; Ottolenghi et al., '55).

SUMMARY

The toxicity of air-oxidized soybean oil to weanling rats was investigated in several aspects.

Growth studies demonstrated that the concentration of the toxic principle corresponded closely to the peroxide concentration of the oil. Moreover, separation of the oxidized oil into high-, medium- and low-peroxide fractions revealed that toxicity again followed peroxide concentration.

Histopathological examination of rats fed oxidized soybean oil gave generally negative findings but indicated that the intestine might be involved. Moreover, absorption of fatty acid peroxides in mature rats, studied by means of thoracic duct cannulation, indicated that although the reduced products of the peroxides were absorbed, the peroxides themselves were destroyed in the intestine and probably had their action at that site. Recovery from the effects of whole-body irradiation, a condition also affecting the intestine, was delayed by diets containing fat with peroxide numbers as low as 100.

Inhibition of intestinal xanthine oxidase by air-oxidized soybean oil and its reversal by exogenous flavin adenine dinucleotide suggest that the specific toxicity of the lipid peroxides may be at the level of the intestinal enzymes.

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THE INFLUENCE OF DIETARY FAT ON THE FATTY ACID COMPOSITION OF LIVER, CARCASS, AND MILK OF RATS[1]

JOYCE L. BEARE

Abstract

Fatty acids of liver, carcass, and milk of rats fed corn oil, rapeseed oil, partially hydrogenated herring oil, or margarine were examined by gas-liquid chromatography. Appreciable quantities of linoleic acid were maintained in the tissues and milk, even when the hydrogenated herring oil with a low level of linoleic acid was fed. The proportion of C_{20} and C_{22} acids deposited or secreted was related to that of the diet, and was highest with rapeseed oil. In the livers of rats fed each diet, long-chain, polyunsaturated acids were observed. The fatty acids of milk more closely reflected the dietary pattern than did those of the tissues.

Introduction

Since rapeseed oil in the diet was found to depress appetite (1, 2) and to change the fatty acid composition of the tissues of rats (3), it was decided to examine the effect of other dietary fats containing long-chain fatty acids. Hydrogenated herring oil and a margarine containing marine oil were compared with corn oil and rapeseed oil during periods when the rats grew rapidly or nursed their young. By means of gas-liquid chromatography, the influence of these dietary fats on liver, carcass, and milk fatty acids was investigated.

Method

A purified diet (2) containing 20% by weight of corn oil, rapeseed oil, hydrogenated herring oil, or a margarine possessing some marine oil was fed ad libitum to male, weaning, Wistar rats of the Food and Drug colony. Ten animals per group, randomized within blocks of individual cages according to initial weights, were maintained on their respective regimens for 6 weeks. At the termination of this period, the rats were anesthetized with ether, bled, and the liver and adrenals removed and weighed. The Abell method (4) was used to determine the serum cholesterol levels.

Lipid samples were obtained from livers and ground degutted carcasses by the extraction method of Bligh and Dyer (5), methylated, and subjected to gas chromatography as previously described (6).

To obtain milk samples, seven mothers were fed each of the diets employed in the first study for 14 to 15 days following parturition. The stomach contents of the young rats were removed and the fatty acids analyzed as in the previous study (6).

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Results and Discussion

A chromatogram of each of the dietary fats is shown in Fig. 1. Their chief characteristics are: the high concentration of C_{18} acids, particularly of linoleic acid, in corn oil; the high levels of eicosenoic and erucic acids in rapeseed oil; the low content of linoleic acid, the odd-numbered fatty acids, and the series of C_{20} acids in partially hydrogenated herring oil; the apparent mixture of vegetable and marine oils with prominent C_{16}, C_{18}, and C_{20} acids in margarine.

From Table I it is seen that rapeseed oil was associated with a decreased food intake, whereas the other dietary oils were not. The total C_{20} and C_{22} acids in rapeseed oil, hydrogenated herring oil, and margarine varied in the ratio of

TABLE I

Food intake, weight gain, liver and adrenal weight, and serum cholesterol level of rats fed corn oil (CO), rapeseed oil (RSO), hydrogenated herring oil (HH), or margarine (M) for 6 weeks (10 rats per group)

Dietary fat	Food intake (g)	Wt. gain (g)	Adj. wt. gain* (g)	Liver wt. (g)	Adrenal wt. (mg/pair)	Serum cholesterol (mg/100 ml)
CO	339 ± 8†	134 ± 3	130	6.96 ± 0.20	26.9 ± 1.1	97.8 ± 5.8
RSO	253 ± 5	88 ± 2	119	6.18 ± 0.21	27.9 ± 1.6	71.3 ± 1.6
HH	398 ± 16	144 ± 7	118	8.57 ± 0.81	26.5 ± 1.2	62.0 ± 3.8
M	338 ± 6	114 ± 3	111	6.93 ± 0.13	27.6 ± 1.0	62.7 ± 2.7

*Weight gain adjusted for food consumption.
†Mean ± S.E.

4:2:1. Since a diet containing 10% instead of 20% rapeseed oil did not depress appetite (7), it was assumed that the other fats did not have the same effect as rapeseed oil because of their lower concentration of long-chain fatty acids. The rats receiving the hydrogenated herring oil consumed more food than did the other groups, and also gained more weight. When all weight gains were adjusted for food consumption by covariance analysis, the diet containing corn oil appeared to be the most efficiently utilized, and the diet containing margarine the least.

The livers of rats fed hydrogenated herring oil were significantly heavier than those of other rats which did not differ from each other. There were no significant differences in adrenal weights. As found previously in rats (8), the highest values for serum cholesterol were obtained in the animals fed corn oil.

In Fig. 2 are shown typical chromatograms of esters of fatty acids of liver tissue. The results of fatty acid analyses of individual livers and carcasses and their standard deviations are shown in Table II. Dietary fatty acids had more influence on the carcass fat than on the liver fat where a large proportion of the fatty acids are constituents of phospholipids which have a relatively constant composition.* The proportion of myristic acid was higher in hydrogenated herring oil and margarine and in the carcass fats of the rats fed those

*B. M. Craig, C. G. Youngs, J. L. Beare and J. A. Campbell. Unpublished data.

FIG. 1. Chromatograms of methyl esters of fatty acids of corn oil (CO), rapeseed oil (RSO), partially hydrogenated herring oil (HH), and margarine (M).

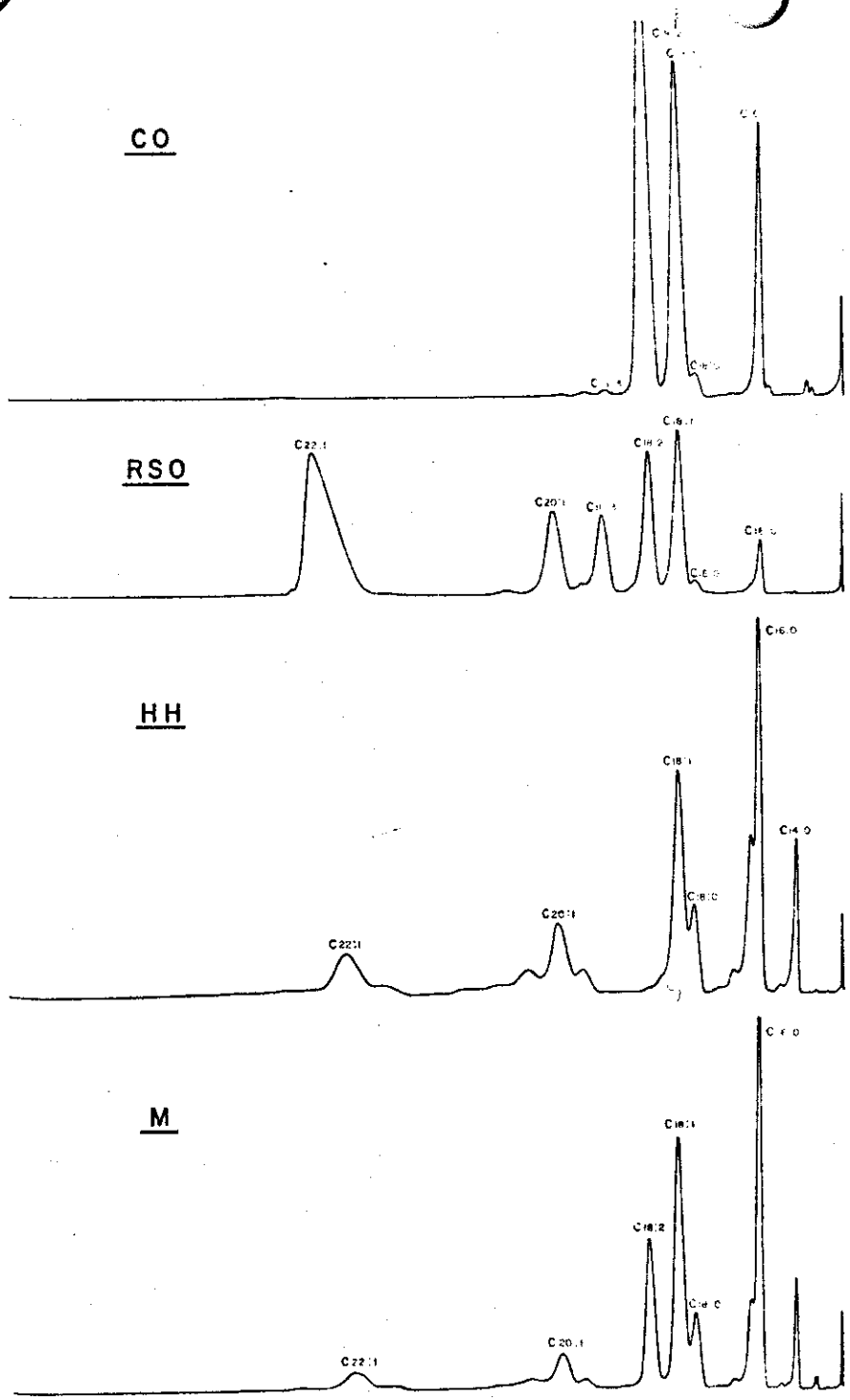

FIG. 1.

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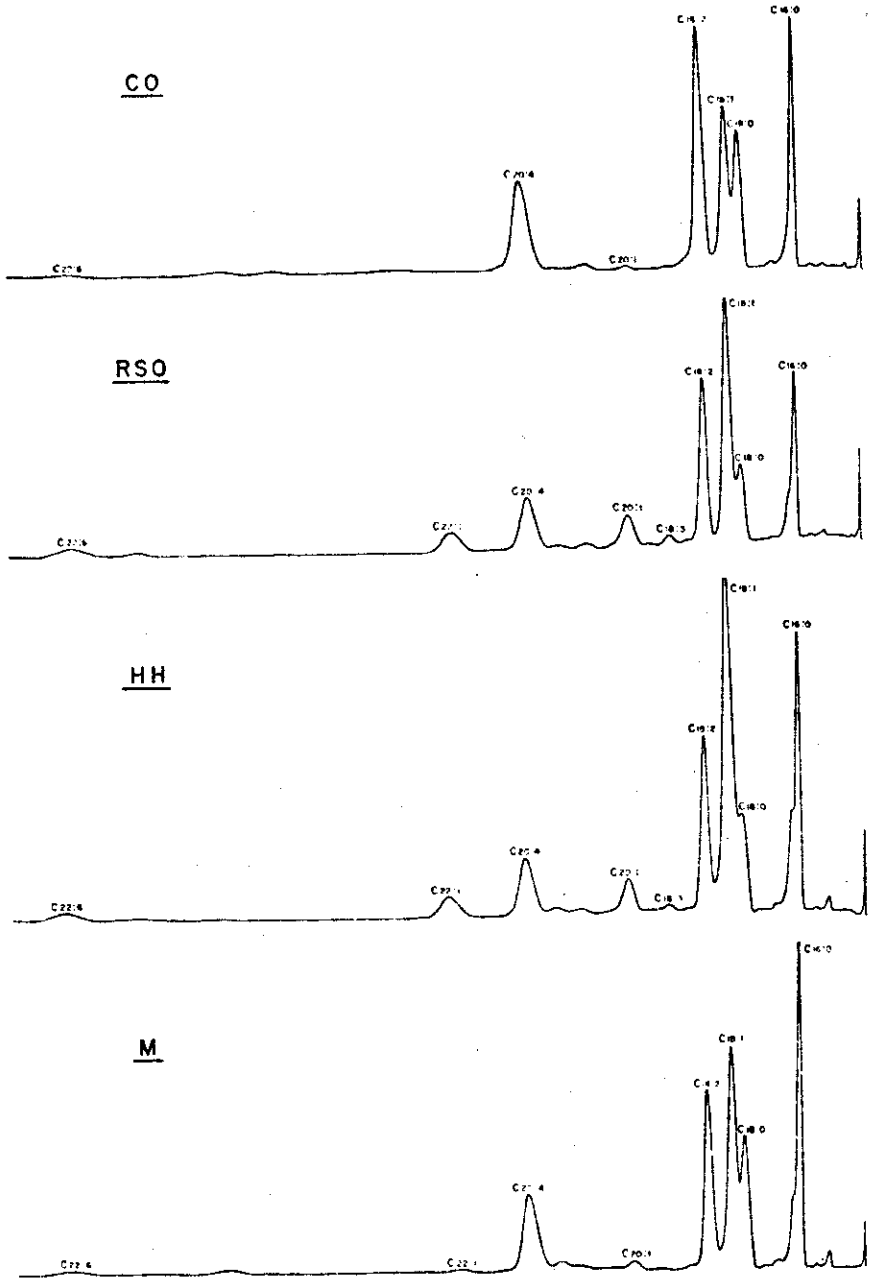

FIG. 2. Chromatograms of methyl esters of livers of rats fed corn oil, rapeseed oil, partially hydrogenated herring oil, or margarine.

TABLE II

Fatty acids of diet, liver, and carcass

Fatty acid	Source	% of total fatty acids			
		CO	RSO	HH	M
$C_{14:0}$	Diet	0.3	0.1	5.0	3.1
	Liver	0.2±0.1	3.7±0.4	0.8±0.2	0.7±0.3
	Carcass	0.9±0.2	1.2±0.3	4.6±0.7	3.9±0.8
$C_{15:0}$	Diet			0.4	0.2
	Liver	0.1±0.0	0.2±0.1	0.2±0.1	0.2±0.1
	Carcass	0.1±0.1	0.1±0.1	0.5±0.2	0.3±0.2
$C_{16:0}$	Diet	12.5	2.7	26.8	35.2
	Liver	16.0±1.4	8.8±2.2	14.4±1.4	18.5±2.2
	Carcass	13.5±1.7	10.9±1.7	19.3±3.9	20.9±2.6
$C_{16:1}$	Diet		1.1	13.1	8.0
	Liver		3.8±1.1	7.9±1.3	5.2±1.3
	Carcass	2.5±0.5	5.0±0.8	11.7±2.8	14.5±2.0
$C_{17:0}$	Diet			1.9	0.7
	Liver	0.6±0.7	0.1±0.1	0.9±0.8	0.5±0.1
	Carcass	0.2±0.1		0.9±0.4	1.0±0.4
$C_{17:1}$	Diet			0.3	0.3
	Liver				
	Carcass			0.4±0.3	0.3±0.2
$C_{18:0}$	Diet	2.1	1.4	6.0	6.5
	Liver	15.1±0.4	7.7±2.5	11.1±2.0	14.9±1.6
	Carcass	3.1±0.6	1.5±0.6	3.4±0.8	2.8±0.8
$C_{18:1}$	Diet	28.3	15.1	16.8	20.9
	Liver	19.7±3.2	34.2±5.8	27.6±1.1	20.9±5.0
	Carcass	35.5±1.1	34.7±2.4	36.4±2.4	36.0±1.9
$C_{18:2}$	Diet	54.2	13.9	0.5	11.8
	Liver	25.2±1.7	17.8±1.9	17.4±2.4	18.0±1.5
	Carcass	42.6±2.2	16.4±1.1	13.4±2.0	12.5±1.7
$C_{18:}$*	Liver	0.2±0.3	0.1±0.2	0.2±0.3	
$C_{18:3}$	Diet	2.0	9.4		
	Liver	0.4±0.4	1.2±0.3	0.3±0.3	0.4±0.4
	Carcass	0.8±0.3	5.2±0.7	0.8±1.5	0.2±0.2
$C_{20:0}$	Diet	0.4		3.0	
	Liver	0.1±0.1	0.1±0.2	0.1±0.2	1.1
	Carcass			0.6±0.2	0.8±0.4
$C_{20:1}$	Diet	0.2	12.7	9.0	4.7
	Liver	0.8±0.4	6.7±1.8	2.0±1.4	1.1±0.3
	Carcass	0.6±0.2	10.2±0.8	4.4±0.7	3.9±0.6
$C_{20:2}$	Diet		0.7	4.2	2.1
	Liver	0.5±0.5	0.3±0.4	0.1±0.2	0.5±0.3
	Carcass	0.1±0.1	0.8±1.1	1.1±0.3	0.9±0.3
$C_{20:}$*	Liver		0.1±0.2	0.8±0.5	0.5±0.3
$C_{20:3}$	Diet			1.6	
	Liver	0.2±0.3	0.3±0.4	1.6±1.1	2.0±0.5
$C_{20:4}$	Diet			0.4	
	Liver	17.5±1.9	8.1±2.1	10.6±0.6	14.2±1.5
	Carcass	2.0±0.8	1.0±0.5	1.1±0.3	1.0±0.5

*Unknown.

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TABLE II (*Concluded*)

Fatty acids of diet, liver, and carcass

Fatty acid	Source	% of total fatty acids			
		CO	RSO	HH	M
$C_{22:0}$	Diet			1.6	0.7
$C_{22:1}$	Diet		42.9	8.6	3.7
	Liver	0.3±0.6	5.6±1.6	1.5±1.3	0.3±0.3
	Carcass	0.1±0.2	11.3±0.9	0.8±0.5	0.7±0.6
$C_{22:2}$	Diet		0.4		
	Carcass		1.4±0.5		
$C_{22:3}$	Liver	0.7±0.5	0.2±0.5		
$C_{22:4}$	Liver	0.9±0.6	0.2±0.5	0.8±0.7	1.0±0.6
$C_{22:5}$	Liver	0.2±0.5	0.9±1.1	0.2±0.2	
$C_{22:6}$	Liver	1.2±0.8	3.0±0.8	1.5±0.5	1.5±0.5

*Unknown.

oils than in corn oil or rapeseed oil or the rats fed those oils. As expected from previous work with animal fats (9, 10, 11), oils of marine origin and rat liver and carcass fats contained pentadecanoic acid. The levels of palmitic and palmitoleic acids in the diet influenced the tissue levels of those constituents. Heptadecanoic acid occurred in herring oil and margarine, in carcasses of the rats fed those fats, and in some livers of each group. Heptadecenoic acid, found in musk-ox fat by Chisholm and Hopkins (12), might have been present, but the chromatogram peak in question might also be identified as isooctadecanoic acid (13). Liver lipids contained relatively high proportions of stearic acid (14), mostly in the phospholipid fraction (15). Although the dietary level of oleic acid varied from 15.1 to 28.3%, the tissue levels appeared to be independent. The high level of linoleic acid in corn oil increased the content of that acid in tissue fat, particularly in the carcass. As shown in the depot fat of mice by Tove and Smith (16), and of rats by Mead (17), linoleic acid was inversely related to palmitoleic acid, but linolenic acid did not increase with linoleic acid. Despite the small quantity of dietary linoleic acid available to rats fed the hydrogenated herring oil, the level of that acid in the body fat was similar to that of rats fed rapeseed oil or margarine. This finding could be indicative of a preferential retention of the sparsely available linoleic acid. In some livers there appeared to be an isomer of the common linoleic acid which emerged immediately after it from the chromatographic column, as was observed previously (8). Rats fed rapeseed oil which contained 9.4% linolenic acid had appreciably more of that acid in their tissues than other rats.

The combined C_{20} and C_{22} fatty acids in the diet and in the carcass of rats fed corn oil was 0.6 and 2.8% respectively; rapeseed oil, 56.7 and 24.7%; hydrogenated herring oil, 28.4 and 8.0%; margarine, 11.2 and 7.3%. Small amounts of arachidic acid in the diet had little influence on its deposition in the animals, whereas eicosenoic acid was readily deposited, as shown by

Hopkins *et al.* (18), and reflected the dietary levels. Eicosadienoic acid was found in some of the livers and particularly in the carcasses of those animals supplied with it. In the livers there were also small amounts of polyunsaturated C_{20} acids and an eicosatrienoic acid, observed in beef liver by Klenk and Montag (19). Arachidonic acid, which was synthesized from linoleic acid (20, 21), was found to be most pronounced in the rats fed corn oil, and to be present in lower and similar concentrations in the other animals. Although linolenic acid was believed to be converted to eicosapentaenoic acid (22), such an acid was not detected on the chromatograms. The dietary behenic acid seemed not to be in the rat tissue. Of particular interest was the tissue erucic acid, which amounted to 11.3% of the carcass fatty acids in rats fed rapeseed oil, and was about 26% as concentrated as in the diet. The small amount of docosadienoic acid of rapeseed oil appeared to be easily retained by the rat. On the basis of relative retention times on the chromatographic column, the liver was found to contain C_{22} acids with three, four, five, and six double bonds, the last being the most pronounced. By studying the oxidative degradation products of liver fatty acids of rats fed labelled acetate, Klenk (21, 23) identified these highly unsaturated, long-chain fatty acids which originated from dietary linoleic and linolenic acids.

Table III gives the data of the young rats from which the stomach contents were obtained. One rat fed margarine failed to produce a litter, and two others on the same diet and one fed hydrogenated herring oil ate their offspring. The

TABLE III

Litters nursed by rats fed different dietary oils

Dietary fat	No. of litters	No. of young per litter	Litter wt. (g)	Mean wt. of young (g)
CO	7	10.6±0.5	252±12	24.1±1.5
RSO	7	9.7±0.6	200±8	21.0±1.2
HH	6	8.8±0.6	169±8	19.3±0.7
M	4	9.5±0.8	220±11	23.2±0.8

weights of the 14- to 15-day-old litters of mothers supplied with corn oil were significantly greater ($P = 0.01$) than of those receiving rapeseed oil or hydrogenated herring oil. Rats fed margarine had significantly heavier litters than those fed hydrogenated herring oil.

In Fig. 3 the values for the milk fatty acids with their standard deviations are shown beside the dietary levels of fatty acids. The proportion of myristic in the total fatty acids was greater in milk than in the tissues, but was again influenced by the dietary oil. The quantities of palmitic, palmitoleic, stearic, oleic, linoleic, linolenic, eicosenoic, eicosadienoic, and erucic acids reflected the dietary pattern of fatty acids, generally to a greater extent than the carcass fatty acids. Arachidonic acid, not provided in the diet, was related to the varying amounts of dietary linoleic acid. Within the pattern of fatty acids

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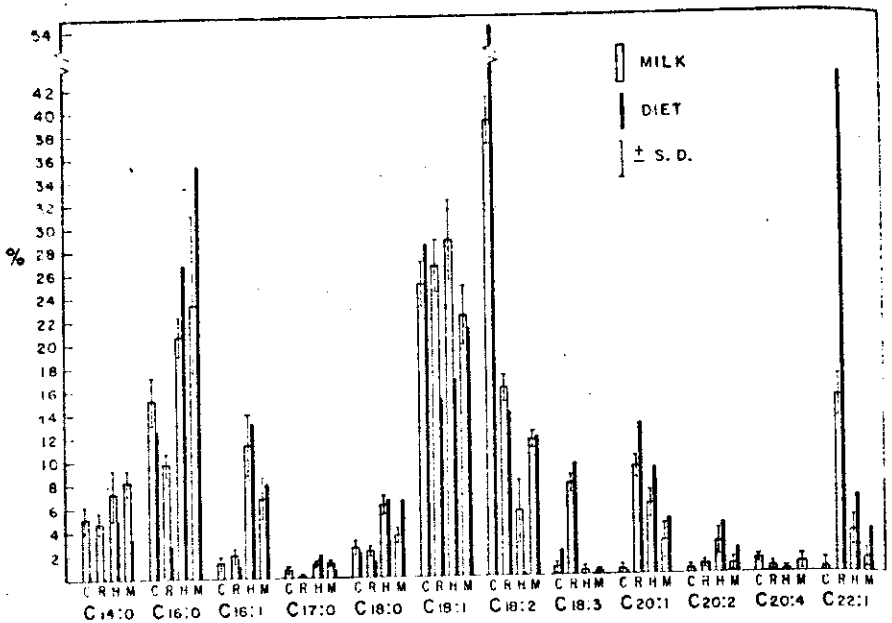

FIG. 3. The percentage of fatty acids in the total fatty acids of milk and diet of seven rats fed corn oil (C), seven rats fed rapeseed oil (R), six rats fed hydrogenated herring oil (H), and four rats fed margarine (M).

secreted by the rat, appreciable variations could be imposed by changes in the dietary fat.

It was of interest that the rats which had been fed erucic acid for several generations (6) secreted milk containing somewhat less than half of the amount of that fatty acid produced by these rats which had received rapeseed oil only while nursing the young. From these findings, it appeared that over long periods of time there was some adaptation in the utilization of erucic acid.

The effect of long-chain fatty acids on food intake and the composition of fatty acids deposited or secreted by the rat appeared to be related to the quantities of such acids present in the diet. Milk showed the best reflection of the dietary pattern of fatty acids.

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

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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

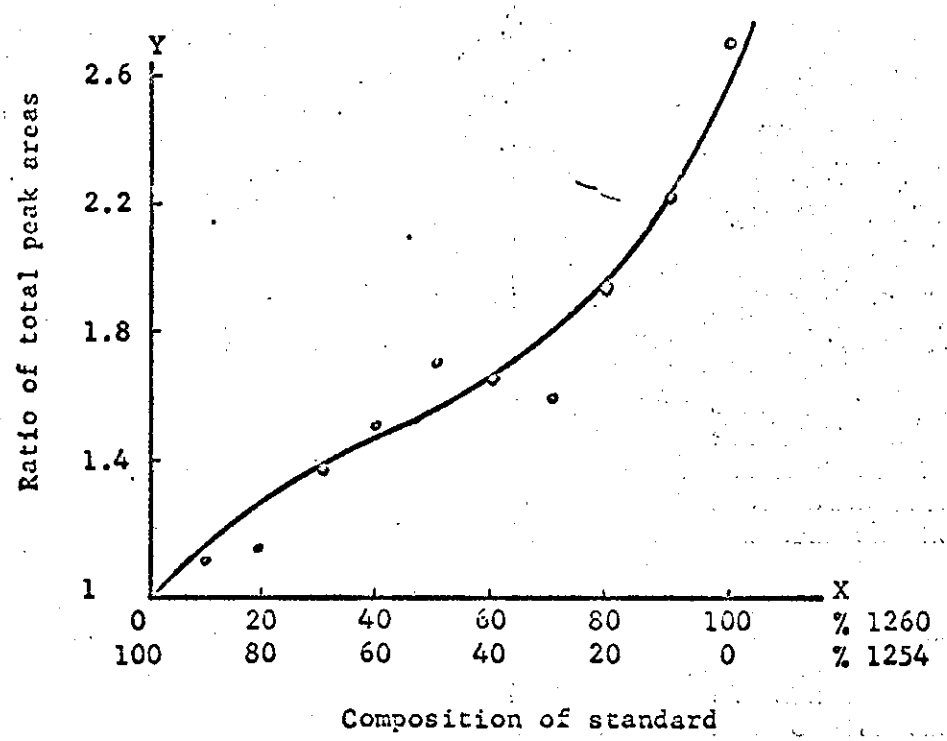

Figure 1. Dependence of total peak area on the relative amounts of Aroclors 1254 and 1260 in a mixed standard. (Total peak area = g paper/ng. PCB's injected).

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

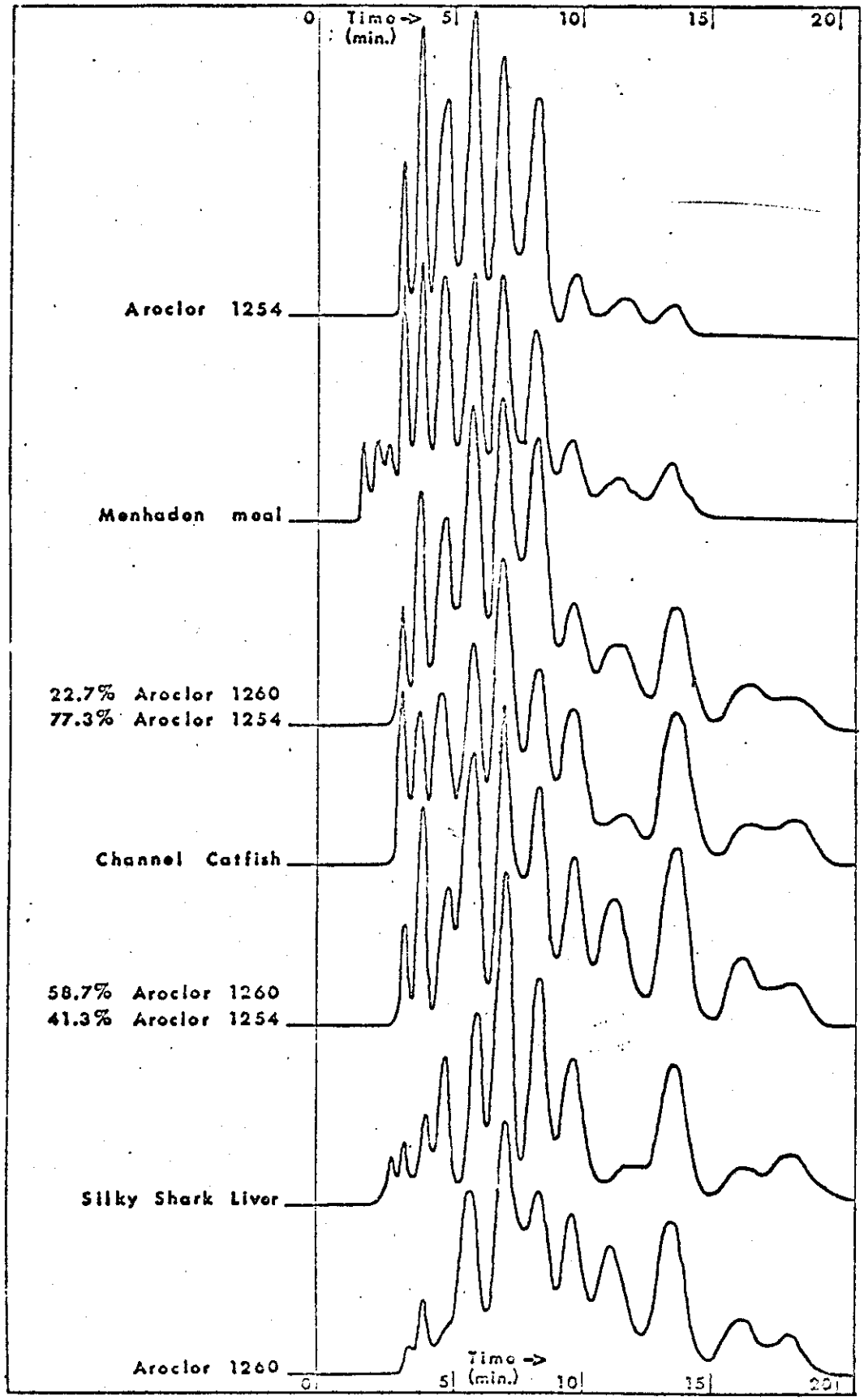

Figure 2. Gas chromatograms of Aroclor standards and three fishery products. Single vertical line represents solvent peaks.

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

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3,455,699

LIQUID SHORTENING PROCESS

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Int. Cl. A23d 5/02
U.S. Cl. 99—118 17 Claims

ABSTRACT OF THE DISCLOSURE

A process for the manufacture of a liquid shortening including forming a molten blend of an oil, fat having a melting point in excess of approximately 100° F. and an emulsifier containing mono- or diglycerides; rapidly cooling the blend to a temperature near the cloud point of the emulsifier; agitating the blend in excess of approximately 30 minutes while maintaining the blend at a temperature between approximately that reached by the heat of crystallization of the blend and the capillary melting point of the emulsifier; rapidly cooling the blend to a temperature near the cloud point of the fat; and agitating the blend in excess of approximately 30 minutes while maintaining the blend at a temperature between approximately that reached by the heat of crystallization of the blend and the capillary melting point of the crystals existing in the oil and fat prior to the formation of the molten blend.

In the preparation of certain baked goods such as cakes and breads using shortening as one of the ingredients, it is necessary during the batter preparation to incorporate the liquid ingredients into the dry material in the form of small droplets surrounded by a thin layer of fat and to mix air in the form of tiny bubbles through the batter. This results in a cake or bread of good volume and texture. To accomplish the formation of small air and liquid droplets, it is necessary to add a material that exhibits surface activity between the aqueous and fat surfaces. This material is commonly known as an emulsifier.

Mono- and diglycerides and mixtures thereof are commonly used as emulsifiers.

Mono- and diglyceride emulsifiers are normally, but not always, solid at room temperature and when such emulsifiers are blended with a liquid base oil the crystals in the emulsifier and those that are present in the liquid base oil tend to settle and the viscosity of the blend tends to change, that is, the blend is unstable. An excellent liquid shortening utilizing mono- and diglyceride emulsifiers is a stable suspension of solid discrete particles or crystals of the emulsifier and the liquid base oil in the liquid base oil at a temperature range of about 60° F. to about 100° F. The suspension should remain fluid throughout this temperature range without any significant change in viscosity and without any significant separation or settling of the solid fraction. These are rigid requirements and it is exceedingly difficult to prepare a liquid shortening which will meet them.

It is a major object of the present invention to provide a process of preparing such a liquid shortening utilizing mono- and/or diglyceride emulsifiers which shortening comprises a stable suspension of solid discrete crystals of the emulsifier and of the liquid base oil in the liquid base oil in a temperature range from about 60° F. to 100° F. and which remains liquid throughout this range without any significant change in viscosity or significant separation or settling of the solid fraction.

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It is known that glycerides may be crystallized in any one of a number of forms depending on the temperature and speed at which crystallization is permitted to take place. The three general classifications of crystalline forms of glycerides in the art are the alpha, the beta and the beta prime form. As is well known in the art, the alpha crystalline form is the least stable and has the lowest melting point of these forms. The beta crystalline form is generally the most stable and has the highest melting point. The beta prime crystalline form is intermediate between the alpha and beta crystalline forms in stability and melting point.

Liquid shortenings having the most desirable properties are believed to have their glycerides, or a predominate portion thereof, in the beta crystalline form. Applicants believe that in their process of preparing liquid shortening the crystals of both the emulsifier and the liquid base oil are predominately in the beta form in the liquid shortening produced, but whether this is true or not, the method of the applicants does produce a stable liquid shortening containing emulsifiers of mono- and diglyceride esters.

It is a further object of the present invention to provide a method for the manufacture of liquid shortenings containing mono- and diglyceride emulsifiers which may be practiced by utilizing conventional equipment.

Other and further objects, features and advantages will be apparent from the following description of the presently preferred examples of the invention given for the purpose of disclosure.

In general, the process of the invention is based upon the discovery that there results a liquid shortening having a stable suspension of discrete particles or crystals of (i) emulsifier of mono- or diglycerides and mixtures thereof and (ii) liquid base oil, in liquid base oil in a temperature range between about 60° F and 100° F., the emulsifier and liquid base oil being different melting point components of the liquid shortening, by carrying out the steps of:

(a) Forming a liquid base oil by mixing a vegetable oil containing solid crystals somewhere in the temperature range of approximately 32° F. to 100° F. with a fat that has a melting point in excess of approximately 100° F., said mixing taking place at temperatures sufficient to completely melt all crystals in the fat and vegetable oil,

(b) Heating the emulsifier until all crystals contained therein are melted,

(c) Forming a blend of melted emulsifier and liquid base oil,

(d) Rapidly cooling the blend to a temperature between about 5° F. above and a temperature slightly below the cloud point of the higher melting point component,

(e) Agitating the blend in excess of approximately 30 minutes while maintaining the blend at a temperature between approximately that reached by heat of crystallization of the blend and the capillary melting point of the higher melting point component,

(f) Rapidly cooling the blend to a temperature between about 5° F. above and a temperature slightly below the cloud point of the lower melting point component, and

(g) Agitating the blend in excess of approximately 30 minutes while maintaining the blend at a temperature between approximately that reached by heat of crystallization of the blend and the capillary melting point of (i) the crystals referred to in step (a) when the liquid base oil is the lower melting point component and (ii) the emulsifier when the emulsifier is the lower melting point component.

If desired, rather than forming the liquid base oil before mixing it with the emulsifier, the vegetable oil, the fat, and the emulsifier may all be heated and blended simultaneously, they may all be heated separately and

then blended together, or any two of them may be heated and then blended with the third.

The emulsifier is normally solid and when it is it will be the higher melting point component of the liquid shortening. If the emulsifier is the lower melting point component then the process set forth above can be varied by having it comprise the steps of:

(a) Forming a liquid base oil by mixing a vegetable oil containing solid crystals somewhere in the temperature range of approximately 32° to 100° F. with a fat that has a melting point in excess of approximately 100° F., said mixing taking place at a temperature sufficient to completely melt all crystals in the fat and oil,

(b) Rapidly cooling the liquid base oil to a temperature between about 5° above and a temperature slightly below the cloud point of the liquid base oil,

(c) Agitating the liquid base oil in excess of approximately 30 minutes while maintaining the liquid base oil at a temperature between approximately that reached by its heat of crystallization and the capillary melting point of the liquid base oil,

(d) Heating the emulsifier until all crystals contained therein are melted,

(e) Forming a blend of the emulsifier and liquid base oil,

(f) Rapidly cooling the blend to a temperature between about 5° above and a temperature slightly below the cloud point of the emulsifier, and

(g) agitating the blend in excess of approximately 30 minutes while maintaining the blend at a temperature between approximately that reached by its heat of crystallization and the capillary melting point of the emulsifier.

The vegetable oils which may be used in accordance with this invention include those which are commonly used in liquid shortenings such as cottonseed oil, soybean oil, peanut oil, rapeseed oil, sesame seed oil and sunflower oil, and mixtures thereof which are liquid at 60° F.

The mono- or diglyceride emulsifiers and emulsifiers which are mixtures thereof include those commonly used in shortenings. Some examples are those (i) made by superglycerinating vegetable oils, lard, or tallow, and (ii) the reaction product resulting from an ester interchange reaction of glycerides of edible fat, propylene glycol and glycerin in accordance with Patent No. 3,097,098, issued July 9, 1963 for a "Shortening and Agent Therefor."

As used herein the term "cloud point" of a liquid base oil or emulsifier is a temperature which is equal to the temperature of the emulsifier or liquid base oil determined in the following manner. A sample of the liquid base oil or emulsifier is heated to approximately 130° C., filtered, and placed in a cloud point beaker having parallel sides and made of precision bore Pyrex tubing having inside dimensions between the parallel sides of 1.751±.001 inches and outside dimensions of 1.92±.01 inches. Such a cloud point beaker is a standard cloud point beaker, is well known in the art, and no further description thereof is necessary. The temperature of the sample in the beaker is adjusted to 70–75° C. and the beaker placed in a container having a circulating cold water bath with a temperature of 35°±1° F. and sufficient flow of water to cool the sample from 60° C. to 40° C. in 70±5 seconds. The container of the circulating water bath receives the beaker in such a manner that opposite portions of the container contact the beaker at opposite points on the parallel sides of the beaker so that a light source may be directed against the beaker on one side and a photoelectric cell may receive the light passing through the beaker without the light passing through any water in the water bath. During the cooling from 60° C. to 40° C. and continued cooling thereafter the sample is continuously agitated. When the temperature of the sample is 50° C. the distance between the light source and the photoelectric cell is adjusted so that the cell detects 30 lumens of light. The light source and cell are then left at this distance with respect to each other during the continued cooling of the sample and when the sample in the beaker becomes so cloudy that the amount of light passing through it is reduced to 5 lumens the temperature of the sample is read and this is used as the cloud point.

The cloud point of either the emulsifier or the liquid base oil is believed to be below its alpha melting point as the alpha melting point is near the lower limit to which glycerides can be supercooled, but applicants are not so be bound by this theory.

The capillary melting point was determined by the capillary tube melting point determination which is the American Oil Chemists' Society official method Cc 1–25. This method is well known in the art and no further description thereof is necessary. In order to determine the capillary melting point of the crystals in the vegetable oil these crystals were removed by conventional fractional crystallization and filtration of the vegetable oil prior to having their capillary melting point determined.

Some vegetable oils such as cottonseed oil have solid crystals naturally occurring somewhere in the temperature range of approximately 32° to 100° F. Other oils such as soybean oil need to be partially hydrogenated to obtain these crystals. The amount of solid crystals in the vegetable oil and the liquid base oil does not itself constitute the present invention as the desirability of such crystals in liquid shortenings is well known, the amount varies considerably, and any of the conventionally employed amounts are suitable for the purpose of this invention and so no further description thereof is necessary.

The rapid cooling of the blend, emulsifier, or liquid base oil referred to herein means cooling in less than approximately three minutes.

As used herein "temperature slightly below the cloud point" means a temperature below the cloud point but not so far below the cloud point that the liquid involved is substantially solidified.

EXAMPLE 1

The liquid base oil of the shortening was prepared by blending 93.7 parts of partially hydrogenated soybean oil having an iodine value of 109 with 6.3 parts of hydrogenated soybean oil having a titer of 63 which resulted in a liquid base oil having an iodine value of 104, a cloud point of 88.9° F. and a capillary melting point of 129.6° F. The 109 iodine value hydrogenated soybean oil had crystals in it at room temperature and these crystals had a capillary melting point of approximately 100° F. The emulsifier was a solid material which was superglycerinated 52 titer tallow which had an alpha monoglyceride content of 40%. Its cloud point was 122° F. and its capillary melting point 126.4° F. Four parts of the emulsifier were blended with 100 parts of the liquid base oil and the mixture heated to 160° F. under agitation until all crystals were completely melted. The blend was then cooled to 111° F. in approximately 1½ minutes by one pass through a "Votator" type chilling machine which is a scraped wall heat exchanger of the type well known in the art. The temperature of the blend was allowed to rise to about 120° F. through heat of crystallization and was agitated for one hour at this temperature. The blend was then cooled to 80° F. in approximately 1½ minutes in a single pass through the Votator and then heated to 90° F. and held at that temperature for approximately one hour under agitation. Agitation was stopped and separate samples of the shortening were collected and held at temperatures of 60°, 70°, 80°, 90°, and 100° F. for one month. During this period of time each of these samples remained pourable and the suspension stable.

EXAMPLE 2

The liquid base oil was prepared from the same ingredients as the liquid base oil in Example 1 above except that 99.5 parts of the 109 iodine value soybean oil were blended with 0.5 parts of the 63 titer hydrogenated

soybean oil. This gave a liquid base oil having an iodine value of 108.2, a cloud point of 57.6° F. and a capillary melting point of 106.2° F. The capillary melting point of the crystals of the 109 iodine value soybean oil was approximately 100° F. The emulsifier was prepared by blending 3.6 parts of superglycerinated 63 titer hydrogenated peanut oil having 40% alpha monoglycerides with 8.6 parts of an emulsifier made in accordance with the process for making the shortening agent of Patent No. 3,097,098 issued July 9, 1963 for a Shortening and Agent Therefor and contained 65% monoesters of propylene glycol, 20% monoglycerides, and the remainder being unreacted triglycerides, diglycerides, and di-esters of propylene glycol. This emulsifier had a cloud point of 116.6° F. and a capillary melting point of 116.8° F. This emulsifier was a solid at room temperature. 100 parts of liquid base oil and 12.2 parts of the emulsifier were mixed together and heated to 150° F. under agitation resulting in a complete melting of all crystals. The blend was then cooled to 100° F. in approximately 1½ minutes and one pass through a Votator and then allowed to rise by heat of crystallization to a temperature of 108° F. where it was agitated for one hour. The blend was then cooled to 60° F. in approximately 1½ minutes by passing it through a Votator and then warmed by heat of crystallization to 80° F., held at the latter temperature for one hour and agitated during that period of time. After the agitation was stopped samples of the liquid shortening were collected and held at temperatures of 60°, 70°, 80°, 90° and 100° F. for one month. During this period of time the samples of shortening remained pourable and the suspension stable.

EXAMPLE 3

The liquid base oil of the shortening was prepared by blending 99.5 parts of refined bleaced cottonseed oil with 0.5 parts of hydrogenated soybean oil having a titer of 63 which resulted in a liquid base oil having an iodine value of 109.2, a cloud point of 61.2° F. and a capillary melting point of 112.4° F. The refined bleached cottonseed oil had crystals naturally occurring in it at room temperature and these crystals had a capillary melting point of approximately 80° F. A solid emulsifier was prepared by blending 3.5 parts of superglycerinated 52 titer hydrogenated blend of 60% lard and 40% tallow (40% alpha monoglyceride content) with 8.5 parts of an emulsifier made in accordance with the process for making the shortening agent of patent No. 3,097,098 issued July 9, 1963 for a Shortening and Agent Therefor and containing 65% monoesters of propylene glycol, 19% monoglycerides, and the remainder being unreacted triglycerides, diglycerides, and di-esters of propylene glycol. This emulsifier had a cloud point of 116.6° F. and a capillary melting point of 116.8° F. 100 parts of liquid base oil and 12 parts of the emulsifier were blended and subjected to the same treatment as the blend of emulsifier and liquid base oil of Example 2 and resulted in a liquid shortening which had essentially the same characteristics of stability and remained pourable with the same range of temperatures as that produced by Example 2.

EXAMPLE 4

In this example 99.5 parts of the same 109 iodine value hydrogenated soybean oil and .5 parts of the same 63 titer hydrogenated soybean oil as used in Example 2 and 12 parts of the same emulsifier as used in Example 3 were used to make the liquid shortening. The emulsifier and the 63 titer hydrogenated soybean oil were heated together to 140° F. causing them to be completely melted. This mixture was added to the 109 iodine value soybean oil which has been heated to 95° F. and the entire blend agitated. The temperature of the blend was 104° F. which resulted in an almost instantaneous cooling of the 63 titer soybean oil and the emulsifier to this 104° F. The blend was homogenized in a Votator without refrigeration and then allowed to rise to 105° F. through heat of crystallization and agitated for approximately one hour. Next, the blend was cooled to 60° F. with a single pass through a Votator and allowed to rise from heat of crystallization to 84° and held at that temperature for one hour under agitation. Separate examples of this liquid shortening were collected and held at temperatures of 60°, 70°, 80°, 90° and 100° F. for one month and during this time the blend remained pourable and the suspension stable.

EXAMPLE 5

The liquid base oil was made of 92.7 parts 109 iodine value hydrogenated soybean oil and 7.3 parts of 63 titer hydrogenated soybean oil. The emulsifier was superglycerinated 100 iodine value hydrogenated soybean oil which had 40% alpha monoglycerides. The liquid base oil had an iodine value of 102.8, a cloud point of 91° F. and a capillary melting point of 131.4° F. The emulsifier had a cloud point of 83.3° F. and a capillary melting point of 113.4° F. so that the emulsifier had a lower melting point than the liquid base oil. In this example the liquid base oil was heated to 140° F. and agitated until all the crystals were melted and then cooled to 86° F. in approximately one minute in one pass through a chilled Votator. The liquid base oil was heated and agitated for about 1 hour at a temperature of 115° F. The emulsifier was heated sufficiently to melt all crystals in it, added to the liquid base oil, agitated to blend them, and the blend cooled to 75° in approximately one minute in a single pass through a chilled Votator. The blend was then heated and agitated for approximately one hour at a temperature of 103° F., agitation stopped, and separate samples of the shortening collected. These separate samples were held at temperatures of 60°, 70°, 80°, 90° and 100° F. for one month. During this time the liquid shortening remained pourable and the suspension stable.

The present invention is well suited and adapted to obtain the objects and ends and has the advantages mentioned as well as others inherent therein.

While only presently preferred examples of the invention have been given for the purpose of disclosure, the invention is to be limited by the scope of the appended claims.

In the following claims the term "emulsifier" means emulsifiers containing monoglycerides diglycerides, and mixtures thereof.

What is claimed is:

1. In a process of preparing liquid shortening which is liquid between about 60° F. and 100° F. and having a stable suspension between about 60° F. and 100° F. of crystals of emulsifier containing mono- or diglycerides or mixtures thereof and crystals of liquid base oil in liquid base oil, the emulsifier and liquid base oil being higher and lower melting point components of the liquid shortening, the improvements comprising the steps of:

(a) forming a liquid base oil by mixing a vegetable oil containing solid crystals somewhere in the temperature range of approximately 32° F. to 100° F. with a fat that has a melting point in excess of approximately 100° F., said mixing taking place at a temperature sufficient to melt all crystals in thte fat and vegetable oil,

(b) heating the emulsifier until all crystals contained therein are melted,

(c) forming a blend of the melted emulsifier and liquid base oil, the heating of the vegetable oil, the fat, and the emulsifier and their blending being carried out in any order,

(d) rapidly cooling the blend to a temperature between about 5° above and a temperature slightly below the cloud point of the higher melting point component,

(e) agitating the blend in excess of approximately 30 minutes while maintaining the blend at a temperature between approximately that reached by heat of crys-

tallization of the blend and the capillary melting point of the higher melting point component,

(f) rapidly cooling the blend to a temperature between about 5° F. above and a temperature slightly below the cloud point of the lower melting point component, and

(g) agitatitng the blend in excess of approximately 30 minutes while maintaining the blend at a temperature between approximately that reached by heat of crystallization of the blend and the capillary melting point of (i) the crystals in the vegetable oil referred to in step (a) when the liquid base oil is the lower melting point component and (ii) the emulsifier when the emulsifier is the lower melting point component.

2. The process of claim 1 in which the agitation in each of steps (e) and (g) is for approximately 60 minutes.

3. The process of claim 1 in which the vegetable oil includes soybean oil.

4. The process of claim 2 in which the vegetable oil includes soybean oil.

5. The process of claim 1 in which the vegetable oil includes cottonseed oil.

6. The process of claim 5 in which the agitation of each of steps (e) and (g) is carried on for approximately 60 minutes.

7. The process of claim 1 in which the emulsifier is the higher melting point component.

8. The process of claim 7 in which the vegetable oil includes soybean oil.

9. The process of claim 8 in which the agitation in each of steps (e) and (g) is for approximately 60 minutes.

10. The process of claim 1 in which the vegetable oil includes cottonseed oil.

11. The process of claim 10 in which the agitation in each of steps (e) and (g) is carried on for approximately 60 minutes.

12. In a process of preparing liquid shortening which is liquid between about 60° F. and 100° F. and having a stable suspension between about 60° F. and 100° F. of discrete particles of emulsifier containing mono- or diglycerides or mixtures thereof and particles of liquid base oil in liquid base oil, the liquid base oil having a higher melting point than the emulsifier, the improvement comprising the steps of:

(a) forming a liquid base oil by mixing a vegetable oil containing solid crystals somewhere in the temperature range of approximately 32° F. to 100° F. with a fat that has a melting point in excess of approximately 100° F., said mixing taking place at a temperature sufficient to melt all crystals in the fat and vegetable oil,

(b) rapidly cooling the liquid base oil to a temperature between about 5° above and a temperature slightly below the cloud point of the liquid base oil,

(c) agitating the liquid base oil in excess of approximately 30 minutes while maintaining the liquid base oil at a temperature between approximately that reached by its heat of crystallization and the capillary melting point of the liquid base oil,

(d) heating the emulsifier until all crystals contained therein are melted,

(e) forming a blend of the emulsifier and liquid base oil,

(f) rapidly cooling the blend to a temperature between about 5° above and temperatures slightly below the cloud point of the emulsifier, and

(g) agitating the blend in excess of approximately 30 minutes while maintaining the blend at a temperature between approximately that reached by its heat of crystallization and the capillary melting point of the emulsifier.

13. The process of claim 12 in which external heat is applied in each of the steps (c) and (g).

14. The process of claim 12 in which the vegetable oil includes soybean oil.

15. The process of claim 13 in which the vegetable oil includes soybean oil.

16. The process of claim 1 in which the fat in step (a) is present in an amount between approximately 0.5 and 7.0% of the liquid base oil.

17. The process of claim 12 in which the fat in step (a) is present in an amount between approximately 0.5 and 7.0% of the liquid b[illegible]il.

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MAURICE W. GREENSTEIN, Primary Examiner.

Aetiology of Multiple Sclerosis

by
JOSEPH BERNSOHN
LEO M. STEPHANIDES
Neuropsychiatric Research Laboratory,
Veterans Administration Hospital,
Hines, Illinois, and
Department of Biochemistry,
Northwestern University Medical School,
Chicago, Illinois

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[12]. 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

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The Nutrition of the Young Ayrshire Calf

14. Some Effects of Natural and Synthetic Anti-oxidants on the Incidence of Muscular Dystrophy Induced by Cod-liver Oil

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The muscular dystrophy of calves caused by giving cod-liver oil (Blaxter, Wood & MacDonald, 1953), the unsaturated acids of cod-liver oil (Blaxter, Brown & MacDonald, 1953*b*) or rations containing lard (Blaxter, Watts & Wood, 1952) may be prevented by giving α-tocopherol.

The mode of action of α-tocopherol in these diseases is unknown. A reasonable supposition, however, is that it acts simply as an anti-oxidant, possibly protecting the animal against the toxicity of unsaturated fatty acids. If this were so, other anti-oxidants might be equally effective. The efficacies of ascorbic acid, ethyl gallate, methylene blue and biotin have therefore been compared with that of α-tocopheryl acetate in preventing the muscular dystrophy induced by giving cod-liver oil to calves.

It has been claimed (Hjärre & Lilleengen, 1936*a*, *b*) that enzootic muscular dystrophy is due to ascorbic-acid deficiency. This is unlikely, for reasons discussed elsewhere (Blaxter & Brown, 1952–3). It is of interest, however, that ascorbic acid is

partially effective in preventing signs of vitamin E deficiency in c[illegible] given rations containing cod-liver oil (Zacharias, Goldhaber & Kinsey, 1950; Dam, Kruse, Prange & Søndergaard, 1948). Further, a decline in serum ascorbic acid occurs in horses affected by myoglobinuria paralytica, a disease in which a muscle degeneration, similar to that of calves deficient in vitamin E, takes place.

Methylene blue has been shown to be of considerable value in treating the above disease of the horse (Elzén, 1932*a*, *b*), and recent experimental studies by Dam and his colleagues have shown that methylene blue can replace vitamin E in preventing many of the signs of vitamin E deficiency in rats and chicks (Dam, Kruse, Prange & Sondergaard, 1951; Christensen & Dam, 1951; Aaes-Jørgensen, Dam & Granados, 1951; Dam & Granados, 1951, 1952; Dam, Prange & Sondergaard, 1951, 1952*a*, *b*). This work has recently been confirmed and extended by Moore and his colleagues working with rats; they have found that some, but not all, manifestations of vitamin E deficiency are prevented by methylene blue, and that the effect is specific, not being shared by a range of redox dyestuffs and anti-oxidants (Moore, Sharman & Ward, 1953*a*, *b*).

Biotin deficiency in the calf (Wiese, Johnson & Nevens, 1946) has been associated with spasticity of gait and prostration, signs similar to those observed in our calves given cod-liver oil. Further, Sullivan, Kolb & Nicholls (1942) have observed muscle changes in biotin deficiency of rats similar to those in vitamin E deficiency, and in vitro studies (Pavcek & Shull, 1942) have indicated that biotin is inactivated by rancid fats. Experiments with calves by Kon & Porter (1951), however, have failed to confirm the observations of Wiese *et al.* (1946).

EXPERIMENTAL

Animals. Forty-two bull calves, purchased when a few days old, were used as experimental animals. Their housing and management were as previously described, save that sawdust was used as bedding instead of peat moss, since the latter was found to contain a substance reacting as a tocopherol in the Emmerie & Engel (1938) test for tocopherol.

Diets. The diet of dried skim milk was prepared and given throughout the experiment in the amounts described by Blaxter, Wood & MacDonald (1953). Each calf received 1 fluid ounce of cod-liver oil daily. This basal ration may be regarded as a standard dystrophy-producing diet. It contained approximately 7·5 mg α-tocopherol, of which 0·5 mg was supplied in the dried skim-milk powder and 7 mg in the cod-liver oil (Brown, 1953). Each calf received a supplement of iron, copper, manganese, cobalt and magnesium. Ten ml. of the solution used in previous experiments (Blaxter, Watts & Wood, 1952) were given once weekly.

Experimental treatment. The effects of α-tocopheryl acetate, ascorbic acid, biotin, ethyl gallate and methylene blue were studied according to the scheme of treatment shown in Table 1. Two other calves, not mentioned in Table 1, were used in studies of urinary excretion and serum concentration of ascorbic acid.

Methods. The calves listed in Table 1 were first grouped according to the centration of tocopherols found in their blood serum, and then the animals w

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each group were allocated at random to treatment groups. The calves were examined twice daily and were weighed and exercised once weekly, observations being recorded as previously described (Blaxter, Wood & MacDonald, 1953). Blood samples were taken weekly for the first 4 weeks and at longer intervals thereafter. The first sample taken on arrival of the calf was examined for the presence of colostral globulins by

Table 1. *Experimental calves (laboratory numbers) and their treatment*

Date of purchase	Basal ration only (controls)	α-Tocopheryl acetate	Ascorbic acid	Biotin	Ethyl gallate	Methylene blue
27. ii. 51	79	—	—	87*	—	—
	80	—	—	88*	—	—
15. v. 51	105	107†	109‡	—	—	—
	106	108†	110‡	—	—	—
1. vi. 51	112	114§	116‖	—	—	—
	113	115§	117‖	—	—	—
7. viii. 51	122	—	125¶	—	—	—
	123	—	126¶	—	—	—
	124	—	127¶	—	—	—
16. x. 51	143	—	—	—	140**	139††
	146	—	—	—	144**	141††
	147	—	—	—	145**	142††
8. i. 52	159	—	—	—	160**	158††
	161	—	—	—	162**	163††
	166	—	—	—	164**	165††
Total no. of calves	15	4	7	2	6	6

* 0·4 mg/day biotin by subcutaneous injection.
† 200 mg/day DL-2-tocopheryl acetate by mouth in a single dose.
‡ 1·6 g/day ascorbic acid by mouth in a single dose.
§ 400 mg/2 days DL-2-tocopheryl acetate by intramuscular injection.
‖ 3·2 g/2 days ascorbic acid by intravenous injection.
¶ 1·6 g/day ascorbic acid by mouth in two doses.
** 2·0 g/day ethyl gallate by mouth in a single dose.
†† 1·0 g/day methylene blue by mouth in a single dose.

the quantitative method of Aschaffenburg (1949), using the Hilger Spekker photoelectric absorptiometer, as well as for tocopherols. In the blood serum of calves receiving ascorbic acid and that of their corresponding controls ascorbic acid was determined by the method of King (1946). Tocopherol in blood serum was determined by the method of Emmerie & Engel (1938), as modified and briefly described by Blaxter, Brown & MacDonald (1953 *a*).

Calves that developed gross and easily recognized signs of muscular dystrophy were slaughtered. Those calves that did not continued on experiment until it appeared unlikely that such signs would develop, when they also were killed. The carcasses of all calves, whether they died, were killed *in extremis* or were killed without showing gross signs of dystrophy, were dissected and examined, particular attention being paid to the musculature. The chemical methods employed for determining creatine and tocopherol contents of muscles and other tissues and the histological methods employed have been described by Blaxter, Brown & MacDonald (1953 *b*).

RESULTS

The details for each calf are not given, results being summarized in Tables 2 and 3.

The effect of α-tocopheryl acetate. Fig. 1 shows the mean concentrations of tocopherols in the blood serum of control calves and of those given α-tocopheryl acetate. Although 200 mg α-tocopheryl acetate by mouth increased the blood concentration, when 400 mg α-tocopheryl acetate were injected intramuscularly every 2 days, the

Table 2. *Mean creatine content of groups of skeletal muscles and the hearts of experimental calves expressed as mg/100 g fresh muscle*

Treatment group	Muscle or muscle groups: Shoulder	Forearm	Lower arm	Rectus femoris	Heart	Conclusion
α-Tocopheryl acetate by mouth	355·2	350·8	382·9	408·2	236·7	Marked difference
Controls	297·9*	312·0*	334·0	326·4*	179·0*	
α-Tocopheryl acetate by injection	378·1	407·1	378·1	417·6	245·7	Marked difference
Controls	229·9*	283·4*	349·1	384·5	179·2*	
Ascorbic acid	302·7*	329·5*	328·9*	403·7	189·9*	No difference
Controls	283·2*	308·9*	319·5*	302·1*	198·8*	
Ethyl gallate	299·1*	319·0*	312·7*	355·4*	212·2*	No difference
Controls	292·9*	308·9*	319·5*	403·4	210·6*	
Methylene blue	373·6	382·8	378·9	440·4	255·0	Marked difference
Controls	292·9*	308·9*	319·5*	403·4	210·6*	
Biotin	229·3*	325·1*	368·5	413·0	207·2*	No difference
Controls	299·4*	331·4*	333·4*	339·1*	149·7*	
Normal†	373	375	372	429	258	—

Shoulder muscles = infraspinatus and supraspinatus.
Forearm muscle = long and lateral head of triceps.
Lower-arm muscles = coracoradialis, anterior brachial and anterior extensor of metacarpus.

* These values are judged to be lower than the normal (Blaxter, Wood & MacDonald, 1953).
† Mean values for normal calves given α-tocopheryl acetate and no cod-liver oil in previous experiments (Blaxter, Wood & MacDonald, 1953).

concentration of tocopherols in the serum did not differ from that observed in control calves, nor did analysis of the blood reveal the presence of the acetate ester, suggesting very slow or negligible absorption of the vitamin from the injection sites. At post-mortem examination of the two calves dissection of the injection sites revealed the presence of oily droplets of the ester within the muscles. Histological examination of the sites showed fine droplets of the vitamin between muscle bundles and a marked reaction on the part of fibrous tissue, effectively sealing off each droplet. There was a distinct eosinophilic infiltration with some polymorphonuclear reaction at the periphery of each droplet.

Clinical and post-mortem examination of the calves showed no dystrophy in those given α-tocopheryl acetate by mouth. In one of the calves given the vitamin by injection, however, histological examination revealed typical hyaline lesions o muscle and sarcolemmal proliferation in the region where the vitamin had injected. No other lesions were found, and chemical analysis showed that

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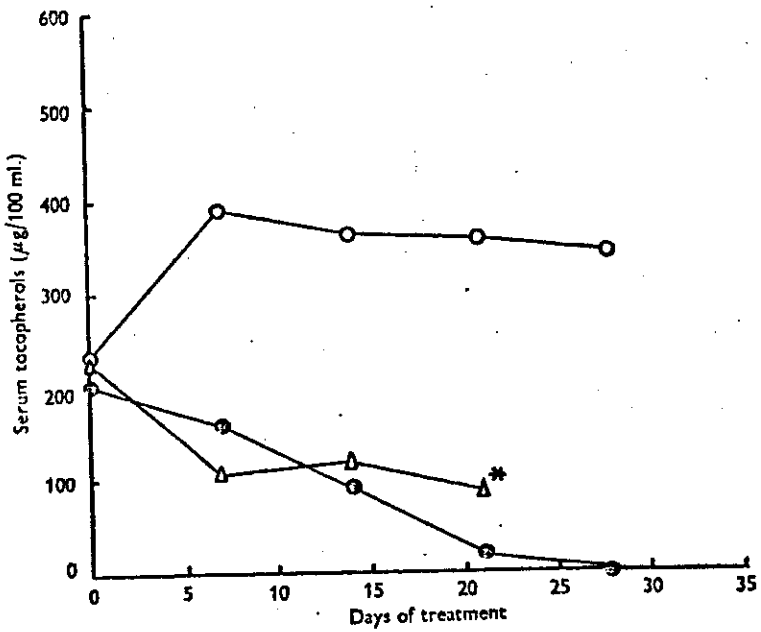
Table 3. *Summary of incidence of muscular dystrophy in the calves*

tment group	No. of calves*	No. that died or were killed *in extremis*	Clinical signs observed in	Mean age at death (days)	Mean score indicating severity of dystrophy†	Conclusion
copheryl acetate outh	2	0	0	—	0·5	Complete prevention of muscular dystrophy
ols	2	2	2	23	24·0	
copheryl acetate jection	2	0	0	—	8·5	Considerable prevention of muscular dystrophy
ols	2	2	2	32	24·5	
bic acid	6	2	3	36	12·5	Slight prevention of muscular dystrophy
ols	7	6	6	33	22·1	
gallate	6	3	3	44	15·8	Ineffective in preventing muscular dystrophy
ols	4	3	4	50	20·5	
ylene blue	5	1	1	38	1·6	Complete prevention of muscular dystrophy
ols	4	3	4	50	20·5	
n	2	2	2	33	21·5	Ineffective in preventing muscular dystrophy
ols	2	2	2	28	27·0	

The calves that died as the result of causes outwith the experiment were not included.
The scoring system was based on the classification of the muscle groups at post-mortem. The wing numerical values were used:

Normal = 0	Slight = 4
? Normal = 1	Severe = 6
Very slight = 2	Very severe = 8

the values given represent the mean of the sums of scores for each of five groups of muscles. mpletely normal animal would thus have a score of 0·0 and a completely affected animal a score of Errors of differences in scores are approximately ±8·0 units.



g. 1. Tocopherol content of blood serum of calves receiving cod-liver oil and vitamin E. Δ, cod-liver oil alone; O, vitamin E by mouth and cod-liver oil; ●, vitamin E by injection and cod-liver oil.

* Both animals dead.

muscles of both groups of calves given tocopheryl acetate could be regarded as normal, unlike those of the controls.

The effect of ascorbic acid. Ascorbic acid given by mouth or by injection had no effect on the rate of tocopherol disappearance from the blood. A high concentration of ascorbic acid in the blood serum was not, however, attained by the methods of administration employed, nor did α-tocopherol administration result in any elevation

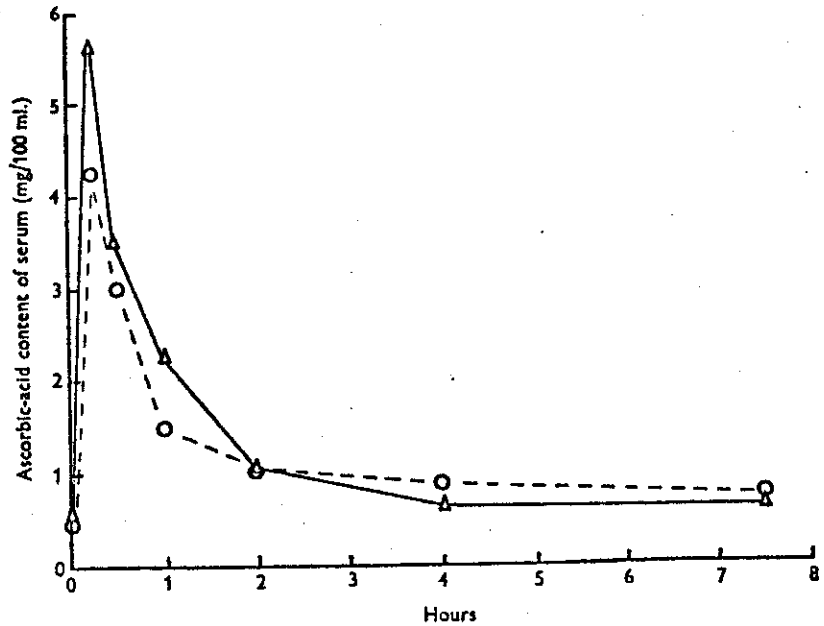


Fig. 2. Effect of an intravenous injection of 1·6 g ascorbic acid on the ascorbic-acid content of the blood serum of each of two calves.

of the ascorbic-acid concentration. Serum ascorbic acid was determined 24 h or sometimes 12 h after dosage or injection. Rapid excretion of ascorbic acid and its rapid destruction in the gut or tissues were possible reasons for the low levels of ascorbic acid found.

When 1·6 g ascorbic acid were given intravenously to each of two calves maintained on a diet not containing cod-liver oil, it had all left the blood stream within 2–4 h, as shown in Fig. 2. The urine concentration was extremely high during the initial period. When 1·6 g ascorbic acid were given by mouth, the highest serum concentration occurred during the 1st hour in one calf and at the 4th hour in the other. The concentration was still elevated at the 8th hour, but had returned to normal by the 24th hour. Again the urine concentration of ascorbic acid was elevated. These results with the calf differ from those obtained with the adult cow in which oral administration of large doses of ascorbic acid does not increase the blood or urine concentration (Knight, Dutcher, Guerrant & Bechdel, 1941), owing to rapid destruction of tl vitamin in the rumen. The present results in the calf suggest, however, that hig concentrations of ascorbic acid in the blood serum, and presumably the tissues, wer

hat there were highly significant
n the serum of calves purchased
months being decidedly lower.
Donald, 1953*a*), that the serum
pherols rather more than it does
, the ingestion of which can be
colostral globulins are absorbed
nates of the serum concentration

al globulins in the blood serum
d

opherol. ith its error ml.)	Colostral globulins in serum. Values with their standard errors. Spekker absorptiometer reading of turbidity (arbitrary units)
33·0	—
36·2	—
33·0	—
27·0	0·621 ± 0·081
36·2	0·392 ± 0·130
27·0	0·141 ± 0·081
25·6	0·086 ± 0·076
25·6	0·420 ± 0·076
10·4	—

n that groups of calves with low
values of colostral globulins.
of tocopherol concentration on
1); when the tocopherol means
content, no seasonal differences
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ter is less than that of those born
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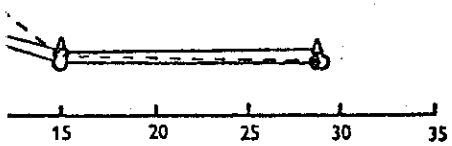
twice that obtaining in summer.

nted in Table 3. It was evident
vented muscular dystrophy. The
jection, since negligible absorp-
e was found that encapsulation
ascorbic acid, chemical analysis
otection; clinical appraisal and
that the severity of the diseas
atever protection was afforded
t the conclusions of Hjärre &

ments owing to the rapid excretion of it in the
gut. It can therefore be assumed that the tissues
peak of ascorbic-acid saturation throughout the
n ascorbic acid was killed after severe diarrhoea
severe enteritis. *Bacterium coli* was isolated from
en excluded from the results given in Tables 2
lar dystrophy occurred despite massive dosage



ne blue on the tocopherols of the blood serum of calves
one; O, ethyl gallate and cod-liver oil; ●, methylene blue

alves, however, no gross lesions were observed,
by their marked pallor. Histological examination
e muscles confirmed that ascorbic acid had little

lves given ethyl gallate did not thrive as well as
even given no supplements. They developed an
he ethyl gallate was given by capsule and was not
ic disturbance of appetite was responsible rather
n abnormal taste in the daily allowance of food.
n abnormal soapy, semiputrid smell, but whether
ormality of appetite was not ascertained. Food
uld generally be coaxed to consume the whole of

contents of the blood serum of calves given ethyl

gallate and those of similar control calves during the course of the ment. There were no differences to be ascribed to treatment. Two of the calves acting as controls to those receiving methylene blue and ethyl gallate died of infectious enteritis. The remainder were, with one exception, dystrophic. As shown in Tables 2 and 3, ethyl gallate was ineffective in preventing the muscular dystrophy induced by cod-liver oil. In one of the calves given ethyl gallate the auricles of the heart were involved in the cardiac dystrophy. This has been the sole case so far observed of dystrophy involving the auricles.

The effect of methylene blue. The dose of 1 g daily of methylene blue to the calves caused some alimentary disturbance with consequent dehydration. The faeces they passed were colourless, turning dark blue on exposure to the air. Their urine was always dark blue. The urine and faeces were collected quantitatively from three calves that received the drug. They showed, of the 1 g of methylene blue ingested, 182, 226 and 295 mg excreted in the urine and 274, 356 and 168 mg in the faeces. An average of approximately 500 mg was not accounted for and was presumably destroyed.

As shown in Fig. 3, between the mean tocopherol contents of the blood serum of the control calves and of those receiving methylene blue there was no difference to be ascribed to treatment.

Two calves succumbed, one to an acute diarrhoea, which did not respond to phthalylsulphathiazole, and the other to an inhalation pneumonia, also associated with diarrhoea. Examination of the muscles of these calves showed them to be normal. Apart from occasional alimentary disturbance, the remaining calves appeared quite healthy. After slaughter the musculature was conspicuously deep red in colour and in one calf only was any evidence of muscular dystrophy found. Histological examination of the affected supraspinatus muscle of this calf showed some hyaline fibres, an early sarcolemmal reaction and some interstitial oedema. Examination of the remaining calves of this group revealed no gross histological abnormality. Since the control calves in this series and the ethyl-gallate calves had shown considerable evidence of muscular dystrophy, it was evident that methylene blue had afforded protection. As shown in Table 2, the creatine contents of the muscles and muscle groups of these calves were normal, indistinguishable in fact from those of calves given no cod-liver oil and adequate vitamin E in previous experiments and decidedly greater than those of the negative control calves.

The effect of biotin. The injection of biotin intramuscularly in two calves did not counteract the effect of cod-liver oil. Both calves died, as did their contemporary controls, and all were severely dystrophic on post-mortem examination. As shown in Table 2, the creatine contents of their muscles were very low. Though the controls had more severe cardiac lesions, it was obvious that those given biotin were in general as severely dystrophic.

Tocopherol content of the tissues. Table 4 presents the amounts of tocopherol found in the tissues of the calves at slaughter. Tissues were taken only where it was certain that less than 60 min had elapsed between death and the excision of the tissue. will be noted there were no differences in the tocopherol contents of the tissu

ascribable to an effect of the supplements. Even the supplement of α-tocopherol by mouth had no effect on the amounts found in the muscles. The livers of those calves given α-tocopherol were not examined.

Table 4. *Tocopherol content of tissues*

Treatment group	Values for individual calves	Mean
	Muscle tocopherol (mg/kg fresh weight)	
Controls	9·9; 11·5; 5·6; 10·4; 9·8; 12·2; 9·0	9·8
α-Tocopheryl acetate by mouth	12·3; 7·8	10·1
α-Tocopheryl acetate by injection	18·4*; 8·7*	13·6
Ascorbic acid	8·8; 8·9; 8·4; 3·6; 8·2	7·6
Ethyl gallate	11·0; 6·4; 11·0; 3·7; 8·8	8·2
Methylene blue	11·0; 14·3; 7·7; 4·2	9·3
	Liver tocopherol (mg/kg fresh weight)	
Controls	7·7; 12·8; 11·9; 14·0; 14·9; 17·0	13·1
α-Tocopheryl acetate by injection	10·9; 11·8	11·4
Ascorbic acid	13·4; 14·5; 13·1	13·7
Ethyl gallate	15·9; 10·7; 9·9	12·2
Methylene blue	8·4; 12·3; 11·6; 12·4	11·2
	Perinephric-fat tocopherol (mg/kg extracted lipid)	
Controls	44·0; 101·8	—
Ethyl gallate	33·0; 49·4; 55·9; 58·7	49·2
Methylene blue	56·0; 45·8; 50·8	50·9

* Triceps muscle, others rectus femoris.

The wide variation in the tocopherol content of the tissues of the calves making up each group was not associated with variation in the severity of dystrophy in individuals; in fact, the worst affected muscles tended to contain a greater concentration. Nor was it closely related to the length of time the calves had been subject to experiment. There was, however, a slight correlation between the concentration of tocopherols in the blood serum when the calves were purchased and the concentration in the muscles at death. The regression was significant ($P=0·05$) and suggests that initial reserves of tocopherols are of importance in maintaining muscle concentration of tocopherols, even after several months' subsistence on a diet of low vitamin E activity and containing unsaturated fat.

The relation of the concentrations of tocopherols and of colostral globulins in the blood serum with the season of the year in which the calves were born. Previous work suggested that the incidence of experimental dystrophy was greatest in calves born in the spring months (Blaxter, Wood & MacDonald, 1953), and it was later found that even apparently normal calves born in the spring had muscles lower in creatine than those of calves born in the autumn (Blaxter, Brown & MacDonald, 1953*a*). The calves used in these experiments were purchased at different times of the year, and figures for concentrations of tocopherols in their blood serum at purchase are presented in Table 5. Results obtained on calves used as experimental animals in studies of the toxicity of cod-liver oil fractions (Blaxter, Brown & MacDonald, 1953*b*) are also included.

Statistical analysis of the data of Table 5 showed that there were highly significant differences among the concentrations of tocopherols in the serum of calves purchased at different times, values obtained during the winter months being decidedly lower. It has been shown, however (Blaxter, Brown & MacDonald, 1953*a*), that the serum level of tocopherols reflects the recent intake of tocopherols rather more than it does tissue reserves. The first food of the calf is colostrum, the ingestion of which can be detected by analysis of the blood for globulins, since colostral globulins are absorbed intact through the mucosa of its gut. In Table 5 estimates of the serum concentration

Table 5. *Concentration of tocopherols and of colostral globulins in the blood serum of calves when purchased*

	Date of purchase	No. of calves	Serum tocopherol. Value with its standard error (μg/100 ml.)	Colostral globulins in serum. Values with their standard errors. Spekker absorptiometer reading of turbidity (arbitrary units)
	15 May	6	334·7 ± 33·0	—
Summer	12 June	5	259·0 ± 36·2	—
	7 August	6	257·3 ± 33·0	—
	16 October	9	285·6 ± 27·0	0·621 ± 0·081
	30 October	5	174·0 ± 36·2	0·392 ± 0·130
Winter	8 January	9	182·4 ± 27·0	0·141 ± 0·081
	4 March	10	148·6 ± 25·6	0·086 ± 0·076
	25 May	10	183·5 ± 25·6	0·420 ± 0·076
	All calves	60	220·8 ± 10·4	—

of colostral globulins are also given, and it will be seen that groups of calves with low serum concentrations of tocopherols also had low values of colostral globulins. Analysis of covariance showed that the regression of tocopherol concentration on globulin concentration was significant ($0·05 > P > 0·01$); when the tocopherol means were adjusted to take into account variation in globulin content, no seasonal differences of statistical significance were observed. This suggests that the total intake of colostrum by calves born on commercial farms in winter is less than that of those born in summer. The reason for this may be economic rather than physiological, since the price the farmer receives for milk in winter is nearly twice that obtaining in summer.

DISCUSSION

The main conclusions of the experiments are presented in Table 3. It was evident that α-tocopheryl acetate when given by mouth prevented muscular dystrophy. The same was not true of α-tocopheryl acetate given by injection, since negligible absorption took place from the injection sites, and evidence was found that encapsulation of the ester within the muscle was beginning. With ascorbic acid, chemical analysis of selected muscles showed no evidence of any protection; clinical appraisal and pathological examination of the animals suggested that the severity of the disease was only slightly less than in the control calves. Whatever protection was afforded however, must have been extremely slight, so that the conclusions of Hjärre &

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Lilleengen (1936*a*, *b*), that muscular dystrophy is due to vitamin C deficiency, are wrong. Ethyl gallate was completely ineffective in preventing muscular dystrophy, despite the fact that dosage was sufficient to cause obvious signs of a systemic disturbance reflected in failure of appetite. Biotin given by injection was also ineffective. Methylene blue, however, afforded considerable protection of the calves against the toxicity of cod-liver oil. This result supports those obtained by Dam and his colleagues in the study of similar 'vitamin E deficiency states' in rats and chicks, as previously outlined.

These results are extremely difficult to explain. One explanation is that in some unknown way the effect of tocopherols in preventing muscular dystrophy is due to their action in the tissues as 'anti-oxidants', that is, they are more readily oxidized than the substrate they protect. Possibly they prevent the formation from dietary unsaturated acids of epoxides and other peroxides that might cause tissue damage. There are several facts to suggest that this is not the explanation. The natural tissue constituents, ascorbic acid and biotin, which could also compete at the site of fat peroxidation, have no effect in preventing the disease, and ethyl gallate, a fat-soluble anti-oxidant, given in large doses also had no effect. Differences in the redox potential of the compounds might explain these findings. Blood and tissue analysis, affords no evidence that these compounds prevent the loss of tissue tocopherols. It is true that methylene blue might protect in the manner outlined, since some is presumably present in the tissues as the leuco compound. It certainly does not prevent muscular dystrophy by protection of tocopherols in the tissues, because the blood concentration of tocopherol declined at similar rates whether methylene blue was given or not, and at slaughter tissue concentrations of tocopherol were the same in both groups.

No metabolic role has yet been assigned to tocopherols. The normal levels of tocopherol in muscles of dystrophic animals might suggest that tocopherol is not intimately concerned with normal muscular metabolism as coenzyme or prosthetic group. It is clear, however, that dietary unsaturated acids in the absence of large amounts of either tocopherol or methylene blue in the diet cause in muscle a sequence of events culminating in dystrophic degeneration. These events all appear contingent upon a primary uncoupling of phosphorylation from respiration, in fact a role in oxidative phosphorylation was originally suggested by Weil-Malherbe (1948) as a possible mode of action of tocopherol. Thus, the increased heat production of the intact calf, the decreased excretion of creatinine and the increased excretion of creatine in the urine (Blaxter, Watts & Wood, 1952), together with the loss of myosin, potassium and creatine from the muscle cell and the infiltration of sodium and water (Blaxter & Wood, 1952), all suggest a decrease in the rate of formation of high-energy phosphate bonds. The simplest explanation of the value of tocopherols and of methylene blue is that they can form a part of some system that either prevents the formation of toxic products from unsaturated fats capable of uncoupling phosphorylation from respiration or alternatively facilitates their dissimilation or deposition as triglycerides.

SUMMARY

1. Experiments with forty-two calves were carried out to determine the value of α-tocopheryl acetate, ascorbic acid, biotin, ethyl gallate and methylene blue in preventing the muscular dystrophy resulting when a diet of dried skim-milk powder and cod-liver oil is given. This diet produced clinically recognizable muscular dystrophy in 87% of animals given no supplement. Protection was judged by clinical signs, post-mortem appearance, chemical analysis of the muscles and histological examination.

2. DL-α-tocopheryl acetate given by mouth increased the concentration of tocopherols in the blood serum and protected the animal against the toxic action of the unsaturated acids of the cod-liver oil. Intramuscular injection of the same amount of DL-α-tocopheryl acetate caused no increase in the tocopherol concentration of the serum and resulted in only slight protection of the animals, since it was poorly absorbed from the injection sites.

3. Ascorbic acid in massive doses by mouth or by intravenous injection had no effect on blood or tissue concentrations of tocopherols and did not prevent muscular dystrophy.

4. Large daily doses of ethyl gallate by mouth caused a systemic disturbance in the calves, but had no effect in preventing dystrophy.

5. Biotin given by intramuscular injection had no effect on the development of muscular dystrophy.

6. Daily administration of 1 g methylene blue resulted in protection of the calves against the toxicity of cod-liver oil. This was not associated with higher concentrations of tocopherols in serum, muscle, liver or perinephric fat than in control calves which succumbed.

7. The concentration of tocopherols in the blood serum of calves at the beginning of the experiments was highly correlated with the amount of colostrum they had been given, as judged by the concentration of colostral globulins in the blood.

8. The results are discussed in relation to explanations of the effect of α-tocopherol in terms of anti-oxidant activity.

We are grateful to Dr E. C. Slater for helpful discussions about the possible role of tocopherols in muscular metabolism. We are also indebted to Mr A. L. Wilson, M.R.C.V.S., for the bacteriological examination of certain calves. Our thanks are due to Miss Mary Hutcheson for the care of the animals, and Misses Grace Breckenridge, Margaret Cunningham and Elsie Rennie for their assistance in the chemical studies.

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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.

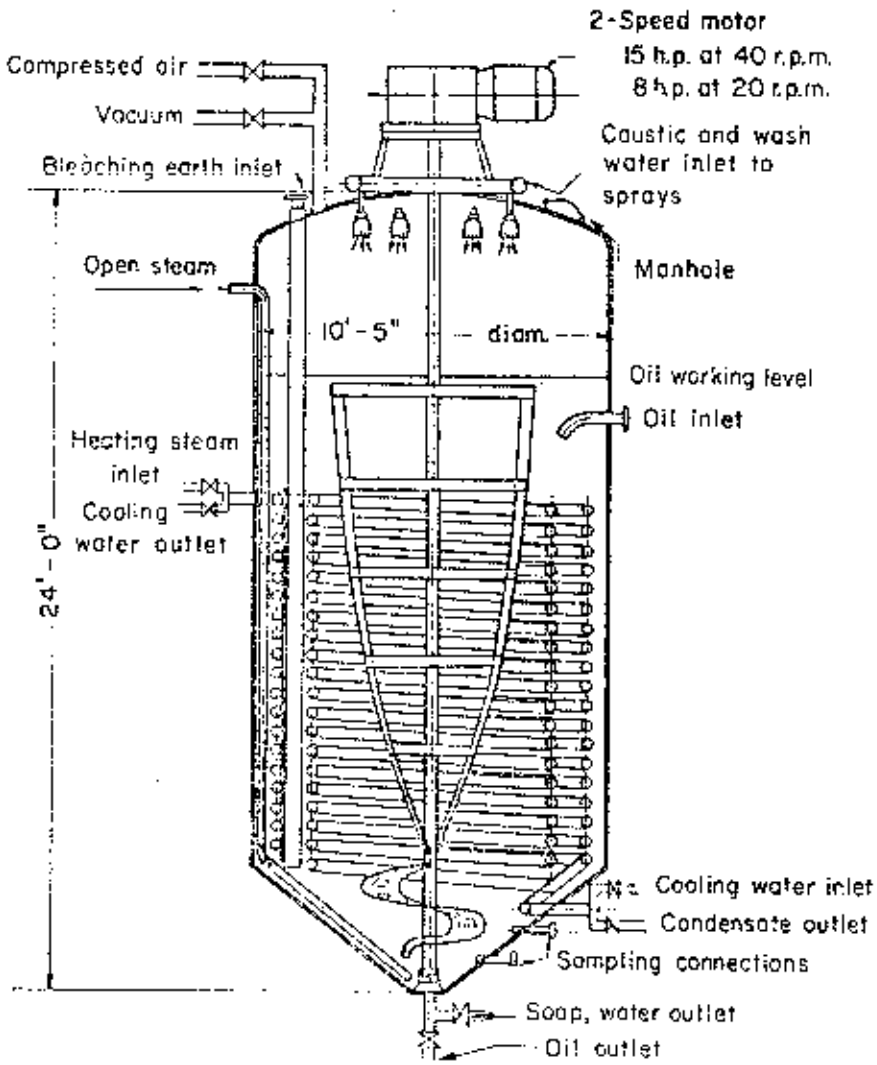
Alkali refining is the treatment of the oil with an aqueous alkaline solution. In this process, the free fatty acids combine with the alkali to form soaps, and the phosphatides and mucilaginous materials absorb alkali and are forced to coagulate through hydration or degradation. This process also removes 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



30 TON NEUTRALIZER-BLEACHER

Courtesy of Pergamon Press, Ltd.

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 . 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, Ganglitz 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

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

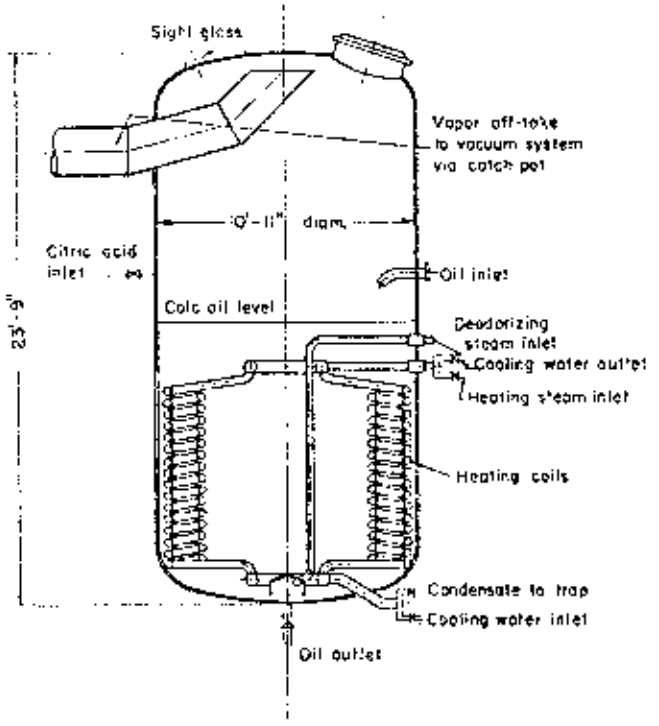
Deodorization is the removal by vacuum steam distillation of small quantities of the more volatile components which are responsible for any odor or taste in fats and oils. The object is to yield a bland product. Before deodorization, the fats and oils may contain the volatile odor and flavor components 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 deaerated 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 3 h the amount of steam used is more than necessary

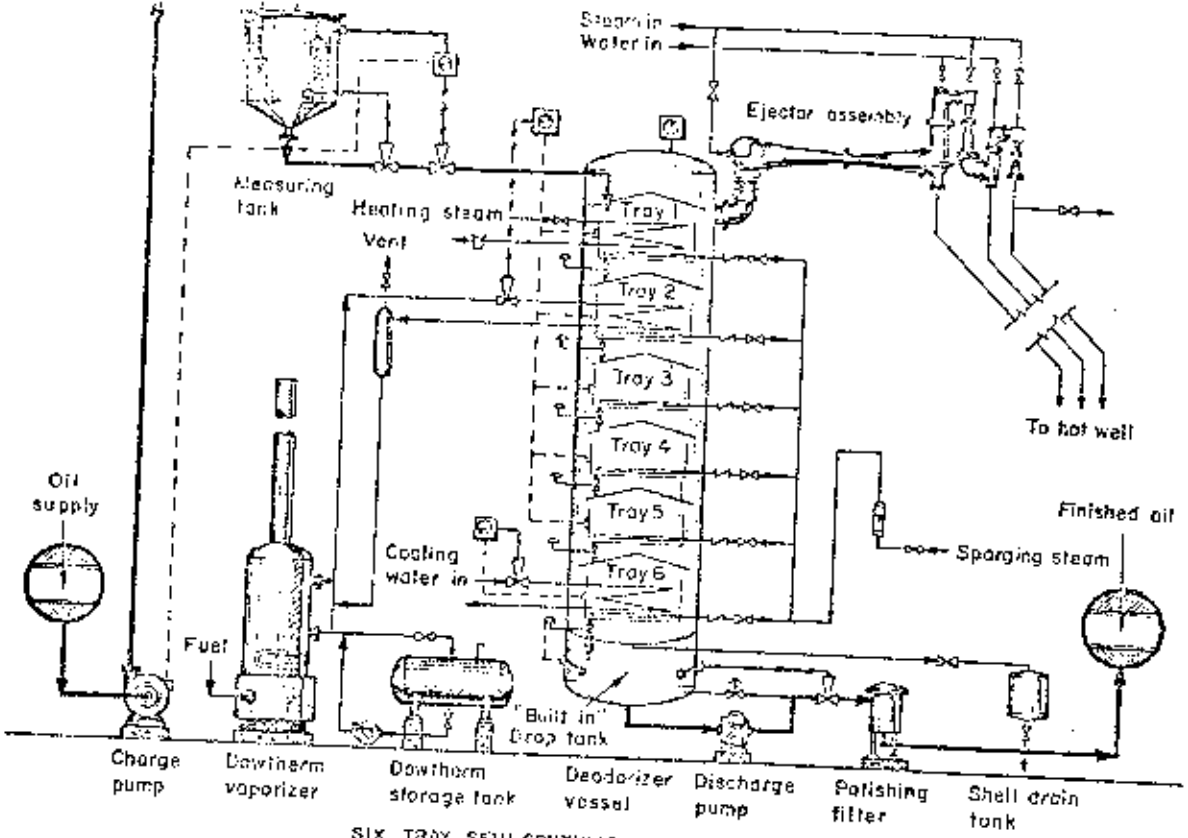


25 TON DEODORIZER

Courtesy of Ferguson Press, Ltd.

FIG. 39. BATCH-TYPE DEODORIZER

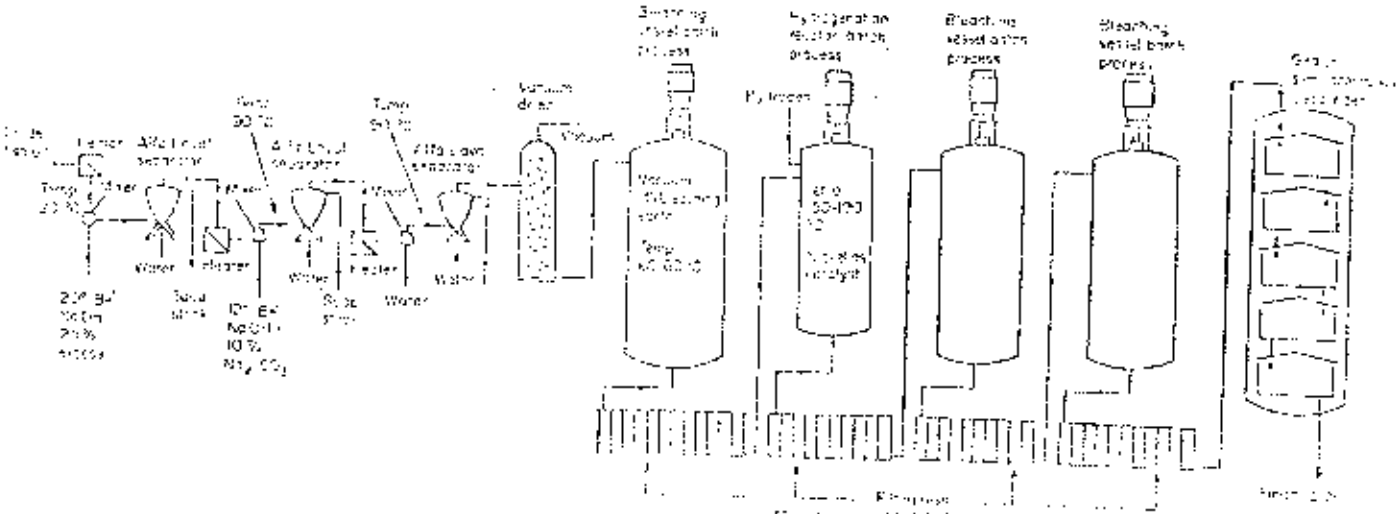
From Devine and Williams, "Chemistry and Technology of Edible Oils and Fats" 1961.



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 FOR FLAT-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 Aktiebolaget Karlshamns Oljefabr. , Karlshamn, Sweden. The flow sheet shows the processing procedure of fish oil in their plant.

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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.

Joint FAO/WHO Food Standards Programme
CODEX ALIMENTARIUS COMMISSION

CAC/RS 20-1969

RECOMMENDED INTERNATIONAL STANDARD FOR EDIBLE SOYA BEAN OIL

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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 Standard for Edible Soya Bean Oil (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

(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.

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Commission are 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 STANDARD FOR EDIBLE SOYA BEAN OIL

1. DESCRIPTION

Soya Bean Oil (synonym: Soybean Oil) is derived from soya beans (the seeds of *Glycine max* (L.) Merr.)

2. ESSENTIAL COMPOSITION AND QUALITY FACTORS

2.1 Identity Characteristics

2.1.1 Relative Density (20°C/water at 20°C)	0.919 - 0.925
2.1.2 Refractive Index (n_D 40°C)	1.466 - 1.470
2.1.3 Saponification Value (mg KOH/g oil)	189 - 195
2.1.4 Iodine Value (Wijs)	120 - 143
2.1.5 Unsaponifiable Matter	not more than 15 g/kg

2.2 Quality Characteristics

2.2.1 Colour: Characteristic of the designated product.	
2.2.2 Odour and Taste: Characteristic of the designated product and free from foreign and rancid odour and taste.	
2.2.3 Acid Value	not more than 0.6 mg KOH/g oil
2.2.4 Peroxide Value	not more than 10 milliequivalents peroxide oxygen/kg oil

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3. FOOD ADDITIVES

3.1 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
3.1.1 Beta-carotene	Not limited
3.1.2 Annatto (*)	Not limited
3.1.3 Curcumin (*)	Not limited
3.1.4 Canthaxanthine	Not limited
3.1.5 Beta-apo-8'-carotenal	Not limited
3.1.6 Methyl and ethyl esters of Beta-apo-8'-carotenoic acid	Not limited

3.2 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. (*)

3.3 Antioxidants

	Maximum level of use
3.3.1 Propyl, octyl, and dodecyl gallates	100 mg/kg individually or in combination

(*) Temporarily endorsed.

	Maximum level of use
3.3.2 Butylated hydroxytoluene (BHT) Butylated hydroxyanisole (BHA)	200 mg/kg individually or in combination
3.3.3 Any combination of gallates with BHA or BHT, or both	200 mg/kg but gallates not to exceed 100 mg/kg
3.3.4 Ascorbyl palmitate) 3.3.5 Ascorbyl stearate)	200 mg/kg individually or in combination
3.3.6 Natural and synthetic tocopherols	Not limited
3.3.7 Dilauryl thiodipropionate	200 mg/kg

3.4 Antioxidant Synergists

3.4.1 Citric acid	Not limited
3.4.2 Sodium citrate	Not limited
3.4.3 Isopropyl citrate mixture) 3.4.4 Monoglyceride citrate) 3.4.5 Phosphoric acid (*))	100 mg/kg individually or in combination

3.5 Anti-foaming Agent

Dimethyl polysiloxane (syn. Dimethyl silicone) singly or in combination with silicon dioxide (*)	10 mg/kg

3.6 Crystallization Inhibitor

Oxystearin (*)	1250 mg/kg

(*) Temporarily endorsed.

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4. CONTAMINANTS	Maximum level
4.1 Matter volatile at 105°C	0.2% m/m
4.2 Insoluble impurities	0.05% m/m
4.3 Soap content	0.005% m/m
4.4 Iron (Fe)	1.5 mg/kg
4.5 Copper (Cu)	0.1 mg/kg
4.6 Lead (Pb)	0.1 mg/kg
4.7 Arsenic (As)	0.1 mg/kg

5. 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).

6. 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:

6.1 The Name of the Food

6.1.1 All products designated as soya bean oil or soybean oil must conform to this standard.

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

6.2 List of Ingredients

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

6.2.2 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.

6.3 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.

6.4 Name and Address

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

6.5 Country of Origin

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

6.5.2 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.

7. METHODS OF ANALYSIS AND SAMPLING

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

7.1 Determination of Relative Density

According to the FAO/WHO Codex Alimentarius Method (FAO/WHO Methods of Analysis for Edible Fats and Oils, CAC/RM 9-1969, Determination of Relative Density at t/20°C).

Results are expressed as relative density at 20°C/water at 20°C.

7.2 Determination of Refractive Index

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

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Results are given as the refractive index relative to the sodium D-line at 40°C ($n_D 40°C$).

7.3 Determination of Saponification Value (I_S)

According to the IUPAC (1964) method (IUPAC Standard Methods for the analysis of Oils, Fats and Soaps, 5th Edition, 1966, II.D.2 Saponification value (I_S)).

Results are expressed as the number of mg KOH/g oil.

7.4 Determination of Iodine Value (I_J)

According to the (Wijs) IUPAC Method (1964) (IUPAC Standard Methods for the Analysis of Oils, Fats and Soaps, 5th Edition, 1966, II.D.7.1, II.D.7.2 and II.D.7.3 The Wijs Method).

Results are expressed as % m/m absorbed iodine.

7.5 Determination of Unsaponifiable Matter

According to the IUPAC (1964) diethyl ether method (IUPAC Standard Methods for the analysis of Oils, Fats and Soaps, 5th Edition, 1966, II.D.5.1 and II.D.5.3).

Results are expressed as g unsaponifiable matter/kg oil.

7.6 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 (IA)).

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

7.7 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 oil.

7.8 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.

7.9 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.

7.10 Determination of Soap Content

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

Results are expressed as % m/m sodium oleate.

7.11 Determination of Iron (*)

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

Results are expressed as mg iron/kg.

7.12 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.

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

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7.13 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.

7.14 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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- Supplement 1966 to the above

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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-

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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 Saiflowerseed 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

INFLUENCE OF ANTIOXIDANTS AND REDOX SUBSTANCES ON SIGNS OF VITAMIN E DEFICIENCY

HENRIK DAM Pharmacol Rev 9:1 Jan 1957

Department of Biochemistry and Nutrition, Polytechnic Institute, Copenhagen, Denmark

Vitamin E, originally discovered as a substance necessary for reproduction in rats, possesses the properties of an antioxidant, that is, a substance which inhibits autoxidation of fats in contact with molecular oxygen.

Speculation has, therefore, arisen concerning whether or how far the biological effects of vitamin E can be explained on the basis of an antioxidant effect *in vivo* (64).

The view that at least some manifestations of vitamin E deficiency are related to the lack of an antioxidant effect has received support from the observation that certain signs of vitamin E deficiency occur only in the presence of dietary highly unsaturated fatty acids, which easily undergo autoxidation, and that under such conditions lipoperoxides can be detected in the body fat. Further, some antioxidants and redox substances unrelated to vitamin E may afford a partial protection against certain signs of vitamin E deficiency. These problems will be more fully discussed in the following sections.

The principal effect of an antioxidant is the neutralization of a free radical representing the initial stage in the autoxidative chain reaction (Fig. 1), the following stages of which are the formation of a peroxidic free radical, hydroperoxides, keto and hydroxy compounds, and polymerization products. The reaction between the antioxidant and the free radical involves the transformation of the antioxidant into an unstable free radical form which easily undergoes irreversible changes, whereby the antioxidant is destroyed. In the case of vitamin E the free radical is the semiquinone radical demonstrated in 1949 by Michaelis and Wollman (67).

Among the seven known tocopherols (alpha, beta, gamma, delta, epsilon, zeta, and eta) the physiologically most important, alpha, has the least pronounced antioxidant effect *in vitro*; it is, however, the one that accumulates to the largest degree in animal tissue. The tocopherols must be in the free phenolic form in order to act as antioxidants. The esters are inactive until they become hydrolyzed.

In the *in vitro* test for antioxidant activity a certain concentration of the antioxidant is required to obtain maximal effect. Higher concentrations of the antioxidant may result in diminished activity. This also applies to the antioxidant effect of alpha tocopherol *in vitro*.

Certain substances may act as synergists for an antioxidant; that is, they may increase its effect. Synergists for the antioxidant effect of tocopherol *in vitro* are, for instance, ascorbic acid and phosphoric acid. The mechanism by which the synergism is brought about has not been completely elucidated, but may be related to a stabilization of the antioxidant.

The signs of vitamin E deficiency known at present are very numerous. A

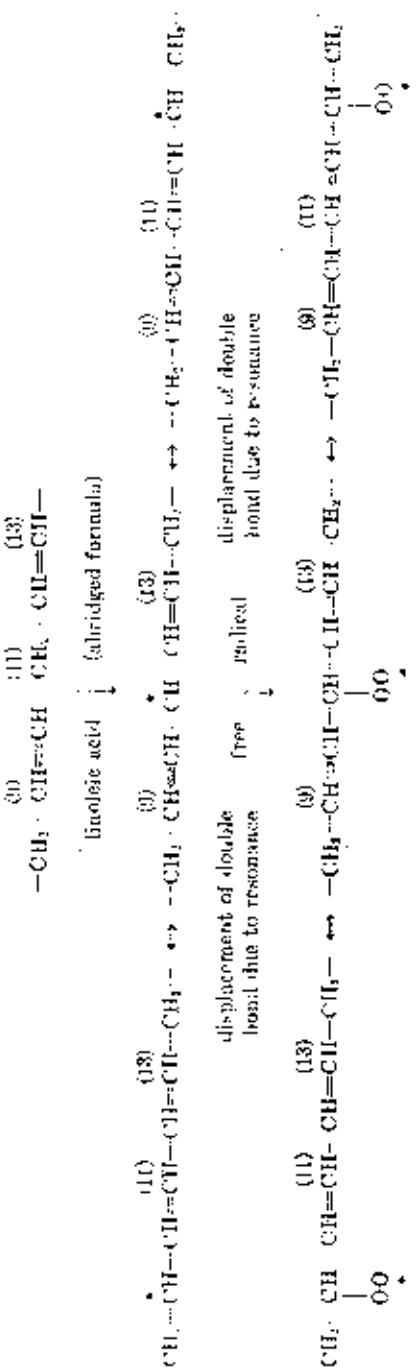

FIG. 1. Some of the initial stages in the autoxidation of linoleic acid

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brief description of some of them may be necessary for the understanding of the present problem.

The *exudative diathesis* in chicks was the first instance of a sign of vitamin E deficiency in which peroxidation of fat *in vivo* was demonstrated. This disease (14) affects preferentially the adipose tissue but also muscles and skin. The initial stage is reddening of the tissue due to diffuse hemorrhage. In the second stage plasma exudes from the capillaries of the affected region. The exudate is usually green colored from destruction products of hemoglobin. During the third stage the green color fades and the exudate is absorbed. Sometimes the condition leads to the death of the animal.

The exudative diathesis does not occur when the diet contains sufficient vitamin E, provided fat is not present in the diet, but an edematous condition of a different type may occur in chicks reared on fat-free diets; this condition, however, is unrelated to vitamin E deficiency. Both cod liver oil and lard may provoke the exudative diathesis. The effect is connected with the highly unsaturated fatty acid fraction. The saturated acids, oleic acid, and the non-saponifiable fraction are inactive. The total fatty acid fraction from linseed oil may also produce exudative diathesis in chicks. Cod liver oil is the most active among the fats mentioned, and when it is used a yellow-brown color develops in the affected adipose tissue after the exudate is absorbed.

If the highly unsaturated fatty acids are autoxidized to such an extent that the iodine value decreases sufficiently before the fat is ingested, the fat will not give rise to this disorder.

TABLE I

Example of a diet which produces exudative diathesis (and encephalomalacia)

Casein, vitamin test[1]	200 g	
Sucrose	547.5 g	
Gelatine	80 g	
Salt mixture[2]	50 g	
Sodium chloride	20 g	
Choline chloride	1.5 g	
Vitamin mixture no. 4[3]	1 g	
Cod liver oil[4]	100 g	
Vitamin E substitute[5]		10 mg
	1000 g	

[1] From British Chemicals & Biologicals Ltd., 43 Regent Street, Loughborough, England.

[2] McCollum Simmonds' salt mixture no. 185, supplemented with 13.5 mg KI, 159 mg $CuSO_4$, $5H_2O$, 556 mg $MnSO_4$, $4H_2O$ per 100 g.

[3] Thiamine hydrochloride 3 mg, riboflavin 4 mg, niacin 50 mg, Ca pantothenate 12 mg, pyridoxine hydrochloride 3.5 mg, biotin 0.1 mg, folic acid 2 mg, sucrose 925.4 mg, total 1000 mg.

[4] The cod liver oil must be incorporated in the diet every day and the unfed portion of the ready-made diet kept in the ice box up to 24 hours.

[5] Dicalcium salt of 2-methyl 1,4-naphthohydroquinone diphosphoric acid ester (Synkavit®, Roche).

The circumstance that the disease primarily affects adipose tissue is particularly favorable to an examination of the affected tissue for abnormal oxidation products of fatty acids, for instance, lipoperoxides. Dam and Granados (13) undertook such an examination using the iodometric method of King *et al.* (50), slightly modified for the purpose.

It was found that the disorder began approximately at the time when the presence of peroxides could be detected, whereas higher peroxide values were found in the later stages of the disease, *viz.*, when the yellow-brown color of the fat appeared. In chicks receiving vitamin E or no fat the adipose tissue did not contain peroxides. Dam *et al.* (19) found peroxides in the adipose tissue of chicks reared on vitamin E-deficient diets containing 10% lard and no cod liver oil.

These results show that vitamin E may exert an antioxidant effect *in vivo*, and that changes of tissue may occur when this effect is lacking, provided a sufficient amount of easily autoxidizable fat is present. (The fact that dietary unsaturated fatty acids are deposited in the adipose tissue had already been demonstrated in 1901 by Henriques and Hansen (41); that they are deposited in the phospholipids was shown by Sinclair (66).)

The question then arises whether the formation of abnormal oxidation products of fat is the cause of the exudative diathesis or merely a sign of the disappearance of vitamin E from the tissue. Since the disorder has not been observed in the absence of dietary highly unsaturated fatty acids, even when the diet contains a substance such as 2,6-dichlorophenolindophenol which oxidizes free tocopherols, the first alternative seems likely. The explanation may be that some primary autoxidation product of the fatty acids (not necessarily the peroxides, but more likely the free radical) reacts with substances in the wall of the capillaries, and thereby damages the latter.

The diet which produces the exudative diathesis often gives rise to another sign: *encephalomalacia*. This condition was first described by Pappenheimer and Goettsch (77) and extensively studied by them. Clinically there is ataxia, spasm or paralysis. The chicks may die or recover without any change in the diet. Usually the disease begins in the cerebellum. Later the cerebrum may become affected. Localized or diffuse areas of edema, fine hemorrhages and necrosis are usually seen in the cerebellum or the cerebrum. According to Pappenheimer and Goettsch (77), the cerebellar lesions are characterized histologically by edema, with disorganization of the fibrillar and granular elements, degeneration of the Purkinje cells and those of the granular layer, small hemorrhages scattered in both white and grey matter, and hyaline thrombosis of capillaries in and around the necrotic areas. Probably the condition is due to interference with the development of certain structures in the brain. It occurs in the period when the growth of the brain is rapid, but is rare after about two months of age (75).

It is possible to compose a diet which produces encephalomalacia, usually without signs of exudative diathesis, such as the original "diet no. 108" of Pappenheimer and Goettsch (77). A simplified diet which produces encephalomalacia as the main sign is indicated in Table II.

The characteristic features of this diet are: high lard, low carbohydrat

TABLE II

Simplified diet which produces encephalomalacia as the main sign

Casein, vitamin test[1]	200 g	
Gelatine	80 g	
Salt mixture[2]	40 g	
Sodium chloride	5 g	
Vitamin mixture[3]	1 g	
Choline chloride	1.5 g	
Vitamin K substitute[4]		10 mg
Corn starch	372.5 g	
Lard	300 g	
	1000 g	

[1] From British Chemicals & Biologicals Ltd., 43 Regent Street, Loughborough, England.

[2] McCollum Sherman's salt mixture no. 185 supplemented with 13.5 mg KI, 120 mg $CuSO_4 \cdot 5H_2O$, 555 mg $MnSO_4 \cdot 4H_2O$ per 100 g.

[3] Thiamine hydrochloride 3 mg, riboflavin 4 mg, niacin 50 mg, Ca pantothenate 12 mg, pyridoxine hydrochloride 3.5 mg, biotin 0.1 mg, folic acid 2 mg, sucrose 925.4 mg, total 1000 mg.

[4] Disodium salt of 2-methyl 1,4 naphthohydroquinone diphosphoric acid ester (Synkavit®, Roche).

absence of vitamin E. Peanut oil deprived of vitamin E by suitable absorption may be used instead of lard.

Although vitamin E prevents encephalomalacia (12, 78), and easily oxidizable fat is necessary for the development of this sign, the search for peroxides in the lipids of brains from chicks with encephalomalacia has, hitherto, been unsuccessful.

Encephalomalacia and exudative diathesis are probably two variations of the same metabolic defect. Certain changes of the diet will favor one or the other of the two disorders. Thus, addition of cholesterol to an encephalomalacia producing diet will depress the tendency to encephalomalacia and favor exudate in fat tissue.

Rats fed diets which produce exudative diathesis and encephalomalacia in chicks fail to develop these conditions. However, vitamin E deficient diets containing a sufficient amount of cod liver oil will produce yellow-brown coloration of the adipose tissue and disappearance of the normal brown color of the incisors in rats.

The yellow-brown fat in the adipose tissue shows a yellow-green fluorescence under the ultraviolet lamp. It has been demonstrated (43) that the development of yellow-brown fat is associated with peroxidation of the fat *in vivo*. Cod liver oil favors the development of the brown fat much more than lard (30). The yellow-brown color of the fat is due to the presence of two brown components, one of which is fat-soluble while the other is insoluble in fat solvents as well as in water. The non-fat-soluble component stains acid-fast with fuchsin and resembles thereby the pigment "ceroid" (31, 32, 59) which occurs in liver, lymph nodes and other tissue of rats reared on diets causing liver damage and con-

taining cod liver oil. There is, in fact, no definite distinction between "ceroid" and the non-fat-soluble component of the brown adipose tissue.

A colorimetric method for the determination of fat-peroxides based on the oxidation of leuco dichlorophenolindophenol has been developed by Hartmann and Glavind (38).[1] This method can be adopted for use on frozen sections of adipose tissue (26). It is thereby possible to demonstrate the location of the peroxides in the affected adipose tissue. The initial stage is characterized by a homogenous peroxidation of the fat content of some of the cells, but the fat-insoluble component which appears as granules does not necessarily contain peroxides (31).

The pigments in the yellow-brown fat tissue must have originated from the abnormal oxidation of the highly unsaturated fatty acids occurring in the adipose tissue. The fat-soluble component must be assumed to represent the least transformed oxidation products while the non-fat-soluble component consists of higher polymerization products. Cod liver oil autoxidized *in vitro* will also give rise to yellow-green fluorescent fat-soluble and non-fat-soluble reaction products, the latter staining acid-fast with fuchsin. Copolymerization of autoxidized lipids with protein, and catalysis of the autoxidation by hemoglobin has been considered by Tappel (88) as a cause of the pigment formation.

Contrary to the findings in chicks, the hemorrhagic and exudative stages do not occur in rats. The reason could be sought in differences in the fine structure of the capillaries in the two species. Further histochemical studies must decide whether this explanation is correct.

Yellow fat and edema of the adipose tissue have been found in mink reared on fish scraps (30). This adipose tissue also stains acid-fast with fuchsin (62). Vitamin E prevents the development of this effect in mink and linolenic acid provokes it (51). The condition has been studied further by Dalgaard-Mikkelsen *et al.* (10). Robinson and Couy (79) have found brown fat in pigs reared on a vitamin E-deficient diet containing cod liver oil. The rare disease sclerodema which affects the adipose tissue in premature infants has been said to respond to vitamin E treatment (25) and thus is believed to be related to the exudative diathesis in chicks.

Martin and Moore (58, 59) have described a *brown discoloration of the uterus* in female rats deprived of vitamin E. Moore and Wang (75) are of the opinion that the brown substance contains oxidized protein. It is likely that the brown pigment of the uterus is closely related to that in the yellow-brown fat tissue. A similar fuchsinophil pigment was found by Escudero *et al.* (24) in the adrenal cortex and by Mason and Emmel (60, 61) in the sex glands and musculature of vitamin E-deficient rats. A fluorescent, acid-fast staining substance has also been found in the nervous system of rats with chronic vitamin E deficiency (23).

Presence of lipoperoxides has been demonstrated in adipose tissue from patients suffering from *peripheral venous disease* (40), and in the arteriosclerotic aorta (27). A relationship of these observations to vitamin E has not been shown experi-

[1] By this method it is sometimes possible to detect traces of peroxide in fat from chicks and rats reared on diets containing 10% cod liver oil and 10 mg % d,l-α-tocopherol acetate (20 and unpublished data from the reviewer's laboratory).

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mentally, although some authors have claimed a beneficial effect of vitamin E on certain forms of such disorders (6).

The *depigmentation of rat incisors* was first described by Davies and Moore (22). The fact that fat is necessary for the appearance of this sign was demonstrated by Grandois and Dam (32). The normal brown pigment of the incisors is a ferric compound, probably ferric phosphate. The maxillary incisors which are normally more colored than the mandibular, are the last to lose their color when the rats are placed on a vitamin E-deficient diet containing suitable fat. Lard, as well as cod liver oil, will produce incisor depigmentation. The effect is associated with the highly unsaturated fraction of the fatty acids. The iron content of the enamel decreases very much and manganese rises somewhat under the influence of the "depigmenting" diet (14). This sign could be in some way related to the lack of an antioxidant effect against autoxidation of fatty acids in the mantal organ.

Hove and Harris (48) have shown that vitamin E increases the normal utilization of a sub-optimal level of essential fatty acids in the diet of rats. The explanation is, apparently, that vitamin E protects these metabolites against abnormal oxidation. Thus, when highly unsaturated fatty acids are given in small amounts they disappear in the absence of vitamin E. When given in higher amounts abnormal oxidation products may accumulate.

Moore (70) showed that the feeding of vitamin A to rats (1000 I.U. per week in the form of halibut liver oil on a diet containing 10% lard) results in higher vitamin A levels in the liver when the diet contains vitamin E than when the diet is vitamin E-free. Hickman *et al.* (42) and Miles *et al.* (68) found that the utilization of vitamin A is more efficiently increased by free tocopherol than by its esters. The latter authors also found that increase of the vitamin E content beyond a certain level results in suboptimal utilization of vitamin A. This latter observation agrees with the general experience that there is a certain optimal concentration for the action of an antioxidant. Beyond this concentration its action decreases or may even be reversed. Regarding the sparing effect on vitamin A, Hickman *et al.* (42) found alpha-, beta-, and gamma-tocopherols equally active.

Dam *et al.* (19, 20) found no significant effect of vitamin E on vitamin A storage in the liver of chicks within 5 weeks and of rats within 14 weeks when the diet contained no cod liver oil (vitamin A being given as natural ester), whereas the effect was marked on diets containing cod liver oil. They also found that the vitamin A level in the liver of rats decreased from the 5th to the 14th week when the cod liver oil-containing diet was vitamin E-free. All these observations are in agreement with the assumption of an *in vivo* destruction of vitamin A coupled to the autoxidation of highly unsaturated fatty acids in the absence of antioxidants.

The circumstance that the antioxidant activity of the tocopherols *in vitro* increases from alpha to delta, whereas the biological activity as measured in the fertility test with female rats decreases in the same order is no hindrance for the assumption of an antioxidant effect *in vivo*. Barnes *et al.* (2) and Lundberg *et al.* (55) have shown that the biopotency of the various tocopherols as antioxidants

(measured in the rendered fat of the experimental animals (rats)) is related to the amounts deposited in the fat, alpha being deposited to a larger extent than gamma.

The following signs of vitamin E deficiency occur even when the diet does not contain easily oxidizable fat: 1) reproduction failure in female rats (the classical symptom of vitamin E deficiency); 2) massive liver necrosis in rats (this is greatly accentuated by easily oxidizable fat); 3) hemolysis of erythrocytes in rats; 4) muscular degeneration in chicks. Therefore, these signs do not seem to be due to autoxidation of fat.

Massive liver necrosis (33, 82) and fatal lung hemorrhage (47) occur in vitamin E deficiency under certain dietary conditions, partly in relation to the amount and quality of the protein. Fat with a high content of highly unsaturated fatty acids greatly accelerates the onset of the two pathological effects (15), but liver necrosis has been developed with fat-free diets (66).

When the dietary protein consists of "damaged" casein (82) or is provided by certain types of yeast (37), the incidence of hepatic necrosis is particularly high. Low sulfur amino acids, especially low cystine, is one of the conditions for the development of hepatic necrosis (28, 35, 41, 85). A more detailed review of this subject has been given by Hinsworth (43) and McLean and Beveridge (65). However, the dietary conditions are still more complicated, since Schwarz (83, 84) has demonstrated the existence of a "factor 3" in non-necrogenic yeast and in casein which protects against this effect. The factor is not identical with cystine or methionine which, under suitable conditions, will protect against liver necrosis in vitamin E deficiency. Lindan and Work (54) have shown that both reduced and total glutathione as well as ascorbic acid are very much lowered in the necrotic livers of vitamin E-deficient rats. While cystine protects, ascorbic acid does not.

In connection with the role of vitamin E in maintaining the normal state of the liver, its ability to minimize the damage of this organ by carbon tetrachloride should be mentioned (46). It might be that the effect of vitamin E in preventing liver necrosis consists in the protection of cystine, glutathione and some other metabolites which are essential to the normal state of the liver. Accumulation of peroxides in the liver has not been demonstrated, and probably does not occur to any appreciable extent.

György and Rose (36, 80) found that alloxan and dialuric acid produce hemolysis of erythrocytes in vitamin E-deficient rats. From this finding they developed a test for vitamin E deficiency. The effect was believed to be caused by the lack of an antioxidant effect against a free radical representing the initial stage of the autoxidation of dialuric acid. The free radical is supposed to react with a substance in the erythrocytes whereby the latter are damaged. Hemolysis of erythrocytes from vitamin E-deficient rats also occurs in isotonic saline provided oxygen is not excluded (8). Rose and György (81) reported that when administered *in vivo* the effectiveness of the tocopherols in inhibiting dialuric acid hemolysis *in vitro* decreased in the order alpha, beta, gamma, delta, similarly to their activity measured by fetal resorption.

Muscular degeneration in chicks (21) occurs even when the diet does not

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contain fat. This condition is greatly ameliorated by increase of dietary cystine. It is possible, therefore, that it is connected with some abnormal metabolism of sulfur amino acids.

Degeneration of the convoluted tubules of the kidney (39) is a sign of vitamin E deficiency which has not yet been studied sufficiently to judge its possible dependence on dietary highly unsaturated fatty acids. Lack of essential fatty acids leads to a similar condition (4, 69).

Studies on the influence of the constant feeding of antioxidants and redox substances on the aforementioned signs of vitamin E deficiency have shown the following results (compare Table III):

The industrially important antioxidant *nordihydroguaiaretic acid* (NDGA) affords some protection against exudative diathesis and encephalomalacia in chicks when fed at a 0.5% level (17, 18). It also increases vitamin A storage in the liver on diets with 10% cod liver oil. At the 0.5% level the substance retards growth. NDGA does not act against muscular degeneration in chicks.

Disulfiram (Antabuse, tetraethylthiuram disulfide) which shows a moderate antioxidant effect in the Swift test, protects fairly well against exudative diathesis in chicks (0.025% of the diet) but not against encephalomalacia (18). It protects vitamin A in the liver of chicks and rats (17, 18), and acts against yellow-brown fat and incisor depigmentation in rats (1), but not against muscular degeneration in chicks.

Ascorbic acid (0.5% of the diet) affords a fairly good degree of protection against encephalomalacia, but much less against exudative diathesis in chicks (15, 16), and no protection of vitamin A in the liver (Dam, Prange and Snoodgrass, unpublished). It does not protect against muscular degeneration in chicks.

Cystine (as already mentioned) protects against liver necrosis and lung hemorrhage in rats and against muscular degeneration in chicks. This effect is probably not due to antioxidant properties but rather to a supplementation of a too low level of sulfur amino acids.

Methylene blue (0.126% of the diet) provides fairly good protection against exudative diathesis and encephalomalacia in chicks (18), and protects vitamin A in the liver of chicks and rats to a very high degree (19, 20). It also protects fairly well against yellow-brown fat and peroxidation of the adipose tissue in rats (1), and against hepatic necrosis and lung hemorrhage in rats receiving cod liver oil (16). The effect of methylene blue against liver necrosis in rats has been confirmed by Schwarz (85a). Methylene blue will counteract to a high degree the unfavorable effect on growth of chicks exerted by cod liver oil in vitamin E deficient diets. As judged from the effect on growth, methylene blue is better tolerated by chicks than by rats, and by rats better than by hamsters.

Whereas methylene blue did not act against muscular degeneration in chicks (21), Blaxter and associates (3) found that it protected against muscular dystrophy in calves induced by the feeding of cod liver oil. The toxic effect of cod liver oil was found to be associated with the polyethenoic acids but did not consist in a lowering of the tocopherol content of the muscles. Methylene blue accelerated the condition without resulting in higher concentrations of tocopherol

TABLE III

The action of vitamin E deficiency diet used in the tests of article and their response to various substances

Sign	Necessary for occurrence of sign	Aggravates or accelerates condition	Nordihydroguaiaretic acid		Disulfiram		Ascorbic acid		Cystine		Methylene blue		Thiol thiol amine		Natural diet*		Diphenyl p-phenylenediamine	
			Early by hydrolizable fat															
			a	b	a	b	a	b	a	b	a	b	a	b	a	b	a	b
Exudative diathesis (chicks)	+			(+)		+		(+)				+		+				
Encephalomalacia (chicks)	+			(+)		0		(+)				+		+				
Peroxidation and brown coloration of adipose tissue (chicks and rats)	+			+		+		(+)		0		+		+				
Brown uterus (rats)																+		
Depigmentation of incisors (mice)				0		0		0		+		+						
Low vitamin A in liver (rats, chicks)	+			+		+		0		0		+						+
Reproduction failure (female rats)	0			(+)				0		0		0†						
Massive liver necrosis (rats)	0(?)	+																
Lung hemorrhage (rats)	0	+						0		+		NB						
Hemolysis with dialuric acid (rats)												(+)						
												NB						
Muscular degeneration (chicks)	0	+		0		0		0		+		0						
Muscular degeneration (calves)		+												+				
Degeneration of renal tubules (rats)														+				

a refers to diets without easily oxidizable fat.
b refers to diets containing such fat.
+ means fairly good protection, (+) less marked protection.
NB = disagreement among various investigators.
* New methylene blue, Rindscheder's green, malachite green, methylviolet, rosaniline.
† formerly found positive with less thoroughly depleted rats and with diets containing traces of vitamin E.

Fish Oil, ... Brunsby Ed., 196.

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

in serum, muscle, liver, or perinephric fat. It seems, therefore, that there was a direct protective effect of methylene blue against the toxicity of the polyethenoic acids of cod liver oil. Ascorbic acid and ethyl gallate did not show this effect. The experiments of Baxter *et al.* (3) show that it may be necessary to distinguish between the effect of methylene blue against a condition as such and against the effect of a noxious substance provoking the condition. Regarding muscular dystrophy caused by lack of vitamin E it is worthwhile to remember that tocopheryl hydroquinone has been assumed to be the specific remedy for this condition (56).

Conflicting statements occur in the literature concerning the influence of methylene blue on reproduction failure in vitamin E-deficient female rats. Thus, Dam and Granados (15) and Markees (57) found that methylene blue counteracts this condition when the substance is added to the diet on which the rats are reared. When methylene blue is given as one single dose at the beginning of pregnancy it has no such effect (57). Moore *et al.* (71) were unable to find any effect of methylene blue on failure of reproduction. Recently, Christensen *et al.* (9) have shown that when the diet is thoroughly freed from traces of vitamin E, which requires that its content of lard is purified by suitable adsorption, methylene blue has no effect on reproduction failure, whereas a marked effect of methylene blue is observed when traces of vitamin E are present in the diet. This must mean that methylene blue retards the depletion by stabilizing traces of vitamin E otherwise insufficient for protection. Such an interpretation is in agreement with the observation of somewhat higher values for tocopherol in adipose tissue of chicks (18) and in blood of rats (57), on vitamin E-deficient, methylene blue-containing diets than found on similar diets without methylene blue. The same interpretation may hold for the effect of methylene blue on incisor depigmentation and diduric acid hemolysis in rats, items on which there have also been different opinions in the literature (1, 7, 40, 71, 72, 73, 74).

Moore and associates (40, 71, 72, 73, 74) were unable to find any effect of methylene blue on these signs, whereas they found effects on brown coloration of the uterus and against tubular degeneration in the rat. Methylene blue was found in their studies to be effective against brown coloration of the uterus when given in the amount of 0.032% of the diet.

Several *other redox dyes and related substances* such as thioniphenylbrine (18), new methylene blue, Binkschuller's green (which does not contain sulfur), the triphenylmethane dyes malachite green (apparently toxic to chicks), methyl violet and rosaniline have effects in rats similar to those of methylene blue (72, 73, 74). Binkschuller's green and toluidine blue also act against encephalomalacia when given in suitable amounts (Dam, unpublished studies). Lately, diphenyl-*p*-phenylenediamine (DPPD) has been found effective against encephalomalacia in chicks (5, 87), and capable of increasing vitamin A in liver and plasma in chicks (63). DPPD was also found effective against encephalomalacia when injected intramuscularly, and the same applied to alpha- and gamma-tocopherols which were nearly equally effective, whereas delta-tocopherol was ineffective. Several antioxidants such as 2,6-di-t-butyl-4-methoxyphenol (BHT),

dibutyl- and diamylhydroquinones, diisobutyl-p-phenylenediamine, and 1,2-dihydro-2,2,4-trimethyl-6-ethoxyquinoline (Santoquine®) given orally counteracted encephalomalacia. As far as the reviewer knows, no effect of redox dyes or antioxidants has yet been demonstrated against muscular degeneration in chicks.

When interpreting the observed effects of antioxidants and redox dyes on signs of vitamin E deficiency the following points must be taken into account: A. The effects of the substances in question seem to be most pronounced against symptoms occurring in the presence of dietary polyenoic fatty acids. B. 1) As already mentioned, the substances may act by protecting small amounts of vitamin E present in the diet or in the animal, thereby improving the action of otherwise insufficient amounts of the vitamin. 2) The substances may act as antioxidants in cases where vitamin E does the same. 3) In the case of methylene blue and of some related redox dyes, the known ability of these substances to substitute for the cytochrome system may play a part. 4) Such substances may also act in a way similar to that recently reported for vitamin E in the respiratory chain, in which the vitamin is believed to act as a carrier in one path of electron transfer from flavin systems to cytochrome c (76). 5) A combination of some of the possibilities mentioned under B. 1) to 4) may apply.

An obvious requirement for a positive effect following oral ingestion of a redox substance *in vivo* is that the substance be absorbed from the intestine. Furthermore, it must be carried to the proper places within the organism. If the substance is not absorbed, it may at most exert a protective effect as an antioxidant for traces of vitamin E or of other essential metabolites in the food or in the intestine. It has long been known that methylene blue is absorbed from the intestine (89), and its profuse excretion in the bile of chicks after being absorbed is easy to demonstrate. Ingested diphenyl-*p*-diphenylamine is also excreted in the bile of chicks (Dam and Sonderegaard, unpublished). Ingested NDGA and Antabuse exert a growth-depressing effect on rats and chicks and therefore must also be absorbed.

In order to stabilize vitamin E in the body directly, an absorbable redox substance must be assumed to have a redox potential lower than that of alpha-tocopherol, but it cannot be excluded that a similar substance with a redox potential somewhat higher than that of α-tocopherol may act as antioxidant against the autoxidation of polyenoic fatty acids. The redox potential of alpha-tocopherol is not known with great accuracy. According to Golumbic and Mattill (29) the E of alpha-tocopherol measured at 75°C in 95% ethanol containing 0.2 N HCl is between the redox potentials of *p*-toluquinone and *p*-xyloquinone, i.e., between 650 and 597 mV; at pH 7 this probably means between 256 and 177 mV. In the Thunberg tube α-tocopherol in 80% ethanol at room temperature without addition of acid will reduce 2,6-dichlorophenolindophenol (+217 mV) but not tolylene blue (+115 mV), thionine (+62 mV), toluidine blue (+11 mV) or methylene blue (+11 mV).

Possibility B. 1), stabilization of vitamin E, probably applies to the effect of methylene blue observed by some investigators against reproduction failure in female rats, and possibly to mouse depigmentation and diduric acid hemolysis in rats.

Possibility B. 2), antioxidant effect corresponding to that of vitamin E, seems to be the explanation of the effect of methylene blue, thiodiphenylamine and Antabuse on liver storage of vitamin A, and of the effect of the same substances and NDGA on brown adipose tissue and peroxidation of body fat in chicks and rats, and of the effect of redox dyes on brown uterus in rats. The effect of methylene blue on cod liver oil-induced muscular dystrophy in calves and on cod liver oil-accelerated liver necrosis in rats may be of the same nature.

A combination of the two aforementioned modes of action may apply to the exudative diathesis and encephalomalacia in chicks.

It seems, at present, less easy to apply possibilities B. 3) and 4), rather in the respiratory chain, to the problems under discussion, since there has not been any indication of diminished respiration in connection with the signs of vitamin E deficiency mentioned. On the contrary, increased oxygen uptake has been reported for muscles of vitamin E-deficient animals (45).

Finally, the question may be raised whether practical application may be made of the observed effects of antioxidants and redox substances on signs of vitamin E deficiency. To this may be said that those of the aforementioned substances which are unphysiological are more or less toxic. The color of the dyes is also an obstacle. A substance like NDGA which in some countries is permitted in low concentrations as an additive to certain foods is not active enough to replace vitamin E in artificial diets. The same probably applies to other artificial antioxidants. Consideration has, however, been given to the use of such substances in chicken rations containing fish oils. According to Singsen *et al.* (87), an amount of 0.0125% of DPPD would be sufficient to prevent encephalomalacia under most field conditions. It remains to be seen whether this practice will hold its own in the future.

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Alimentary Production of Gallstones in Hamsters

17. Influence of butter fat and the fat of a dietetic margarine rich in linoleic acid on gallstone formation and composition of the bladder bile*)

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With 3 figures and 5 tables

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Previous work from our laboratory (1) has shown that fats counteract the development of cholesterol gallstones in young hamsters fed a diet containing a high level of glucose. When the fats were given in low amounts, it could be shown that soybean oil and cod liver oil, i. e. fats rich in polyunsaturated fatty acids, are more efficient than lard in counteracting the development of this type of gallstones.

The present work concerns the development of gallstones under the influence of two different fats used in human nutrition, viz. butter fat and the fat of a dietetic margarine having a high content of linoleic acid.

Experimental

The hamsters (Mesocricetus auratus auratus) were young from out stock colony, about one month old at the beginning of the experiment. They were caged individually, and each of them received 0.1 ml of a 0.5% solution of Mepacrine hydrochloride (3-Chloro-7-methoxy-9-(1-methyl-4-diethylaminobutylamino)acridine dihydrochloride) by mouth on 3 consecutive days before the experimental feeding started, as a measure to prevent spreading of a protozoal intestinal infection.

The results were based only on animals living through the entire experimental period of 6 to 7 weeks and not having diarrhea.

At the end of each experiment, the animals were killed with chloroform and autopsied immediately thereafter. Examination for the presence of gallstones and determination of the type of stones found were carried out as described earlier (1).

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In one of the experimental series (G 117), bile was collected from the gallbladder at autopsy for quantitative determination of cholesterol, bile acids and lipid soluble phosphorus as described in an earlier communication (2). It was not possible to obtain bile from all the animals in this series, and in several cases it was necessary to pool bile from 2 or 3 animals in order to get enough material for the determinations.

The composition of the fat-free basal diet is shown in table 1. Vitamins A and D_2 were included in the vitamin mixture instead of being given separately as in most of our previous studies. Further, the salt mixture contained added trace elements as shown in the footnote to table 1.

Table 1. Composition of the fat-free basal diet

Casein[1])	20.0 g
Glucose	62.3 g
Rice starch	12.0 g
Salt mixture[2])	5.0 g
Vitamin mixture[3])	0.5 g
Choline chloride	0.2 g
	100.0 g

[1]) "Vitamin Test Casein" from Genatosan Ltd., Loughborough, England.

[2]) Sodium chloride, 0.3 g; magnesium subcarbonate, 0.25 g; potassium chloride, 0.35 g; dipotassium phosphate, 0.85 g; monosodium phosphate (2 aq.), 0.5 g; monocalcium phosphate (1 aq.), 0.85 g; calcium lactate (approx. 5 aq.), 1.8 g; ferric citrate (16.7% Fe), 0.1 g; potassium iodate, 0.25 mg; zinc carbonate, basic, (56% Zn), 3.6 mg; manganese carbonate (47.8% Mn), 2.1 mg; cupric carbonate (50–55% Cu), 0.95 mg; sodium fluoride, 2.0 mg; sodium molybdate (2 aq.), 0.25 mg; chromium sulfate (15 aq.), 0.055 mg; selenium dioxide, 0.016 mg; total 5.000 g.

[3]) Biotin, 0.05 mg; folic acid, 0.2 mg; ascorbic acid, 10 mg; thiamine hydrochloride, 1.5 mg; riboflavin, 1.5 mg; pyridoxine hydrochloride, 1.5 mg; calcium pantothenate, 5 mg; nicotinic acid amide, 5 mg; inositol, 5 mg; p-aminobenzoic acid, 1 mg; menadiol diphosphate tetrasodium salt (6 aq.) ("Synkavit", Roche), 0.5 mg; α-tocopherol acetate ("Epiphal", Roche), 5 mg; cyanocobalamin, 0.003 mg (furnished in the form of "Bendogen", G12A); vitamin A, 1000 I. U., and vitamin D_2, 100 I. U. (furnished in the form of "Rovimix 50/5", Roche, 20 mg); sucrose to make a total of 500 mg.

The whole amount of butter fat and margarine fat necessary for one experiment was prepared in advance by melting and separation from non-lipid material. The fats so prepared were stored in several 0.5 liter plastic containers at minus 20 °C until use.

The fats were incorporated into the basal diet instead of an equal amount by weight of glucose. Two kg of diet lasting for 4–5 days were prepared at a time and stored at 4 °C when not in use.

The dietetic margarine was of the brand "MIFU" which is commonly used in Denmark. It contains 40% or more of linoleic acid in the fat which according to information obtained from the manufacturer consists of cottonseed oil, 82.7%; hydrogenated palm oil, 17%; and sesame oil, 0.3%; plus the prescribed addition of vitamins A and D*).

In two experimental series (G 112 and G 117), the effect of this margarine fat was compared with that of butter fat at the dietary level of 10%. In a third experimental series (G 113), the effect of the fats were compared at the 3% dietary level.

The fatty acid composition of the fats used in each of the experimental series was determined by gas liquid chromatography of the methyl esters. The results of these determinations are shown in table 2.

*) In Denmark, margarine shall contain 14 i.u. preformed vitamin A, 6 i.u. vitamin A in the form of carotene, and 0.5 i.u. vitamin D per g.

Table 2. Fatty acid composition of the fats used.
Per cent of the methyl esters of the individual fatty acids in the methyl esters of the total fatty acids from each fat

Fatty acid[1] (Number of carbon atoms and double bonds)	Butter fat			Margarine fat "MIFU"		
	G 112	G 113	G 117	G 112	G 113	G 117
4:0	0.6	0.9	t			
6:0	0.9	0.7	t			
8:0	0.8	1.1	0.3			
10:0	1.7	2.4	2.2			
11:0	0.6	0.8	0.2			
12:0	2.9	3.2	4.0	0.3	0.2	0.4
13:0	0.6	0.8	0.8			
14:0	8.8	9.3	15.3	0.7	0.7	0.9
14:1	3.2	2.3				
			3.6			
15:0	2.2	2.0				
16:0 br.?[2])	0.8	0.6	0.4			
16:0	22.9	26.4	38.2	24.4	22.4	30.3
16:1	5.2	6.6	6.6	2.6	2.7	1.1
17:0	2.1	1.3	0.9			
18:0 br.?[2])	0.5	0.5	t			
18:0	11.3	10.6	6.7	3.1	3.2	3.6
18:1	27.7	25.6	17.6	26.6	26.4	21.0
18:2	3.8	3.0	1.9	41.1	43.3	40.0
18:3	2.5	2.0	0.8	1.0	0.9	2.6
20:1	0.7	t	t			
22:1				0.3	0.3	t

[1]) The amounts of fatty acids of 8:0 and lower are undoubtedly low because of high volatility of esters of these acids.
[2]) br. = branched.

Results and Discussion

The incidences of the various types of gallstones are shown in table 3.

The results of the analyses of bladder bile from animals in the experimental series G 117 are shown in tables 4 and 5 and in figs. 1, 2, and 3.

In each of the two experimental series (G 112 and G 117), in which the fats of butter and dietetic margarine were compared at the 10% level, the incidence of cholesterol gallstones was much less with the margarine fat than with butter fat. For each sex this difference is significant with a probability of more than 99% [the method for evaluating the significance was that described by Kolmer (3)].

In the experimental series (G 113) in which the two fats were compared at the 3% level, the incidence of cholesterol gallstones was also less with the margarine fat than with the butter fat, but the significance of the difference was lower, viz. 95% for the females, and also for the two sexes considered together.

The incidences of amorphous pigmented gallstones and of "mixed gallstones" were low in each of the experimental series of the present study.

The growth of the animals was about the same with the two fats and faster at the 10% than at the 3% fat level.

The cause of the lower incidence of cholesterol gallstones obtained with the fat of the dietetic margarine as compared with butter fat is, apparently, the much higher content of linoleic acid in the margarine fat.

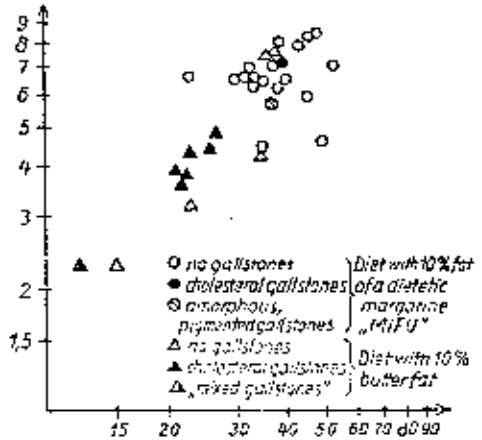

Fig. 1. Molar ratios bile acids/cholesterol (horizontal) and lipid-soluble phosphorus/cholesterol (vertical) in bladder bile of hamsters from Experimental Series G 117. Logarithmic scale.

Linoleic acid, however, is not the only polyenoic fatty acid capable of keeping the incidence of cholesterol gallstones low, since in previous experiments drastic reduction of the formation of cholesterol gallstones has been obtained also with cod liver oil (1), cod liver oil freed from vitamin A, and with ethyl esters of the fatty acids from menhaden oil (4).

The analyses of the bladder bile from animals receiving butter fat and margarine fat, resp., at the 10% dietary level (tables 4 and 5 and figs. 1, 2, and 3) show the following:

The ratios bile acids/cholesterol and lipid P/cholesterol are particularly scattered in the butter fat group. In more than half of the cases in the butter fat group, these ratios are lower than the lowest value obtained in the margarine fat group (fig. 1).

The concentrations of cholesterol are also particularly scattered in the butter fat group, but in more than half of the cases higher than the highest cholesterol concentration found in the margarine fat group (figs. 2 and 3).

Furthermore, in the butter fat group there is a general trend for the values of the two ratios to decline with increasing concentrations of cholesterol (figs. 2 and 3).

The data recorded in figs. 1, 2, and 3 may represent part of the explanation why the occurrence of cholesterol gallstones is much more frequent in the 10% butter fat groups than in the 10% margarine fat groups. The validity of an explanation based on these data is, of course, dependent upon the correctness of the assumption that low values of the two ratios together with high cholesterol concentration *within the ranges observed* are incompatible with keeping

Table 3. Incidence of gallstones and other data for the three experimental series

	Age of animals at beginning of feeding, days	Duration of feeding period, days	Diet characteristics	Sex and number of animals		Number of animals having: Cholesterol gallstones	Mixed gallstones	Amorphous pigmented gallstones	No gallstones	Percentage of animals having: Cholesterol gallstones	Mixed gallstones	Amorphous pigmented gallstones	No gallstones	Mean weight gain during first 42 days g	Range
G 112	29–37	42–43	10% butter fat	m	22	13	0	0	9	59.2	0	0	40.8	40.5 ± 2.7	22–62
				f	23	14	0	1	8	60.9	0	4.3	34.8	34.6 ± 1.8	16–48
				m + f	45	27	0	1	17	60.0	0	2.2	37.8		
			10% margarine fat (MIFU)	m	19	0	0	0	19	0	0	0	100.0	39.1 ± 2.3	23–91
				f	25	2	0	1	23	8.0	0	4.0	88.0	36.1 ± 2.5	19–78
				m + f	44	2	0	1	41	4.6	0	2.3	93.1		
G 113	34–37	43–45	3% butter fat	m	21	19	0	0	2	90.5	0	0	9.5	26.8 ± 2.8	2–54
				f	32	25	0	0	7	78.2	0	0	21.8	21.3 ± 1.7	1–36
				m + f	53	44	0	0	9	83.0	0	0	17.0		
			3% margarine fat (MIFU)	m	22	16	0	0	6	77.7	0	0	22.3	25.8 ± 1.9	6–37
				f	25	12	0	0	13	48.0	0	0	52.0	26.2 ± 2.5	7–48
				m + f	47	28	0	0	19	59.6	0		40.4		
G 117	27–33	48–50	10% butter fat	m	15	13	0	0	2	86.7	0	0	13.3	30.7 ± 2.3	20–57
				f	19	12	2	0	5	63.2	10.5	0	26.3	42.9 ± 2.4	25–64
				m + f	34	25	2	0	7	73.5	5.9	0	20.6		
			10% margarine fat (MIFU)	m	20	2	0	1	17	10.0	0	5.0	85.0	43.9 ± 2.7	15–61
				f	18	2	0	1	15	11.1	0	5.5	83.4	43.7 ± 3.1	32–57
				m + f	38	4	0	2	32	10.5	0	5.3	84.2		

Two figures connected with a solid vertical line on their right-hand side are considered to be different with a probability of more than 99%. Two figures connected with a dotted vertical line on their right-hand side are considered to be different with a probability of 95%

Table 4. Data for bladder bile of hamsters (Experimental series G 117) on the diet with 10% butterfat from the age of 27–33 days. All concentrations are given as millimolarities

Group no./ hamster no.	Type of stones	Days on diet	Cholesterol	Lipid phosphorus	GC	GCD	GD	TC	TCD + TD	Total bile acids	G	T	Trl	Dt	G/T	Dt/Trl	Total bile acids/ cholest.	Lipid phosphorus/ cholest.
females:																		
1226/2	C	48	6.1	17.0														2.8
1226/19	C	48	5.8	—	63.1	51.2	14.5	12.8	25.9	167.5	128.8	39.7	75.9	91.6	3.3	1.2	28.9	—
1226/30	C																	
1226/68	C																	
1226/17	O	48	8.0	18.6	28.3	57.8	8.5	7.6	17.1	119.3	94.6	24.7	35.9	83.4	3.8	2.3	14.9	2.3
1227/39	O	50	4.9	15.5	61.6	18.9	11.6	8.7	8.4	112.2	95.1	17.1	70.3	41.9	5.6	1.6	22.9	3.2
1227/94	C	50	8.0	18.3	40.1	29.3	14.7	8.1	4.2	96.4	84.1	12.3	48.2	48.2	6.8	1.0	12.1	2.3
1227/83	C	50	4.5	20.6	25.1	57.4	8.8	8.4	16.4	116.1	91.3	24.8	33.5	82.6	3.7	2.5	27.0	4.8
1227/86	C																	
1228/13	C																	
1228/41	O	50	2.0	8.3	12.2	44.3	3.5	1.9	7.1	69.0	60.0	9.0	14.1	54.9	6.7	3.9	34.5	4.2
1228/97	O																	
1228/47	C	50	3.1	11.6	22.9	29.8	6.5	3.8	5.4	68.4	59.2	9.2	26.7	41.7	6.4	1.6	22.1	3.8
1230/21	O	50	2.4	18.3	37.4	9.3	20.6	9.5	11.5	88.3	67.3	21.0	46.9	41.4	3.2	0.9	36.8	7.6
1230/90	M	50	2.7	20.1	38.4	23.4	13.6	7.3	14.4	97.1	75.4	21.7	45.7	51.4	3.5	1.1	36.0	7.4
1230/15	O	50	5.7	25.3	31.9	67.4	11.9	10.0	25.5	146.7	111.2	35.5	41.0	104.8	3.1	2.5	25.7	4.4
1230/57	C																	
1230/91	O																	
males:																		
1226/32	C	48	7.1	26.3														3.7
1226/10	C																	
1226/68	C	48	8.3	27.6														3.3
[illegible]/46	O	48	8.1	20.4														2.5
[illegible]/33	C																	
[illegible]7/10	C	49	8.4	33.6	50.6	60.2	11.5	10.1	20.2	170.0	130.7	48.3	65.1	100.0	2.7	1.6	21.3	3.9
1228/12	C																	
1228/43	C																	
1228/80	C	49	6.3	22.7	[illegible]	[illegible]	13.9	8.2	30.0	138.1	69.9	38.2	33.9	104.2	2.6	3.1	21.9	3.6
1229/25	C																	
1229/81	C	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	9.7	17.3	[illegible]	[illegible]	126.3	[illegible]	84.2	[illegible]	2.3	1.1	[illegible]	6.9

C = cholesterol gallstones; M = [illegible]; O = [illegible] gallstones.

Table 5. Data for bladder bile of hamsters (Experimental series G 117) on the diet with 10% margarine fat (MIFU) from the age of 27–33 days. All concentrations are given as millimolarities

Group no. of hamster	Type of stones	Days on diet	Cholesterol	Lipid phosphorus	OB	GCD	GD	TC	TCB + TD	Total bile acids	C	T	TG	PL	G/T	TG/Tel	Total bile acids/cholest.	Lipid phosphorus/cholest.
female:																		
1230/07	O	48	2.6	21.4	32.9	54.9	7.3	7.7	17.7	120.5	95.1	25.4	40.0	79.9	3.7	2.9	46.3	8.2
1230/18	O	48	3.8	17.3	60.1	41.5	24.6	14.0	14.8	155.5	126.1	29.1	74.7	80.8	4.3	1.1	40.9	4.5
1230/82	O																	
1230/79	O																	
1213/59	A	50	3.5	19.1	49.1	35.8	19.9	8.8	17.8	130.4	103.8	26.6	57.9	72.5	3.0	1.3	37.3	5.5
1231/16	O	50	2.0	11.6	7.2	65.1	4.4	3.7	10.8	91.2	76.7	14.5	10.9	80.3	5.3	7.4	45.6	5.8
1211/37	O	50	2.9	10.9	50.3	40.6	14.9	12.6	25.1	152.5	114.8	37.7	71.9	60.6	3.0	1.1	52.6	0.9
1231/85	O																	
1233/10	O	50	2.4	16.3	17.4	38.6	8.5	4.0	8.9	77.4	64.5	12.9	21.4	56.0	5.0	2.6	32.3	6.8
1233/20	O																	
1233/52	O	50	1.8	11.5	6.5	21.8	28.0	0.0	2.8	60.9	57.2	3.7	7.4	53.5	15.5	7.2	33.8	6.4
1233/191	O																	
1232/46	O	50	2.5	16.0	7.2	37.8	13.3	5.4	10.9	74.6	59.3	16.3	12.6	62.0	3.0	4.9	29.8	6.4
1233/84	O	50	2.9	18.8	8.6	26.5	15.7	5.0	11.4	67.2	50.8	16.4	13.0	53.6	3.1	3.9	23.2	6.5
males:																		
1230/75	O	48	3.7	30.7	40.4	36.5	16.2	34.0	36.6	172.5	101.1	71.4	84.3	88.2	1.4	1.0	46.6	8.2
1231/76	O																	
1230/10	O	48	4.3	27.4	30.3	51.4	26.2	21.6	9.5	148.0	110.9	31.1	60.9	87.1	3.8	1.4	34.4	6.4
1240/09	O																	
1231/26	O	49	3.9	25.0	38.0	44.3	18.3	9.2	12.6	122.4	100.6	21.8	47.2	75.2	4.6	1.6	31.4	6.4
1232/5	O																	
1233/24	O	49	3.1	21.2	27.7	40.8	10.0	8.9	15.8	112.2	87.5	24.7	36.6	75.6	3.5	2.1	33.0	6.2
1232/8	O																	
1233/30	O	49	3.8	20.4	62.1	43.2	11.7	23.7	22.1	162.8	117.0	45.8	57.8	77.0	2.6	0.0	42.8	7.7
1210/11	O																	
1233/35	O	49	1.7	13.4	26.8	10.3	8.5	6.1	7.5	65.2	61.6	13.6	32.9	32.3	3.8	0.9	38.4	7.9
1233/73	O																	
1232/55	O	49	2.9	18.5	39.3	31.1	29.1	6.3	10.6	116.4	99.5	16.9	45.6	70.9	5.9	1.6	40.1	6.4
1233/58	O																	
1233/82	O	49	4.2	25.8	32.0	35.6	6.1	42.9	42.6	169.2	73.7	85.5	74.9	84.3	0.9	1.1	37.9	6.1
1231/7	O																	
1231/14	C	50	3.8	16.9	44.5	38.4	12.4	15.0	22.0	132.3	95.3	37.0	50.5	72.8	2.6	1.2	34.8	4.4
		50	3.1	21.9	35.2	46.7	13.6	8.4	14.4	118.3	95.5	22.8	43.6	74.7	4.2	1.7	38.2	7.1

C = cholesterol gallstones, A = amorphous pigmented gallstones, O = no gallstones

cholesterol in solution in the bile. An exact settlement of this question will require suitable *in vitro* solubilization experiments.

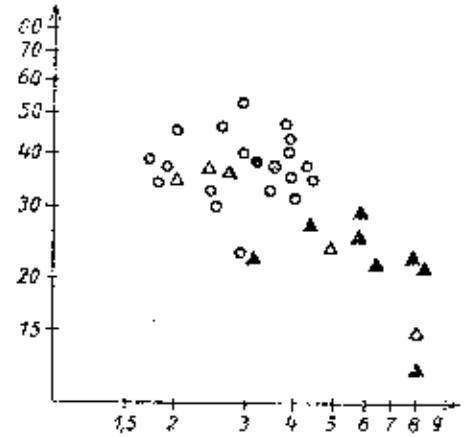

Fig. 2. Molar ratios bile acids/cholesterol (vertical) and millimolar concentrations of cholesterol (horizontal), of bladder bile of hamsters from Exp. Series G 117. Logarithmic scale. Symbols as or fig. 1

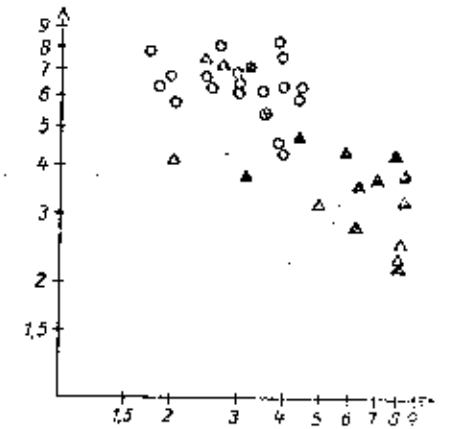

Fig. 3. Molar ratios lipid-soluble phosphorus/cholesterol (vertical) and millimolar concentration of cholesterol (horizontal) of bladder bile of hamsters from Exp. Series G 117. Logarithmic scale. Symbols as for fig. 1

From the figs. it is evident, that in the butter fat group there is not in every case correlation between the trend of the analytical data and the occurrence or absence of cholesterol gallstones. The absence of cholesterol gallstones in some of the cases having high cholesterol concentrations and low ratios bile acids/cholesterol and lipid-P/cholesterol is, however, not surprising since precipitated cholesterol or small gallstones may have passed out of the gallbladder. More interesting is the occurrence of cholesterol gallstones in one or two cases in the butter fat group having data within or close to the range of the corresponding data in the margarine fat group. Further, there is eve

one case of cholesterol gallstones in the margarine fat group. This suggests that other features besides those hitherto considered may play a role in determining the occurrence of cholesterol gallstones.

In a previous series of experiments (5), young hamsters received diets with 10% butter fat and with 10% of the fat from a high linoleic acid margarine of the same brand and essentially the same fatty acid composition as that used in the present experiments. (The fat-free basal diet was one that did not greatly favor the occurrence of cholesterol gallstones.) It was then found that the bile lecithin of the animals receiving the margarine fat contained much more linoleic acid and correspondingly less oleic acid than did the bile lecithin from the animals receiving butter fat.

The fatty acid compositions of the bile phosphatides of the butter and margarine fat groups in the present experiment were not examined, but there can hardly be any doubt that they have had differences similar to those found in the earlier study.

The question therefore arises whether the bile phosphatides characteristic of the margarine fat group are more efficient in preventing formation of cholesterol gallstones than are the bile phosphatides characteristic of the butter fat group.

Attempts to decide this question must be made in future studies, i. a. by comparing the solubilization properties of phosphatides with different fatty acid patterns, towards cholesterol in the presence of bile acids.

Summary

Young hamsters were reared on diets containing butter fat and fat of a high linoleic acid margarine, respectively.

The basal diet without the fat had previously been shown to produce a very high incidence of cholesterol gallstones in young hamsters.

In two trials, in which the fats were compared at the 10% dietary level, the incidence of cholesterol gallstones was much less with the diets containing margarine fat than with the diets containing butter fat. The difference was significant with a probability of more than 99%.

In a third trial, in which the fats were compared at the 3% dietary level, the incidence of cholesterol gallstones was also less with margarine fat than with butter fat. Here the difference was significant with a probability of more than 95% but less than 99%.

The superior effect of the margarine fat in counteracting the development of cholesterol gallstones is ascribed to its high content (about 40%) of linoleic acid.

In one of the trials in which the two fats were given at the 10% dietary level the bladder bile was analyzed for cholesterol, bile acids and lipid soluble phosphorus, with the following results:

For the animals receiving the butter fat the concentrations of cholesterol were found to be more scattered and, in more than half of the cases, higher than for animals receiving margarine fat.

The ratios between the molar concentrations of bile acids and cholesterol and between the molar concentrations of lipid soluble phosphorus and cholesterol were also more scattered for animals receiving the butter fat diet, but in more than half of the cases in the butter fat group these ratios were lower than in the margarine fat group.

In the butter fat group the values for the two aforementioned ratios tended to decrease with increasing concentrations of cholesterol.

A more exact correlation of the composition of the bladder bile with the occurrence of gallstones must take into account not only the analytical data summarized above, but, probably, also a previous finding according to which the bile lecithin from hamsters receiving a diet with 10% of the high linoleic acid margarine fat contained much more linoleic acid and correspondingly less oleic acid than did the bile lecithin from hamsters receiving a diet with 10% butter fat.

Zusammenfassung

Junge Hamster wurden mit Nahrungen, die Butterfett bzw. das Fett einer linolsäurereichen Diätmargarine enthielten, gefüttert.

Frühere Versuche hatten gezeigt, daß die fettfreie Basal-Nahrung eine hohe Incidenz von Cholesterin-Gallensteinen in jungen Hamstern hervorruft.

In zwei Versuchen, in welchen die zwei Fette in der Höhe von 10% der Nahrung gegeben wurden, war die Incidenz von Cholesterin-Gallensteinen viel niedriger in den mit Margarinefett gefütterten als in den mit Butterfett gefütterten Gruppen von Tieren. Die Differenz war mit mehr als 99% Sicherheit signifikant.

In einem dritten Versuch, in welchem die Fette in der Höhe von 3% gegeben wurden, war die Incidenz von Cholesterin-Gallensteinen auch niedriger in der mit Margarinefett gefütterten Gruppe als in der mit Butterfett gefütterten. Die Differenz war signifikant mit 95% aber nicht mit 99% Sicherheit.

Die Überlegenheit des Margarinefettes in bezug auf die Unterdrückung der Cholesterin-Gallensteinbildung wird dem hohen Linolsäuregehalt (ungefähr 40%) dieses Fetts zugeschrieben.

In einem der Versuche, in welchem die Fette in der Höhe von 10% gegeben wurden, wurde die Blasengalle auf Cholesterin, Gallensäuren und lipid-löslichem Phosphor analysiert. Die Hauptergebnisse waren die folgenden:

In der Butterfett-Gruppe waren die Einzelwerte der Konzentrationen des Cholesterins besonders zerstreut und, in mehr als der Hälfte der Fälle höher als in der Margarinefett-Gruppe.

Die Einzelwerte der Verhältnisse der molaren Konzentrationen von Gallensäuren zu Cholesterin und von lipid-löslichem Phosphor zu Cholesterin waren auch besonders zerstreut in der mit Butterfett gefütterten Gruppe. In mehr als der Hälfte der Fälle in der Butterfett-Gruppe waren diese Verhältnisse niedriger als in der Margarinefett-Gruppe.

In der Butterfett-Gruppe zeigten die beiden Verhältnisse eine Neigung zur Abnahme mit zunehmenden Cholesterin-Konzentrationen.

Eine genauere Korrelation der Zusammensetzung der Blasengalle mit dem Vorkommen von Gallensteinen in den zwei Gruppen muß neben den obenerwähnten analytischen Befunden wahrscheinlich auch die in früheren Untersuchungen gefundene verschiedene Fettsäurezusammensetzung der Gallenphosphatide in Betracht ziehen. Nach diesen Untersuchungen enthält das Gallenlecithin der mit 10% linolsäurereicher Diätmargarine gefütterten Hamster viel mehr Linolsäure und entsprechend weniger Ölsäure als das Gallenlecithin der mit 10% Butterfett gefütterten Hamster.

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Fat Versus Sucrose as the Nonprotein Calorie Portion of the Diet of Rats[1]

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ABSTRACT Diets with a uniform ratio of protein to nonprotein calories of 1:4, have been used to study the comparative effects of sucrose and lard on protein retention. In a nitrogen balance study on rats, the lard diet gave better N economy than did the sucrose diet. Liver glucose-6-phosphate dehydrogenase enzyme levels were found to be relatively high in the case of rats fed the sucrose diet, but when the lard diet was fed the liver levels of these enzymes were as low as those of starved rats. Short-term in vivo protein synthesis as measured by labeled amino acid incorporation into liver protein indicated increased arginine incorporation when the fat diet was fed. The observations indicate an improved protein utilization and nutritional status when the high fat carbohydrate-free diet was fed. J. Nutr. 103: 342-346, 1973.

INDEXING KEY WORDS protein retention · nitrogen · protein utilization · sucrose · fat · glucose-6-phosphate dehydrogenase

Three liver enzymes involved in glycolysis, glucose-6-phosphate dehydrogenase (EC 1.1.1.49) (G6PD), 6-phosphogluconate dehydrogenase (EC 1.1.1.44) (6PGD) and pyruvate kinase (EC 2.7.1.40) (PK), have been shown to be carbohydrate dependent (1). This dependence appeared to be mediated in liver cytoplasm through glycogen levels, in a manner consistent with regulation of each enzyme by its specific substrate. Inhibition experiments indicated a requirement of new RNA synthesis for those enzyme increases (2).

We now report further studies comparing the effects of balanced diets containing sucrose as principal nonprotein energy source with diets containing the same protein/calorie ratio but with the nonprotein calories supplied as lard. Protein utilization and certain liver enzyme levels after prolonged feeding of these diets at "steady state" levels have been studied. In view of the extensive literature on the cardiovascular effects of high fat diets, it is interesting to note that rats grow as well and appear at least as healthy on a carbohydrate-free high fat diet containing sufficient protein as on a "conventional" carbohydrate-containing diet.

MATERIALS AND METHODS

Animals. Male rats were used. Management, environment and principal diets have been described (1, 2). S diet contained: (g/100 g diet) 69 sucrose, 5 corn oil, 20 casein, and 6 minerals plus vitamins; F diet contained 51 lard, 8 corn oil, 33 casein, and 8 minerals plus vitamins. These two diets provided the same caloric ratios of protein to nonprotein energy source, similar and sufficient essential fatty acids per calorie, and similar amounts of minerals and vitamins per calorie. A third diet, M diet, was used in one study; it contained: (g/100 g diet) 25.3 lard, 6.7 corn oil, 34.9 sucrose, 26.7 casein, 4.9 salt mix (1), 0.64 vitamin mix (1), 0.15 each choline and DL-methionine.

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TABLE 1

Nitrogen balance studies[1]

Parameters	Units	S diet[2]	F diet[2]	SEM[3]	$P <$
Body weight at start	g	328	328	6	NS[4]
Body weight gain during study	g/day	4.7	9.1	0.7	0.01
Food intake	g/day (kcal)	18.3 (73.9)	14.0 (98.3)	0.7 (4.2)	0.01 (0.01)
Nitrogen intake	g/day	0.542	0.800	0.034	0.01
Nitrogen excreted in feces	g/day	0.024	0.022	0.002	NS
Nitrogen excreted in urine	g/day	0.363	0.463	0.025	0.01
Nitrogen retained	g/day	0.154	0.315	0.027	0.01
Nitrogen absorbed	g/g	0.955	0.972	0.003	0.01
Nitrogen—retained/consumed	ratio	0.284	0.394		
Nitrogen—retained/absorbed	ratio	0.298	0.403	0.003	0.05
Protein consumed	g/day	3.39	5.00		
Daily body weight gain/protein intake	g/g	1.40	1.81		
Protein retained	g/day	0.963	1.969		
Protein contribution to body weight gain	percent	20.4	21.7		
Nitrogen retained per kcal of intake	mg/kcal	2.86	3.19	0.36	0.01
Daily body weight gain/total energy of intake	g/kcal	0.064	0.092		

[1] Started after 11 days on diet. [2] Nine rats per group; S diet energy supplied by sucrose; F diet by lard. [3] Standard error of the difference between the means for the S and F diets. [4] Not significant.

The following laboratory methods were used in addition to those already described (1, 2):

Nitrogen balance. Urine and feces were collected separately and quantitatively from rats kept singly in metabolism cages. After removal of hair, the feces were homogenized[4] using silicone[5] to eliminate foam. Nitrogen content of urine and of feces was determined by the Kjeldahl procedure.

Radioarginine and radioleucine incorporation. Each liver was homogenized (1); 0.5 ml of homogenate was precipitated with 10% trichloroacetic acid, washed twice with 10% trichloroacetic acid and the precipitate was dissolved in 1 ml NCS solution.[6] Radioactivity was counted in scintillation solvent as described by Bruno and Christian (3).

Liver G6PD assays were carried out by the method previously reported (4).

RESULTS

To compare the effects of S and F diets on protein utilization, 18 3-month-old Charles River rats previously fed cereal stock diet were divided into two groups of equal average body weight and were fed S or F diet ad libitum for 13 days. For the last 2 days the rats were placed in metabolism cages to determine nitrogen balances. Table 1 shows that the F diet was not poorer but in fact appeared better than the S diet in terms of intake, thrift, economy of protein utilization, and economy of energy utilization. Acceptance of S diet was immediate, but most rats took about 3 days to accept the F diet fully; in neither case did acceptance diminish once established. The nitrogen absorption value of about 0.96 is close to the values that can be calculated from the data of Bunce and King (table 2 of reference (5)) but differs considerably from values given by Mitchell and Bert (6).

At the end of the balance study each rat received intraperitoneally, at 8:00 AM, 0.5 ml water containing 5 μCi each of generally labeled L-arginine-^{3}H and L-leucine-^{14}C. Three rats per diet group were killed 3, 12, and 24 hours later. The results given in table 2 indicate that more ^{3}H (arginine) was found at all times in the total liver protein of the rats fed F diet than S diet, while less ^{14}C (leucine) was found in the total liver protein after 12 and 24 hours in the rats fed F diet. The relatively heavier livers of the rats fed S diet were attributed,

[4] Omni Mixer, Ivan Sorvall Inc., Norwalk, Conn.
[5] Antifoam A Spray, Dow Corning Corp., Midland, Mich.
[6] Nuclear Chicago Corp., Des Plaines, Ill.

TABLE 2

Incorporation of label from L-arginine-^{3}H and L-leucine-^{14}C into liver protein of rats fed F or S diets[1]

Intervals after injection, hr	S diet 3	S diet 12	S diet 24	F diet 3	F diet 12	F diet 24	SEM	$P<$[2]
Body wt at death, g	419	437	407	432	416	448	11	0.01
Liver wt/body wt, g/100 g	3.55	3.56	3.41	2.65	2.75	2.86	0.12	0.01
^{3}H (arginine) present in total liver protein, 10^3 dpm	216	213	218	287	245	294	6	0.01
^{14}C (leucine) present in total liver protein, 10^3 dpm	11.5	14.4	11.6	11.1	8.3	8.9	0.8	0.01

[1] S diet nonprotein energy supplied by sucrose, F diet nonprotein energy supplied by lard; average three rats per subgroup.
[2] Between treatments; differences between times within diet groups were not significant in the case of arginine.

from inspection and previous experience (7, 8), to glycogen and lipid.

Because the table 1 findings were unexpected, in view of the increased synthesis of particular enzyme proteins found in rats fed S diet in short-term experiments (1, 2, 7, 8), the levels of one of these proteins, G6PD, were determined in the livers of rats which had been fed either F or S diet for 9 months from weaning and for comparison in the livers of S diet rats that were starved for 7 days. The results given in table 3 show the great difference in liver level of this enzyme when S diet was fed as compared to when F diet was fed. The level of G6PD after 9 months of the F diet is similar to the level after 7 days' starvation.

DISCUSSION

The reasons for contrasting single dietary energy sources have been given (1, 2). Sucrose was chosen as carbohydrate because it had produced the most rapid increases of G6PD and PK (8, 9); fructose produces similar enzyme increases (10–16) but some of its effects are untypical (17). Lard was chosen for fat, partly because of the ill repute of animal fats and partly to avoid known vitamin E problems with less saturated vegetable oils (18). The protein/calorie ratio was the same for both diets and protein oxidized (i.e., excess amino acids) contributed a relatively small portion of the calories of the diets (1, 2).

While the calories per gram of the two diets could have been equalized with, e.g., methylcellulose, we wished to avoid the one-sided effects on digestion, absorption and excretion introduced by roughage addition. Higher calorie intakes on F diet are probably related to the higher caloric density of this diet. Since, however, the data are expressed in terms of efficiency of utilization it is difficult to believe this efficiency would be improved by the somewhat higher intake and in fact the opposite would be expected. Any regulation of food intake is complicated by the disparity of rates of stomach emptying and hence absorption of dietary components, between F diet (very slow) and S diet (rapid). Hence we infer that the apparent metabolic superiority of F diet (table 1) owed little to the higher caloric intakes.

The possibility that calories from dietary fat might be metabolically superior has long been recognized. Munro (19) has pointed out the lack of evidence that dietary carbohydrate enhanced protein utilization by growing animals except immediately after increasing the carbohydrate content of the diet. Maignon (20), Forbes and co-workers (21), and Samuels and co-workers (22) found nitrogen balances to be no less favorable when fat-rich mixed diets were fed than when carbohydrate-rich mixed diets were fed. When Swendseid and co-workers (23) more recently

TABLE 3

Liver G6PD in rats fed for 9 months high fat as compared to high sucrose diets

Diet	No. of rats	G6PD enzyme[1] units per 100 g body weight	SE[2]
F diet	6	2.35	0.11
S diet	6	18.02	2.13
Starved 7 days	6	2.65	0.11

[1] A unit of G6PD enzyme activity is defined as 1 μmole of $NADP^+$ reduced/minute. [2] $P < 0.001$ when S diet is compared to F diet or to 7 days starved rats.

Effect of hydrogenated soybean fat on rat adipose tissue and on liver acetyl-CoA carboxylase and fatty acid synthetase activities[1,2]

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The cytoplasmic malonyl-CoA[4] pathway for fatty acid biosynthesis, catalyzed by acetyl-CoA carboxylase and fatty acid synthetase multienzyme complex, is now generally recognized as the mean de novo biosynthetic pathway for fatty acids (1, 2). Several workers have advanced evidence to show that acetyl-CoA carboxylase is probably the key enzyme of this pathway (3–5), and the numerous properties of this enzyme that are consistent with the properties of regulatory enzymes (6–8) tend to support this idea. These two enzymes and the hexose monophosphate shunt dehydrogenases have been reported to respond to various nutritional or metabolic stress conditions, i.e., starvation (5), fasting and refeeding a high carbohydrate diet (9), meal feeding (10), and biotin deficiency (11). It has also been known for a long time that fat feeding leads to a depressed acetate incorporation into fatty acids (12), and this has been indirectly attributed to a block at the acetyl CoA carboxylase step (13). Allmann et al. (14), using liver cytoplasmic enzyme preparations from starved rats that were refed a fat-free diet over a 48-hr period, found that this dietary change brought about a marked elevation in the activities of acetyl CoA carboxylase and fatty acid synthetase, as measured, respectively, by acetate-1-^{14}C or acetyl-1-^{14}C CoA and malonyl-2-^{14}C CoA incorporation into long-chain fatty acids. Similar results were also obtained for these two enzymes during early linoleate deficiency in mice (15). Chu et al. (16) recently reported that dietary linoleate, α-linolenate, or arachidonate at daily milligram amounts depressed the activity of liver fatty acid synthetase. The activities of the three shunt dehydrogenases, as well as that of citrate cleavage enzyme, have all been studied following fat feeding (17) and were found to parallel the depressed acetate incorporation seen in adipose tissue and liver of rats fed antecedent fat diets.

In the course of studies on the metabolic effects in rat tissues of the ingestion of partially hydrogenated soybean fat containing substantial amounts of *trans* fatty acids we noticed that this fat was a better depressor of acetate incorporation into fatty acids than corn oil. We report here the results of our attempt to relate this observation to the activities of the two key lipogenic enzymes acetyl CoA carboxylase and fatty acid synthetase, of both adipose tissue and liver of male rats fed the two kinds of fat.

Experimental

Twenty-one-day-old male rats of the Holtzman strain were divided into six groups of four animals.

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[illegible] The rodent stock fat-free diet, purchased from the Nutritional Biochemicals Corp., Cleveland, Ohio, is given in Table 1. Exhaustive extraction of samples from this diet with chloroform–methanol (2:1 v/v) showed that it was essentially devoid of fat. Rats in groups 1 and 2 received, respectively, the fat-free diet in which 10 and 20% (w/w) of sucrose was replaced by equivalent [illegible] partially hydrogenated soybean fat (prepared and supplied by Humko Co., Memphis, Tennessee) respectively (diet groups 10% HF and 20% HF). Diets, at the lower and higher levels of fat supplementation, the content of sucrose in the diet was 64 and 54%, respectively. Hill et al. (18) had suggested that the carbohydrate level in fat diets should be higher than 50% to ensure that any observed effects of fat feeding are attributable to the fat content of the diet only, and not to low levels of carbohydrate in the diet. Fatty acid analysis by gas–liquid chromatography (GLC) of the methyl esters, prepared from the partially hydrogenated fat, showed that it contained about 1.0% $C_{16:0}$ and 76.5% $C_{18:1}$, 47.5% of which was the *trans* isomer (elaidate). Groups 3 and 4 received the same diets as the rats in groups 1 and 2, respectively, with the only difference that 2% corn oil (CO) was also added at the expense of sucrose (diet groups 2% CO + 10% HF, 2% CO + 20% HF). Groups 5 and 6 (controls) received corn oil at 12 and 22% levels, added at the expense of sucrose (diet groups 12% CO and 22% CO). The percent calorie equivalents of the fat in the different diets are summarized in Table 2.

The different groups of rats were started on their diets sequentially at 2-day intervals. Food and water were provided ad libitum, except for the two groups on rather high fat diets (22% CO and 2% CO + 20% HF), which were meal-fed for 2 hr daily to encourage a greater food intake. Meal feeding is known to lead to enhanced activity of a number of lipogenic enzymes (10). However, as the 2% CO + 20% HF group and its immediate control group (22% CO) were fed similarly, a comparison of the enzyme activities in these two groups would still be valid.

TABLE 1

Composition of fat-free stock diet used in formulation of the different fat diets

Ingredient	Weight, %
Vitamin free casein	16.0
Alphacel cellulose	4.0
sucrose	74.0
salt mixture USP XIV	4.0
	g/100 g
vitamins	
Choline chloride	212.50
Niacin	27.25
Inositol	13.75
Vitamin A concentrate (2 × 10[illegible] units/g)	4.50
Vitamin D concentrate (4 × 10[illegible] units/g)	3.00
Alpha tocopherol	10.225
Menadione	0.0025
Thiamin hydrochloride	1.00
Pyridoxine hydrochloride	1.00
Riboflavin	1.00
Calcium pantothenate	2.05
Vitamin B[illegible]	0.015

TABLE 2

Calorie equivalent of diets

Diet	Percent of calories[a]		
	Protein	Carbohydrate	Fat
FFD	19.1	80.9	
2% CO[b]	18.5	76.4	5.1
12% CO	16.1	55.3	26.6
22% CO	14.1	43.5	42.5
2% CO + 10% HF	16.5	60.7	22.7
10% HF	14.6	45.2	40.2
20% HF			

FFD = fat free diet; CO = corn oil; HF = partially hydrogenated fat.

[a] Calculated on the basis that 1 g sucrose, protein, and fat, respectively, equals 3.94, 4.30, and 9.46 calories. [b] Fat added at the expense of carbohydrate.

The animals, housed in an air-conditioned room, temperature 25°C, were inspected and weighed weekly. Feeding was for 6.5 weeks and the average body weight for all the groups at the end of this period was 187 g. Growth rate, as indicated by body weight, was generally satisfactory (Table 3), and over the 6.5-week feeding period, there were minor differences in the body weights of rats within each group or in the different diet groups.

The meal-fed rats were killed after their daily 2-hr meal, whereas the others had access to food and water up until the time they were killed. Animals in each group were decapitated under light ether anesthesia, exsanguinated, and about 3 g pieces of liver and epididymal adipose tissue (taken from both sides) were quickly excised and washed clear of all blood in excess of cold 0.9% saline (0°C). Separate pieces of each tissue type were homogenized for each of the enzymes, using a Potter-Elvehjem glass homogenizer equipped with a Teflon pestle. For fatty-acid synthetase assay, 2.0- to 2.5-g portions of adipose tissue or 1.0- to 1.5-g portions of liver were taken from the pooled tissues of the rats in each group and homogenized at 0°C in 2 and 10 volumes, respectively, of a buffer (pH 7.0–

[5] Fatty acids are designated as number of carbon atoms in the chain:number of double bonds.

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TABLE 1

Composition of the dietary fats

Analysis	Dietary fats A	B	C	D	E	F	Egg yolk	G*
Iodine value	108.5	101.7	83.8	81.4	36.0	66.8		59.2
Saponification value	191.3	192.2	191.1	193.1	193.9	191.9		204.0
Total fatty acid, %	95.0	95.8	95.6	95.0	96.1	95.4		94.2
Unsaponifiable, %	0.7	0.8	0.8	0.8	0.8	0.7		0.5
Tocopherol, %	0.16	0.03	0.11	0.04	0.04	0.04		0.03
Fatty acid composition, % of total acids†								
Palmitic	10.6	11.9	15.9	15.8	24.6	26.0	25.9	23.2
Palmitoleic	0	0.9	0.2	0.4	0.6	0.7	1.1	2.6
Stearic	5.5	5.8	15.9	16.2	35.1	11.1	11.3	13.0
Oleic	45.7	45.1	13.1	12.9	34.9	47.2	45.5	13.5
Cis	38.0	45.1	37.1	12.9	34.9	47.2	45.5	38.5
Trans	7.7	0	6.0	0	0	0	0	5.2
Linoleic	33.6	34.7	21.9	24.6	3.1	12.8	12.1	10.1
Cis, cis	30.7	34.7	19.5	24.6	3.1	12.8	12.1	9.3
Cis, trans	2.9	0	2.4	0	0	0	0	0.8
Linolenic	3.1	0.9	2.3	0	0	0.7	0	1.3
Total trans fatty acids	10.6	0	8.4	0	0	0		6.0
Conjugated diene	0.4	0.1	0.3	0.2	0	0.2		.3

* In addition, Fat G contained the following short-chain fatty acids: caprylic, 0.1; capric, 0.5; lauric, 0.8; myristic, 3.9; myristoleic, 1.1.

† Determined by gas-liquid chromatography and infrared analysis after fractionation of the mercuric acetate derivatives.

Since the process of hydrogenation, as customarily used in the preparation of edible fats, causes little or no increase in the content of saturated fatty acids, any question would be limited to the effects produced by the conversion of polyunsaturated fatty acids to monounsaturated acids or by isomerization of the unsaturated fatty acids. In brief, there is disagreement concerning the effect of hydrogenated fats on the serum cholesterol level.

In view of the contradictory reports concerning the effects of various types of dietary fats on the plasma cholesterol level, the following study was conducted. The role of long chain saturated and polyunsaturated fatty acids and the ratio of these were investigated by feeding vegetable fats of different fatty acid compositions. The effect of hydrogenation was studied by comparing the plasma lipid response of subjects to a partially hydrogenated fat and to a blend of unhydrogenated fats having the same fatty acid content but containing no isomeric fatty acids. The quantity of trans isomers provided by the partially hydrogenated fat was slightly greater than that found in the average American diet.[1]

[1] A laboratory blend of animal and vegetable fats (Fat G) similar in composition to that of all fats found in the average American diet (27) was analyzed for trans fatty acids and served as a basis for estimating the average intake of trans fatty acids.

For several years dietary cholesterol was held to have little effect on the serum cholesterol level. However, recent reports have demonstrated significant elevations in the serum cholesterol and phospholipid levels (28, 29) of subjects receiving cholesterol in the form of egg yolk. In the present study, the partially hydrogenated fat and the blend of unhydrogenated fats were fed with and without dried egg yolk powder in order to determine whether there is a difference in the plasma lipid response to these fats when fed in diets containing none or a substantial quantity of dietary cholesterol.

Methods

Subjects. The subjects were male inmates of the Philadelphia County Prison at Holmesburg, Pennsylvania, ranging in age from 29 to 45 years. The median age was 35 years. All of the subjects were examined before the study and found to be in good health with no known metabolic disorders.

Diet. The composition of the formula diets and the method of their preparation have been described (19). The fats and the P/S ratios of diets containing these fats were as follows: Fat A (P/S = 1.6), a partially hydrogenated soybean oil; Fat B (P/S = 1.5), a vegetable fat mixture composed of 53.5% olive oil, 39.5% safflower oil, and 8.0% cocoa butter (this fat was similar to Fat A in saturated and monounsaturated fatty acid content but contained no hydrogenated fats); Fat C (P/S = 0.7), a mixture composed of Fat A and cocoa butter; Fat D (P/S = 0.7), a mixture composed of Fat

TABLE II

Composition of the complete dry mixes

Ingredient	Dry Mix No. 1				Dry Mix No. 2			
	Amount	Protein*	Carbohydrate*	Fat*	Amount	Protein*	Carbohydrate*	Fat*
	g/100 g				g/100 g			
Dried egg white†	18.1	15.2			21.0	17.3		
Dextrose	57.5		52.1		57.5		52.1	
Fat	16.8			16.8	16.8			16.8
Dried egg yolk powder‡	6.1	2.1		3.8				
Fat F§					3.8			3.8
Sodium chloride‖	0.5				0.5			
Imitation vanilla	0.4				0.4			
Total	100.0	17.3	52.1	20.6	100.0	17.3	52.1	20.6
Calories supplied		69	208	194		69	208	194
% of total calories		15	44	41		15	44	41

* Expressed on a moisture-free basis.
† Biotin added at a level of 0.1 mg per 100 g of diet to inactivate avidin in the egg white.
‡ Provided 141 mg cholesterol per 100 g of dry mix.
§ Fat F in Dry Mix 2 simulated the fatty acid composition of egg yolk lipid present in Dry Mix 1.
‖ Contained 0.01% potassium iodide.

B and cocoa butter; and Fat E (P/S = 0.1), cocoa butter. Fats A and B were fed both with and without dietary cholesterol, added in the form of dried egg yolk powder. To compensate for the fatty acids provided by the dried egg yolk powder, a fat mixture (Fat F) having a fatty acid composition similar to that of all the lipids of egg yolk was included in all of the diets not containing dried egg yolk powder. Fat F consisted of 26.0% olive oil, 31.0% palm oil, 25.0% cocoa butter, and 8.0% cottonseed oil. The fatty acids provided by the egg yolk or Fat F were included in calculating the P/S ratios of the dietary fats. In all cases the experimental fats contributed 84.6% of the total dietary fat and thus accounted for the main bulk of fatty acids. Fat G was a mixture of animal and vegetable fats and was fed to all groups during the transition from a mixed diet to a formula diet. Analytical data obtained on the experimental fats and the fat isolated from the dried egg yolk powder are given in Table I. Fatty acid composition was determined by gas-liquid chromatography (30). The level of geometric isomers in the experimental fats was established by infrared spectroscopy (31) of the fractions obtained after column chromatography of the mercury acetate addition complexes (32). The remaining analytical values were obtained by the usual methods (33).

The compositions of the dry mixes, which were used for preparation of the formula diets, are shown in Table II. Dried egg white was used as a source of protein.[2] Since two of the diets contained egg yolk powder, it was necessary to use two different dry mixes so that the level of carbohydrate, fat, and protein would be the same in all diets. The complete Dry Mix No. 1 represents the composition of diets containing 6.4% of dried egg yolk powder. Subjects ingesting the diets containing egg yolk received an average of 742 mg of cholesterol per day. The composition of diets from which dried egg yolk powder was omitted is represented by the complete Dry Mix No. 2. Fat F was included in this mix to compensate for the egg yolk lipid fatty acids present in Dry Mix No. 1. The total fat in the diets supplied 41% of the total calories.

The preparation of dry mixes and their ultimate use in emulsion diets were similar to those described by McOsher, Mattson, Swerdsen, and Lippman (19).

Dietary supplement and feeding procedure. The subjects were fed four times daily. At each feeding each subject received one-fourth of his daily allotment of emulsion. Initially, the emulsion diets were fed at a level of 35 calories per kg of body weight. However, some individual adjustment in calorie intake was necessary to maintain constant body weight. Subjects registered slight gains and losses in body weight; the average weight change was an increase of 2.6 pounds per subject.

As described previously (19), the subjects also received vitamin-mineral capsules, salt tablets, methylcellulose waters, and one small serving of a low-fat, low-calorie food item per day.

Blood samples and plasma lipid analyses. Blood samples were drawn twice weekly before the morning meal. The blood was collected in oxalated Vacutainer[3] tubes and the plasma obtained after centrifugation. Duplicate portions of each plasma sample were analyzed for total cholesterol (33) and triglyceride (34) content. The triglyceride values are expressed in terms of mg of tripalmitin per 100 ml of plasma. In addition, a single phospholipid determination (35) was made on each plasma sample. To establish the reliability of the analytical procedures, replicate samples of a desiccated serum

[2] At the suggestion of Dr. E. H. Ahrens of the Rockefeller Institute.

[3] Becton, Dickinson and Company, Rutherford, N. J.

sample were analyzed in conjunction with the experimental plasma samples during the entire study. The standard deviations for the analytical methods, in mg per 100 ml of plasma, were: 7.3 for cholesterol, 3.4 for phospholipid, and 7.9 for triglyceride.

Experimental design. A standard seven-treatment, four-replication, incomplete Latin square design was used. This design made it possible to feed all of the diets in each of the four periods. Any period or time effects were thus self-counterbalancing. The experimental design did not, however, permit the feeding of all diets to all groups, and since all groups do not respond identically to a given dietary fat, statistical adjustments in the calculated treatment means were necessary (36). The design of the experiment is shown in Table III.

During an initial 28-day pre-experimental period, all 42 subjects received the normal prison diet. On this regimen, the average plasma lipid levels, in mg per 100 ml, were as follows: cholesterol, 218.5; phospholipid, 216.3; and triglyceride, 10.8. At the end of this period the 42 subjects were divided into seven groups of six subjects each. The subjects were distributed so that the average body weight of each group was similar. In addition, the average cholesterol level of each group was essentially equal, and the range of plasma cholesterol levels was similar within each group. During the next 10 days the diet was gradually changed from solid food to the liquid formula. This changeover period was necessary to prevent gastrointestinal disorders. During this changeover, all of the subjects received a formula diet containing a blend (Fat G) of animal and vegetable fats similar in composition to that of all fats, both visible and inaccessible, that are present in the average American diet (27).

The 20-week experiment was divided into four periods of 5 weeks each. The first 2 weeks of each period served as an equilibration period during which the subjects attained their new plasma lipid levels. Data obtained previously (16), and confirmed here, indicate that 2 weeks is sufficient time for the serum cholesterol to reach a steady state after a change in the dietary fat. The data collected during the final 3 weeks of each period were used to determine the effect of each diet on the plasma lipid levels.

Of the original 42 subjects, 36 completed the study. The six who failed to complete the experiment dropped out for reasons unrelated to the study. The number of subjects completing the study in each group is shown in Table III.

The data obtained during the final 3 weeks of each experimental period were analyzed for statistically significant differences by an analysis of variance technique described by Cochran and Cox (36). Only the values obtained on the subjects who completed the entire study were used. The analysis of variance indicated that significant differences did exist. Therefore, the Newman-Keuls technique for multiple comparisons (37) was used to establish which of the adjusted treatment means were significantly different from one another.

One restriction inherent in the incomplete Latin square design is that a valid comparison of treatment effects cannot be made before the completion of the experiment. Thus, the only cholesterol values that are comparable are the adjusted cholesterol levels that are calculated from the values obtained for each fat over the entire study.

Results

The combined, adjusted, average plasma cholesterol, phospholipid, and triglyceride values for the final 3 weeks of each experimental period are shown in Table IV. Those values not significantly different from each other at the 95% probability level are mutually underlined.

The addition of egg yolk cholesterol to diets containing the partially hydrogenated soybean oil (Fat A) and the blend of unhydrogenated fats (Fat B) significantly increased the plasma cholesterol level, 23.8 and 26.6 mg per 100 ml, respectively. The plasma cholesterol levels obtained when Fat A or Fat B was fed together with dietary cholesterol were also significantly higher than those produced by the cholesterol-free diets containing fats with lower P:S ratios (Fats C, D, and E).

TABLE III

Feeding sequence of the various dietary fats

Experimental period	Length of period	Dietary fat received by each group*						
		1	2	3	4	5	6	7
Pre-experimental	28 days	Mixed†	Mixed	Mixed	Mixed	Mixed	Mixed	Mixed
Changeover	10 days	G	G	G	G	G	G	G
1	5 weeks	A	A+	B	D	E	C	B+
2	5 weeks	B+	A	A+	C	D	B	E
3	5 weeks	D	E	B+	A+	B	A	C
4	5 weeks	E	B	D	A	C	B+	A+
No. of subjects‡		4	6	5	6	5	5	5

* A plus symbol indicates that the diet contained cholesterol.
† Normal prison diet.
‡ Initially, each group contained six subjects.

TABLE IV

Adjusted average plasma lipid levels produced by the dietary fats and egg yolk cholesterol

Dietary fat*	A+	B+	E	A	C	B	D
P/S	1.6	1.5	0.1	1.6	0.7	1.5	0.7
Adjusted average plasma cholesterol, mg/100 ml†	217	215	195	193	190	188	188
Dietary fat	B+	A+	E	D	C	A	B
P/S	1.5	1.6	0.1	0.7	0.7	1.6	1.5
Adjusted average plasma phospholipid, mg/100 ml†	211	210	201	194	192	192	189
Dietary fat	B+	A+	E	A	C	D	B
P/S	1.5	1.6	0.1	1.6	0.7	0.7	1.5
Adjusted average plasma triglyceride, mg/100 ml†	110	110	107	106	101	99	96

* A plus symbol indicates that egg yolk cholesterol was fed in conjunction with the dietary fat. The hydrogenated fats contained a partially hydrogenated soybean oil.

† Those values not mutually underlined are significantly different from each other at the 95% confidence level ($p = 0.05$ or less); no proof of difference exists for those values mutually underlined.

This elevating effect was caused by an average daily intake of 742 mg of cholesterol per subject.

The difference between the plasma cholesterol levels obtained with the partially hydrogenated fat (Fat A) and the blend of unhydrogenated vegetable fats (Fat B) was not significant whether or not cholesterol was present in the diet. Further, a reduction in the P/S ratio of the partially hydrogenated fat and blend of unhydrogenated vegetable fats (Fats C and D), by the addition of cocoa butter, had no significant effect on the plasma cholesterol response in comparison to that obtained with Fat A or Fat B alone; the fats involved in these latter comparisons were fed only in the cholesterol-free diets.

A comparison of the results obtained with all of the cholesterol-free diets shows that variations in the P/S ratio of the dietary fats had no effect on the plasma cholesterol response. The diet containing Fat E (cocoa butter) with a P/S ratio of 0.1, for instance, produced a plasma cholesterol level of 195.0 mg per 100 ml. This value is not significantly different from those produced by the diets with higher P/S ratios such as the diet containing Fat A, which had a P/S ratio of 1.6, and which gave a plasma cholesterol level of 192.8 mg per 100 ml.

The plasma phospholipid response was similar to that of the cholesterol. The adjusted plasma phospholipid levels were increased, 18.4 and 22.1 mg per 100 ml, respectively, by the addition of cholesterol to diets containing Fats A and B. As in the case of the plasma cholesterol, this elevation was statistically significant. The differences in the plasma phospholipid values obtained with the partially hydrogenated soybean oil (Fat A) and unhydrogenated blend (Fat B) were not significant whether or not cholesterol was present in the diet. Relative to the cholesterol-free diets, however, the plasma phospholipid level produced by Fat E (cocoa butter) was significantly greater than that resulting from the four fats with higher P/S ratios. There were no significant differences among the plasma phospholipid values produced by the diets containing Fats A, B, C, and D even though the P/S ratios ranged from 1.6 to 0.7.

The plasma triglyceride response to the various dietary treatments did not follow a definite pattern as did the response of plasma cholesterol and phospholipid. This is shown by the underlying bracket lines indicating statistical significance in Table IV. The inconsistencies can be seen in a comparison of the effects of the hydrogenated and unhydrogenated fats. When the partially hydrogenated fat (Fat A) and the blend of unhydrogenated vegetable fats (Fat B) were fed in the presence of dietary cholesterol or in conjunction with cocoa butter (Fats C and D), there was no significant difference between the effects of the hydrogenated fat and unhydrogenated blend on the plasma triglyceride level. In the cholesterol-free diets, however, the plasma triglyceride response was slightly but significantly higher with the partially hydrogenated fat (Fat A) than with the blend of unhydrogenated vegetable fat (Fat B).

Discussion

The change from the normal prison diet to the cholesterol free liquid diets resulted in a decrease in the plasma cholesterol level. This type of change has been observed previously (9, 14, 19) and might be regarded as evidence that formula diets per se are hypocholesterolemic. However, the plasma cholesterol levels produced by the cholesterol containing formula diets were not greatly different from those produced by the normal prison diet. Thus, the reduced plasma cholesterol level is probably due to the removal of cholesterol from the diet rather than the change from a solid to a liquid diet.

The reports in the literature do not agree as to whether hydrogenation of a vegetable oil alters its hypocholesterolemic action (8, 9, 17, 20, 26, 38, 39). Since the process of hydrogenation, as normally used in the preparation of edible fats, results in the conversion of polyunsaturated to monounsaturated fatty acids and in the formation of some isomeric unsaturated fatty acids, either one or both types of change might possibly modify the plasma cholesterol response. In many of the experiments already reported, it is impossible to determine which of these changes in composition is responsible for the observed alterations in the serum cholesterol level. To measure the effects of the isomeric acids, it is necessary to feed pairs of hydrogenated and unhydrogenated fats, each similar with regard to content of saturated and mono- and polyunsaturated fatty acids, but differing in level of isomeric acids. Any difference between the cholesterol levels caused by the two fats would then be a function of the isomeric unsaturated fatty acids present in the partially hydrogenated fat. This was done in the present study.

The results obtained with the cholesterol-containing diets revealed that no interaction occurred between dietary cholesterol and the isomeric unsaturated fatty acids present in the partially hydrogenated fat. The quantity of trans fatty acids provided by the partially hydrogenated fat was slightly greater than that calculated to be present in the average American diet. These data suggest, therefore, that the present level of trans fatty acids in the average American diet does not alter the plasma cholesterol level relative to that obtained by a corresponding intake of the cis isomers.

A lightly hydrogenated soybean oil and a blend of unhydrogenated vegetable oils were also compared with regard to their effect on the plasma lipid response in human subjects by Grasso and co-workers (21). The fats were approximately equal in saturated and mono- and polyunsaturated fatty acid content but differed in that the hydrogenated fat contained 20% of trans fatty acids. In comparison to an equal intake of coconut oil both fats produced a marked depression in the plasma cholesterol and phospholipid levels. Although the data are qualitatively similar to those presented here, direct comparisons are not possible, since the two subjects studied by Grasso and associates differed quantitatively in their response to the pair of hydrogenated and unhydrogenated fats.

Anderson, Grande, and Keys (2) have also studied the effect of hydrogenation on the hypocholesterolemic action of dietary fats. The serum cholesterol levels obtained in two experiments, when examined by their prediction equation, led to the conclusion that trans fatty acids in the partially hydrogenated fats caused either a decrease or no change in the serum cholesterol level. The plasma cholesterol response to a pair of hydrogenated and unhydrogenated fats, each similar in fatty acid content, was studied in a third experiment. In this case the isomeric unsaturated fatty acids of the partially hydrogenated fat were reported to have a hypercholesterolemic effect. Although in two instances the effect of trans fatty acids was determined indirectly, the conflicting observations suggest that the trans fatty acids had no consistent effect on the serum cholesterol level.

In an earlier study conducted in this laboratory, McOsker and co-workers (26) showed that the hypocholesterolemic action of several partially hydrogenated vegetable fats was equal to that of unhydrogenated cottonseed oil. It might be asked if the same would be true of other hydrogenated and unhydrogenated fats, if the system were stressed by the presence of a relatively large amount of saturated fatty acids. In the present experiment this aspect of the problem was studied by reducing the P/S ratios of diets containing the partially hydrogenated soybean oil or blend of unhydrogenated vegetable fats by the addition of cocoa butter. We observed that even in the presence of the relatively large amount of saturated

fatty acids there was no significant difference between the effects of the partially hydrogenated and unhydrogenated fats on the plasma cholesterol response.

The diets used in this study afford a rigorous test of the concept that blood cholesterol is a function of the P/S ratio of the dietary fat, since comparisons were made among P/S ratios of 0.1, 0.7, 1.5, and 1.6. We found that a high ratio, 1.6, or an extremely low ratio, 0.1, resulted in essentially identical plasma cholesterol levels. These results are thus incompatible with such a concept. Jolliffe's recommendation (13) of a P/S ratio in excess of 1.25 was based on experiments in which not only the dietary fats were varied but other dietary components as well. The effectiveness of a P/S ratio of 1.25 thus may be fortuitous or the result of an interaction of this factor with the other dietary components not controlled in his experiments.

In many experiments the dietary fats producing elevations in serum cholesterol have been composed principally of coconut oil or animal fats such as butter, beef tallow, and lard. Although these fats are relatively saturated, their hypercholesterolemic effects are complicated by the presence of variable amounts of cholesterol or saturated fatty acids of short and intermediate chain length. Verification in this regard is provided in the observations of Ahrens and co-workers (1), who found the dietary butterfat in comparison to cocoa butter had a significant elevating effect on the serum cholesterol level. In the present study fats with various P/S ratios were fed in the absence of dietary cholesterol, and since none of the fats contained cholesterol or any appreciable quantity of short or intermediate chain length fatty acids, the final results were not complicated by either of these factors. These findings thus indicate that long-chain saturated fatty acids per se are not hypercholesterolemic. However, the effect of ingesting cholesterol in conjunction with variations in the P/S ratio of dietary fats on the plasma cholesterol response is unknown.

The plasma phospholipid response to variations in the diet followed a pattern similar to that of the cholesterol. Hence, the plasma phospholipid level was responsive to the presence or absence of cholesterol in the diet but was not significantly affected by partial hydrogenation of the dietary fat. The plasma phospholipid level was apparently more sensitive than the plasma cholesterol to variations in the P/S ratio, because the dietary fat with the lowest P/S ratio, 0.1, caused a significantly higher level of plasma phospholipid than those produced by the fats with higher P/S ratios.

The complexity of the plasma triglyceride response to the various dietary treatments makes it impossible to ascertain accurately the relative importance of dietary cholesterol, partial hydrogenation of the dietary fat, or variations in the P/S ratio on the plasma triglyceride level.

On the basis of studies previously reported in the literature and the one described above, it appears that with cholesterol-free formula diets the plasma cholesterol is not affected by variations in the P/S ratio of the dietary fat. The results produced by partial hydrogenation indicate that fats prepared by this process have an effect on the plasma cholesterol level similar to that of unhydrogenated fats containing equal quantities of saturated and mono- and polyunsaturated fatty acids. Furthermore, in the present study the efficacy of a partially hydrogenated vegetable fat to maintain or lower the plasma cholesterol level was not affected by either dietary cholesterol or the simultaneous consumption of a relatively saturated fat.

Summary

Forty-two healthy men were divided into seven groups and maintained for 20 weeks on formula diets. During this time the effects of the following dietary fats on plasma lipid levels were determined: Fat A, a partially hydrogenated soybean oil; Fat B, a blend of vegetable fats similar to Fat A in saturated and mono- and polyunsaturated fatty acid content but containing no hydrogenated fat; Fat C, a mixture composed of Fat A and cocoa butter; Fat D, a mixture composed of Fat B and cocoa butter; and Fat E, cocoa butter. The diets containing these fats had P/S ratios of 1.6, 1.5, 0.7, 0.7, and 0.1, respectively. Fats A and B were fed both with and without dietary cholesterol in the form of dried egg yolk powder. All of the other fats were fed only in cholesterol-free diets.

Addition of cholesterol (average of 742 mg daily per subject) to diets containing Fat A or Fat B significantly increased the plasma cholesterol levels, 23.8 and 26.6 mg per 100 ml, respectively.

The plasma cholesterol response to the partially hydrogenated fat (Fat A) was identical to that of the unhydrogenated fat (Fat B), whether or not cholesterol was included in the diet. A reduction in the P/S ratios of cholesterol-free diets containing Fats A and B from 1.6 and 1.5, respectively, to 0.7 by the addition of cocoa butter had no effect on the plasma cholesterol response.

In the cholesterol-free diets, cocoa butter produced an average plasma cholesterol level not significantly different from that obtained with the fat having the highest P/S ratio. We conclude that with a cholesterol-free formula diet the plasma cholesterol level is unaffected by variation in the P/S ratio of the diet from 1.6 to 0.1.

The plasma phospholipid values, like the values for cholesterol, were highest when the diet contained cholesterol. With the cholesterol-free diets the plasma phospholipid values produced by the ingestion of cocoa butter were somewhat higher than those obtained with the other fats. There were no significant differences among the other fats in their effect on the plasma phospholipid level.

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Chemistry of Butter and Margarine

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An extensive description of the chemistry of butter and margarine can hardly be found in the published literature. A striking factor is that the otherwise quite complete handbook on the milk industry published by Grimm, Weigmann, and Winkler, contains only two pages on the subject "Chemical Composition of Butter" and gives no information on fatty acids present in butter fat. In regard to margarine, the well-known Swedish physiologist Prof. Dr. Widmark (1) writes that he was unable to locate any comprehensive publications on the composition of margarine, although excellent work precisely in this field has been done in recent years by contemporary fat research. Particularly as a result of the thorough and extensive studies of Hilditch and his associates on butter and of Kaufmann and Grossfeld in margarine research, we are now quite well informed about the chemistry of these two important food materials.

A review of the chemistry of butter and margarine, obtained from the published literature, will be given here. In view of my many years of experience and activity as a specialist in the field of butter and margarine, I have been able to make a critical judgement and evaluation of the published data and to supplement the latter by means of personal studies.

A. Butter

As we know, the term butter is used to designate a product obtained by churning milk or cream and subjected to further treatment, consisting mostly of milk fat intimately mixed with smaller amounts of other milk components. The composition of the usual salted butter is approximately

the following: fat 83.0%, water 14%, proteins 0.6%, salts 2.0%, milk sugar and lactic acid 0.4%, lecithin 0.05%. In addition, butter contains dyes, vitamins, diacetyl, and sterols.

Butter fat, which is of greatest interest in our case, contains the following fatty acids, according to the studies of Schmidt-Nielsen and Frog (2), Vivario and van Beneden (3), Holland et al. (4), and Hilditch and Sleightholme (5):

	Range (%)	Average Content (%)
Butyric acid	2.23-4.23	3.0
Caproic acid	1.29-2.40	1.9
Caprylic acid	0.53-1.7	1.1
Capric acid	1.19-3.8	2.0
Lauric acid	4.53-7.69	5.9
Myristic acid	6.9-22.62	19.8
Palmitic acid	5.78-29.0	15.2
Stearic acid	4.8-20.37	15.0
Arachidic acid	0.9-1.2	1.0
Oleic acid	25.27-40.9	31.9
Linoleic acid	2.4-4.0	3.3
Linolenic acid	0.1-0.2	0.1

Thus, the literature data given show a considerable variation, and Grossfeld (6) even reports a content of 3.4% stearic acid and up to 44.4% oleic acid. Already Schmidt-Nielsen (2) had determined the presence of higher unsaturated acids, and his findings were confirmed by later studies; however, these acids are present in very small amounts.

A recently published article by Schmidt-Nielsen and Astad (7) gives a good review of the general constants of butter fat. The following figures are given:

Refractive index n_D40:	1.4523-1.4552;		average 1.4536
Refractive index in degrees Zeiss:	39.9-44.0	;	average 41.8
Saponification number (SN):	388.9-422.4	;	average 408.0
Kottstoffer number (SN):	218.2-237.0	;	average 228.9
A-number:	3.8-9.8	;	average 6.9
B-number:	28.8-36.6	;	average 32.3
Reichert-Meissl number:	22.4-30.6	;	average 27.3
Polenske number:	1.15-3.43	;	average 2.18
Iodine number:	28.1-46.1	;	average 36.4

According to the above authors, these figures can be derived from each other by the following equations:

1. Polenske number = 0.125 x SN - 46.6

2. A-number = 0.22 x SN - 80.9

3. Iodine number = 6080 x n_D - 8801.9

3a. Iodine number = 4.4 x refractive index (in degress Zeiss) - 147.8

Values of the new constants recommended by Grossfeld in his investigation of butter will be reported later in this article. Some physical properties of butter fat will also best be mentioned during discussion of the same properties of margarine fat.

B. Margarine

1. Chemical Properties. The term margarine is used to designate a butter-like emulsion, the fat content of which does not originate exclusively from milk. From this definition it follows that the composition of margarine fat varies within a very broad range. Therefore, it can be expected already in advance that the relations found in margarine are much more complex than in butter.

The evolution of the composition of the fat content in Norwegian margarine can be seen from the following table:

	1930	1936
"Old-animal" raw materials	6.9%	1.0%
Hardened fat	18.6%	31.1%
Vegetable oils	24.4%	17.1%
Coconut fat	50.1%	50.8%

The published literature shows that a similar evolution in the fat content of margarine took place in other margarine-producing countries. In order to learn something about the chemistry of margarine, we must therefore study the chemistry of coconut fat, hardened fats, and other oils.

a. Coconut fat. According to Collin and Hilditch (8) and Grossfeld (9), the distribution of fatty acids in coconut fat is as follows:

	Collin and Hilditch	Grossfeld
Caproic acid	traces	0.46%
Caprylic acid	7.9%	8.7%
Capric acid	7.2%	4.9%
Lauric acid	48.0%	45.0%
Myristic acid	17.5%	17.4%
Palmitic acid	9.0%	7.1%
Stearic acid	2.1%	2.9%
Oleic acid	5.7%	7.1%
Linoleic acid	2.6%	0.9%

The constants of coconut fat (according to different sources) are as follows:

Iodine number	9.4-10.7
Saponification number	246-258
Reichert-Meissl number	5.6-7.4
Rhodan number	5.7-8.3
Refraction, in degrees Zeiss	33.5-36.3
A-number	27.7
B-number	2.75
Lauric acid number	126
Caprylic acid number	19
"Total number"	38.0
Butyric acid number	0.9
"Residue number"	37.1

b. Hardened fats. Among such fats, hardened walrus oil plays by far the most important role, and we shall therefore be concerned only with this hard fat in this brief survey. Specific information on the constants of hardened fats used in the margarine industry is only scarce in the pertinent literature. Even in the recently published issue on walrus capture of this journal, where the chemical composition of walrus oil is described in great detail, practically no figures are given on hardened walrus oil used in the margarine industry. The walrus oils used almost exclusively in this industry include a so-called light-hardened oil with a melting point of 31-33°C and a high-hardened oil of melting point 40-42°C. On the basis of my own studies and some scattered published data, the following figures are presented:

	Oil with m.p. 31-33°C	Oil with m.p. 40-42°C
Refraction at 50°C (in degrees Zeiss)	45.5	43
Iodine number	72	55
Rhodan number		51
Partial iodine number		30
Iso-oleic acid	35%	30%
Saponification number	192	192

We wish to point out at once the high content of iso-oleic acids, which are important in the analysis and determination of margarine. The content of iso-oleic acids in other naturally occurring fats is very small; according to Grossfeld (9), it is equal to 2.9% (after a single crystallization) in butter fat, beef dripping, and lard.

c. Vegetable Fats. At the present time, margarine made in Nordic countries contains almost only peanut and soybean oil. The fatty acid distribution of peanut oil has been studied by H. P. Kaufmann

(10), Hilditch and Jones (11), and Jamieson et al. (12), who obtained the following results:

	Hilditch and Jones	Jamieson et al. Spanish oil	Jamieson et al. Virginia oil	Kaufmann	
Oleic acid	60.4%	52.9%	60.6%	58.5%	55.1%
Linoleic acid	21.9%	24.7%	21.6	22.5%	23.1%
Palmitic acid	8.2%	8.2%	6.3%		
Stearic acid	3.4%	6.2%	4.9%		
Arachidic-lignoceric acid	6.1%	--	--	19.0%	21.2%
Arachidic acid	—	4.0%	3.3%		
Lignoceric acid	—	3.1%	2.6%		

According to literature data, peanut oil has the following constants:

Iodine number	85.0-98.7
Rhodan number	70.0-78.5
Saponification number	189.8-191.5
Reichert-Meissl number	0.4-1.6
Refraction at 20°C, in degrees Zeiss	63.2-69.5

The first accurate analyses of soybean oil were performed by H. P. Kaufmann and his associates (13). Later, Hilditch and Jones (11) found the following composition:

Myristic acid	0.6%
Palmitic acid	7.0%
Stearic acid	5.8%
Oleic acid	60.6%
Linoleic acid	20.8%
Linolenic acid	5.2%

The constants of soybean oil are as follows:

Iodine number	129-137
Rhodan number	75-80.8
Reichert-Meissl number	0.1-0.7
Refraction at 15°C, in degrees Zeiss	76.8-78.5
Saponification number	about 192

Additives. It is impossible, in a brief survey such as this one, to study in greater detail the chemistry of margarine additives. We merely wish to point out the role of salt in margarine preservation (storage); it is true that salt acts as a preservative, but, on the other hand, it exerts, especially when small amounts of metal ions are present, a detrimental effect (fishy taste).

2. Physical Properties. Under physical properties of margarine, only the melting point and consistency shall be discussed here. We are indebted to Th. Lexow (15) for a very detailed study of the melting point of margarine and margarine raw materials. Among the generally used methods for determining the flow point, drip point, and melting point, this author finds that only the flow point determination according to Ubbelohde (designated by Lexow as A.1) and the clear melting point (C.III) are suitable for this purpose. However, the difference A.1-C.III, which Lexow calls the melting range (SI), has been found to be an extremely characteristic value for every margarine raw material. The following values are taken from Lexow's tables:

	A.1	C.III	A.1-C.III=SI
Soybean oil	-3°C	4.5°C	7.5°C
Peanut oil	-3°C	6.5°C	9.5°C
Cottonseed oil	-1.5°C	9.5°C	11.0°C
Coconut fat	24.3°C	25.7°C	1.4°C
Oleomargarine	24.5°C	30.3	6.1°C
Hardened walrus oil m.p. 40-42°C	36.0°C	39.2°C	3.2°C
Tallow (Premier Jus)	39.6°C	49.0°C	9.4°C
Lard	31.7°C	46.1°C	14.4°C
Butter	---	---	10.0°C

It has been shown that the consistency of a margarine is very closely related to the melting range: raw materials with a high SI value impart to

margarine a sticky and smooth consistency, whereas others (and primarily coconut fat) make the margarine hard, brittle and not smooth. As a result of this fact, raw material mixtures of the same melting range (isotectic mixtures) may have different SI values and thus different consistency imparting properties. Thus, for example, the following 3 fat mixtures all melt at 38°C:

1. 42% Premier Jus + 29% coconut fat + 29% cottonseed oil.
2. 40% Premier Jus + 40% coconut fat + 20% cottonseed oil.
3. 34% Premier Jus + 32% coconut fat + 34% cottonseed oil.

The melting ranges of these mixtures are 13.4, 8.0, and 17.4, respectively. Thus, the last mixture (#3) will yeild the smoothest margarine.

C. Mixtures of Margarine and Butter.

At present, four methods are known for the qualitative analysis of margarine in butter:

1. Fish oil reaction according to Tartelli-Jaffe (or other authors).
2. Bomer's phytosterol acetate test.
3. Microchemical identity reaction.
4. Luminescence analysis.

In regard to microchemical identity reactions, we wish to refer to an excellent Norwegian publication (16). According to our own personal studies, luminescence analysis is also suitable to some extent for the quantitative determination of both components in a butter-margarine mixture, since differences of 10% can be determined by this method, although with some difficulty at the extreme outward points.

The quantitative determination of butter and margarine in mixtures of both components can be presently carried out by using one or several of the five methods listed below:

1. Determination of the butyric acid and residue numbers, known as the "Butyric acid number-residue number method".

2. Determination of the lauric acid and residue numbers, known as the "lauric acid number-residue number method".

3. Determination of the A- and B- numbers, known as the "A- and B- number method".

4. Determination of the iso-oleic acid content, known as the "Iso-oleic acid method".

5. Determination of the rhodan number and partial iodine number, known as the "rhodan number-PI-method".

Methods 1-3 refer to properties of butter fat or coconut fat, while methods 4 and 5 refer to hardened fats.

1. Butyric acid number-residue number method. According to Grossfeld (17, 18), the average values for total number and butyric acid number, as well as for the so-called residue number derived from them, are as follows:

	Total number	Butyric acid number	Residue number
Coconut fat	38.0	0.9	37.1
Butter fat	39.7	20.4	19.3

All three numbers are equal to zero for other fats, especially for lard and cocoa butter. According to Grossfeld, the following relations are observed:

% butter fat = 5.12 x butyric acid number - 0.12 x residue number.

% coconut fat = 2.77 x residue number - 2.3 x butyric acid number.

2. Residue number-lauric acid number method. According to Grossfeld, the lauric acid number of coconut fat is equal to 111-140 (average 126) and of butter fat 5-18 (average 11); % coconut fat = 0.79 (lauric acid number-0.5 butyric acid number). The difference between the two fats is even more striking when the lauric acid and residue numbers are multiplied:

	Lauric acid number	Residue number	Product
Butter fat	11	19	209
Coconut fat	126	37	4862

The ratio between the two products is equal to 23, and this figure can be of valuable help during quantitative determination.

3. A- and B- number method. These figures are known to be approximately equal to:

	A-number	B-number
Butter fat	6.7	33.4
Coconut fat	27.7	2.75
Other fats	0.6	0.6

Determination of the A- and B- numbers is an excellent method for determiring the coconut fat content of a fat mixture, while determination of the B- number and butyric acid number is useful in determining the butter fat content. According to Schmidt-Nielsen (7), the B-number is least dependent upon the season and therefore most suitable for the determination of the butter fat content in margarine.

4. Iso-oleic acid method. According to Grossfeld (9), the iso-oleic acid content of some fats (in percent) is as follows:

	Single Crystallization		Double Crystallization	
Hardened fat, not specified	24.8-38.8	31.8 (average)	20.5-35.4	26.0 (average)
Butter fat	0.7-2.1	1.6	0.6-1.4	0.9
German margarine fat	9.3-17.0	13.1	4.1-9.9	7.3
Other fats, maximum value		2.9		1.5

By this method it is possible to determine 10% hardened fat after a single crystallization and 1-2% fat after a double crystallization. The method can also be used for the quantitative determination of margarine, provided that the contnet of hardened fat and also its iso-oleic acid content are known.

5. Rhodan number-PI-method. According to Kaufmann (19), naturally occurring and non-hardened fats have about the same rhodan number and partial iodine number, as can be seen from the following table:

	Iodine number	Partial iodine number	Rhodan number
Coconut fat	10.7	8.4	8.3
Milk fat	25.6	20.7	21.7
Lard	49.5	44.9	44.2
Peanut oil	89.3	71.1	69.5
Cod liver oil	156.8	99.7	101.0

However, different relations are observed in the hardened fats in view of the formation of iso-oleic acids during the hardening process. Thus, Kaufmann (19) found the following values:

	Iodine number	Partial iodine number	Rhodan number
Hardened coconut fat	8.3	4.8	8.0
Hardened peanut oil	69.8	41.5	66.8
Hardened sunflower oil	62.0	35.8	61.3
Hardened fish oil	34.4	18.0	34.0
Hardened walrus oil	54.8	30.2	51.0

Therefore, if a discrepancy between the rhodan and partial iodine numbers is found during the study of a fat mixture, the latter probably contains a hardened fat.

In addition to the methods reported above, the systematic analysis of a fat mixture for the determination of butter or margarine fat will be mentioned.

A. Butter in Margarine

1. Luminescence study. Comparison with samples containing 10, 20, 30%, etc. butter.

2. Eventual identity reactions, for example a microchemical reaction according to Kolle (16).

3. Determination or calculation of butyric acid and residue numbers, followed by the use of Grossfeld's formula: percent butter fat = 5.12 x butyric acid number - 0.12 x residue number.

4. Control: determination of the B-number, and, if necessary, also of the A-number.

B. Margarine in Butter

1. Luminescence study as stated above, followed by qualitative testing for the presence of coconut fat (a) or hardened fats (b).

2a. Positive phytosteral reaction indicates the presence of vegetable fats or oils, which can be detected by microchemical reactions.

2b. When the Tortelli-Jaffe reaction is positive, hardened fish oil is present.

3a. Determination of butyric acid, lauric acid and residue numbers, and calculation by means of the equations: percent coconut fat = 2.77 x residue number - 2.63 x butyric acid number, or percent coconut fat = 0.79 (lauric acid number - 0.5 butyric acid number).

3b. Determination of the rhodan number and partial iodine number, and determination of the iso-oleic acid content. From these results, calculate the probable content of hardened fat.

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6. J. Grossfeld: The saponification number of edible fats as a function of the content of palmitic acid and other fatty acids, Z. Unters. Lebensmittel 60, 64-92 (1930) (in German).

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Zur Chemie der Butter und Margarine

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Eine umfassende Darstellung der Chemie der Butter und Margarine läßt sich kaum im Schrifttum finden. Auffallend ist, daß das sonst sehr umfassende Handbuch der Milchwirtschaft von Grimmer-Weigmann-Winkler nur etwa 2 Seiten über das Thema „Chemische Zusammensetzung der Butter" enthält und zwar nichts über die im Butterfett enthaltenen Fettsäuren. In bezug auf Margarine schreibt der bekannte schwedische Physiologe Prof. Dr. Widmark[1]), daß es ihm nicht gelungen ist, zusammenfassende Veröffentlichungen über die Zusammensetzung der Margarinefette zu finden. Und doch hat die neuzeitliche Fettforschung gerade auf diesem Gebiete in den letzten Jahren schöne Arbeit geleistet. Besonders dank der gründlichen und umfassenden Untersuchungen von Hilditch und Mitarbeitern über Butter und von Kaufmann und Großfeld auf dem Gebiete der Margarineforschung sind wir jetzt recht gut über die Chemie der beiden genannten wichtigen Lebensmittel orientiert.

Im folgenden soll eine aus dem Schrifttum entnommene Übersicht über die Chemie der Butter und Margarine gegeben werden. Dank langjähriger Erfahrung und Betätigung sowohl als Butter- wie als Margarinefachmann habe ich die Zahlen und Angaben kritisch beurteilen und bewerten und durch eigene Untersuchungen ergänzen können.

A. Butter

Unter Butter verstehen wir bekanntlich ein aus Milch oder Rahm durch Kirnen ausgeschiedenes und später bearbeitetes Produkt. Sie besteht zum größten Teil aus Milchfett in inniger Mischung mit geringeren Mengen der übrigen Bestandteile der Milch.

Die Zusammensetzung einer gewöhnlichen, gesalzenen Butter ist etwa:

Fett	83,0 %
Wasser	14,0 „
Proteinstoffe	0,6 „
Salze	2,0 „
Milchzucker und Säure	0,4 „
Lecithin	0,05 „

[1]) E. M. P. Widmark u. Marit Neymark: Ein Vergleich zwischen dem nahrungsphysiologischen Wert von Butter und Margarine auf Grund einer Zusammenstellung der diesbezüglichen Literatur (Schwedisch). — Årsskr. Alnarp, Malmö 1938. 48 S.

Außerdem enthält sie Farbstoffe, Vitamine, Diacetyl und Sterine.

Das Butterfett, das im vorliegenden Fall am interessantesten ist, besteht nach den Untersuchungen von Schmidt-Nielsen und Frog[2]), Vivario und van Beneden[3]), Holland und Mitarbeitern[4]) und Hilditch und Sleightholme[5]) aus den folgenden Fettsäuren:

	Grenzwerte	Mittelwert
Buttersäure	2,23— 4,23	3,0
Capronsäure	1,29— 2,10	1,9
Caprylsäure	0,53— 1,7	1,1
Caprinsäure	1,19— 3,8	2,0
Laurinsäure	4,53— 7,69	5,9
Myristinsäure	6,9 —22,62	19,8
Palmitinsäure	5,78— 30,0	15,2
Stearinsäure	4,8 —20,57	15,0
Arachidinsäure	0,9 — 1,2	1,0
Ölsäure	25,27—40,9	31,9
Linolsäure	2,4 — 4,1	3,3
Linolensäure	0,1 — 0,2	0,1

Die Angaben schwanken also recht beträchtlich, ja Großfeld[6]) gibt sogar nur 3,4 % Stearin-, dafür aber bis zu 44,4 % Ölsäure an. Schon Schmidt-Nielsen[2]) hat die Anwesenheit von höher ungesättigten Fettsäuren nachgewiesen, und spätere Untersuchungen konnten diesen Befund bestätigen. Die Mengen dieser Säuren sind aber sehr gering.

Von den allgemeinen Kennzahlen des Butterfettes gibt eine kürzlich von Schmidt-Nielsen und Astad[7]) veröffentlichte Arbeit einen guten Überblick. Sie fanden:

Brechungsindex n_D^{40}	1,4523—	1,4552	im Mitt.	1,4536
in ° Zeiss	39,9	— 44,0	„	41,8
Verseifungszahl	388,9	—422,4	„	408,0
Köttstorferzahl	218,2	—237,0	„	228,9
A-Zahl	3,8	— 9,8	„	6,9
B-Zahl	28,8	— 36,6	„	32,3
Reichert-Meißl-Zahl	22,1	— 30,6	„	27,6
Polenske-Zahl	1,15	— 3,43	„	2,18
Jodzahl	28,1	— 46,1	„	36,4

Die Zahlen sind nach den Verfassern durch folgende Gleichungen auseinander ableitbar:

1. Polenske-Zahl = 0,125 mal VZ — 46,6
2. A-Zahl = 0,22 mal VZ — 80,9
3. Jodzahl = 6080 mal n_D — 8301,9

3a. Jodzahl = 4,4 mal Brech. Ind. in °Zeiss — 147,8

Die Werte der von Großfeld bei der Butteruntersuchung empfohlenen neuen Kennzahlen sollen später angeführt werden. Auch einige physikalische Eigenschaften des Butterfettes werden am besten bei der Besprechung der gleichen Eigenschaften beim Margarinefett erörtert.

B. Margarine

1. Chemische Eigenschaften. Unter Margarine verstehen wir eine butterähnliche Emulsion, deren Fett nicht ausschließlich der Milch entstammt. Es folgt aus dieser Definition, daß die Zusammensetzung des Margarinefettes innerhalb sehr weiter Grenzen wechselt. Man kann deshalb schon im voraus erwarten, daß die Verhältnisse bei der Margarine weit verwickelter als bei der Butter sind.

Die Entwicklung der Zusammensetzung des Fettansatzes norwegischer Margarine geht aus der folgenden Tabelle hervor:

	1930:	1936:
„Alt-animalische" Rohstoffe	6,9 %	1,0 %
Gehärtetes Fett	18,6 „	31,1 „
Vegetabilische Öle	21,4 „	17,1 „
Cocosfett	50,1 „	50,8 „

Aus dem Schrifttum geht hervor, daß die Entwicklung auch in anderen margarineherstellenden Ländern ähnlich gewesen sei.

Um die Chemie der Margarine kennenzulernen, muß man deshalb die Chemie des Cocosfettes, der gehärteten Fette und der übrigen Öle zuerst studieren.

a) Cocosfett

Die Fettsäureverteilung im Cocosfett ist nach Collin und Hilditch[8]) und Großfeld[6]) die folgende:

	C. & H.	Gr.
Capronsäure	Spuren	0,46 %
Caprylsäure	7,9 %	8,7 „
Caprinsäure	7,2 „	4,9 „
Laurinsäure	48,0 „	45,0 „
Myristinsäure	17,5 „	17,4 „
Palmitinsäure	9,0 „	7,1 „
Stearinsäure	2,1 „	2,9 „
Ölsäure	5,7 „	7,1 „
Linolsäure	2,6 „	0,9 „

Seine Kennzahlen sind (nach verschiedenen Quellen):

Jodzahl	9,4— 10,7
Verseifungszahl	246 —258
Reichert-Meißl-Zahl	5,6— 7,4
Rhodanzahl	5,7— 8,3
Refraktion in ° Zeiss	33,5— 36,3
A-Zahl	27,7
B-Zahl	2,75
Laurinsäurezahl	126
Caprylsäurezahl	19
„Gesamtzahl"	38,0
Buttersäurezahl	0,9
„Restzahl"	37,1

b) Gehärtete Fette

Unter den gehärteten Fetten spielt das gehärtete Walöl die weitaus größte Rolle. Wir werden uns daher in dieser kurzen Übersicht nur mit diesem Hartfett befassen. Nähere Auskünfte über die Kennzahlen der in der Margarine-Industrie verwendeten gehärteten Fette finden sich nur spärlich im einschlägigen Schrifttum. Selbst in dem kürzlich erschienenen Walfang-Heft dieser Zeitschrift, wo die chemische Zusammensetzung des Walöls sonst sehr eingehend behandelt worden ist, finden sich fast keine Zahlenangaben über das gehärtete

[2]) S. Schmidt-Nielsen u. F. Frog: Die Fettsäureverteilung des Butterfettes. — Biochem. Z. 127, 168 [1922].

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Walöl für die Margarine-Industrie. Die dort fast ausschließlich verwendeten gehärteten Walöle sind das sogenannte leichtgehärtete vom Schmp. 31/33° und das hochgehärtete vom Schmp. 40/42°. Auf Grund eigener Untersuchungen sowie einiger vereinzelten Angaben im Schrifttum seien die folgenden Zahlen angeführt:

	31/33°:	40/42°:
Refraktion bei 50° (in ° Zeiss)	45,5	48
Jodzahl	72	55
Rhodanzahl		51
Partielle Jodzahl		30
Isoölsäure	35 %	30 %
Verseifungszahl	192	192

Wir bemerken sofort den hohen Gehalt an Isoölsäuren, die für Nachweis und Bestimmung von Margarine wichtig sind. Der Gehalt an Isoölsäuren in anderen natürlich vorkommenden Fetten ist sehr gering, nach Großfeld[9]) unter 2,9 % (nach einmaliger Kristallisation) in Butter-, Rinder- und Schweinefett.

c) Pflanzliche Fette

In nordischer Margarine kommen z. Z. fast nur Erdnuß- und Sojaöl vor.

Die Fettsäureverteilung des Erdnußöles ist von H. P. Kaufmann[10]), Hilditch und Jones[11]), sowie von Jamieson und Mitarbeitern[12]) mit dem folgenden Ergebnis untersucht worden:

	H. & J.	Jam. u. Mitarb. Span. Öl	Jam. u. Mitarb. Virginia-öl	Kaufmann	Kaufmann
Ölsäure	60,4 %	52,9 %	60,6 %	58,5 %	55,7 %
Linolsäure	21,9	21,7	21,6	22,5	23,1
Palmitinsäure	8,2	8,2	6,3		
Stearinsäure	3,4	6,2	4,9		
Arachidin-Lignocerins.	6,1	—	—	19,0	21,2
Arachinsäure	—	4,0	3,3		
Lignocerinsäure	—	3,1	2,6		

Die Kennzahlen des Erdnußöles sind nach den Schrifttumsangaben:

Jodzahl	85,0— 98,7
Rhodanzahl	70,0— 78,5
Verseifungszahl	189,8—191,5
Reichert-Meißl-Zahl	0,4— 1,6
Refraktion bei 20° in ° Zeiss	63,2— 69,5

Die ersten genauen Analysen von Sojaöl stammen von H. P. Kaufmann und seinen Mitarbeitern[13]). Später fanden Hilditch und Jones[11]) folgende Zusammensetzung:

Myristinsäure	0,6 %
Palmitinsäure	7,0 „
Stearinsäure	5,8 „
Ölsäure	60,6 „
Linolsäure	20,8 „
Linolensäure	5,2 „

Seine Kennzahlen sind:

Jodzahl	129 —137
Rhodanzahl	75 — 80,8
Reichert-Meißl-Zahl	0,1— 0,7
Refraktion bei 15° in ° Zeiss	76,8— 78,5
Verseifungszahl	etwa 192

Zutaten. In einer kurzen Übersicht wie der vorliegenden ist es unmöglich, auf die Chemie der Zusatzstoffe der Margarine näher einzugehen. Nur soll auf die Rolle des Salzes beim Aufbewahren aufmerksam gemacht werden. Zwar wirkt es konservierend, aber auf der anderen Seite übt es, besonders in Gegenwart geringer Mengen von Metallionen, einen verschlechternden Einfluß (Fischigwerden) aus[14]).

2. Physikalische Eigenschaften. Unter den physikalischen Eigenschaften der Margarine seien an dieser Stelle nur Schmelzpunkt und Konsistenz besprochen.

Th. Lexow[15]) verdanken wir eine sehr gründliche Untersuchung vom Schmelzpunkt der Margarine und der Margarine-Rohstoffe. Unter den allgemeinen Fließ-, Tropf- und Schmelzpunktmethoden findet er nur den Fließpunkt nach Ubbelohde (von Lexow als A. 1 bezeichnet), sowie den Klarschmelzpunkt (C. III) für diese Zwecke geeignet. Aber auch die Differenz A. 1—C. III, die er Schmelzintervall (SI) nennt, hat sich als eine für jeden Margarine-Rohstoff überaus charakteristische Größe gezeigt. Seinen Tabellen entnehmen wir folgendes:

	A. 1	C. III	A. 1—C. III = SI
Sojaöl	—3,0°	4,5°	7,5°
Erdnußöl	—3,0	6,5	9,5
Baumwollsamenöl	—1,5	9,5	11,0
Cocosfett	24,3	25,7	1,4
Oleomargarin	24,5	30,3	6,1
Gehärtetes Walöl 40/42	36,0	39,2	3,2
Talg (Premier Jus)	39,6	49,0	9,4
Schweineschmalz (Lard)	31,7	46,1	14,4
Butter			10,0

Es hat sich nun gezeigt, daß die Konsistenz einer Margarine in sehr enger Beziehung zum Schmelzintervall steht: Rohstoffe mit großem SI verleihen der Margarine eine zähe und geschmeidige Konsistenz, während die übrigen, vor allem Cocosfett, die Margarine hart, spröde und wenig geschmeidig machen. Eine Folge davon ist, daß gleichschmelzende (isotektische) Rohstoffmischungen verschiedenes SI und damit verschiedene konsistenzverleihende Eigenschaften besitzen können. So schmelzen z. B. folgende 3 Fettmischungen alle bei 38°:

1. 42 % Premier Jus + 29 % Cocosfett + 29 % Baumwollöl,
2. 40 % Premier Jus + 40 % Cocosfett + 20 % Baumwollöl,
3. 34 % Premier Jus + 32 % Cocosfett + 34 % Baumwollöl.

Ihre Schmelzintervalle sind aber 13,4, 8,0 bzw. 17,4. Die letzte Mischung wird also die geschmeidigste Margarine geben.

[9]) J. Großfeld u. J. Peter: Nachweis von Margarine und gehärteten Ölen in Lebensmitteln. — Z. Unters. Lebensmittel 68, 345—358 (1934).

[10]) Arch. Pharm. 263, 718 (1925).

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C. Gemische von Margarine und Butter

Zum qualitativen Nachweis von Margarine in Butter besitzen wir jetzt 4 Methoden:

1. Tranreaktion nach Tortelli-Jaffe (oder anderen),
2. Phytosterinacetat-Probe nach Bömer,
3. Mikrochemische Identitätsreaktionen,
4. Lumineszenzanalyse.

In bezug auf die mikrochemischen Identitätsreaktionen sei auf ein vorzügliches norwegisches Werk verwiesen [16]). Die Lumineszenzanalyse eignet sich nach eigenen Untersuchungen auch einigermaßen zur quantitativen Bestimmung der beiden Komponenten einer Butter-Margarine-Mischung, indem Unterschiede von 10 % mit dieser Methode bestimmbar sind, wenn auch etwas schwierig bei den Außenpunkten.

Die quantitative Bestimmung von Butter und Margarine in Mischungen der beiden Komponenten läßt sich z. Z. durch Verwendung einer oder mehrerer der unten genannten 5 Methoden durchführen:

1. Ermittelung von Buttersäurezahl und Restzahl. — „Buttersäurezahl-Restzahl-Methode."
2. Ermittelung von Laurinsäurezahl und Restzahl. — „Laurinsäurezahl-Restzahl-Methode."
3. Ermittelung von A- und B-Zahl. — „A- und B-Zahl-Methode."
4. Bestimmung des Gehaltes an Isoölsäure. — „Isoölsäuremethode."
5. Ermittelung von Rhodanzahl und partieller Jodzahl. — „Rhodanzahl-PJ-Methode."

Die Methoden 1 bis 3 beziehen sich auf Eigenschaften des Butterfettes oder Cocosfettes, die Methoden 4 und 5 dagegen auf das gehärtete Fett.

1. Buttersäurezahl-Restzahl-Methode. Nach Großfeld [17]), [18]) sind Gesamtzahl und Buttersäurezahl sowie die hieraus abgeleitete sogenannte Restzahl im Mittel folgende:

	Gesamtzahl	Buttersäurezahl	Restzahl
Cocosfett	38,0	0,9	37,1
Butterfett	30,7	20,4	19,3

Alle drei Zahlen sind bei anderen Fetten, besonders bei Schweine- und Kakaofett = 0. Nach Großfeld bestehen folgende Beziehungen:

% Butterfett = 5,12 mal Buttersäurezahl — 0,12 mal Restzahl.
% Cocosfett = 2,77 mal Restzahl — 2,63 mal Buttersäurezahl.

2. Restzahl-Laurinsäurezahl-Methode. Die Laurinsäurezahl ist nach Großfeld:

bei Cocosfett	111—140, im Mittel 126
bei Butterfett	5— 18, im Mittel 11

% Cocosfett = 0,79 (Laurinsäurezahl — 0,5 Buttersäurezahl).

Noch schärfer tritt der Unterschied zwischen den beiden Fetten bei einer Multiplikation von Laurinsäure- und Restzahl hervor:

	Laurinsäurezahl	Restzahl	Produkt
Butterfett	11	19	209
Cocosfett	126	37	4662

Das Verhältnis zwischen den beiden Produkten ist = 23, und diese Zahl kann bei quantitativer Bestimmung wertvolle Hilfe leisten.

3. A- und B-Zahl-Methode. Diese Zahlen sind bekanntlich etwa:

	A:	B:
Butterfett	6,7	33,4
Cocosfett	27,7	2,75
Andere Fette	0,6	0,6

Die Ermittelung der A- und B-Zahlen ist eine vorzügliche Methode zur Bestimmung des Cocosfett-Gehaltes einer Fettmischung, die Ermittelung von B-Zahl und Buttersäurezahl zur Bestimmung des Butterfett-Gehaltes. Nach Schmidt-Nielsen [7]) soll die B-Zahl am unabhängigsten von der Jahreszeit und deshalb am geeignetsten zur Bestimmung des Butterfettgehaltes einer Margarine sein.

4. Isoölsäure-Methode. Nach Großfeld [9]) ist der Isoölsäuregehalt einiger Fette wie folgt:

	Einmal. Kristall.		Zweimal. Kristall.	
Gehärtetes Fett, nicht näher bezeichnet	24,8—38,8	31,8	20,5—35,4	28,0
Butterfett	0,7— 2,1	1,6	0,6— 1,4	0,9
Deutsches Margarinefett	9,3—17,0	13,1	4,1— 9,9	7,3
Andere Fette, Höchstwert		2,9		1,5

Nach dieser Methode lassen sich 10 % gehärtetes Fett nach einmaliger und 1 bis 2 % gehärtetes Fett nach zweimaliger Kristallisation erkennen. Die Methode läßt sich auch zur quantitativen Bestimmung von Margarine verwenden, vorausgesetzt, daß man den Gehalt an gehärtetem Fett und außerdem dessen Isoölsäuregehalt kennt.

5. Rhodanzahl-PJ-Methode. Nach Kaufmann [19]) sind bei natürlich vorkommenden und nicht gehärteten Fetten die Rhodanzahl und die partielle Jodzahl etwa gleich, wie aus der folgenden Tabelle hervorgeht:

	Jodzahl	Part. JZ	Rhodanzahl
Cocosfett	10,7	8,1	8,3
Milchfett	25,6	20,7	21,7
Schweinefett	19,5	44,9	44,2
Erdnußöl	89,9	71,1	69,5
Lebertran	156,8	99,7	101,0

Aber auf Grund der Bildung von Isoölsäuren während des Härtungsprozesses sind die Verhältnisse in den gehärteten Fetten anders. So fand Kaufmann [19])

	Jodzahl	Part. Jodzahl	Rhodanzahl
Geh. Cocosfett	8,3	4,8	8,0
Geh. Erdnußöl	69,8	41,5	66,8
Geh. Sonnenblumenöl	62,0	35,8	61,3
Geh. Tran	34,4	18,0	34,0
Geh. Walöl	54,8	30,2	51,0

Findet man deshalb bei der Untersuchung des Fettgemisches eine Diskrepanz zwischen Rhodanzahl und partieller Jodzahl, so enthält es wahrscheinlich ein gehärtetes Fett.

Im Anschluß an die erwähnten Methoden soll schließlich die systematische Analyse eines Fettgemisches zur Bestimmung von Butter- oder Margarinefett angegeben werden.

A. Butter in Margarine

1. Lumineszenzuntersuchung. Vergleich mit Proben, die 10, 20, 30 % usw. Butter enthalten.

2. Etwaige Identitätsreaktionen z. B. mikrochemisch nach Kolle [16]).

3. Ermittelung bzw. Berechnung von Buttersäurezahl und Restzahl und Verwendung der

[16]) Finn Kolle: Qualitative pharmazeutische Mikroanalyse (Norwegisch). — Oslo 1926.

[17]) J. Großfeld u. F. Battay: Über die Bestimmung der Buttersäure und Capronsäure in Speisefetten. — Z. Unters. Lebensmittel 62, 99—126 [1931].

[18]) J. Großfeld: Prüfung auf Butter- und Margarinefett in der Lebensmittelkontrolle. — Fette u. Seifen 43, 100—103 [1936].

[19]) H. P. Kaufmann, Studien auf dem Fettgebiet. Verlag Chemie, Berlin 1935.

Großfeldschen Formel: % Butterfett = 5,12 mal Buttersäurezahl — 0,12 mal Restzahl.

4. Kontrolle: Bestimmung von B-Zahl, wenn nötig auch von A-Zahl.

B. Margarine in Butter

1. Lumineszenzuntersuchung wie oben. — Dann qualitative Prüfung auf Cocosfett (a) oder gehärtete Fette (b).

2. a) Positive Phytosterinreaktion zeigt pflanzliche Fette oder Öle; durch mikrochemische Reaktionen erkennbar.

2. b) Wenn Tortelli-Jaffe-Reaktion positiv: gehärteter Tran.

3. a) Ermittelung von Buttersäure-, Laurinsäure- und Restzahl und Berechnung nach den Gleichungen: % Cocosfett = 2,77 mal Restzahl — 2,63 mal Buttersäurezahl, oder % Cocosfett = 0,79 (Laurinsäurezahl — 0,5 Buttersäurezahl).

3. b) Ermittelung von Rhodanzahl und partieller Jodzahl und Bestimmung des Isoölsäuregehaltes. Hieraus den wahrscheinlichen Gehalt an gehärtetem Fett berechnen.

Über das Reaktionsgleichgewicht der Fettspaltung

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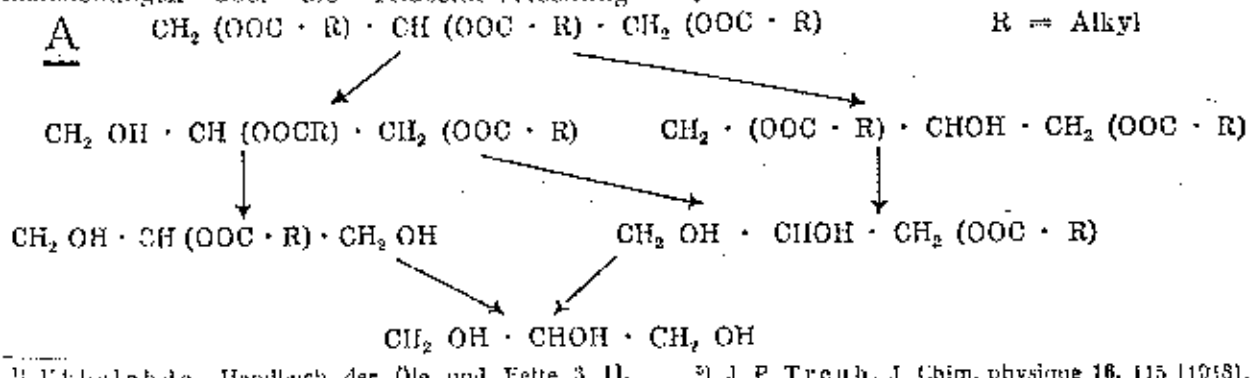
Über den Verlauf der Spaltung und der Verseifung von Fetten liegen zahlreiche theoretische und experimentelle Arbeiten vor, doch besitzen nur wenige praktische Bedeutung. Selbst die wichtigen Resultate der sehr gründlichen Untersuchungen der Kinetik der sauren Verseifung von Triacetin lassen sich nur bis zu einem gewissen Grad auf die höheren Fettsäureester des Glycerins übertragen, da die geringere Löslichkeit der letzteren ein heterogenes Reaktionssystem bedingt, welches eine genaue mathematische Erfassung des Problems wenigstens vorläufig als aussichtslos erscheinen läßt.

Technisch von Interesse ist die Berechnung des theoretisch zu erwartenden Höchstspaltgrades bei der Spaltung von Fetten, das heißt also des während des Spaltprozesses maximal erreichbaren Prozentsatzes an freien Fettsäuren, bezogen auf die Gesamtfettsäure des ursprünglichen Neutralfettes. Die theoretische Lösung dieser Aufgabe verspricht Erfolg, weil es sich dabei nur um die Berücksichtigung von chemischen Gleichgewichtszuständen handelt und nicht um den zeitlichen Verlauf der Reaktion. Tatsächlich gelingt es, eine für praktische Zwecke genügend genaue und verblüffend einfache Formel zur Berechnung des Höchstspaltgrades aufzustellen, wenn einige bei der Triacetin-Verseifung gefundene Gesetzmäßigkeiten auf die Fettspaltung übertragen werden.

Eine Zusammenstellung der grundlegenden Untersuchungen über die Triacetin-Verseifung findet sich in Ubbelohdes Handbuch der Öle und Fette[1]). Die in dieser Arbeit berücksichtigten Ergebnisse sollen kurz erwähnt werden:

Nach den Untersuchungen von R. Wegscheider[2]), A. C. Geitel[3]) und J. Meyer[4]) verläuft die saure Verseifung des Triacetins in sehr verdünnter Lösung über Di- und Monoacetin zum Glycerin. Die einzelnen Reaktionen haben monomolekularen Charakter und die Geschwindigkeitskonstanten dieser Stufen stehen unter sich im gleichen Verhältnis wie die Zahlen der verseifbaren Estergruppen der verschiedenen Moleküle, also wie 3 : 2 : 1, welches Verhältnis mit ziemlicher Genauigkeit durch das Experiment bestätigt wurde. Die Arbeiten von J. P. Treub[5]) und E. Yamasaki[6]) führten zu den gleichen Ergebnissen und bestätigten außerdem noch die Voraussetzung der erstgenannten Autoren, daß die drei Estergruppen des Triacetins praktisch gleiche Verseifbarkeit zeigen. — E. Abel[7]) hat für eine Reaktion mit n aufeinanderfolgenden Stufen 1. Ordnung, deren Geschwindigkeitskonstanten sich wie n : n—1 : n—2 : ... : 2 : 1 verhalten, die erschöpfende mathematische Behandlung durchgeführt und gezeigt, daß sie im Gesamtverlauf einer Reaktion 1. Ordnung über eine Stufe gleicht, was früher zu der Erklärung verleitete, daß die erste Estergruppe mit endlicher, die beiden andern aber mit unendlicher Geschwindigkeit abgespalten würden[8]). Desgleichen gilt für die alkalische Verseifung des Triacetins, daß drei bimolekulare Reaktionen mit den Geschwindigkeitskonstanten $k_1 : k_2 : k_3 = 3 : 2 : 1$ aufeinander folgen[9]).

Bei der Berechnung unserer Formel gingen wir vom allgemeinen Schema A aus, welches sich unter Annahme der Identität der drei Estergruppen im Triglycerid, bzw. der Hydroxylgruppen im Glycerin zu B vereinfachen läßt.



[1]) Ubbelohde, Handbuch der Öle und Fette 3 II, 1—26.

[2]) R. Wegscheider, Mh. Chem. 29, 81 [1907].

[3]) A. C. Geitel, J. prakt. Chem. 55, 429 [1897]; 57, 113 [1898].

[4]) J. Meyer, Z. Elektrochem. angew. physik. Chem. 13, 485 [1907]. Z. physik. Chem. 66, 115 [1909]; 67, 302 [1909].

[5]) J. P. Treub, J. Chim. physique 16, 115 [1918].

[6]) E. Yamasaky, J. Amer. Chem. Soc. 42, 1456 [1920]; 44, 428 [1922].

[7]) E. Abel, Z. Elektrochem. angew. physik. Chem. 12, 681 [1906]; Z. physik. Chem. 56, 558 [1906].

[8]) Ulzer-Klimont (E. Abel), Allgemeine und Physiologische Chemie der Fette, S. 220 [Berlin 1906].

[9]) R. Kremann, Mh. Chem. 27, 607 [1906].

Effects of Diet on Fish Oil Toxicity in the Rat

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It is well established that the prolonged administration of fish oils will induce or aggravate symptoms of vitamin E deficiency in a number of animal species (Schuhr, '39a, b, '39; Madsen et al., '37; Jam, '43; Simpson et al., '53a). This effect has been ascribed to one or more of the following mechanisms: (1) destruction or inactivation of vitamin E in the diet, the gastrointestinal tract or the tissues by the autoxidation of the polyunsaturated fatty acids of fish oil; (2) an increased requirement for or an impaired utilization of tocopherol induced by (a) toxic substances in or made in metabolism of fish oil, or (b) toxic substances produced by the interaction of fish oil with other substances in the diet.

Symptoms of vitamin E deficiency induced by fish oil administration such as exudative diathesis in herbivora or encephalomalacia in chicks can readily be interpreted by the concurrent feeding of tocopherol (Dam et al., '38; Mackenzie and McGibbon, '39). They can also be counteracted, however, by various non-tocopherol supplements. Thus, Ni ('36, b, '38, '40) found that a water-soluble material derived from Chinese sclerotia of Sclerotinia prevented the occurrence of muscular dystrophy and encephalomalacia in rabbits containing fish liver oils. Jukes and Babcock ('38) described a paralytic disorder in chicks which could be prevented either by a fat-soluble fraction prepared from soybean oil (presumably vitamin E) or by a water-soluble fraction prepared from fat-extracted alfalfa meal.

More recently, Simpson et al. ('53, '51) observed that a number of apparently unrelated compounds, including methylene blue, ascorbic acid and nordihydroguaiaretic acid had significant activity in preventing chick encephalomalacia induced by diets containing cod liver oil. Similar findings were reported by Simpson et al. ('53b) with N, N' - diphenyl - p - phenylenediamine (DPPD) and by Burrell et al. ('55) with other antioxidants including 2, 6-dextrtiary butyl-4-methylphenol (BHT), dicumyl and diamyl hydroquinone, disecondary p-phenylenediamine, and 1,2-dihydro-2,4-trimethyl 6 ethoxyquinoline (Santoquin). Methylene blue, ascorbic acid and tetraethylthiuram disulfide (antabuse) were also active in protecting against the development of such symptoms of vitamin E deficiency as fat edematous and peroxidation, incisor depigmentation, hepatic damage and sterility in rats fed diets containing cod liver oil (Dam et al., '49). Methylene blue was also active in preventing dystrophy in calves fed a diet containing cod liver oil (Blaxter et al., '53). Since all the materials indicated above have antioxidant activity, it would appear that their activity was due either to their sparing effect on the destruction of vitamin E in the diet, gastrointestinal tract or tissues or to their ability to replace vitamin E, at least in part, in the tissues.

There is evidence, however, that the toxic effects of fish oil administration are not confined to the induction or accentuation of a vitamin E-deficiency state. Thus, Scott ('51a) observed that the addition of fish liver oil to the diet of poults resulted in a high incidence of enlarged hock disorder. Vitamin E deficiency does not appear to be the cause of this condition since high levels of α-tocopheryl acetate (50 mg/pound of diet) failed to prevent the disorder (Scott, '51b). Intramuscular injections of α-tocopheryl phosphate (10 mg per poult weekly) were also ineffective in this respect (Scott, '51b). The high incidence of enlarged-hock disorder in poults fed diets containing fish liver oil could be prevented completely however, by the concurrent feeding of dried brewers' yeast (Scott, '51b). In the present communication further data are presented indicating that toxic effects may occur following fish oil administration due to factors other than the induction of a vitamin E deficiency state.

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PROCEDURE AND RESULTS

A series of experiments was designed to study the effects of various dietary supplements on the growth of immature rats fed a purified low-fat ration supplemented with 10% of fish oil. The basal low-fat ration used in these experiments consisted of sucrose, 71%; casein,[1] 24%; and salt mixture,[2] 5%. To each kilogram of the above diet were added the following synthetic vitamins: (in milligrams) thiamine·HCl, 20; riboflavin, 20, pyridoxine·HCl, 20; Ca pantothenate, 60; nicotinic acid, 100; ascorbic acid, 200; biotin, 4; folic acid, 10; p-aminobenzoic acid, 400; inositol, 800; 2-methyl-1,4-naphthoquinone, 5 mg; and vitamin B_{12}, 150 mμ; choline chloride, 2 gm, vitamin A, 5000 U.S.P. units,[3] vitamin D_2, 500 U.S.P. units;[3] and α-tocopheryl acetate, 100 mg. The vitamins were added in place of an equal amount of sucrose. Fish oils and the various test supplements were incorporated in the above diet in the amounts listed in tables 1-4, replacing equal amounts of sucrose.

[1] Vitamin-Free Test Casein, General Biochemicals, Inc., Chagrin Falls, Ohio.

[2] Wesson Modification of Osborne-Mendel Salt Mixture, General Biochemicals, Inc., Chagrin Falls, Ohio.

[3] Crystalline 360 A—50 D, crystalline vitamin A acetate and crystalline vitamin D_2 stabilized with gelatin and sugar, 500,000 U.S.P. units vitamin A and 50,000 U.S.P. units vitamin D_2 per gm, Chas. Pfizer and Co., Inc., New York.

TABLE 1

Effects of fish oil administration on the weight increase of immature male rats fed a purified low-fat diet and a similar ration supplemented with cottonseed oil (6 animals per group)[1,2]

Dietary group	Average gain in body weight after following days of feeding			
	7th	14th	21st	28th[3]
	gm	gm	gm	gm
Basal low-fat diet	27	64	93	124 ± 6.1
Basal low-fat diet plus following supplements:				
10% Crude tuna oil	2	14	29	38 ± 3.4
10% Refined tuna oil	1	9	21	37 ± 2.9
10% Crude sardine oil	−3	3	13	28 ± 2.1
10% Menhaden oil	−1	4	15	21 ± 4.2
10% Cod liver oil	6	34	45	61 ± 3.1
Basal low-fat diet plus 10% cottonseed oil	33	80	118	153 ± 5.8
Basal low-fat diet plus 10% cottonseed oil plus following supplements:				
10% Crude tuna oil	29	68	97	138 ± 4.4
10% Refined tuna oil	26	59	89	119 ± 3.9
10% Crude sardine oil	29	56	84	120 ± 4.6
10% Menhaden oil	30	58	88	124 ± 4.1
10% Cod liver oil	32	66	98	147 ± 2.9

[1] The average initial body weight of rats in the various groups ranged between 46.4 and 48.6 gm.

[2] The crude and refined tuna oil and the crude sardine oil were kindly provided by the late Dr. E. Geiger and Mr. H. J. Dunn of the Van Camp Sea Food Co., Inc., Terminal Island, California. The menhaden oil was obtained from the Pacific Vegetable Oil Corp., Los Angeles; the cod liver oil from E. R. Squibb and Sons, New York. The cod liver oil had a vitamin A content of 1700 U.S.P. units/gm and a vitamin D_2 content of 170 U.S.P. units/gm. When fed at a 10% level in the diet it supplied 170,000 U.S.P. units of vitamin A and 17,000 U.S.P. units of vitamin D_2/kg of ration. The tuna oils contained approximately 200 U.S.P. units of vitamin A and 50 U.S.P. units of vitamin D_2/gm, and the sardine oil approximately 300 U.S.P. units of vitamin A and 100 U.S.P. units of vitamin D_2/gm. No data are available on the vitamin A and D content of the menhaden oil.

[3] Including standard error of the mean calculated as follows:

$$\sqrt{\sum d^2/n}/\sqrt{n}$$

where "d" is the deviation from the mean and "n" is the number of observations.

TABLE 2

Comparative effects of cottonseed oil, methyl linoleate and other sources of dietary fat on the growth retardation of immature rats fed a purified low-fat ration supplemented with 10% of crude tuna oil (6 animals per group).[1]

Dietary group	Average gain in body weight after 24 days of feeding
	gm
Basal low-fat diet	63
Basal low-fat diet + 10% cottonseed oil	81
Basal low-fat diet + 10% crude tuna oil	17
Low-fat diet + 10% crude tuna oil plus following supplements:	
10% Cottonseed oil	54
5% Cottonseed oil	26
2% Cottonseed oil	14
10% Soybean oil	67
5% Soybean oil	51
2% Soybean oil	22
10% Sesame oil	68
5% Sesame oil	34
2% Sesame oil	21
10% Wheat germ oil	72
10% Corn oil	62
10% Olive oil	28
10% Coconut oil	18
10% Lard	15
10% Butter fat	14
10% Hydrogenated cottonseed oil	24
10% Methyl linoleate	−12
5% Methyl linoleate	−9
1% Methyl linoleate	−2
Dextrin (substituted for sucrose in diet)	−4
Corn starch (substituted for sucrose in diet)	2

[1] The average initial body weight of rats in the various groups ranged between 45.2 and 46.4 gm.

[2] The fats were obtained from the following sources: cottonseed oil, Wesson Oil and Snowdrift Sales Co., New Orleans, La.; wheat germ oil, Vitalis Corporation, Monticello, Ill.; corn oil, Corn Products Refining Co., Argo, Ill.; stripped lard, Hygrade Food Products Industries, Rochester, Minn. (tocopherol content of this material according to manufacturer's analysis was less than 5 mg/gm); hydrogenated cottonseed oil, H. Wolf Foods, Inc., New York, and Spry, Lever Brothers Company, Los Angeles. The butter fat was prepared by heating the butter and removing the oil layer. The sesame oil, olive oil and coconut oil were obtained from the Lesile Vegetable Oil Co., Los Angeles. The methyl linoleate was kindly provided by Dr. Evelyn B. Allin-Slater, Department of Home Economics, University of California, Los Angeles. The purity of methyl linoleate as determined as described in a subsequent section of this paper was 92%.

Male rats of the Holtzman strain were selected, 21 to 24 days old, having a body weight between 40 and 54 gm for the present series of experiments. The rats were housed in metal cages with raised screen bottoms (two animals per cage) and were provided with feed and water ad libitum. Diets were made up biweekly and stored under refrigeration when not in use. The animals were fed daily (6 rats per group) and all feed not consumed 24 hours after feeding was discarded. Feeding was continued for either 14 or 28 days as indicated in tables 1–4. Animals were weighed once weekly during the course of the experiment.

Experiment 1

Deleterious effect of fish oil on the growth of immature rats fed a purified low-fat diet. Considerable data are available indicating that animals fed low-fat diets are particularly sensitive to a number of stressor agents including X-irradiation (Derbes et al., '50; Cheng et al., '52), thyroid administration (Greenberg and Hegsted, '50, Greenberg, '52), mineral oil administration (Bloom et al., '52; Bastedt and Greenberg, '54) and administration of succinylsulfathiazole, atabrine and other drugs (Rossbardt et al., '50; Rossbardt and Huff, '53). In each of the experiments indicated above a protective effect was obtained by increasing the fat content of the diet.

Present findings indicate that immature rats fed a purified low-fat diet supplemented with 10% of fish oil exhibited a highly significant retardation in growth which was evident during the very first week of feeding. During this period rats fed the above diets exhibited an almost complete cessation of growth or actual loss in body weight accompanied by diarrhea. Subsequent to this period a small increase in body weight occurred, particularly during the third and 4th week of feeding. With the exception of their smaller size, however, rats fed the low-fat diet supplemented with fish oils appeared grossly normal in all respects. Similar findings were obtained with both crude and refined

[3] The fish oil in these experiments were either a crude fish oil with a high content of unsaturated fatty acids or methyl linoleate per se.

tuna oil, crude sardine oil and menhaden oil. Growth also was retarded using diets containing 10% of cod liver oil but to a lesser extent than with the other fish oils used. In contrast to the above, the growth-retarding effect of the various fish oils as well as the occurrence of diarrhea was largely if not completely counteracted by the concurrent administration of cottonseed oil at a 10% level in the diet.

Experiment 2

Comparative effects of cottonseed oil, methyl linoleate, and other sources of dietary fat on the growth retardation of immature rats fed a purified low-fat ration supplemented with 10% of crude tuna oil. In agreement with experiment 1 the incorporation of crude tuna oil at a 10% level in the basal purified low-fat ration resulted in a significant retardation in growth accompanied by diarrhea which was largely counteracted by the concurrent administration of 10% of cottonseed oil. Soybean oil, sesame oil, corn oil and wheat germ oil when fed at a 10% level in the diet were active also in this regard but olive oil, coconut oil, hydrogenated cottonseed oil, butter fat and lard at a 10% level of feeding had little if any growth-promoting effect. The above findings indicate that the fats most active in counteracting fish oil toxicity under conditions of the present experiment were vegetable oils with a high content of unsaturated fatty acids, whereas the more saturated fats (such as hydrogenated cottonseed oil, vegetable oils of low unsaturated fatty acid content or animal fats) had little if any activity in this regard.

Methyl linoleate, however, when incorporated at levels of 1, 5 or 10% in the diet was without protective effect. On the contrary the rats fed these supplements lost weight during the course of the experiment in contrast to the small weight increase of rats fed the basal low-fat ration with 10% of crude tuna oil. Studies conducted with cottonseed oil, soybean oil and sesame oil at levels of 2, 5 and 10% of the diet indicate that the effects obtained were proportional to the level fed. At the 2% level of fat in diet findings were comparable to those obtained in the low-fat diet [illegible] of crude tuna oil ration. A significant increase in body weight over that using the latter diet occurred in rats fed the 5% soybean oil or sesame oil supplements and to a lesser extent in rats fed the diet containing 5% of cottonseed oil. A still further increase in body weight resulted when these supplements were fed at a 10% level. Replacing the sucrose in the purified low-fat diet with dextrin or corn starch did not improve the growth of rats fed the crude tuna oil supplement. On the contrary the weight increase of rats fed dextrin or corn starch as the source of carbohydrate in a low fat diet supplemented with 10% of crude tuna oil was significantly less than that of rats fed a similar ration containing sucrose as the dietary carbohydrate.

Experiment 3

Comparative effects of α-tocopherol, α-tocopheryl acetate, DPPD, Santoquin and sesamol on the growth retardation of immature rats fed a purified low-fat ration supplemented with 10% of crude tuna oil. Findings indicate that α-tocopherol, when fed at levels of 0.025, 0.05 or 0.1% in the diet, partially counteracted the growth retardation (and completely prevented the diarrhea) of immature rats fed a purified low-fat ration supplemented with 10% of crude tuna oil. Its growth-promoting effect was more marked at the 0.05% than the 0.025% level; no further increase in weight occurred, however, when fed at a higher (i.e., 0.1%) level. In contrast to the effects obtained with α-tocopherol, α-tocopheryl acetate, when fed in comparable amounts, was without protective effect. Intraperitoneal injections of α-tocopheryl phosphate (10 mg/rat/day, 3 times weekly) were also without growth-promoting effect. DPPD and Santoquin, when fed at a 0.05% level in the diet and sesamol (dihydrofuranone ether of oxyhydroquinone, a constituent of sesame oil with antioxidant properties), when administered at a 0.05 or 0.01% level in the ration, counteracted completely the growth retardation caused by crude tuna oil when incorporated at a 10% level in the purified low-fat diet. Results are summarized in table 3.

TABLE 3

Comparative effects of [illegible] and other materials on the growth retardation of immature rats fed a purified low-fat ration supplemented with 10% of crude tuna oil (6 animals per group)[1]

Dietary group	Average gain in body weight after 14 days of feeding
	gm
Basal low-fat diet	61
Basal low-fat diet + 10% cottonseed oil	76
Basal low-fat diet + [illegible]	54
Basal low-fat diet + 10% crude tuna oil + following supplements:	
0.[illegible]% α-tocopherol	32
0.[illegible]% α-tocopherol	39
0.1% α-tocopherol	11
[illegible] α-tocopherol acetate	34
0.[illegible] α-tocopherol acetate	13
0.1% α-tocopheryl acetate	12
Tocopherol phosphate (Phosphate at day 6 [illegible] I.P.)	4
0.1% DL-Methionine	65
0.1% Sarcosine	70
0.01% [illegible]	72
0.01% Sarcosine	74

[1] The average initial body weight of rats in the various groups ranged between [illegible] and 46.9 gm.

[2] Test supplements were obtained from the following sources: [illegible] and tocopherols: Nutritional Biochemicals Corporation, Cleveland; α-tocopheryl acetate: Dry Vitamin E Acetate Powder 33% [illegible], Roche Inc., Nutley, N. J.; DL-α-tocopherol: Merck & Co., Chemicals and Synthetics, Merck Chemical Div., St. Louis. The [illegible] used in the present experiment was a [illegible] product prepared by the [illegible] supplied by the Western Utilization Research [illegible] Department, Division of Agricultural Research Service, U. S. Department of Agriculture, Albany, [illegible].

Experiment 4

Comparative effects of known nutrients and other materials of plant and animal origin on the growth retardation of immature rats fed a purified low-fat ration supplemented with 10% of crude tuna oil. Previous studies indicate that a number of natural source materials other than unsaturated vegetable oils or substances present therein were also effective in promoting the growth and preventing diarrhea of immature rats fed a purified low-fat diet supplemented with 10% of crude tuna oil, alfalfa meal at a 20% level in the diet and defatted liver N.F. and Torula yeast at a 10% level of feeding were particularly effective in this regard. Both liver residue at a 10% level and liver concentrate powder at a 2.5% level of supplementation had growth-promoting and anti diarrheal activity. Increasing the cane sugar content of the diet by an additional 10% of the ration or supplementing the basal ration with a fat-free fish flour or a mixture of crystalline amino acids to the extent of 10% of the diet also resulted in a significant increase in body weight, although less than that obtained with the 20% alfalfa meal or 10% liver and yeast supplements. DL-Methionine when fed at a 0.6% level in the diet was similar to the casein fish meal and amino acid supplements in activity. Doubling the vitamin or salt mixture content of the basal ration or adding 0.5 or 1% of ascorbic acid, 2% of mixed flavonoids, 10% of cellulose or 1 gm of Aureomycin-HCl per kilogram of diet had little if any protective effect. A combined supplement of 20% of casein, 5% of salt mixture, 2 gm of choline chloride per kilogram of diet plus the various vitamins in an amount equal to the level at which they were incorporated in the basal low-fat ration counteracted completely, however, the growth retardation and diarrhea of immature rats fed the basal low fat ration supplemented with 10% of crude tuna oil. Results are summarized in table 4.

DISCUSSION

Present findings indicate that immature rats fed a purified low-fat diet supplemented with 10% of fish oil (tuna, crude or refined tuna oil, crude sardine oil, menhaden oil or cod liver oil) showed a highly significant retardation in growth accompanied by diarrhea which was evident during the first week of feeding. These effects were counteracted largely by the concurrent administration of cottonseed oil at a 10% level in the diet. Soybean oil, sesame oil, corn oil and wheat germ oil at a 10% level of feeding were also active in counteracting the growth retardation and diarrhea of immature rats fed the basal low fat ration supplemented with 10% of crude tuna oil. Olive oil, coconut oil, hydrogenated cottonseed oil, butter

fat and lard, when fed at a 10% level in the diet, had little if any protective effect. These observations indicate that the fats most active in counteracting fish oil toxicity under conditions of the present experiment were vegetable oils with a high content of unsaturated fatty acids, whereas the more saturated fats (such as hydrogenated cottonseed oil, vegetable oils of low unsaturated fatty acid content or animal fats) had little if any activity in this regard.

TABLE 4

Comparative effects of known nutrients and other materials of plant and animal origin on the growth retardation of immature rats fed a purified low-fat ration supplemented with 10% of crude tuna oil (6 animals per group)[1]

Dietary group	Average gain in body weight after following days of feeding	
	14th[2]	28th
	gm	gm
Basal low-fat diet	61	132
Basal low-fat diet + 10% cottonseed oil	60	151
Basal low-fat diet + 10% crude tuna oil	12	45
Basal low-fat diet + 10% crude tuna oil + following supplements:		
Vitamins A, D and E[3]	12	41
Vitamins B, C and K[4]	16	40
5% Salt mixture[5]	23	61
10% Casein[5]	38	93
Combined supplements[6]	88	155
0.5% Ascorbic acid	23	54
1% Ascorbic acid	20	53
2% Mixed flavonoids[7]	18	41
Aureomycin-HCl (1 gm/kg of diet)	22	54
10% Cellulose[8]	30	65
10% defatted fish flour[9]	50	116
10% Amino acid mixture[10]	41	101
0.6% DL-methionine	55	91
20% Alfalfa meal	83	178
10% Alfalfa meal	51	89
Dehydrated liver N.F.	87	160
10% Liver residue[11]	57	129
2.5% Liver concentrate powder N.F.	51	99
10% Torula yeast[12]	67	131

[1] The average initial body weight of rats in the various groups ranged between 43.8 and 49.6 gm.

[2] The following vitamins were added per kilogram of diet: 5000 U.S.P. units of vitamin A, 500 U.S.P. units of vitamin D_2 and 100 mg of α-tocopheryl acetate.

[3] The following were added per kilogram of diet: thiamine-HCl, 20 mg; riboflavin, 20 mg; pyridoxine-HCl, 20 mg; Ca pantothenate, 60 mg; nicotinic acid, 100 mg; ascorbic acid, 200 mg; biotin, 4 mg; folic acid, 10 mg; p-aminobenzoic acid, 400 mg; inositol, 800 mg, vitamin B_{12}, 150 μg; 2-methyl 1,4 naphthoquinone, 5 mg; and choline chloride, 2 gm.

[4] Wesson Modification of Osborne-Mendel. See footnote 2 in text.

[5] Vitamin free Test Casein. See footnote 1 in text.

[6] These include the supplements indicated in footnotes 2, 3, 4 and 5.

[7] The following flavonoids were each fed at a 0.5% level in the diet: calcium flavonate dihydrate, rutinose (naringenin 5-rhamnosidoglucoside), hesperidin complex, hesperidin methyl chalcone and lemon bioflavonoid complex.

[8] Solka Floc BW 200, Brown Co., Boston.

[9] Vitalife Corp., Aurora, Illinois.

[10] Synthetic amino acid mixture (Schulze, '57) consisting of the following: L-arginine monohydrochloride, 7.43; L-histidine monohydrochloride, 5.21; DL-isoleucine, 10.94; L-leucine, 16.57; L-lysine hydrochloride (95%), 11.66; DL-methionine, 5.10; DL-phenylalanine, 8.00; DL-threonine, 12.00; DL-tryptophan, 2.98; DL-valine, 21.30; DL-aspartic acid, 9.12; DL-glutamic acid, 8.05; L-cystine, 5.01; L-glutamine, 3.30; 50.76; L-tyrosine, 3.90; and glycine, 6.70.

[11] Dehydrated liver residue, Wilson Laboratories, Chicago. This consists of the coagulated, water insoluble material remaining after the removal of the extractable water soluble substances.

[12] Dried Torula Yeast U.S.P., Lake States Yeast Corp., Rhinelander, Wisconsin.

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(methyl linoleate, however, when fed at levels of 1, 5 or 10% of the diet was without protective effect, suggesting thereby that the beneficial effects of the more unsaturated vegetable oils were caused by some factor or factors other than their essential fatty acid content. Since tocopherol is present in considerable quantity in a variety of unsaturated vegetable oils and in view of the extensive literature on the destruction or inactivation of vitamin E by fish oils, experiments were conducted to determine the effects of (also) supplying of vitamin E (in addition to that present in the basal ration) on the growth of immature rats fed the basal low-fat diet supplemented with 10% of crude tuna oil. Findings indicate that α-tocopherol, when fed at levels of 0.025, 0.05 or 0.1%, partially counteracted the growth retardation (and completely prevented diarrhea) on the above diet. Its growth promoting effect was more marked at the 0.05% than the 0.025% level; no further increase in weight occurred, however, when fed at a higher (i.e., 0.1%) level.

In contrast to the effects obtained with α-tocopherol, however, α-tocopheryl acetate, when fed in comparable amounts, was without protective effect. Intraperitoneal injections of α-tocopheryl phosphate (10 mg/rat day, 6 times/week) were also without growth promoting or antidiarrheal effect. These findings suggest that the beneficial effects of α-tocopherol (which has marked antioxidant activity in contrast with that exhibited by α-tocopheryl acetate) were due not to its vitamin E activity per se but rather to its antioxidant properties, either in the diet itself or in the intestinal tract. Further evidence that the beneficial effect of α-tocopherol was due to its antioxidant activity was the observation that DPPD and Santoquin (both potent antioxidants) were also active in counteracting the growth retardation and diarrhea of immature rats fed the basal low-fat diet supplemented with 10% of crude tuna oil.

Available data indicate, however, that the beneficial effect of the unsaturated vegetable oils in counteracting the toxic effects of tuna oil administration were due, at least in part, to some factor other than its tocopherol content. This is indicated by (1) findings that cottonseed oil, soybean oil, sesame oil, corn oil and wheat germ oil, when fed at a 10% level in the diet, were more effective than the α-tocopherol supplements in counteracting the growth retardation caused by crude tuna oil under conditions of the present experiment, despite the fact that they contained less tocopherol; and (2) the demonstration that sesamol, a constituent of sesame oil with antioxidant properties, was completely effective when fed in the sole supplement, even at 1/50th the dosage of α-tocopherol used, in counteracting the growth-retarding effect of fish oil toxicity on the purified low-fat diet. These findings suggest that the unsaturated vegetable oils indicated above contained, besides tocopherol, one or more additional materials with significant antioxidant activity equal to or exceeding that of tocopherol itself.

In addition to the protective effect of unsaturated vegetable oils and substances present therein, a number of natural source materials of both plant and animal origin, as well as some of the known complex lipid nutrients, were also active in preventing growth retardation and diarrhea in immature rats fed the purified low-fat ration supplemented with 10% of crude tuna oil. Alfalfa meal at a 20% level in the diet and desiccated liver N.F. and Torula yeast at a 10% level of feeding were particularly effective in this regard. A supplement of 10% of casein, 10% of fish meal or a mixture of crystalline amino acids at a 10% level in the diet, as well as DL-methionine alone at a 0.6% level of feeding, also resulted in a significant increase in body weight, although less than that obtained with the 20% of alfalfa meal, 10% of liver or 10% of Torula yeast supplements. A combined supplement of 10% of casein, 5% of salt mixture, 2 gm of choline chloride per kilogram of diet plus the various vitamins in an amount equal to the level at which they were incorporated in the basal low-fat ration were also active in counteracting the growth retardation (and diarrhea) of immature rats fed the basal low-fat diet supplemented with 10% of crude tuna oil. No data are available as to the cause of the toxic effects produced by fish oils when incorporated in the purified low-fat diet nor the mechanism (or mechanisms) whereby α-tocopherol, sesamol, DPPD, Santoquin, DL-methionine and the other supplements indicated above exerted their protective effect.

SUMMARY

Immature rats fed a purified low fat diet supplemented with 10% of fish oil (i.e., crude or refined tuna oil, crude sardine oil, menhaden oil or cod liver oil) showed a highly significant retardation in growth accompanied by diarrhea. These effects were largely counteracted by the concomitant administration of cottonseed oil at a 10% level in the diet. Soybean oil, sesame oil, corn oil and wheat germ oil at a 10% level of feeding were also active in this regard but olive oil, coconut oil, hydrogenated cottonseed oil, butter fat and lard when fed at a 10% level in the diet had little if any protective effect. Methyl linoleate when fed at levels of 1, 5 or 10% of the diet was without protective effect. α-Tocopheryl acetate when incorporated at levels of 0.025, 0.05 or 0.1% in the diet was also inactive. Intraperitoneal injections of α-tocopheryl phosphate (10 mg/rat day, 6 times/week) were likewise ineffective. α-Tocopherol, however, when administered at levels of 0.025, 0.05 or 0.1% in the diet had a significant protective effect although less than that obtained with DPPD (N,N'-diphenyl-p-phenylenediamine) and Santoquin (6-ethoxy-2,2,4-trimethyl-1,2-dihydroquinoline) when fed at a 0.05% level in the diet or sesamol (the methylene ether of oxyhydroquinone, a constituent of sesame oil with antioxidant properties) when fed at 0.05 or 0.017% levels in the diet. In addition to the materials indicated above, alfalfa meal at a 20% level in the diet, desiccated liver N.F. and Torula yeast at a 10% level of feeding and supplements of casein, fish meal or a mixture of crystalline amino acids at a 10% level in the diet or DL-methionine at a 0.6% level of feeding also had significant activity in preventing growth retardation and diarrhea in immature rats fed a purified low-fat diet supplemented with 10% of crude tuna oil.

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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% [illegible] in 1961. 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	2.35
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 24 years. The low in recent years was 4.0¢ 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 [illegible] on 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[3]

[1] Peruvian semirefined, industry practice.
[2] NPFL Association raw material index, October 1965.
[3] Representative samples entering U.S. in 1962 and 1964 (courtesy of Archer-Daniels-Midland Company).
[4] Data not available.

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[5]	Rapeseed[2]	Tall[3]
Iodine value	170–200[1] 177, min.[2] 188, min.	120–141	120–135[4] 125–136[4]	160–175	97–108	127–132
Sap value	189–195	189–195	188–194	189–195	170–180	—
FFA, %	2 max.	[6]	[6]	[6]	[6]	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	[6]	[6]	[6]		[6]	0.6

[1] ASTM, D 234-46 for raw oil.
[2] AOCS, various.
[3] Distilled fatty acids only. Hauser (1961).
[4] Mass et al. 1962.
[5] Reported in various oil lists.
[6] Not applicable or not available.

TABLE 22

RANGES OF FATTY ACIDS FROM FISH OILS[1]

	Per cent Composition			
	Menhaden	Herring	Sardine (Pilchard)	Anchovy[2]
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	35–48[4]	26–29
Dienes	2.4–7.6	2.6–4.3		3.4–7.5
Trienes	0.9–1.4	0.6–4.1	[3]	0.3–3.2
Tetraenes	3.2–3.8	0.9–3.2		
Pentaenes	11.8–15.8	2.0–2.9	29–36[4]	28–30[4]
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_{16} and C_{18}	1.5	0.8–2.2	[3]	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	13.6–19.6	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 Mass et al. 1962; Jart 1965; Perlet et al. 1966; Lane et al. 1965; Iverson 1958 and 1965; Gruger et al. 1964.
[2] Not applicable or not available.
[3] Data from Ackman-Daniels-McPhan 1 — private communication.
[4] Sum of values for combined monoenes and dienes.
[5] Sum of values for combined trienes, tetraenes, 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	60.7–72	31–37.5
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	8–13.5
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}	—	0–4	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 I.

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.[1]	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[3]	58	[4]	[4]	15, 26 [4,5]	
1965	175[2]	75[3]	[4]	[4]	[4]	[4]	[4]

[1] MM lb. = millions of lb.
[2] Estimate.
[3] Average monthly rate for hydrogenated and edible from the Fatty Acid Producers' Council.
[4] Not separately reported.
[5] Refined and hydrogenated.

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

U.S. statistics (Anon. 1965G) 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 o[illegible]d 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 Elmer 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 in 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 po[illegible] 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 Graham 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; Dozz 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 high

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 15.2 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

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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Letters to the Editor

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Fate of Chlorinated Organic Pesticide Residues in the Production of Edible Vegetable Oils

SIR. The Food and Drug Administration (U.S.A.) has established tolerances for residues of certain pesticides, defoliants and herbicides in raw agricultural products. Among these raw agricultural products are the seeds from which crude vegetable oils are obtained for further processing for edible oil uses. It is to be expected that pesticide residues may appear in the crude oils obtained from oil-seeds which have been subject to insecticidal sprays in the course of normal horticultural practices and when grains have been treated in storage.

Of the several classes of pesticides approved for use in the production of raw agricultural commodities, the class of chlorinated organic pesticides is of the most serious concern as a potentially harmful residue in the end-product of vegetable oil processing. There are no established tolerances in the U.S.A. for pesticide residues in crude vegetable oils nor in processed edible vegetable oils. Tolerances have been established in the U.S.A. for one or more chlorinated organic pesticides in one or more of the following seeds: corn, cottonseed, milo, peanuts, rice and soya beans. It was of interest to learn if any of the chlorinated organic chemicals authorized for use on the named raw agricultural products can survive normal processing procedures used in the production of edible vegetable oils.

The following commercial grade, chlorinated organic pesticide chemicals used in formulating pesticidal sprays, *etc.*, were added by us to a crude cottonseed oil in pilot plant work. The highest, established FDA tolerance in parts per million for the named pesticide in one or more of the named seeds is given as the first figure in parentheses following the name of the pesticide while the second figure is the amount of the commercial-grade chemical we added to the crude cottonseed oil.

Aldrin (0·25, 1·0); BHC (5·0, 15·0); Chlordane (0·3, 1·0); DDT (7·0, 21·0); Dieldrin (0·1, 1·0); Heptachlor (0·0, 1·0, Heptachlor Epoxide-99·4%, (0·0, 1·0); Kelthane (0·1, 1·0); Lindane (10·0, 30·0); Methoxychlor (14·0, 42·0); Sesone (6·0, 18·0); Strobane (5·0, 15·0); TDE or DDD (7·0, 21·0) and Toxaphene (7·0, 21·0).

These were added all together in the amounts noted. This afforded a rigorous testing at each stage of alkali-refining, bleaching and deodorisation and the fact that all disappeared upon deodorisation to produce the final edible oil is noteworthy. These results have been confirmed in plant-scale tests in which the crude vegetable oil contained adventitious traces of residues. In one instance, a vegetable oil containing 2·8 p.p.m. Chlordane with DDT and DDD 0·5 p.p.m. each, was subjected to a hydrogenation step during processing whereupon the trace of pesticide residues disappeared, presumably through adsorption by the activated carbon contained in the catalyst or possibly by destruction by dehalogenation in the hydrogenation process.

Standard electron-capture and micro-coulometric methods were used for the analyses.

Although no tolerances have been established in the U.S.A. for processed edible vegetable oils, it appears that none may be required since the processing of these oils inherently involves steps which result in food products free of chlorinated organic pesticide residues.

Yours faithfully,
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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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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 I 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 I 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 maximum activity. It would thus appear that some of the precursor of the active principle is present in the oil as a stable ester, and that only the compound liberated on hydrolysis is susceptible to oxidation and gives rise to the active agent.

VEGETABLE OILS. The presence of the active oil in several marine oils and its apparent production by oxidation of the esterifiable fraction suggested a search in other animal and vegetable fats for a source of the active agent. We have investigated the following oils both in their usually available form, as well as after oxidation and after oxidation of the hydrolyzed material: Almond (sweet), cottonseed, hydrogenated corn, soya bean, peanut, sesame, tall, olive, linseed and grape seed. Oils of animal origin (commercial red oils) were also studied.

Of the above-mentioned oils only tung oil showed unquestionable activity. The other oils investigated manifested none, or at most minimal activity. Some samples of tung oil showed an activity comparable to the most potent fish oils which we have encountered. Since this oil is readily and cheaply available it may offer a source for obtaining the active material superior to that of marine oils. Unfortunately, the yield was not consistently good from all specimens of oil which were obtained at different times. Moreover, the tendency of tung oil to solidify and to precipitate β-eleostearin on exposure to air and sunlight, renders difficult the extraction of the active principle. The use of the oil directly, except in very small doses, is impossible because of its unsavory taste and toxicity.

In Figure 1 are reproduced typical assays on active preparations of tung oil. As seen in the uppermost curve of this figure, the activity was manifest in the unoxidized oil. It may be assumed that the active principle is either present as such in the fresh oil or was produced during the process of refining. Further oxidation of the oil, as in the case of marine oils (fig. 1), appears to increase the activity further but this is not striking and the increased yield, if any, does not compensate for the technical difficulties induced by the undesirable secondary changes occurring during oxidation.

Although tung oil when administered in the small doses used in these experiments is not obviously toxic to rats, it is conceivable that the observed lowering of the blood pressure is a result of intestinal irritation, or other toxic effect of the oil. The improbability of this conclusion is shown by the third curve of Figure 1, in which a comparable effect on the blood pressure was obtained by administering an aqueous extract derived from tung oil by the procedure described in a subsequent section.

CLINICAL STUDIES. Because of the normally great fluctuations in the blood pressure of the hypertensive patient and the spontaneous drops which occur on prolonged bed rest, the evaluation of the effectiveness of therapeutic agents on the blood pressure is difficult and requires many months of hospitalization under carefully controlled and constant conditions. We have thus far studied only three patients adequately to determine the effects of administering active oxidized oil on the blood pressure. Two other patients were given an aqueous concentrate with results which will be reported elsewhere.

In figure 2 is reproduced the blood pressure curve of a twenty-one year [illegible] male patient suffering from hypertension secondary to chronic glomerulo-nep[illegible]

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

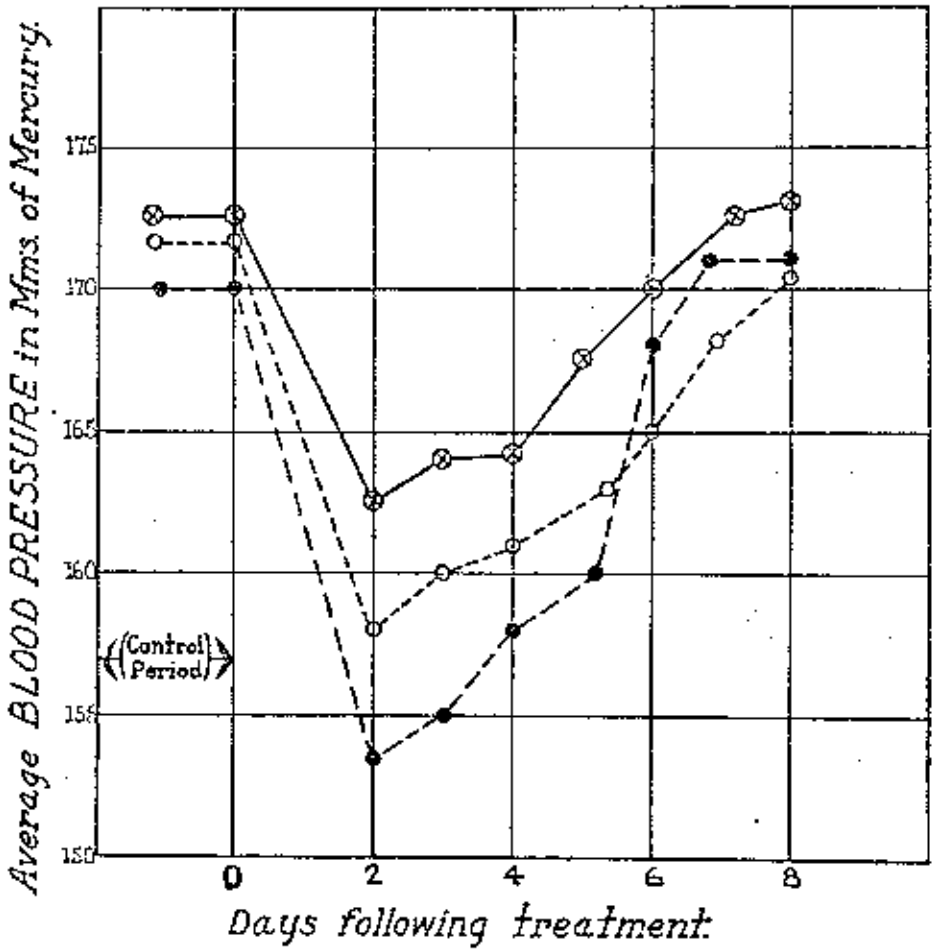

FIG. 1. THE EFFECT OF TUNG OIL ON THE BLOOD PRESSURE OF THE HYPERTENSIVE RAT

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.

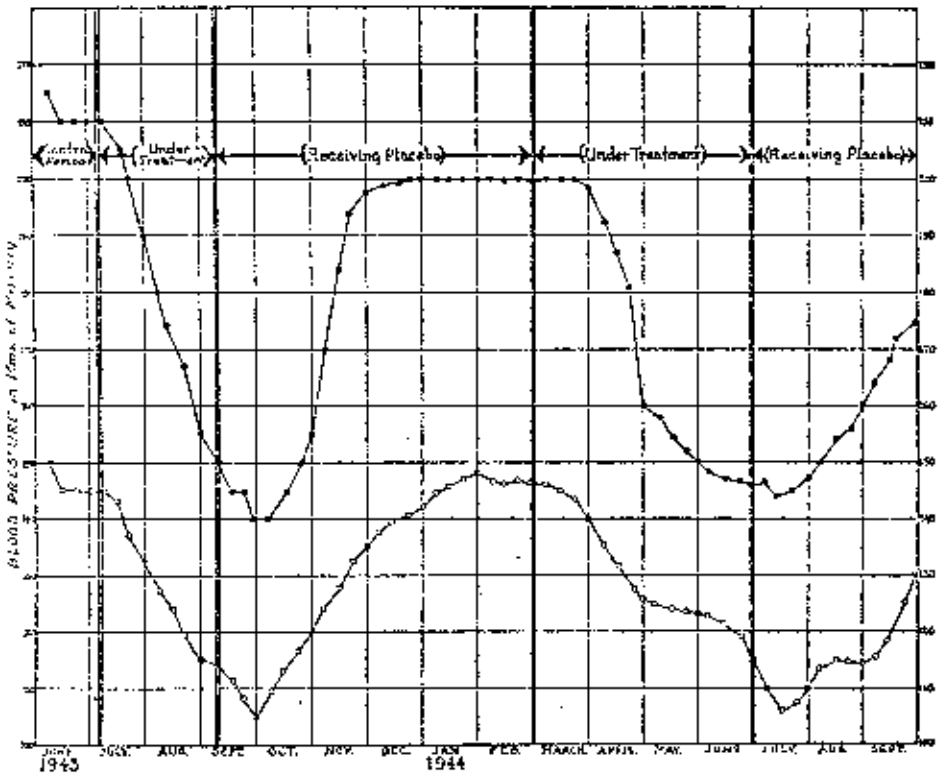

FIG. 2. THE BLOOD PRESSURE OF A HYPERTENSIVE PATIENT TREATED WITH OXIDIZED MARINE OIL

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.

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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

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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How polycyclic hydrocarbons cause cancer

portant as environmental contaminants, some polycyclic hydrocarbons are known to cause cancer in mals and they must therefore be a cancer hazard for man. Many years of research are now culminating to reveal the mechanism by which these chemicals make a normal cell malignant

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Polycyclic hydrocarbons (Figure 1) are relatively inert chemicals. Because they are formed during the partial combustion of organic material, it is not surprising that they occur both as common contaminants of the urban environment and as constituents of tobacco smoke. Following the initial isolation and characterisation of polycyclic hydrocarbons from coal-tar as the first pure cancer-producing chemicals by Ernest Kennaway and his colleagues, working at the Royal Cancer Hospital in London in the 1930s, research workers have been intrigued by this class of carcinogens. They probably represent a very real environmental hazard. Many hundreds of tons of benzopyrene are emitted annually into the atmosphere of a large industrialised country, and cancer can be induced in experimental animals by just a few micrograms of this hydrocarbon. Although it is difficult to obtain direct proof, the polycyclic hydrocarbons are almost certainly carcinogenic in man. For this reason they have come under increasingly intensive scrutiny in recent years and research has now yielded new clues to the way in which they may act.

Following the fundamental work of James and Elizabeth Miller of the McArdle Laboratory, Wisconsin, on the metabolism of the carcinogenic aromatic amines, it became clear that the rather inert but highly carcinogenic polycyclic hydrocarbons also required metabolic activation to exert their biological effects. The Miller's discovered that aromatic amines are metabolised to active electrophilic (positively charged) intermediates capable of reacting with cellular DNA. This activation is now known to occur with polycyclic hydrocarbons, and epoxides have recently been identified as reactive metabolic intermediates derived from the hydrocarbons.

Earlier studies showed that the main metabolites formed from polycyclic hydrocarbons by the drug-metabolising enzymes in the cell's microsomes are hydroxylated derivatives. As long ago as 1950, Professor Eric Boyland of the Chester Beatty Research Institute, London, suggested that epoxides like that shown in Figure 2 are formed in the oxidative metabolism of the individual double bonds of the hydrocarbons. These epoxides could then either rearrange to give phenols, react with water to form dihydrodiols, or react with glutathione to yield conjugates. The idea seemed attractive because these products were the ones that had been found as the main metabolites of the hydrocarbons. The epoxide intermediates were thought to be good candidates, in theory, for the role of the ultimate carcinogenic form of the hydrocarbons.

At the Millers' suggestion, Professor Melvin Newman of Ohio State University succeeded in devising a route for the synthesis of epoxide derivatives on the K-regions of some hydrocarbons. The K-regions are regions of high-electron density associated with the isolated phenanthrenoid double bonds (Figure 1) that have long been suspected of involvement in hydrocarbon carcinogenesis. However, when they were tested in experimental animals, the K-region epoxides turned out, rather disappointingly, to be less active carcinogens than the parent hydrocarbons.

The matter rested there until reapproached by a group led by Dr Peter Sims, again at the Chester Beatty Research Institute. They have recently confirmed that epoxides are formed from hydrocarbons by the action of the microsomal mixed function oxidases present in both animal and human tissues. They have also succeeded in detecting the K-region epoxides of a range of carcinogens, including benzo(a)pyrene and 7, 12- dimethylbenz(a)anthracene in extracts of microsomal incubation mixtures. Sims and his colleagues have synthesised radioactive tritium-labelled epoxides, thus enabling them to demonstrate that K-region epoxides possess properties very relevant to cancer induction. They are electrophilic reagents that react with nucleic acids both in the test tube and in cells in culture. Although the exact sites of nucleic acid alkylation by epoxides have not yet been established, preliminary results suggest that guanine moieties are involved.

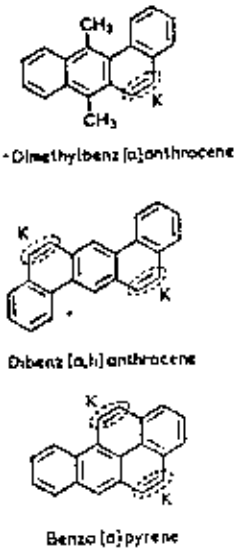

Figure 1 Chemical structures of some carcinogenic hydrocarbons

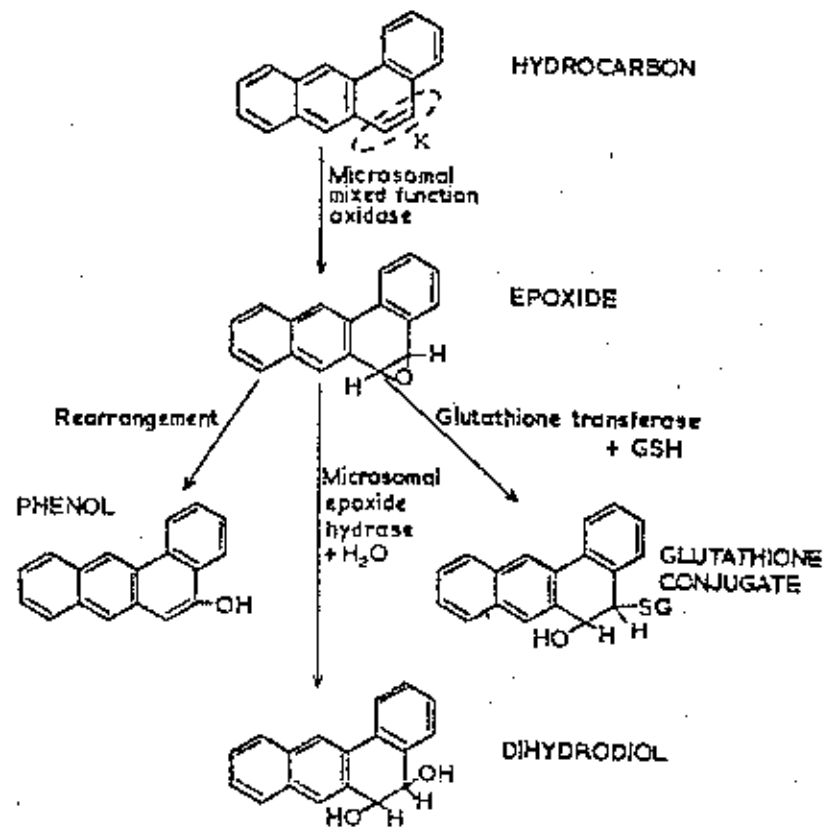

Figure 2 Metabolism at the K-region double bond of benz (a) anthracene

Further evidence that epoxides may be responsible for cancer initiation by polycyclic hydrocarbons comes from collaboration between Sims' group and that headed by Professor Charles Heidelberger of the McArdle Laboratory in Wisconsin. These workers compared the biological activities of epoxides with those of the corresponding hydrocarbons using in vitro transformation systems. In these systems, rodent cells growing in tissue culture are treated with chemicals and the cultures subsequently scored for the number of morphologically abnormal colonies of cells that develop. The transformed colonies are then isolated and tested for malignancy by re-injection into animals. The researchers found that the K-region epoxides are, in general, much more active than the hydrocarbons themselves and that the hydroxylated metabolites are inactive in these systems. At this stage therefore the evidence is that the polycyclic hydrocarbons are metabolised by microsomal enzymes to active epoxides. Although most of the research carried out so far has, for practical reasons, involved K-region epoxides, other double bonds are no doubt oxidised to epoxides in vivo.

The second problem that concerns the whole field of chemical carcinogenesis, and which is quite distinct from the problem of metabolic activation, concerns the mechanism by which chemicals transform normal cells into cancer cells capable of uncontrolled growth. A recent advance, which supports the idea that epoxides are involved in cancer induction and which also throws some light on a possible mutational mechanism for this malignant transformation, has come from the work on the mutagenicity of hydrocarbon derivatives. The somatic mutation theory of cancer —according to which tumours result from genetic changes in the fully differentiated cells of the body—has not been proven. With increased understanding of the mechanisms of metabolic activation, however, closer correlations are now being drawn between the mutagenicity and carcinogenicity of biologically active chemicals. Because the polycyclic hydrocarbon epoxides were found to react with DNA, Heidelberger tested them for mutagenicity. He found them to be active in a system using Chinese hamster cells growing in tissue cultures; he did not characterise the types of mutation induced.

In more specific tests in bacteria, Professor Bruce Ames of the University of California finds that K-region epoxides are potent "frameshift mutagens". These cause the insertion or deletion of one or two bases in the DNA chain, thus throwing the triplet sequence out of order. An early theory of polycyclic hydrocarbon action (which had the dubious advantage that it could neither be proved nor disproved) was based on the ability of the planar hydrocarbon molecules to slot into

DNA between the base-pairs, as shown in Figure 3. People thought that this "intercalation," as it is called, could then give rise to errors in DNA replication. Frameshift mutations are also believed to arise following intercalation of a planar molecule between the base-pairs of DNA. The resultant insertion or deletion of a base in the daughter strand leads to a shift in the reading frame of the triplet codons in the DNA (Figure 4).

In his experiments, Ames found that the hydrocarbons and their hydroxylated derivatives were inactive. He therefore proposed that the mechanism by which the epoxides induced frameshift mutations involved intercalation of an epoxide, followed by a covalent reaction between the epoxide group of the hydrocarbon and a neighbouring nucleophilic centre on a base. Result from work on certain acridine derivatives supports this mutational mechanism. Furthermore Ames, in collaboration with the Millers, has recently shown that reactive derivatives of the carcinogenic aromatic amines also act as frameshift mutagens in bacteria.

The point about frameshift mutations which may have far-reaching implications if they are found to be as common in mammalian cells as they are in bacteria is that, in theory, they can be far more effective biologically than other types of mutation. They can completely inactivate the part of a gene or a series of linked genes subsequent to the site of reaction. The induction of this type of mutation could therefore explain why some polycyclic hydrocarbons are such potent carcinogens, compared with the more aliphatic alkylating agents.

Some of the events that lead to tumour induction by polycyclic hydrocarbons can be predicted on the basis of the incomplete evidence now available. The sequence is likely to involve the uptake of hydrocarbon by a cell and its metabolism by that cell to reactive epoxides which are formed faster than they can be inactivated by further metabolism to hydroxylated derivatives. If, as the evidence suggests, the epoxide then intercalates into and reacts with DNA, this would fix the hydrocarbon molecule in position in the nucleic acid. The reaction with DNA probably needs to occur in a cell which has entered or is about to enter the stage of the cell cycle when DNA is synthesised. Prompt replication of the DNA before the cell's repair enzymes remove the hydrocarbon would then lead to the formation of a daughter strand of DNA carrying the frameshift mutation which would not be detectable as damage by the DNA repair enzymes.

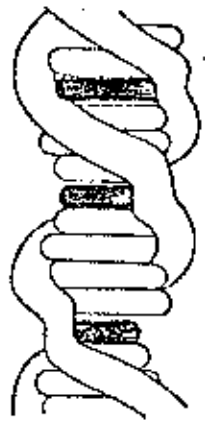

Figure 3 Intercalation of planar molecules (shown in black) into DNA

There seems to be no fundamental reason why mutations induced by chemicals cannot occur in integrated viral genomes. This could perhaps be the mechanism for activating the "oncogenes" that are suggested to exist in otherwise normal cells. Mutagenic chemical compounds may even change an inactive virus into an oncogenic one. On the other hand, if many spontaneous mutations are also of the frameshift type in mammalian cells, then perhaps most cancer results from spontaneous mutations of this type that arise in the absence of specific chemical or viral carcinogens in ageing populations of cells defective in DNA surveillance systems.

The wide range of structure to activity relationships that exist for the polycyclic hydrocarbons, and the relatively simple metabolic transformations they undergo, make them a valuble series of compounds for use in studies of the molecular mechanisms of carcinogenesis. Hopefully, recent progress made in understanding their mode of action will be rapidly extended in the next few years.

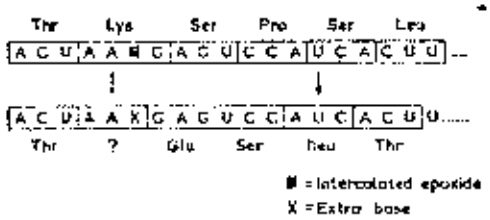

Figure 4 A mechanism involving DNA replication by which a hydrocarbon epoxide may induce a frameshift mutation

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On the Specificity of the Oxygenation of Unsaturated Fatty Acids Catalyzed by Soybean Lipoxidase*

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SUMMARY

The positional and stereochemical specificities of the soybean lipoxidase-catalyzed oxygenation of unsaturated fatty acids have been investigated. It was found that the oxygen function was introduced at position ω6 in all of the fatty acids which reacted. In one case a small amount of the substrate was oxygenated in position ω10.

The structural requirement for substrates is the presence of a *cis,cis*-1,4-pentadiene group with its methylene group located in position ω8. The structure of the hydroperoxy acid formed from 8,11,14-eicosatrienoic acid was 15L-hydroperoxy-8(*cis*),11(*cis*),13(*trans*)-eicosatrienoic acid. By using stereospecifically tritium-labeled 8,11,14-eicosatrienoic acids, it was found that the removal of hydrogen from the ω8 methylene group is stereospecific. Thus, only the hydrogen of the L configuration is removed by the enzyme during the conversion into 15L-hydroperoxy-8(*cis*),11(*cis*),13(*trans*)-eicosatrienoic acid. The conversion of [13-^{3}H,1-^{14}C]8,11,14-eicosatrienoic acid is accompanied by an isotope effect, suggesting that the hydrogen removal occurs as the initial step of the reaction.

The enzyme lipoxidase catalyzes the oxygenation of unsaturated fatty acids by molecular oxygen. The reaction, which seems to be specific for fatty acids possessing methylene group interrupted *cis* double bonds, results in the formation of unsaturated hydroperoxy fatty acids with one pair of conjugated double bonds (1). Earlier studies showed that aerobic incubation of sodium linoleate with lipoxidase yields two isomeric hydroperoxy fatty acids, *viz.* 9-hydroperoxy-10,12-octadecadienoic acid and 13-hydroperoxy-9,11-octadecadienoic acid (2, 3). This finding seemed to indicate that the lipoxidase-catalyzed oxygenation of unsaturated fatty acids is of a relatively unspecific nature; *i.e.* every pair of methylene group interrupted *cis* double bonds should give rise to two isomeric hydroperoxides. According to this view, the number of isomeric hydroperoxy fatty acids should be $2n - 2$, where n equals the number of double bonds in the substrate molecule.

The present study involves characterization of the products formed by the action of soybean lipoxidase on a large number of unsaturated fatty acids. The results show that the enzyme-catalyzed oxygenation is very specific with respect to the position in the fatty acid that is oxygenated. It was also found, by using naturally occurring and synthetic fatty acid analogues, that the *cis,cis*-1,4-pentadiene grouping of the fatty acid, which was previously considered to be the only structural requirement (1), must be located with its methylene group in position ω8 in order to secure reaction. We have studied the stereochemical features of the reaction by determining the absolute configuration of the carbon atom bearing the hydroperoxy group and the stereochemistry of the removal of the hydrogen from the methylene group. Data on isotope discrimination in the reaction also provide information on the initial step of the transformation.

Part of this investigation has been published previously in preliminary form.[1]

MATERIALS AND METHODS

Lipoxidase. Crystalline soybean lipoxidase (20,000 units per mg) purchased from Fluka was used.

Unsaturated Fatty Acids. The substrates were purified by silicic acid chromatography prior to the incubations. The purity was checked by gas liquid chromatography on a polyester column (8%) ethylene glycol succinate S-X supported on Gas-Chrom Pt. The following fatty acids were gifts from the sources indicated: 15-methyl-8,11,14-eicosatrienoic acid from Dr. J. E. Pike, the Upjohn Company; 5,8,11-eicosatrienoic acid from Professor W. Stoffel, Cologne; 8,11-eicosatrienoic acid from Dr. D. A. van Dorp, Unilever, Vlaardingen; and 8,11,14-docosatrienoic acid, 9,12,15-heneicosatrienoic acid, and 9,12,15-tricosatrienoic acid from Dr. D. Kienberg, Karolinska Institutet, Stockholm.[2]

Incubation Procedure. Incubations on a preparative scale were performed at 0° and pH 9.0. The substrates (4 mg [illegible]

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[1] *Biochem. Biophys. Res. Commun.*, 21, 531 (1965).
[2] D. Kienberg, unpublished results.

converted into their ammonium salts by the addition of NH_4OH (0.05 M, 1 ml). The enzyme was dissolved in a buffer solution (1 mg of lipoxidase per ml of 0.1 M sodium borate buffer, pH 9.0). Incubations were started by the addition of 2.5 ml of enzyme solution. After 15 min an additional 0.5 ml of enzyme solution was added. Measurements of the absorbance at 234 mμ of aliquots of the incubation mixture removed every 5 min showed that the conversion was almost complete after 30 min. After this period of incubation, 15 ml of ethanol were added. The solution was immediately diluted with water, acidified to pH 3, and extracted with ether. The ether was washed with water until the reaction was neutral, and was evaporated at room temperature under reduced pressure. Incubations were also performed on an analytical scale at room temperature. The substrates (10 to 50 μg) were transferred into quartz cuvettes and were converted into their ammonium salts by the addition of NH_4OH. After addition of 2 ml of borate buffer, pH 9.0, the incubations were started by the addition of enzyme solution. The absorption at 234 mμ was followed with a Zeiss PMQ II spectrophotometer.

Reduction with Sodium Borohydride—The compounds (4 to 100 mg) were dissolved in methanol (1 to 10 ml), cooled to 0°, and reduced with sodium borohydride (10 to 150 mg). After 20 min at 0° and 10 min at room temperature, the solution was acidified and extracted with ether.

Catalytic Hydrogenation—For complete hydrogenation, the unsaturated compounds (1 to 4 mg) were dissolved in 2 ml of ethanol and were hydrogenated under a stream of hydrogen gas with 10 mg of PtO₂ as catalyst. For selective hydrogenation, the compounds (10 to 20 mg) were dissolved in 2 ml of methanol, and 50 mg of 5% Pd/BaSO₄ catalyst and 50 μl of quinoline were added. The solution was stirred under a stream of hydrogen gas.

Oxidation with Chromic Acid—The compounds (1 to 3 mg) were dissolved in 1 ml of glacial acetic acid and added to 1 ml of 95% acetic acid containing 3.4 mg of CrO_3. After 30 min at 35°, methanol was added. The solution was acidified and extracted with ether.

Oxidative Cleavage—Prior to ozonolysis, the unsaturated hydroxy acids were esterified by treatment with diazomethane and acetylated with acetic anhydride in pyridine. These derivatives (2 to 5 mg) were dissolved in 4 ml of chloroform, cooled to −45°, and treated with an excess of ozone. After 10 min at room temperature, the chloroform was evaporated at reduced pressure. The ozonides were oxidized with 0.2 ml of 30% H_2O_2 in 1 ml of glacial acetic acid. After 18 hours at 50°, the solution was taken almost to dryness at 40° under reduced pressure.

Mass Spectrometry—Mass spectrometry was performed in combination with gas chromatography as described by Hvinge (2) and the LKB 9000 instrument. A column of 1% SE 30 on Chromosorb P, operated at 160–190° at a pressure of 3 kg per cm², was used. In the case the samples were introduced through the direct inlet of the mass spectrometer. This was necessary for the analysis of hydroxy esters, which could not be passed through the gas chromatograph without degradation.

Chromatographic Methods—Silica acid chromatography was carried out on silica acid (Merck, 100 mesh) activated at 115°. The columns were eluted with increasing concentrations of ether in benzene.

Two solvent systems were used for thin layer chromatography. Glass plates coated with Silica Gel G were developed with ether-pentane, 50:50 (5). For the development of glass plates coated with Silica Gel G-silver nitrate (25:1), the organic layer of the following system was used: ethyl acetate-2,2,4-trimethylpentane-water (75:25:100). The compounds were detected with an ultraviolet lamp after spraying with 2′,7′-dichlorofluorescein.

Reversed phase partition chromatography was carried out as described by Norman and Sjovall (6). The solvent systems used were methanol-water, 120:171 (moving phase), and chloroform-isoamyl alcohol, 15:15 (stationary phase).

Gas-liquid chromatography was performed with an F and M Biomedical gas chromatograph, model 400. Part of the effluent was led into an ionization chamber via a stream splitter, permitting continuous measurement of radioactivity in combination with gas chromatography. The phases used were silicone grease (15% on 100 to 120 mesh Celite), SE 30 (1% on Gas-Chrom P), and ethylene glycol succinate 8-X (8% on Gas Chrom P).

Measurement of Radioactivity—A Frieseke Hoepfner FH 570 gas flow counter operated in the proportional range was used for the assay of radioactivity in chromatographic fractions. A Packard Tri-Carb model 3322 liquid scintillation counter was used for the determination of the $^3H/^{14}C$ ratio.

Stereospecifically Tritium-labeled Fatty Acids—The preparations of [15-^{3}H,3-^{14}C]S,11,14-eicosatrienoic acid and [15-^{3}H,3-^{14}C]S,11,14-docosatrienoic acid are described in an accompanying report (7). The reactions used for the preparation of [15-^{3}H,3-^{14}C]S,11,14-eicosatrienoic acid are also described in an accompanying report (8).

Method Used for Analysis of Products—The substrate specificity of the enzyme was examined by incubating fatty acids differing in chain length and in location and degree of unsaturation. The hydroperoxy acids were reduced with sodium borohydride, yielding the hydroxy acids. These were subjected to silicic acid chromatography. The unsaturated hydroxy acids (eluted with ether-benzene, 2:98) were subjected to catalytic hydrogenation. The saturated hydroxy acids were esterified by treatment with diazomethane and were subsequently oxidized with chromic acid, yielding methyl esters of saturated keto acids. The position of the keto group of the esters could be determined by mass spectrometry in combination with gas chromatography. Since there is no rearrangement of the hydroperoxy groups, the position of the keto group in the keto esters is the same as of the hydroperoxy group initially introduced by the enzyme. In the case where mass spectrometry indicated the occurrence of two isomeric keto esters giving one single peak on gas-liquid chromatography; *cf.* Reference 10, the two corresponding isomeric hydroxy esters were separated by preparative thin layer chromatography on silica gel not containing silver nitrate. The compounds were scraped off, eluted from the silica gel, and subjected to gas-liquid chromatography. This procedure permitted determination of the percentage composition of the hydroxy ester mixture.

RESULTS

Positional Specificity—A series of 14 unsaturated fatty acids was incubated with lipoxidase, and the position of the hydroperoxy group in the products was determined as described above. The results are summarized in Table I. The soy-

spectra recorded for the keto esters derived from the products of 9,12-octadecadienoic acid, 8,11,14-eicosatrienoic acid, and 4,7,10,13,16,19-docosahexaenoic acid are given in Figs. 1, 2, and 3, respectively. For comparison the mass spectra of methyl 9-ketostearate, methyl 13-ketostearate, and methyl 11-ketoarachidate are given in Figs. 4, 5, and 6, respectively. The mass spectrum of the keto esters derived from 9,12-octadecadienoic acid (Fig. 1) obviously represents a mixture of methyl 9-ketostearate and methyl 13-ketostearate. The following ions are due to methyl 9-ketostearate: *m/e* 281 (M − 31), 200 (M − 112); β cleavage with loss of $-CH_2-CH=(CH_2)_8-CH_3$, 185 (M − 127); α cleavage with loss of $-(CH_2)_8-CH_3$, 170 (M − 142); α cleavage with loss of $CH_2=CH-(CH_2)_6-COOCH_3$, and 168 (M − 144; 200 − 32). The ions formed from methyl 13-ketostearate are 281 (M − 31), 256 (M − 56); β cleavage with loss of $CH_2=CH-CH_2-CH_3$, 241 (M − 71); α cleavage with loss of $-(CH_2)_8-CH_3$, and 199 (M − 113); β cleavage with loss of $-CH_2-\underset{\underset{O}{\|}}{C}-(CH_2)_8-CH_3$. The relative amounts of the two isomers were determined by thin layer chromatographic separation (silver, ether-pentane) of the two saturated hydroxy esters and subsequent quantitative determination by gas-liquid chromatography on SE-30.

The mass spectrum of the keto ester derived from 8,11,14-eicosatrienoic acid (Fig. 2) shows ions at *m/e* 369 (M − 31), 284 (M − 56); β cleavage with loss of $-CH_2-CH=CH_2-CH_3$, 263 (M − 71); α cleavage with loss of $-(CH_2)_8-CH_3$, 252 (M − 88; 284 − 32), 237 (M − 103; 269 − 32), 227 (M − 113); β cleavage with loss of $-CH_2-\underset{\underset{O}{\|}}{C}-(CH_2)_8-CH_3$, and 195 ($M$ − 145; 227 − 32). By comparing this mass spectrum with the mass spectrum of methyl 11-ketoarachidate (prepared as described in an accompanying report [8]), the absence of the 11-keto isomer could be established. The mass spectrum of the latter compound, given in Fig. 6, shows ions at *m/e* 369 (M − 31), 228 (M − 112); β cleavage with loss of $CH_2=CH-(CH_2)_8-CH_3$, 213 (M − 127); α cleavage with loss of $-(CH_2)_8-CH_3$, 196 (M − 144; 228 − 32), 174 (M − 169); β cleavage with loss of $-CH_2-\underset{\underset{O}{\|}}{C}-(CH_2)_8-CH_3$, 170 ($M$ − 170); β cleavage with loss of $CH_2=CH-(CH_2)_6-COOCH_3$, and 155 (M − 185); α cleavage with loss of $-(CH_2)_6-COOCH_3$. These ions either are absent in the mass spectrum of the keto ester derived from incubated 8,11,14-eicosatrienoic acid, or, if present, can be ascribed to methyl 15-ketoarachidate. The absence of the 8- and 12-keto isomers was established similarly by the absence of ions which, by applying the same type of fragmentation as described above,

TABLE 1

Structures of keto esters derived from products obtained on incubation of unsaturated fatty acids with soybean lipoxidase

After an incubation period of 30 min, ethanol was added, and the solution was acidified and extracted with ether. The product obtained after evaporation of the ether was reduced with $NaBH_4$ and subjected to silicic acid chromatography. The isolated hydroxy acids were hydrogenated and esterified by treatment with diazomethane. Oxidation with chromic acid yielded saturated keto esters, the structures of which were determined by mass spectrometry.

Substrate	Saturated keto ester derived from products
9,12-Octadecadienoic acid	Methyl 13-ketostearate (2 %)
	Methyl 9-ketostearate (8 %)
9,12,15-Octadecatrienoic acid	Methyl 13-ketostearate
9,12-Octadecatrienoic acid	Methyl 13-ketostearate
8,11,14-Eicosatrienoic acid	Methyl 15-ketoarachidate
8,11,14-Eicosatrienoic acid	Methyl 15-ketoarachidate
8,11,14,17-Eicosapentaenoic acid	Methyl 15-ketoarachidate
9,12,15-Heneicosatrienoic acid	Methyl 16-ketoheneicosanate
10,12,16-Docosatrienoic acid	Methyl 17-ketodocosanate
4,7,10,13,16,19-Docosahexaenoic acid	Methyl 17-ketodocosanate

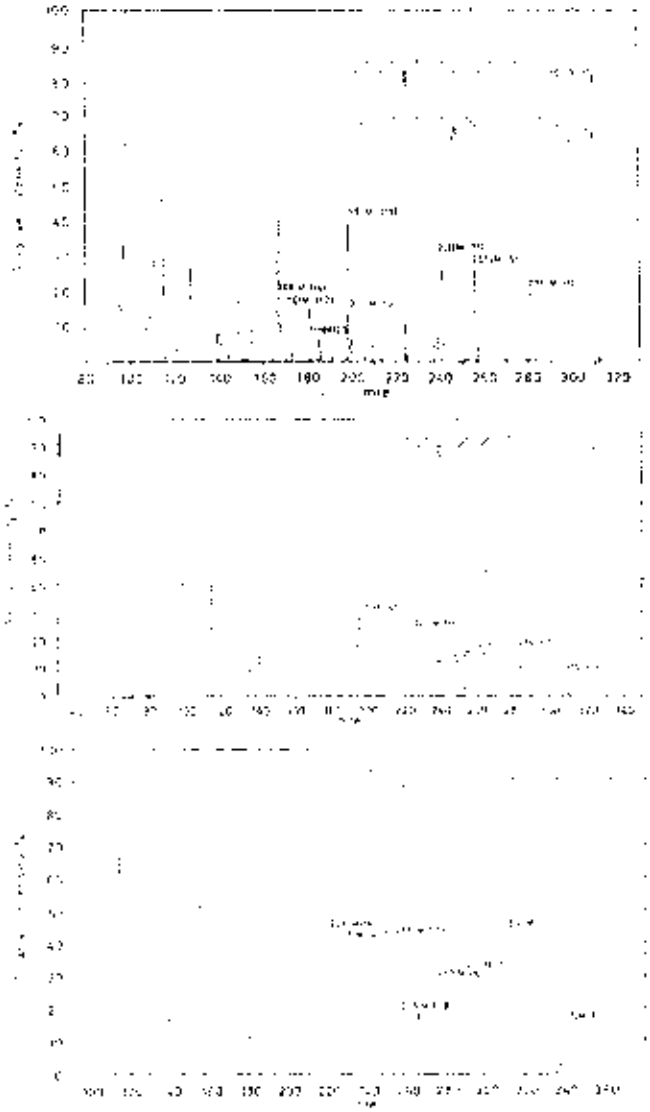


FIG. 1. Mass spectrum recorded for the keto esters derived from 9,12-octadecadienoic acid.

FIG. 2. Mass spectrum recorded for the keto ester derived from 8,11,14-eicosatrienoic acid.

FIG. 3. Mass spectrum recorded for the keto ester derived from 4,7,10,13,16,19-docosahexaenoic acid.

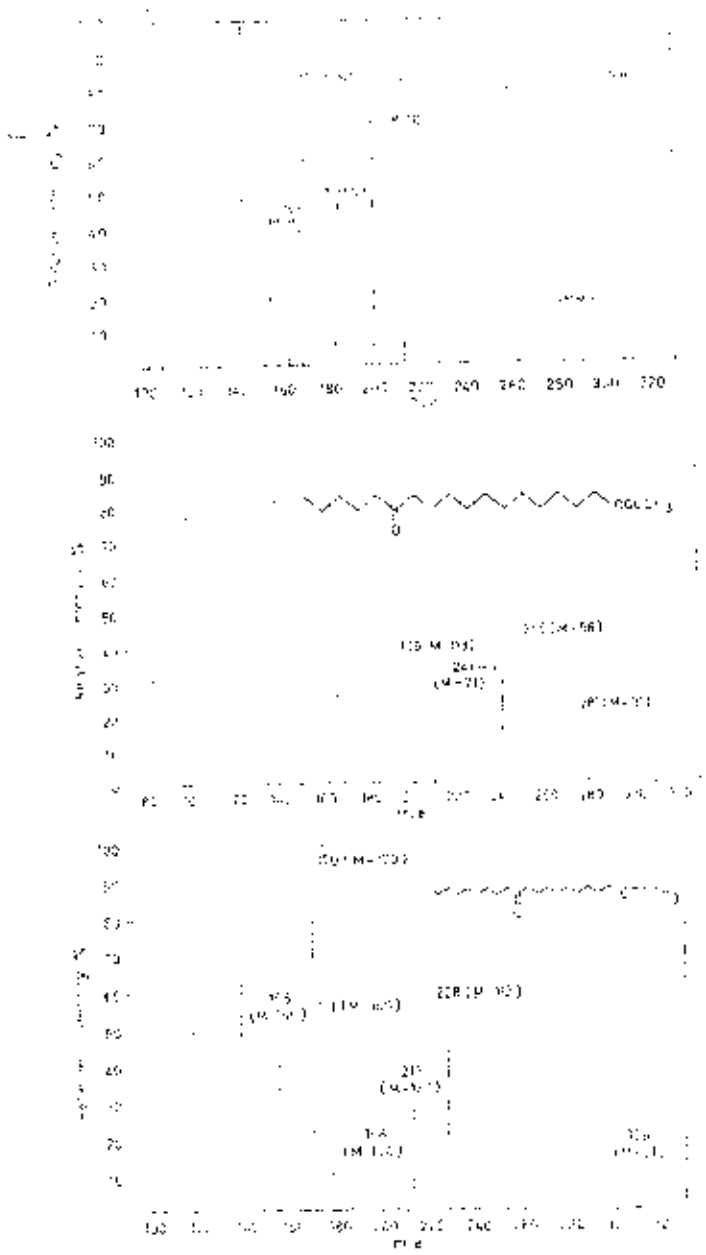


FIG. 4. Mass spectrum of methyl 9-ketostearate
FIG. 5. Mass spectrum of methyl 13-ketostearate
FIG. 6. Mass spectrum of methyl 11-ketoarachidate

could be predicted to be specific for these keto esters (10). The mass spectrum of the keto ester derived from the product obtained by the action of lipoxidase on 8,11,14-eicosatrienoic acid is thus in accordance only with that of methyl 15-ketoarachidate.

Fig. 3 shows the mass spectrum of the keto ester prepared from the product of 4,7,10,13,16,19-decosahexaenoic acid. The identification of the keto ester as methyl 17-ketodecosanate is based on the mass spectrum which showed ions at m/e 337 (M − 31), 312 (M − 56), α cleavage with loss of $CH_3-CH_2-CH_2$, 296 (M − 71), α cleavage with loss of $-(CH_2)_4-CH_3$, 280 (M − 88, 312 − 32), 265 (M − 103, 296 − 32), 255 (M − 113); β cleavage with loss of $-CH_2-\underset{\underset{O}{\|}}{C}-(CH_2)_4-CH_3$, 223 ($M$ − 145; 255 − 32), and 222 (M − 146; β cleavage with loss of $CH_2=C-(CH_2)_4-CH_3$ plus CH_3OH).

OH

In one case, viz. the product from 8,11,14-eicosatrienoic acid, more complete structural work was performed. Oxidative ozonolysis of the methyl ester and acetate of the compound formed by sodium borohydride reduction of the hydroperoxy acid yielded two short chain acids. These were identified, after esterification by treatment with diazomethane with the mass spectrometry in combination with gas chromatography, as methyl 2-acetoxyheptanoate and dimethyl subcrate (the mass spectra of the compounds were identical with those of the authentic compounds). Therefore, the parent hydroperoxy acid was 15-hydroperoxy-8,11,13-eicosatrienoic acid. This structure is independently supported by the structure of the derived saturated keto ester, methyl 15-ketoarachidate. The presence of one pair of conjugated double bonds is in agreement with the presence of a strong absorption band with $\lambda_{max}^{ethanol}$ 2.5 mμ. The molecular extinction coefficient was 30,000 at this wave length. This value is somewhat higher than that reported earlier, viz. 28,000 (11).

The geometrical configuration of the double bonds of 15-hydroperoxy-8,11,13-eicosatrienoic acid has also been determined. Selective hydrogenation of the Δ^{11} double bond of methyl 15-hydroxy-8,11,13-eicosatrienoate yielded methyl 15-hydroxy-8,13-eicosadienoate. This compound was isolated by preparative thin layer chromatography on silica gel containing silver nitrate. The structure of the compound was determined by oxidative ozonolysis performed on the acetate of the compound. Gas-liquid chromatographic analysis of the ozonolysis product treated with diazomethane showed the presence of three main compounds. These were identified, by means of gas-liquid chromatography and mass spectrometry, as dimethyl glutarate, methyl 2-acetoxyheptanoate, and dimethyl subcrate. The infrared spectrum of methyl 15-hydroxy-8,11,13-eicosatrienoate showed absorption bands at 10.13 and 10.50 μ. The presence of these bands indicates that the two conjugated double bonds of methyl 15-hydroxy-8,11,13-eicosatrienoate are *cis-trans* (12). Since there was no absorption at 10.3 μ, the Δ^{11} double bond is *cis*. The infrared spectrum recorded for methyl 15-hydroxy-8,13-eicosadienoate showed an absorption band at 10.34 μ, suggesting the presence of an isolated *trans* double bond. This is therefore the Δ^{11} double bond. The structure of the hydroperoxy acid formed on incubation of 8,11,14-eicosatrienoic acid with lipoxidase is therefore 15-hydroperoxy-8(*cis*), 11(*cis*), 13(*trans*)-eicosatrienoic acid. The identity of this acid with a hydroperoxy acid was confirmed by mass spectrometry of the methyl ester of the compound. The mass spectrum showed ions of high intensity at m/e 334 (M − 18; loss of H_2O) and 318 (M − 34; loss of H_2O_2). Ions of lower intensity were present at m/e 335 (M − 17; loss of $-OH$), 332 (M − 20), 319 (M − 33; loss of $-OOH$), 303 (M − 49; loss of H_2O + $-OCH_3$), and 287 (M − 65; loss of H_2O + $-OCH_3$).

The complete structures of the hydroperoxy fatty acids obtained on incubation of the remainder of the substrates with lipoxidase have not been determined. However, there seems to be no reason why the general mechanism of lipoxidase ac-

tolysis involving isomerization of one double bond should not hold true for the eicosatrienoates also.

Absolute Configuration of Methyl 13-Hydroxystearate and 2-Hydroxyheptanoic Acid Derived from 9,12-Octadecadienoic Acid—The product obtained on a large scale incubation of 9,12-octadecadienoic acid with lipoxidase was subjected to catalytic hydrogenation. The hydroxy acid fraction obtained on silicic acid chromatography was esterified by treatment with diazomethane and was subjected to preparative thin layer chromatography on silica gel not containing $AgNO_3$. Material obtained on elution of the silica gel was subjected to silicic acid chromatography and was subsequently crystallized from petroleum, yielding methyl 13-hydroxystearate. The purity of the compound was checked by gas-liquid chromatography and analysis of thin layer chromatography. The compound exhibited a plain positive optical rotatory dispersion curve, $[\alpha]_{589}$ +0.3, $[\alpha]_{300}$ +2.3 (c, 5.0, in methanol). Methyl 13-hydroxystearate derived from ricinoleic acid gives a plain negative optical rotatory dispersion curve $[\alpha]_{589}$ −0.93 (c, 2.5, in methanol) (13) and has been used for the D configuration by comparison of the optical rotatory dispersion spectrum with that of methyl 12D-hydroxystearate. Therefore, methyl 13-hydroxystearate derived from the product obtained on incubation of 9,12-octadecadienoic acid with lipoxidase should have the L configuration. Additional evidence on the stereochemistry was furnished by the optical rotation of 2-hydroxyheptanoic acid obtained on oxidative ozonolysis of the unsaturated hydroxy ester fraction formed from 9,12-octadecadienoic acid. The optical rotatory dispersion spectrum $[\alpha]_{589}$ −1.38, $[\alpha]_{300}$ +3.50 (c, 1.15, in methanol)) shows a positive Cotton effect (peak at 226 mμ, $[\alpha]_{226}$ +13.10 (c, 1.15, in methanol)). The optical rotatory dispersion spectrum of 2-hydroxyoctanoic acid showed a negative Cotton effect at 227 mμ. The optical rotation of the obtained 2-hydroxyheptanoic acid was also measured on the sodium salt of the compound: $[\alpha]_{589}$ −9.0 (c, 0.6, in N NaOH). According to Baker and Mislow (14), the acid then has the L configuration (*cf.* also Reference 15).

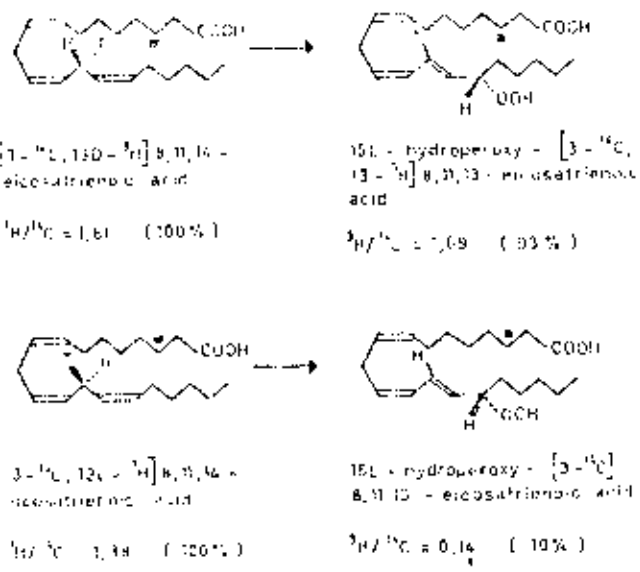


FIG. 7. Stereochemistry of hydrogen removal in the conversion 8,11,14-eicosatrienoic acid into 15L-hydroperoxy-8cis,11trans,13trans-eicosatrienoic acid. The labeled acids (250,000 cpm/mmol) were dissolved in 50 μl of acetone, and 0.1 ml of 1 M NaHCO3 was added. After cooling to 0°, 1 ml of 0.1 M borate buffer, pH 9.0, containing 75 μg of lipoxidase was added. After an incubation of 30 min at 0°, conversion of the substrate was checked, ethanol was added, and the solution was acidified and extracted with ether. The lower 1/10 of the extract was removed with 0.1% Ag^+. The 15-hydroxy-8,11,13-eicosatrienoic acid was purified by silicic acid chromatography, treated with diazomethane, and applied to preparative thin layer chromatography on silica gel containing $AgNO_3$.

TABLE II

Enrichment of tritium in percentage during conversion of [13α-^{3}H,3-^{14}C]8,11,14-eicosatrienoic acid into [3-^{14}C]15L-hydroperoxy-8(cis),11(cis),13(trans)-eicosatrienoic acid

The precursor, 1.1 mg (700,000 cpm, ^{3}H/^{14}C = 1.35), was dissolved in 50 μl of acetone, and 0.3 ml of 0.1 M NaHCO3 was added. After cooling to 0°, 1 ml of 0.1 M borate buffer, pH 9.0, containing 75 μg of lipoxidase, was added. The incubation mixture was kept at 0° for 25 min. At the times indicated, 0.2 ml was withdrawn and added to 2 ml of ethanol. Sodium borohydride, 20 mg, was added to each sample. Remaining 8,11,14-eicosatrienoic acid was isolated by silicic acid chromatography (eluted with ether–hexane, 5:95), and the ^{3}H/^{14}C ratio was determined. Every 5 min, 30 μl of the incubation mixture were withdrawn and added to 2 ml of ethanol. By using an extinction coefficient of 30,000 for the hydroperoxy acid, the percentage conversions of the precursor could be determined.

Reaction time	Conversion	^{3}H/^{14}C in remaining 8,11,14-eicosatrienoic acid
min	%	
2	20	1.51
6	46	2.03
11	59	2.74
15	69	3.44
25	84	4.61

Incubation of [15-^{3}H,3-^{14}C]8,11,14-Eicosatrienoic Acid—The labeled acids (250,000 cpm/mmol) were dissolved in 50 μl of acetone, and 0.1 ml of 1 M NaHCO3 was added. After cooling to 0°, 1 ml of 0.1 M borate buffer, pH 9.0, containing 75 μg of lipoxidase was added. After an incubation of 30 min at 0°, conversion of the substrate was checked, ethanol was added, and the solution was acidified and extracted with ether. The lower 1/10 of the extract was removed with 0.1% Ag^+. The 15-hydroxy-8,11,13-eicosatrienoic acid was purified by silicic acid chromatography, treated with diazomethane, and applied to preparative thin layer chromatography on silica gel containing $AgNO_3$.

Stereochemistry of Reaction—The absolute configuration at C-15 of 15-hydroperoxy-8,11,13-eicosatrienoic acid has been determined. The products obtained on oxidative ozonolysis of 130 mg of methyl 15-acetoxy-8,11,13-eicosatrienoate were subjected to reversed phase partition chromatography. Treatment of the isolated 2-acetoxyheptanoic acid with 0.1 N NaOH for 18 hours at room temperature yielded 18 mg of 2-hydroxyheptanoic acid. After neutralization with NaOH, the optical rotation of the sodium salt was measured. The experimentally found optical rotatory power (corrected to $[\alpha]_{589}$ −9.0 (c, 0.6, in N NaOH)). According to Baker and Mislow (14), the acid then has the L configuration. The complete structure of the hydroperoxy acid is therefore 15L-hydroperoxy-8cis,11trans,13trans-eicosatrienoic acid.

The absolute configuration of the hydrogen removed from C-13 of 8,11,14-eicosatrienoic acid has also been determined. Incubations were performed with [13α-^{3}H,3-^{14}C]8,11,14-eicosatrienoic acid and [13β-^{3}H,3-^{14}C]8,11,14-eicosatrienoic acid. The ^{3}H/^{14}C ratios of the derived hydroxy methyl esters were determined. Fig. 7 shows the percentage retentions of tritium in the products relative to the precursors. These experiments indicate that the 13β hydrogen is selectively removed from the methylene group at C-13, whereas the 13α hydrogen is retained. This removal is accompanied by a pronounced isotope effect. The magnitude of the isotope effect is shown in Table II.

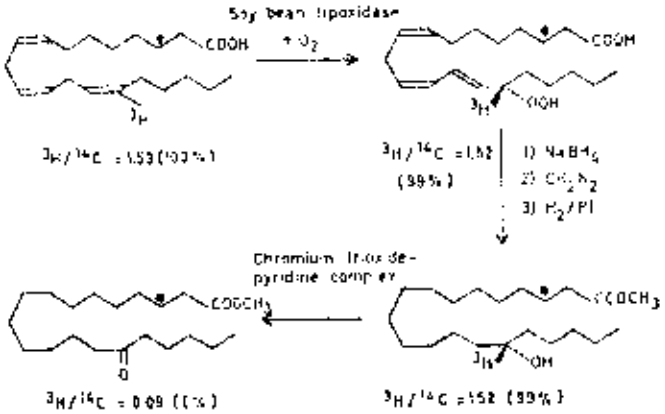
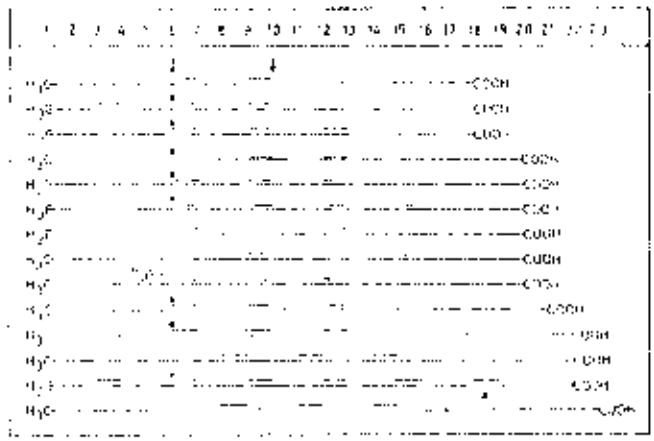


FIG. 8. Incubation of [15-^{3}H,3-^{14}C]8,11,14-eicosatrienoic acid with lipoxidase. The labeled acid (10^6 cpm, 0.5 mg) was dissolved in 50 µl of acetone and 0.5 mg of 0.1 M NH_4OH was added. Lipoxidase, 0.1 mg, dissolved in 2 ml of borate buffer, pH 9.0, was added. After 30 min at 0°, the solution was acidified and extracted with ether. The ether was washed until neutral and was evaporated. The labeled 15-hydroperoxy-8,11,14(*cis*),13(*trans*)-eicosatrienoic acid was isolated by silicic acid chromatography. Part of the material was dissolved in scintillation and $^3H/^{14}C$ was determined. The remaining material was reduced with $NaBH_4$, esterified by treatment with diazomethane, and hydrogenated. Labeled methyl 15-hydroxyarachidate was obtained after silicic acid chromatography of the product. Again, part of the material was treated with CH_2N_2. The remaining material was oxidized with chromium trioxide pyridine complex (15). Methyl 15-ketoarachidate was isolated by silicic acid chromatography and $^3H/^{14}C$ was determined.

TABLE III

Positional specificity of soybean lipoxidase-catalyzed oxygenation

The positions of the oxygens introduced are indicated by *arrows*. The carbon of the methyl group of the fatty acids is denoted as ω1.



late of the hydrogen at C-15 of 8,11,14-eicosatrienoic acid during the conversion into 15-hydroperoxy-8,11,13-eicosatrienoic acid seemed to be of interest since a carbon-oxygen bond is created at C-15. Therefore [15-^{3}H,3-^{14}C]-8,11,14-eicosatrienoic acid (8) was incubated with lipoxidase and the 15-hydroperoxy-8,11,13-eicosatrienoic acid formed was isolated by silicic acid chromatography. As shown in Fig. 8, the tritium label is retained in 15-hydroperoxy-8,11,13-eicosatrienoic acid and in methyl 15-hydroxyarachidate. However, the tritium label is lost in methyl 15-ketoarachidate. Thus the hydrogen at C-15 of 8,11,14-eicosatrienoic acid remains in the same position during the conversion into 15-hydroperoxy-8,11,13-eicosatrienoic acid.

DISCUSSION

In the present investigation, the products formed by the action of soybean lipoxidase on a number of unsaturated fatty acids have been analyzed. The procedure involved conversion of the hydroperoxy acids into saturated keto esters, followed by characterization by mass spectrometry. This method permitted determination of the positions of the keto groups in the derivatives and thus the location of the hydroperoxy groups in the enzymatic products. These studies revealed a very high degree of specificity in the oxygenation with respect to the position attacked.

The results summarized in Table III show that in all of the acids that reacted, oxygen was introduced at the carbon in position ω6. In the case of 9,12-octadecadienoic acid, however, a small amount of the substrate was oxygenated in position ω10. These findings seemed to indicate that the structural requirement for substrates in the lipoxidase-catalyzed oxygenation is the presence of a *cis,cis*-1,4-pentadiene group with its methylene group located in position ω8.

In order to test this hypothesis further, acids with the methylene group of the pentadiene structure in positions ω10 (8,11,14-eicosatrienoic acid and 9,12,15-eicosatrienoic acid) or ω11 (5,8,11-eicosatrienoic acid) were tested. None of these acids reacted, nor did 15-methyl-8,11,14-eicosatrienoic acid, which contains a methyl group on the double bonded carbon atom which is oxygenated in the unsubstituted acid (Fig. 9).

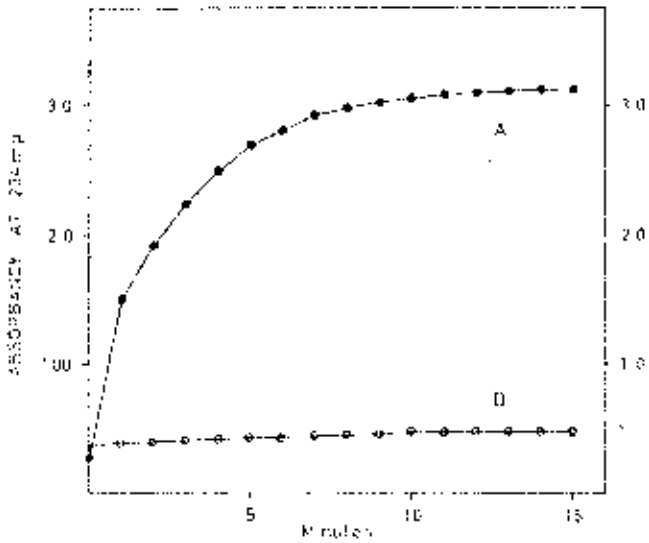


FIG. 9. Incubation of 8,11,14-eicosatrienoic acid (A) and 8,11,14-eicosatrienoic acid (B) with lipoxidase at room temperature. The fatty acids, 50 µg, were transferred to quartz cuvettes, and 1 ml of 0.029 M NH_4OH and 1 ml of borate buffer, pH 9.0, were added. Lipoxidase, 2.5 µg dissolved in 50 µl of borate buffer, was added to the cuvettes, and the solutions were stirred for a few seconds with a glass bar. The black cuvette contained 2 µl of NH_4OH, 1 ml of borate buffer, and 2.5 µg of lipoxidase. The absorption at 234 mμ was determined every minute for 15 min.

The structure of 15s-hydroperoxy-*cis*,11,*trans*,13-eicosadienoic acid was established by mass spectrometric analysis of the derived keto ester, oxidative ozonolysis, and partial hydrogenation, as well as by ultraviolet and infrared spectroscopy. The determination of the geometrical configuration of the double bonds is noteworthy, since it has earlier been assumed but never been shown that the 13-conjugated double bond has the *trans* configuration. By determining the optical rotation of 2-hydroxyheptanoic acid formed on oxidative ozonolysis, it could be shown that C 15 has the L configuration. Prostaglandin E_2, which is formed from 8,11,14-eicosatrienoic acid in a reaction having many features in common with the lipoxidase-catalyzed oxygenation (7), has also the L configuration at C 15 (17).

Optical rotatory dispersion spectroscopy performed on methyl 13-hydroxyoctadecadienoate and 2-hydroxyheptanoic acid, both derived from the product formed on incubation of 9,12-octadecadienoic acid with lipoxidase, showed that the two acids, and therefore also the parent hydroperoxy acid, belonged to the L series. It is interesting that C 13 of 13-hydroxy-*cis*,11,*trans*-octadecadienoic acid recently isolated from *Calendula officinalis* Will. seed oil was shown to have the L configuration (18).

The stereochemistry of the hydrogen removed has been studied with 8,11,14-eicosatrienoic acid as substrate. In this acid the transformation involves elimination of hydrogen from C 13 and introduction of oxygen at C 15. The stereochemistry of elimination of hydrogen was determined by using [13s-H, 3-^{3}C]- and [13r-H, 3-^{3}C]8,11,14-eicosatrienoic acids. These acids were found to be stereospecifically deuterated in the 13-position and was determined to be the D-form of the H-label, whereas the 13s-H-label was found in the 15-OH. The isotope data also showed that no isotope effect in the removal of the 13-H was found. Since no deuterium of 13-H was found in the prostaglandin acid, the loss of hydrogen from C 13 and its stereospecific initial change to the proxyl, or free radical form, has been related to a bond between the substrate and oxygen. After the 13s-H hydrogen has been eliminated, π electrons of the 8,9 and 10,11 double bonds are redistributed to form the conjugated pentadienyl free radical. The biradical oxygen is then introduced stereospecifically, and the proxyl radical finally recaptures a hydrogen atom. The hydrogen attached to the carbon atom which is attacked by oxygen retains in the same position in the hydroperoxy acid.

Acknowledgments.—We wish to thank Dr. R. Ryhage for his help with the mass spectrometric analyses. We also wish to thank Mrs. C. Rignell for her capable technical assistance.

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Oil and Soap 20: 133-134, 1943

Detection and Estimation of Dipalmito and Distearo Glycerides in Hydrogenated Oils

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Introduction

The glyceride composition of natural fats has been studied at length by Hilditch and co-workers. Much of their work is included in his book—The Chemical Constitution of Natural Fats. Longenecker's paper entitled The Composition and Structural Characteristics of Glycerides in Relation to Classification and Environment is also of interest and has an excellent bibliography. This paper was given at the April, 1941, meeting of the American Chemical Society in St. Louis and appears in the October, 1941, issue of Chemical Reviews.

Apparently the methods developed by these workers have not been applied at all extensively to vegetable shortenings. Available information as to change in glyceride composition during the course of hydrogenation has been obtained incidentally when hydrogenation was applied to the natural fats as one of the steps in determining original composition.

Compound type shortening depends mostly on trisaturated glycerides for its consistency or physical characteristics. These glycerides are normally a mixture of tristearin and mono-palmito-distearin. The consistency of an all-hydrogenated blend is determined largely by its disaturated-mono-unsaturated glycerides. These will be mostly distearo-monounsaturated, palmito-stearo-unsaturated and dipalmito unsaturated. Iso-oleic is also a factor, but for the time being, has not been considered in our work. The amounts and kinds of disaturated glycerides in an all-hydrogenated blend depend upon oil composition and hydrogenation. Variations in these components probably determine the properties of the product, as far as physical characteristics are concerned.

We have developed a crystallization method, using the principles offered by Hilditch which, when applied to hydrogenated oils, will give some information as to the composition of the unsaturated or partially saturated glycerides. This method has been applied to several samples of widely varying oil composition with results which will be discussed later.

Method

The sample is dissolved in four parts by weight of C.P. acetone and then stored in a tightly capped bottle at 0°C. for three days. The precipitate is separated by rapid filtration on a Buchner funnel. The solvent is evaporated from the filtrate and the amount of fat recovered is obtained. Iodine value, thiocyanogen value, and saponification value are determined on this portion.

The precipitate from the first crystallization is dissolved in ten parts of C.P. acetone and crops obtained after twenty-four hours at 15.0°C. and then after twenty-four hours at 0°C. Yields, iodine, thiocyanogen, and saponification values are obtained on the fat in both precipitates and the filtrate from this second crystallization.

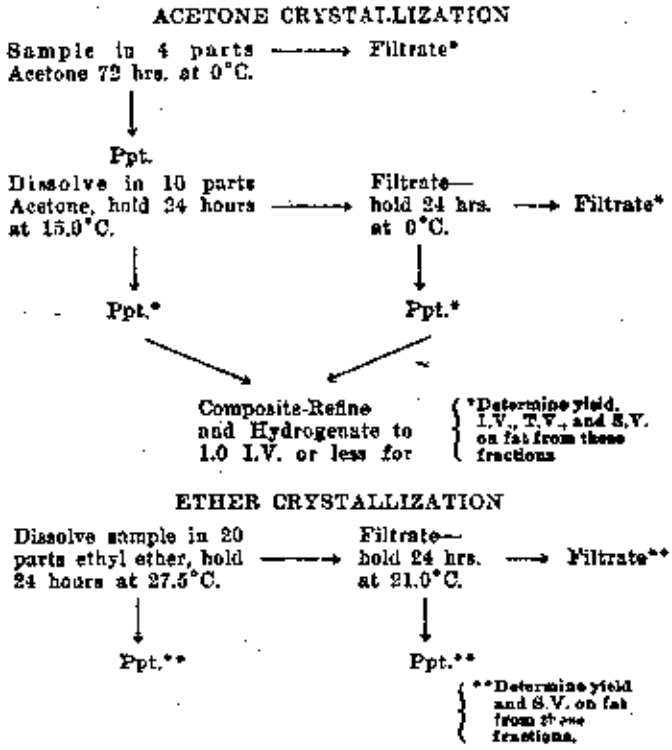
The 15.0°C. and 0°C. precipitates from the second crystallization, which contain most of the disaturated glycerides in the original sample are composited and, after refining, hardened to 1.0 iodine value or less. This stearine is dissolved in twenty parts of ethyl ether and allowed to stand twenty-four hours at 27.5°C. followed by twenty-four hours at 21.0°C. Crops of crystals are separated at both temperatures. Yields and saponification values are obtained on the fat recovered from each precipitate and the filtrate.

The fat from the filtrate contains the di- and tripalmitin. Since any glycerides of shorter chain acids, such as those in coconut oil, are also present in this fraction, it is necessary to determine the palmitic acid content by methyl ester distillation.

A record is kept of the quantities obtained at all points in the procedure. It is desirable for a study of the results to convert all fractions to percentages of the original sample, making the necessary corrections for losses. Olein and linolein in each fraction may be calculated from the iodine and thiocyanogen values.

If a methyl ester distillation for the determination of palmitic acid in the ether filtrate fraction is to be made, an original sample of 500-1000 grams is desirable. If this is omitted, a much smaller amount, possibly 100 to 200 grams, will provide sufficient material from the several separations for analysis.

Crystallization Flow Sheet



Discussion of Results

The olein and linolein figures on the various acetone fractions indicate the degree of separation which has been obtained between saturates and unsaturates. The saponification numbers on the acetone fractions are of little value if coconut oil is present because, while this component appears mostly in the less saturated fractions, it nevertheless influences all the saponification numbers to some extent. On the ether crystallization fractions, the saponification number indicates the disposition of the palmitic acid and in this separation the coconut oil appears only in the most soluble fraction.

There appear to be six points of major interest in the experimental data:

1. There are essentially no trisaturates in hydrogenated shortenings containing no added stearine. The fractions insoluble at 15.0°C. from the second acetone crystallization contain approximately one-third unsaturates or an average of one per glyceride, except in the case of blend No. 4, where the unsaturates are lower by an amount in proportion to the quantity of trisaturates known to be present.

2. The portion insoluble at 27.5°C. in the ether crystallization is an index of the monounsaturated distearine in the original sample. In the blends studied, this component increases in proportion to the amount of soybean oil present (i.e. numbers 1, 2, and 3) and undoubtedly, is derived from the hydrogenation of this oil.

3. The other two ether fractions, after subtraction of the dipalmito glycerides, are composed mainly of glycerides which were palmito-stearo-monounsaturated in the original shortening.

4. The monosaturated-diunsaturated glycerides may have some bearing on the consistency characteristics of a shortening. These appear largely in the filtrate from the second acetone crystallization, but also to some extent in the other fractions. An idea as to the proportions of palmitic and stearic comprising the saturated acids can be gained from saponification number data.

5. The glycerides containing two or three palmito groups are not completely recovered when palm oil is present. Judging from the amount of these components present in palm oil, as indicated by our acetone separation and also the data of Hilditch and co-workers, roughly one-half the amount present in the original sample appeared in the final filtrate from the ether crystallization of blends numbered 2 and 4.

6. More di- and tripalmitin was recovered from hydrogenated cottonseed oil and blend No. 1 than was to be expected. According to the "even distribution" theory of Hilditch and experimental work of Hilditch and Jones (J. Soc. Chem. Ind. 1934, 53, 13T) the amount of these in cottonseed oil is negligible. If the recovery of them in our procedure is assumed to be as poor as for the same components in palm oil, the deviation from the "even distribution" theory is even more pronounced.

This work has been preliminary in nature and the conclusions should be considered as tentative. There are some findings which are not readily understandable, as for instance, the relative recoveries of dipalmito glycerides from cottonseed oil and from palm oil. Variation in the positions at which the palmitic acid groups are attached to the glycerol is an interesting possibility which might account for solubility differences. Another opportunity for further work would be the correlation of glyceride composition with finished product behavior.

TABLE 1

Experimental Data on Various Oils and Blends

Sample	Palm Oil	Hyd. Cotton Oil	Hyd. Blend No. 1	Hyd. Blend No. 2	Hyd. Blend No. 3	Compound Type Blend No. 4
Composition %						
Palm Oil	100.0	.....	.....	20.0	.....	42.0
Cottonseed Oil	.....	100.0	85.0	31.0	31.0	8.0
Soybean Oil	.....	.....	.....	44.0	64.0	50.0*
Coconut Oil	.....	.....	5.0	5.0	5.0	.....
1st Acetone Crystallization						
Fraction soluble at 0°C.—% of original sample	53.0	63.7	66.5	58.0	56.0	63.4
Olein—% in fraction	49.3	64.5	59.2	60.1	67.2	60.2
Linolein—% in fraction	12.3	11.5	12.5	10.6	11.8	17.6
Saponification Number	194.7	190.0	197.2	193.9	194.9	193.8
2nd Acetone Crystallization (Insoluble Portion From 1st Crystallization)						
Fraction insoluble at 15.0°C.—% of original sample	12.0	15.6	13.6	16.5	20.0	14.8
Olein—% in fraction	7.9	42.1	38.2	29.5	30.8	17.1
Linolein—% in fraction	3.1	0.4	0.2	1.2	3.7	2.8
Saponification Number	206.7	197.7	198.5	196.6	193.3	198.2
Fraction insoluble at 0°C.—% of original sample	23.0	10.3	10.7	13.0	14.0	12.2
Olein—% in fraction	30.0	51.4	44.8	47.0	49.7	39.2
Linolein—% in fraction	4.1	1.6	4.3	8.4	5.0	3.2
Saponification Number	203.1	198.6	196.0	195.8	194.0	199.2
Fraction soluble at 0°C.—% of original sample	12.0	10.2	10.2	13.5	10.0	9.8
Olein—% in fraction	.....	56.7	54.9	57.3	62.1	51.5
Linolein—% in fraction	.....	7.8	8.8	9.6	5.2	14.9
Saponification Number	199.5	193.6	197.0	196.7	195.2	195.7
Ether Crystallization (Composite of Insoluble Fractions From 2nd Acetone Crystallization Hydrogenated to 3.0 I. V. or lower)						
Fraction insoluble at 27.5°C.—% of original sample	.....	0.0	0.8	12.1	17.8	7.4
Saponification Number	.....	.....	195.8	191.6	190.8	194.3
Fraction insoluble at 21.0°C.—% of original sample	.....	13.6	13.2	5.4	5.8	10.3
Saponification Number	.....	.....	194.4	193.5	192.4	198.8
Fraction insoluble at 21.0°C.—% of original sample	.....	12.4	10.6	13.0	9.6	9.1
Saponification Number	.....	.....	196.0	198.3	195.5	199.3
% Palmitin in Fraction	.....	42.3	44.6	44.2	34.0	65.6
Di- and Tripalmitin necessarily present—calculated as dipalmitin—% of original sample	.....	9.1	8.7	9.2	0.3	8.8

Tristearine	189.0	Saponification Number
Palmito-distearine	195.5	Saponification Number
Dipalmito stearine	202.0	Saponification Number
Tripalmitin	208.8	Saponification Number

* Includes 4.5% stearine.

J. Hannewijk

Use of Fish Oils in Margarine and Shortening

Fish oils M.E. Stansby (Ed) 1967

INTRODUCTION

In Europe, Canada, Japan, and other countries hardened whale oil has been used in margarine and edible fats for over 50 years. Hardened fish oil has likewise long been used in foods, although to a lesser extent, since until recently the amount of fish oil available was invariably smaller than that of whale oil. Many countries, however, have now greatly increased their fish oil production, whereas the whale-oil production has decreased.

The types of fish oil that are marketed change. Whereas 10 to 15 years ago it was mainly herring oil that was marketed in Western Europe, besides menhaden oil from the United States, at present it is chiefly anchovy oil from Peru. The latter oil is characterized by an exceptionally high iodine value.

Hydrogenation is a necessary process in making marine oils suitable for use in edible fats; with a view to the flavor, the highly unsaturated fatty acids must be removed or at least converted to less unsaturated ones. Polymerization might also be applied, were it not for serious objections to the use of polymerized fats in foods.

HYDROGENATION

General

Catalytic hydrogenation of oils was technically developed by Normann early in this century. Besides vegetable oils, it was mainly whale oil which was then selected for hydrogenation because large amounts became available on account of its being no longer needed for illumination. It appeared that a fat could be prepared from it which, with respect to its consistency, was well suited for use in margarine.

Whereas in the past, the primary objective of hydrogenation of fatty oils was the increase in melting point, at present it is the improvement of taste. Therefore, vegetable oils are sometimes hydrogenated in such a way that the liquid character of the oil is largely maintained. A similar hydrogenation of whale oil and fish oil does not, however, give oils possessing the high standards required for human consumption; the fishy taste is insufficiently removed.

Although the taste is improved as the melting point is raised, there is a certain limit to this rise in melting point, since the fat as such, or in combi-

nations, must be capable of melting in the mouth. It should, however, be noted that the standards set on taste differ in various countries. In Japan, for instance, where whale meat is used for human consumption, fewer objections will be made to slight fishy taste than in Western Europe, with the exception perhaps of Norway where they are accustomed to the odor of whale oil.

In the hydrogenation process, three phases are present: liquid phase (oil), gas phase (hydrogen), solid phase (catalyst).

Liquid Phase (Oil)

If the free fatty acid content is higher than 0.1%, the oil must be neutralized and bleached before hydrogenation in order to avoid poisoning of the catalyst. These treatments are discussed in Chapter 15.

The sulfur compounds, however, which also poison the catalyst, cannot be removed by a normal refining process. Neutralized and bleached whale oil still contains ca. 30 ppm sulfur, fish oil 10–15 ppm.

Other properties of fish oil before hydrogenation will be discussed in the section on the keeping qualities of the hardened fat.

Gas Phase (Hydrogen)

Several procedures may be followed for the industrial preparation of hydrogen which is suitable for the hydrogenation of fish oil.

(a) Steam/Iron Contact Process.—This is one of the oldest and cheapest processes. Iron ore is reduced to iron or FeO; on treating with steam at 600°–800°C. hydrogen is formed. The iron is oxidized to Fe_3O_4 which is again reduced with, e.g., water gas prepared by passing steam over coke. As this hydrogen contains H_2S, purification, e.g., in a caustic scrubbing tower is necessary.

(b) Electrolysis of Water.—Electrolyzers which perform at normal or elevated pressure and produce oxygen as a by-product are commercially available. If a fat-hardening factory is in the vicinity of a plant where NaCl is electrolyzed, the hydrogen released by this process can readily be used for hydrogenation. Hydrogen obtained by electrolysis has a high degree of purity.

(c) Catalytic Steam/Hydrocarbon Process.—A hydrocarbon, e.g., propane, is passed with steam over Ni-catalyst at 800°–1000°C.; this gives H_2, CO, and CO_2. The propane is previously desulfurized by passing over bauxite catalyst at 370°C. Organic sulfur is then converted to H_2S which is removed with monoethanolamine by a scrubber. Removal of CO may be carried out by catalytic conversion into methane and water, using nickel catalyst at about 300°C.

For hydrogenations in the laboratory, electrolytic hydrogen from a cylinder can be used without further treatments.

Solid Phase (Catalyst)

Only those hydrogenation catalysts are discussed which are industrially applied. In Western Europe they are mainly nickel formate or nickel/guhr catalysts, in Russia also nickel/copper catalysts (in which copper serves to lower the reduction temperature of nickel oxide), while in Japan also nickel-free copper catalysts are used, or at least have been suggested.

Application of the nickel formate catalyst is relatively simple; it is, therefore, most attractive for small industries. The nickel formate is suspended in four times its weight of oil and reduced for 30–60 min. at *ca.* 204°C. (so-called wet reduction); a finely divided metallic nickel is then formed. If nickel copper formate is taken as starting material, the reduction temperature can be lower. Since 240°C. is far too high for unhardened fish oil, the nickel formate is best taken up in, for example, hardened fish oil. The hydrogenation activity of such a nickel catalyst is excellent, although the filtering properties are poorer than those of a nickel/guhr catalyst; this results in the filtering of black oil. After reduction, the catalyst suspended in fat can be stored or transported in the form of flakes.

Nickel catalysts on carrier can be prepared by precipitation of nickel hydroxide from a solution of nickel sulfate containing waterglass or guhr, by means of soda. After washing and drying a storable green cake is obtained. Before use, this cake must be subjected to dry reduction with H_2 in, for example, a rotary-drum-type oven at 400°–500°C. The reduced catalyst is pyrophoric; it must, therefore, be kept in inert medium and be taken up in the oil to be hardened as soon as possible. If it is to be stored or transported, it must be taken up in a relatively saturated oil such as hardened marine soil.

The various hydrogenation catalysts commercially available nearly always consist of finely divided nickel, whether or not it is on carrier, taken up in fat. The nickel content is *ca.* 25%.

Apparatus

There are two types of apparatus for batch-hardening, those with and those without recirculation of the hydrogen. Hardening with the former type is called dead-end hardening. Hydrogenation is usually performed at an overpressure of *ca.* 1 atm. in order to prevent penetration of air into the hardening vessel and thus the danger of explosion.

For a smooth-running, dead-end hardening process, pure hydrogen is required. Since volatile substances will evaporate from the oil—some of which are catalyst poisons—the gas in the top of the apparatus must be l

off at intervals; this is, of course, uneconomic owing to the loss of hydrogen.

In the recirculation system, the gases from the hardening vessel are returned via a scrubber. In laboratory experiments, the hydrogen may simply be passed through a vessel with oil, with intensive stirring, and let off in the open air.

In addition to batch-hardening, there is the continuous or semi-continuous system which is sometimes used for vegetable oils. A slurry of oil and catalyst is pumped through a series of 3 or 5 hardening vessels, through each of which hydrogen is passed. This system is applied in Russia for the hydrogenation of sunflower oil (Sokol'skiĭ and Bolkhovitina 1956; Sergeev *et al.* 1956; Okrugov and Kopylenko 1962). The semi-continuous system only performs well if raw materials of constant quality can be supplied. Since in Western Europe the quality varies widely, the batch system is generally also applied for vegetable oils.

As in the hardening process a heterogeneous system is involved, thorough stirring being required in order to bring the three reacting phases into close contact.

Hydrogen Addition

Hydrogen addition is one of the three main reactions occurring in the hardening process. Since the aim of the process is to hydrogenate the highly unsaturated fatty acids and to leave those with one double bond unchanged, selective hydrogenation is required, in which high temperatures must be applied. Should, however, the fish oil be heated to 180°C. after which the hydrogen is introduced, the temperature of the oil would rise too much. This would lead to a temporarily insufficient supply of hydrogen and consequently to excessive isomerization—particularly on hardening with poisoned catalyst—and formation of cyclic compounds, of which the physiological properties are as yet not fully known (Gottenbos and Thomasson 1965). It has been found that at extremely high hardening temperatures aromatic fatty acids (bound to glycerol) may be formed. To determine the amount of these aromatic acids the total fatty acids are treated with urea. Aromatic acids do not form adducts. It has been advised (Coenen *et al.*, to be published) to harden highly unsaturated fish oils in two steps, first at relatively low temperature, e.g., 150°C., and afterwards at higher temperature, preferably below 200°C.

Thorough stirring is required also here, because the reaction at 150°C. must proceed as rapidly as possible.

In Fig. 42 the changes in highly unsaturated, diunsaturated, monounsaturated and saturated fatty acid percentages occurring during hydrogenation with poisoned catalyst are given for three marine oils. These oils

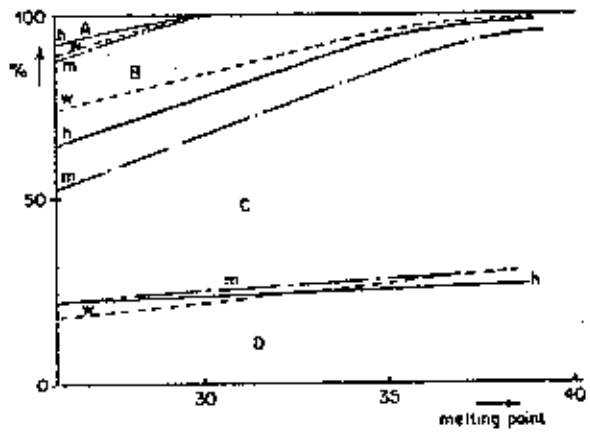


Fig. 42. Composition of Partially Hardened Fats with Different Melting Points

h = Canadian Herring Oil
w = Whale Oil
m = Menhaden Oil
A = Highly Unsaturated Fatty Acid Region
B = Di-unsaturated Fatty Acid Region (not conjugable)
C = Mono-unsaturated Fatty Acid Region
D = Saturated Fatty Acid Region

behaved almost identically. The rise in saturated fatty acid is only *ca.* 1%/h (selective hydrogenation). Near m.p. 35°C. there is hardly any difference between the three oils with respect to their saturated, monounsaturated, and diunsaturated fatty acid percentage.

Isomerization Reactions

Besides hydrogen addition, two other reactions occur, viz. migration of double bond and *cis/trans* isomerism. These reactions are promoted by a low hydrogen coverage of the catalyst or by poisoning of the catalyst with sulfur. No systematic investigation has been made into these reactions, but an idea about the extent to which they occur can be given.

Herring oil with the double bond mainly in the 9-position was partially hardened in the presence of sulfur-containing nickel catalyst at 180°C. to m.p. 33°C. The position of the double bond in the hardened fat was determined by oxidation with $KMnO_4$.[1]

Counting from the COOH-group, the position of the double bond was as follows:

[1] With acknowledgement to Mr. J. G. Keppler of Unilever Research Laboratory Vlaardingen, for these experiments.

Position	5	6	7	8	9	10	
% of double bond	6	8	13	18	18	14	
Position	11	12	13	14	15	16	17
% of double bond	8	6	←		8.5		→

From these values it must not be concluded that, for example, the entire percentage of the double bond found in the 5-position has migrated from the 9th carbon atoms since also double bonds from the highly unsaturated fatty acids have remained. Migration seems, however, to have occurred to a large extent. In a fat hardened to m.p. 38°C., the maxima at the 8- and 9-positions were even lower, which is indicative of even further migration.

No distinction was made between *trans* compounds from monoene, diene, triene, etc.; only the total *trans* content was consiedred. It was found that initially it rose very steeply; in a hydrogenation process of 2 h, 80% of the final *trans* content was obtained after only $^1/_2$ h, and 100% after 1 h.

It is supposed that after one hour approximately as large an amount of new *trans* compounds is formed as disappears by hydrogenation (or as is isomerized back to the *cis* form). Marine fats hardened with fresh nickel catalyst contain *ca.* 40% *trans* compounds, those hardened with sulfur-containing nickel catalyst 55–65%.

Selectivity

For selective hydrogenation both the process conditions and the nature of the catalyst are of importance (Coenen 1960). With respect to the former it is desirable that the hydrogen coverage of the nickel catalyst is low; this can be achieved by not too high a hydrogen pressure, a high catalyst concentration, and a relatively high temperature. A low hydrogen coverage of the catalyst unavoidably leads to strong conjugation, which results in migration of the double bond and formation of *trans* compounds.

The catalyst must have pores wider than 25 Å (Coenen *et al.* 1964). This allows a glyceride molecule to move rapidly. Suppose a glyceride molecule, containing one highly unsaturated fatty acid and two monounsaturated ones, is situated in a catalyst pore, on the nickel surface of which the highly unsaturated fatty acid is hydrogenated. If the molecule is not capable of diffusing out as a result of too narrow pores, the monounsaturated fatty acids will then also be hydrogenated, so that a trisaturated glyceride is formed. In view of the high melting point this is undesirable.

Coenen *et al.* (1964) have found that especially nickel catalysts with pores of 20–25 Å cause nonselective hydrogenation. Pores smaller than 20 Å are likewise undesirable because they are too narrow for triglycerides and do not, therefore, participate in the hydrogenation process.

The pore distribution in catalysts can be determined by gas absorption at *ca.* −200°C.

The melting point at a specific refraction or iodine value may serve as a measure of the selectivity of a hardening process. Under selective conditions this melting point will be lower than under nonselective ones; the difference may be as much as 20°C. This primarily depends on the pore structure of the (nickel) catalyst and only in the second place on the process conditions.

Sulfur poisoning of a nickel catalyst mainly leads to *cis-trans* isomerization. A sulfur-poisoned catalyst is often said to be more selective than a fresh one, but on the selectivity as meant above sulfur has no influence. Fats prepared with a sulfur-poisoned catalyst have, however, a higher *trans* content, and are, therefore, firmer at room temperature as a result of a higher content of solid phase.

Practical Hydrogenation of Marine Oils

A fresh catalyst is first used for hydrogenation of marine oils to m.p. 42°–45°C. For the first three batches an amount of 0.1% nickel calculated on the oil is sufficient for hardening at a final temperature of 180°C. As the degree of poisoning increases the catalyst becomes less active, so that larger amounts are required. If these amounts become too large, e.g., 0.5% nickel, the catalyst is used for another type of hydrogenation, so-called steep-hardening. This also takes place at a final temperature of 180°C., but the oils are hardened to a melting point of only 35°–37°C. Gradually the catalyst becomes less and less active so that by the end of the processing even as much as 2% nickel must be used.

Only 10–15% of the fat obtained from hydrogenation with fresh catalyst can be used in margarine, since a higher percentage would affect the oral response. This fat is especially suitable for the preparation of margarine, because it initiates crystallization.

The second type, viz. the steep-fat, is characterized by a relatively steep dilatation line as a result of a relatively high *trans* content. Both types of fat are used for the preparation of margarine.

The still relatively high melting point of the above fats may be a drawback with respect to melting in the mouth, but hydrogenation to a lower melting point does not sufficiently remove the fishy taste.

It has been suggested to add small amounts (<0.1%) of organic nitrogen compounds (e.g., acetamide or benzylamine), soybean lecithin or dimethylpolysiloxane (Ogami *et al.* 1962; Shimamura *et al.* 1964) during hydrogenation. This would lead to hardened marine oils which, at a relatively low melting point, taste better than the corresponding fats obtained without these additives. The effect of such additions, however, large

depends on the surface and structure properties of the hydrogenation catalyst.

Control of Hydrogenation Process

The simplest method of controlling a hardening process is by determination of the refraction and melting point. If, for example, a fish oil is to be hardened to m.p. 35°C., a trial hardening may be made in the laboratory with 200 gm. oil. Samples are drawn at regular intervals and the refraction of the desired fat is determined. During the industrial hardening process, samples are drawn for control of the refractions and, if necessary, the melting points.

The most common method of determining the melting point is by way of the slip point; this is the temperature at which the amount of solid phase in the melting fat has become so low that an air bubble is forced upward in an open capillary filled with the fat. This takes place when there is only 3–5% solid phase present.

Besides the slip point, determinations may also be made of the complete melting point, the Wiley melting point, the softening point, the critical temperature of dissolution, the aniline point, etc.

The melting point only indicates at what temperature the fat is completely melted; it does not give information about the consistency of fat at temperatures below the melting point. In order to know whether we are dealing with a "steep" or a "flat" fat, we must know the amount of solid phase present at different temperatures. This can be determined by dilatometry as well as calorimetry. Since dilatometry is generally applied for checking the hydrogenation process and since it is of great interest in the preparation of margarine, some more attention will be paid to it in the next section.

DILATOMETRY[4]

If a fat is cooled to complete crystallization and subsequently heated gradually, the volume increases as a result of the expansion of the solid fat. At a specific temperature, when the fat begins to melt, the volume increases relatively more. If it is melted completely, another gradual although smaller expansion occurs due to expansion of oil.

These expansions are diagrammed in Fig. 43. Line *RS* represents the melting expansion of 100% solid fat, *R* being the volume of undercooled oil at temperature T_1 and *S* the volume of solid fat at the same temperature if the melting process could be retarded at B. *PR* is called the dilatation:

[4] Boekenoogen 1964.

it is normally expressed in mm³/25 gm. fat. The percentage of solid phase at *T* is roughly RP/RS × 100%.

This is not quite exact because not all the glycerides from the fat have the same melting dilatation; it depends on the unsaturation and polymorphism of the glyceride. For partially hardened marine oil with m.p. *ca.* 35°C. the melting dilation for 100% solid fat at 0°C. is 1500–1700, dependent on the manner of cooling. For partially hardened marine oil with m.p. 42–45°C. it is 1800–2000. This value is increased by *ca.* 100 mm³/25 gm. per 10°C. temperature rise.

The dilatation of hardened fish oil (m.p., 37°C.) at 20°C. (D_{20}) is 1100, i.e., the amount of solid phase at 20°C. is *ca.* 1100/19 = *ca.* 60%. If D_{30} is 625, the amount of solid phase at 30°C. is *ca.* 625/20 = *ca.* 30%. In hardening industries just as in margarine factories it is unusual to convert dilatation values into percentages of solid phase. Hardened fats and margarine compositions are characterized by dilatation values.

As stated above, we distinguish between steep and flat fats. Examples of steep fats are cocoa butter and coconut fat, as well as the fat obtained from hydrogenation of marine oil with sulfur-poisoned nickel catalyst. The fat owes its steepness at a relatively low melting point to a high content of *trans* compounds. Fats hardened with fresh nickel catalyst also contain *trans* compounds, but during the hydrogenation also saturated fatty acid is formed which gives fats with higher melting points.

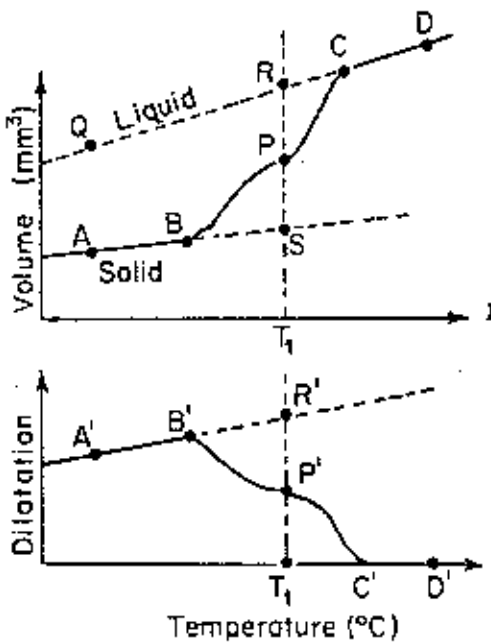


FIG. 43. VOLUME-TEMPERATURE AND DILATION-TEMPERATURE DIAGRAMS

Flat fats of good taste and m.p. below 40°C. can be prepared from vegetable oils by hydrogenation with fresh catalyst. Corresponding hydrogenation of marine oil does also give a flat fat, but its taste is unsatisfactory. In addition, the catalyst absorbs sulfur from the marine oil and forms *trans* compounds, so that on repeated use of the catalyst a steep fat is obtained.

Figure 43 can also be used for representing thermal measurements by plotting the heat content on the vertical axis. The slope of *AB* then no longer represents the expansion coefficient of solid fat but its specific heat, and the slope of *CD* now represents the specific heat of oil. Line *RS* is no longer the melting expansion but the heat of fusion. Thus the same data on, e.g., the percentage of solid phase in a hardened fat can be obtained by calorimetry, although in a more complicated way. Moreover, calorimetry requires a much more complex apparatus than dilatometry.

A dilatometer is shown in Fig. 44. The glass bulb contains *ca.* 5 gm. fat and is connected with a calibrated capillary which contains a sealing liquid, e.g., water, or a solution of calcium chloride.

Dilatation figures of hardened marine oils vary. They depend on the properties of the relevant oil, such as iodine value, place of origin, pretreatment of the oil, properties of the hardening catalyst, etc. When hardened to melting point 33°–37°C., D_{20} varies between 800 and 1200, D_{30} between 300 and 700, D_{35} between 0 and 300.

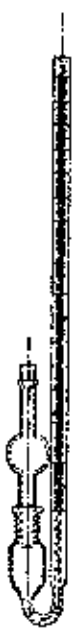


FIG. 44. DILATOMETER

Of a hardened marine oil with a melting point of about 45°C., D_{20} is 1400–1600 and D_{30} 1200–1400.

POST-REFINING AND DEODORIZATION

After hydrogenation and filtration the fat is post-treated at about 100°C. with a weak alkaline solution (0.1 *N*) to remove traces of fatty acid and hardening catalyst. As to the use of active or inactive bleaching earth in the subsequent bleaching process opinions differ. Our experiments showed that in the hardened fish oil post-bleached with active bleaching earth a hardening flavor developed more rapidly during storage than in the same oil post-bleached with inactive bleaching earth. When using nickel guhr catalyst with good filtering properties the remaining nickel content in refined, hardened fats is zero or at any rate lower than 0.5 ppm.

After bleaching, the fat is subjected to steam-deodorization under reduced pressure (e.g., 10 mm Hg). For removal of the volatile components the fat may also be stripped with an inert gas, but the ultimate results obtained by steam-deodorization are better. Steam probably exercises a hydrolytic action on some flavor precursors, as a result of which the keeping qualities of fats treated with steam are in most cases bettter than those of fats stripped with nitrogen.

In our laboratory an investigation was made into the influence of the batch-steaming conditions on the keeping qualities of hardened marine oil. The steaming time was varied from 1 to 23 h, the amount of steam from 25 to 400 wt.% (calculated on the fat), and the temperature from 150° to 220°C. A pressure of 10 mm Hg was invariably applied. It was found that the keeping qualities of an adequately pre-treated, hardened marine oil were good, irrespective of the steaming conditions, and that those of the same oil pre-treated inadequately could in no way be improved. Errors made in the refining process before hydrogenation cannot be rectified by hardening, by post-refining, or by deodorization.

The addition of antioxidants after deodorization has little effect, since the development of the hardening flavor in the deodorized fat cannot be arrested by it. Antioxidants may be effective if the fats have to be stored for more than *ca.* 3 months., but this is generally not the case with margarine; in Western Europe the shelf-life of margarine is much shorter.

Nordihydroguaiaretic acid in the presence of citric acid favorably influenced the keeping qualities of hardened marine oils which had been stored for 3 to 4 months after deodorization, whereas dodecyl gallate and dehydroacetic acid did not.

It has been suggested to add antioxidants to the fish oil during the production of the oil, so immediately after processing, which sometimes is done on the high seas, deterioration of the oil would be prevented prior

the refining process, where the added antioxidants are removed. It is not known whether this method is being applied successfully. Working up the oil to a hardened fat immediately after processing seems to be preferable (Pringuet 1964).

The hardened fat should be deodorized immediately before being worked up to margarine.

FLAVOR AND KEEPING QUALITIES OF DEODORIZED HARDENED FISH OIL

On storage of a hardened marine oil which immediately after deodorization is tasteless, an off-flavor develops. In daylight this flavor develops within a few hours, but also in the dark the fat acquires a certain off-taste.

We found that on storage in the dark the content of free aldehydes and ketones in hardened herring oil and whale oil may rise to *ca.* 5 ppm within two months. For most aldehydes this value is far above the threshold value (Meijboom 1964). The peroxide value generally increases to a value of *ca.* 1 within two months. In a few cases, however, it drops again after an initial slight increase. The content of bound aldehydes, which is 100–200 ppm immediately after deodorization, increases to 200–300 ppm within two months. Since, however, freshly deodorized oils are tasteless, it is assumed that the bound aldehydes do not contribute to the flavor. It may be expected that on storage of hardened marine oils various aldehydes are formed, each having a different threshold value. One of these aldehydes, 6-*trans*-nonenal, has an extremely low threshold value, viz. 0.0003 ppm. This aldehyde was isolated from hardened linseed oil and hardened soybean oil, where it is the carrier of the typical hardening flavor (Keppler *et al.* 1965). In our opinion, this compound is also responsible for the hardening flavor of hardened marine oils. However, when hardened marine oils are tasted indoors, the hardening flavor is sometimes not perceived provided it is present in a relatively low concentration. On inhaling outside air (even half on hour after tasting) it is experienced as an offensive taste in the throat. The sensitivity to this flavor varies individually.

Hardened whale oil and herring oil with a relatively low melting point (e.g., below 30°C.) almost invariably possess this hardening flavor. It can be largely removed by hardening the oils to m.p. 33°C. But even after hardening to m.p. 35°C., highly perceptive tasters will observe this flavor. Highly unsaturated fish oils, such as menhaden and pilchard oil, have to be hardened to an even higher melting point; hardening to a melting point below 40°C., generally is ineffective.

Although herring oil and whale oil hardened to m.p. 35°C. may be free from this particular hardening flavor, they as well as any other oil may develop a certain off-taste during storage. These off-tastes are less objectionable because they can probably be masked when the fats are worked up into margarine (Meijboom 1964).

The content of so-called bound aldehydes, which occur particularly in unhardened marine oils, can be determined by the color reaction with fuchsin/sulfurous acid (Schiff's reagent). The color obtained is compared with that of standard solutions of heptanal and expressed as γ heptanal/gm. The values thus found have only relative significance, because one aldehyde may give a stronger coloration than another. On comparing the aldehyde contents of different marine oils, it should also be borne in mind that the aldehydes formed during autoxidation need not necessarily be all of the same type. For one type of marine oil, however, it may be assumed that the values found are indeed a measure of the aldehyde content of this oil and that, although the concentration may differ, invariably the same compounds are measured.

In the case of unrefined marine oils it was generally impossible to determine the aldehyde content due to the strong color of these oils. As regards bleached oils, it may be observed that the content of bound aldehydes rapidly increases when air is passed through at 70°C. or on exposure to sunlight. Thus, in the case of herring oil the aldehyde content rises from 2 to 14 mg./gm. when air is passed through for three h, and after two hour's exposure to sunlight the total aldehyde content has increased from 2 to 17 mg./gm.

A normally bleached marine oil contains *ca.* 1–5 mg. aldehyde/gm. The aldehyde largely disappears on hydrogenation; after hydrogenation only *ca.* 0.5 mg./gm. is left. As already mentioned, after deodorization only 0.1–0.2 mg. aldehyde/gm. oil is recovered. A correlation seems to exist between the content of bound aldehydes in the oil at the stage just before hydrogenation and the quality of the hardened oil. This is, in any case, true of whale oil and herring oil. As for fish oils with a higher iodine value, such as menhaden and pilchard oil, insufficient data are available.

The rate of hydrogenation, too, correlates with the aldehyde content. Herring oil, for instance, required a very long hydrogenation time after air had been passed through at 70°C. or if it had been exposed to sunlight, both processes giving rise to a high aldehyde content.

The correlation between the aldehyde content and the quality of the hardened fat appears from the values given in Table 34.

A careful pre-treatment of marine oil before hydrogenation, as was said before, is required for the preparation of a hardened fat with good keeping qualities.

Although hardly applicable in practice, it should in this connection be observed that molecular distillation of herring oil (normal quality) gav

TABLE 34

RELATIONSHIP BETWEEN ALDEHYDE CONTENT OF UNHARDENED OIL AND FLAVOR OF HARDENED OIL

Type of Oil	Clarit (%)	Total Aldehyde in Unhardened Oil (Mg./Gm.)	Hardening Time (H)	Assessment of Hardened Fat, 4 Weeks after Deodorization
Herring oil	0.5	4.2	2.25	Fairly good
Herring oil	3	1.7	2.25	Good
Herring oil	5	0.7	1.25	Good
Herring oil	5	0.7	1.25	Good
Whale oil 3 (bad quality)	2	10.0	3	Poor
Whale oil 3 (bad quality)	5	4.0	2.5	Rather poor
Whale oil 3 (bad quality)	10	1.1	2.5	Fairly good
Presstran[1]	0.5	>5.0	3.25	Very poor
Presstran[1]	3	2.3	2.5	Fairly good
Presstran[1]	5	1.3	2.5	Good

[1] Mixture of fish oils, mainly herring oil.

an oil with particularly good color. This oil could be hydrogenated rapidly to m.p. 33°C.; after deodorization it had extremely good keeping qualities. Molecular distillation of Norwegian herring oil, 30% f.f.a., first gave a top fraction of 31%; after raising the temperature of the rotor from 110° to 230°C. (pressure $<10^{-3}$ mm Hg/cm.^{2}) the oil itself distilled. This oil also could be hydrogenated rapidly and gave a good fat, although not as good as that obtained from herring oil of good quality.

It is worth mentioning that the same inferior Norwegian herring oil (30% f.f.a.) could be purified by percolating a solution of the oil in technical hexane through a silica column. The partially hardened fat (I.V. 33) subsequently prepared did not develop a hardening flavor until two months after deodorization.

By steam-deodorization of fish oil before hydrogenation, the quality of the oil can also be greatly improved, which results in relatively rapid hydrogenation.

On ascertaining whether a relationship exists between the content of mucilage, phosphorus, and nitrogen in crude oil and the quality of hardened fat, it appeared that these substances are removed by a normal refining process. So there is no relationship between these contaminations in the crude oil and the hardening flavor of hardened marine oil.

MARGARINE AND SHORTENING

Margarine is at least 80% a fat/oil mixture, in which 16% water or milk has been emulsified, using 0.25% soybean lecithin, calculated on the oil, as emulsifier. A further 0.25% of a mono, di, and triglyceride mixture,

obtained from glycerolysis of, for example, hardened marine oil, is added. The mixture contains *ca.* 40% monoglycerides which bind the water and prevent the margarine from exuding moisture, which otherwise may give rise to the growth of mold on the wrappers.

Further ingredients are: coloring materials (annatto or carotene), flavors (chiefly diacetyl or some specific lactones), vitamins A and D, salt and a preservative (e.g., benzoate). In Western Europe the ultimate aim is to manufacture a salt-poor, nonpreserved margarine. This can be realized provided the water dispersion is sufficiently fine, i.e., if the emulsion droplets are *ca.* 5μ; at any rate no larger than *ca.* 12μ. In these small droplets the bacteria cannot multiply.

For the manufacture of margarine and shortening (Heesch 1963) mainly two procedures are followed, viz. the churn-drum process and the votator process. In the churn-drum process an emulsion (w/o) of fat, oil, and milk or water is prepared in a churn, which is a closed vessel equipped with a rapidly rotating stirrer. The emulsion is then brought onto a rotating drum which is cooled by cold brine. The fat in the emulsion crystallizes and, in the form of flakes, is scraped from the drum by a knife. The flakes are collected in trolleys and after a resting period during which the fat crystallization can proceed, they are kneaded by a crusher or a so-called plodder, which is a tube with a narrowed outlet. Kneading may be applied under vacuum, so that an air-free margarine is obtained, which promotes the keepability. After being kneaded, the margarine is immediately wrapped automatically. A drawback of this method is that the margarine can be infected rather easily.

In modern factories, therefore, a continuous closed system (Votator process) is used. For special shortenings the churn-drum process is still applied, since this system gives fats with a better plasticity than the closed system.

In the Votator system, the fat and water phase, whether emulsified or not, is led through cooling cylinders, so-called A-units, which are cooled by liquid ammonia. Crystallization takes place on the inner wall of the cylinders. The fat is scraped off by knives and fed to a so-called crystallizer, in which the partially crystallized margarine is subjected to a mechanical treatment. This treatment is necessary in order to give the margarine or the shortening its plastic consistency. On leaving the crystallizer, the margarine is again passed through one or more A-units, after which it enters a B-unit, a long resting tube, in which the margarine continues its stiffening process. As soon as it is sufficiently firm, it is packed automatically.

In order to prevent post-crystallization, which makes the margarine crumbly and hard, several methods may be applied, such as variatio

the cooling process. Each fat composition has its own optimum processing conditions.

Besides reducing the danger of infection, the closed system (with its many variations: Votator, Kombinator, Perfektor) has some further advantages, such as requiring smaller floor area and less labor force, and cooling more effectively.

A margarine factory must be extremely clean. The strictest hygiene must be observed. Frequent scrubbing and cleaning are first requisites, just as is air-conditioning. If required, the apparatus must be sterilized with a chlorine-containing bleaching agent.

Very important is the consistency of table margarine (Haighton 1959, 1963, 1965), especially its spreadability at room temperature. A rapid method has been developed to determine the hardness at different temperatures, which is expressed as C-values in gm./cm.^{2}. A margarine is spreadable between C = 150–800 gm./cm.^{2}, although this is also dependent on the firmness of bread. In winter, as well as for northern countries, the spreadability range is lower than in summer and for southern countries. Further it is important to know whether the margarine is to be stored in a refrigerator, in which case it must be spreadable from *ca.* 5° C.

The spreadability is chiefly determined by the solid phase/oil ratio. The work-softening and the size of the fat crystals indeed also influence the spreadability, but of primary importance is the amount of solid phase, of which the dilatation is a measure.

Dilatation figures of margarine depend on many factors, e.g., time of the year, climatic conditions of the relevant country, packaging material (tub or wrapper), way of storage (refrigerator or not), etc.

The amount of solid phase does not determine only the spreadability; it also determines the melting away in the mouth. In order to achieve rapid melting, the dilatation values at 30°–37°C. must be as low as possible, although not too low since otherwise oil exudes from the margarine; so a compromise must always be found.

It should be noted that the operator can easily modify the processing conditions and make the margarine firmer or softer; dilatations are, therefore, not the only standard.

Once the dilatation requirements for a margarine composition have been established, it is still very complicated to determine the ultimate composition since requirements other than the consistency must be fulfilled, such as taste, keepability, unsaturated fatty acid content, etc. Then there are types of margarine in which no animal fat may be worked up, and others which may not contain hardened fats. For all these reasons and on account of the fact that the prices of the different fats vary constantly, modern factories in Western Europe now calculate with the aid of a computer what composition fulfills the various requirements most economically.

The dilatation values for hardened whale oil and hardened fish oil can be handled in the same way as those for other fats. With a view to the hardening flavor which is to be expected, the percentage of hardened whale oil and fish oil may be limited for certain types of margarine. Moreover, as was said before, people in one country are more sensitive to hardening flavors than in another country.

Something remains to be said about the rates of crystallization in the preparation of margarine. In Western Europe, usually 5–15% hardened fat, m.p. 42°–45°C. is used in the fat composition. This fat does not undercool in the Votator or on the cooled drum and, therefore, initiates crystallization. Instead of hardened vegetable oil also hardened whale oil can be used for this purpose. There are, however, some hardened fish oils, notably menhaden oil, which crystallize slower and are, therefore, less suitable. If hardened menhaden oil of say m.p. 35°–37°C. is used, the processing conditions may have to be modified, such as cooling to a lower temperature or circulation in the Votator.

Margarines prepared from hardened vegetable oils sometimes recrystallize on storage. Especially fats with C_{18}- fatty acids, such as soybean and sunflower oil tend to do so. This is caused by the fact that the glycerides which on storage crystallize into the metastable β'-modification, after some time partly pass to the β-modification. Fats with a greater variation in chain length do not display this phenomenon. Since whale oil and fish oil have a widely varied chain length, viz. from C_{14} to C_{22}, margarines with an excellent plastic consistency which is not lost on storage can be prepared from them, e.g., margarines consisting of 70–80% partially hardened whale or herring oil (m.p. 33°–35°C.) and 20–30% liquid vegetable oil. In many countries, however, the taste of this margarine is not considered excellent.

Shortenings and bakery margarines must have other properties than table margarine. Several types can be distinguished, viz. those used for puff pastry, cake, piped doughs, biscuits as well as those used for shallow frying and deep frying.

Fats used for making puff pastry may not intersperse with the dough, because the fat and dough layers must remain separate in order to give a light pastry consisting of many layers. Generally, hardened vegetable fats are more suitable for the preparation of a pastry fat than hardened fish oil. Fats used for pastry are characterized by higher dilatation values than those used for making normal household margarine.

Fats for cakes (pound cakes or high-ratio cakes) must readily take

air and have good creaming property. For these fats, hardened marine oils can be used. These are also suitable for being worked up in biscuit fats.

For shallow frying, e.g., of fish, usually liquid oils are used. Fats used for deep frying must remain stable on repeated use and they must have a sufficiently high smoke point and not foam out of the frying-pan. In most cases a low smoke point is caused by the presence of fatty acid or traces of glycerol which disappear in a good refining process.

Hardened marine oils, if hydrogenated properly, are relatively foam stable. In connection with the taste stability, marine oils must be hydrogenated to over 40°C., which, however, on cooling may give rise to the formation of a fat film on products prepared from these fats.

An ever-increasing amount of pumpable shortening is produced for use in industrial bakeries. These fats are transported to the consumer by tank trailer and collected in vessels which are provided with a stirrer. Also in these pumpable fats, hardened marine oils can be used.

For the preparation of a confectionery fat—as a substitute for cocoa butter—the fractionation of hardened marine oil from solvent has been suggested. In this way a fraction can be obtained with melting properties approximately similar to those of cocoa butter, but on mixing a steep drop in melting point occurs.

SUMMARY

Hardened whale oil has been used in margarine for more than half a century. Fish oils, of which the available quantities increase in contrast to those of whale oil, can also be used for this purpose. Just as is the case with whale oil, there are good and bad batches of fish oil. For good stability of hardened margarine oil, it is necessary to process the oil as fresh as possible and to refine it adequately before hydrogenation. There are no essential differences between the qualities of hardened herring oil and hardened whale oil. The lower the initial melting point and the higher the iodine value of the nonhardened herring oil, the better. Menhaden oil and pilchard oil, which are characterized by a high iodine value and a relatively high initial melting point, must be hardened as high as the oral response permits. Even then the hardening flavor cannot be entirely eliminated. These fish oils with a high iodine value should be hardened in such a way that the temperature does not rise too much, since this leads to undesirable isomerizations.

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Perspectives in Nutrition

Quantitative Consideration of the Effect of Polyunsaturated Fatty Acid Content of the Diet Upon the Requirements for Vitamin E

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It is hoped that Perspectives in Nutrition will review the literature selectively, interpret it moderately and present a spectrum of ideas that will serve as a continual stimulation to nutritional research applied to medical problems.

Studies with poultry,[1] rats,[2] rabbits[3] and cattle[4] have shown that increased levels of unsaturated fat lead to increased requirements for vitamin E. This relationship has recently been extended to man by Horwitt and co-workers.[5,6]

While a number of toxic materials and adverse environmental conditions are known to affect the requirements for vitamin E,[7] Horwitt[8] has concluded that the concentration of auto-oxidizable substances in the diet is the primary factor.

A conclusion that polyunsaturated fatty acid is the principal determinant of vitamin E nutriture seems reasonable to us in the light of present knowledge; the use of this conclusion allows us to compare quantitatively vitamin E researches on human subjects and experimental animals.

POLYUNSATURATED FATTY ACID AND VITAMIN E IN THE UNITED STATES DIET

No widespread deficiency of vitamin E has been found to exist in the United States. Surveys[9] of normal subjects have shown that

Communication No. 300 from the Laboratories of Distillation Products Industries, Division of Eastman Kodak Company, Rochester, New York. Dr. Harris is now Director of the Division of Nutrition, Food and Drug Administration, Department of Health, Education and Welfare, Washington D. C.

TABLE 1

Fat and *d*-α-Tocopherol in Various Food Groups Available for Consumption in the United States in 1960

	Fat* (gm./day)	*d*-α-Tocopherol† Content (mg./100 gm. fat)	*d*-α-Tocopherol† Amount (mg./day)
Visible fats			
Butter	7.19	1.6	0.115
Lard	9.55	2.3	0.220
Margarine	9.42	10.2	0.961
Shortening	15.62	10.0	1.562
Other fats and oils	14.14	50.0	7.063
Total	55.92	...	9.921
Other food fats			
Dairy products	23.31	1.6	0.378
Eggs	5.33	10.7	0.572
Meats, etc.	52.08	1.7	0.893
Beans, peas, nuts, etc.	5.33	9.3	0.496
Fruits and vegetables	1.74	91.7	1.597
Grain products	2.23	48.9	1.092
Total	90.02	...	5.028
Totals	145.94	...	14.949

* From Table 4, National Food Situation, No. 96.[10]
† From Harris, et al.;[11] Macy et. al.;[12] and Agriculture Handbook No. 62.[13]

TABLE II

Fatty Acids in Major Food Groups, U. S. per-Capita Consumption, 1960*

	gm./day	Saturated	Oleic	Linoleic	Other PUFA	Total PUFA
Visible fat						
Butter	7.19	3.06	2.37	0.216	0.216	0.432
Lard	0.55	3.63	4.39	0.055	0.006	1.051
Margarine	9.42	2.45	5.37	0.818	0.377	1.225
Shortening	15.62	6.72	6.41	1.719	0.156	1.875
Other fats and oils	14.14	2.54	3.53	7.351	0.283	7.634
Totals	55.92	19.30	22.07	11.089	1.128	12.217
Invisible fat						
Dairy products	23.31	12.82	7.60	0.699	0.699	1.398
Eggs	5.33	1.71	2.35	0.378	0.528	0.906
Meats, etc.	62.08	19.96	22.55	3.717	3.002	6.719
Beans, peas, nuts	5.33	1.54	2.13	1.249	0.067	1.316
Fruits and vegetables	1.74 (35%)†	...	...	...	...	0.609
Grain products	2.23	0.33	0.66	0.926	0.060	0.986
Totals	90.02	36.36	35.38	6.964	4.361	11.934
Total visible and invisible fats	145.94	55.66	57.45	18.053	5.489	24.151

* The amount of fat supplied by each food group is from Table 1, National Food Situation No. 80.[9] The fatty acid composition of individual foods is from Fatty Acids in Food Fats. Home Economics Research Report No. 7.[14] Food economist E. Cofer of USDA gave valuable assistance and information regarding fat supplied by individual foods comprising the food groups in the invisible fat category.

† Since fatty acid composition of individual fruits and vegetables is not available, an arbitrary content of 35 per-cent was assigned to polyunsaturated fatty acid in the fat for this food group. This percentage is the average of all thirty-four values given for edible plant products listed in Fatty Acids in Food Fats. Home Economics Research Report No. 7.[14]

93 to 96 per cent of them have serum tocopherol concentrations above the deficiency level of 0.5 mg. per 100 ml. Thus for the purposes of this article, the average daily diet can be taken as having generally supplied sufficient vitamin E for the supply of polyunsaturated fatty acid.

The *d*-α-tocopherol content of the 1960 diet in the United States is shown by major classes of foods in Table I; the food available to the average consumer contained 14.9 mg. of *d*-α-tocopherol per day. We believe that this is a useful guide to the vitamin E content of the United States diet; deviations on the one hand from ignoring the slight contribution of the non-α-tocopherols are partly compensated on the other hand by losses of tocopherol during cooking or by losses in absorption.

The fatty acid analysis of the 1960 diet in the United States is presented in Table II: the food available daily to the average consumer contains 24.2 gm. of polyunsaturated fatty acid. Linoleic acid, 18 gm. daily, is the major source of polyunsaturated fatty acids.

To aid the comparison of various diets with respect to milligrams of *d*-α-tocopherol (E) and grams of polyunsaturated fatty acid (PUFA), we shall use the ratio E:PUFA. The data just presented show that the United States diet had a value of E:PUFA of 14.9:24.2, or 0.616. The value of 0.6 will be used hereafter as a reference ratio for an adequate supply of vitamin E.

CONTROLLED EXPERIMENTS IN MAN

In Figure 1 are plotted the data from experiments designed by Horwitt[6] to produce vitamin E deficiency in man. At the start

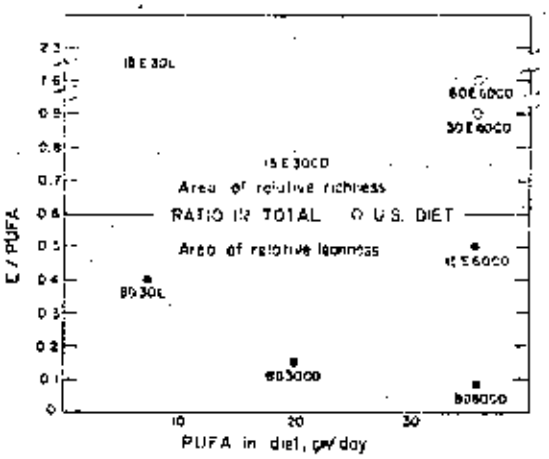

FIG. 1. Comparison of total U.S. diet, 1960, with Elgin experimental diets for ratio of E (milligram d-α-tocopherol) to PUFA (gram polyunsaturated fatty acids).

of this study, in which male adults in the Elgin State Hospital were to be mildly depleted of vitamin E, the daily diet supplied 7.1 gm. of polyunsaturated fatty acid (Table III). Since the diet was found to contain about 3.0 mg. d-α-tocopherol,[6,8] the E:PUFA ratio was 0.4. (This diet, with its ratio well below the reference "adequate" value, appears in Figure 1 as point BD30L.)

Depletion developed, but it proceeded slowly. After the subjects had been on the diet for about two years, Horwitt increased the polyunsaturated fatty acid intake by replacing the 30 gm. of lard (low in vitamin E, relatively low in polyunsaturated fatty acid content) with 30 gm. of treated corn oil (low in vitamin E, relatively high in polyunsaturated fatty acid content). This altered diet (with a ratio of 0.15, shown in Figure 1 as point BD30CO) is still farther from the area relatively rich in vitamin E. The subjects showed a further decrease in plasma tocopherol levels during the ten months on this diet.

Another change in diet, an increase in the quantity of corn oil from 30 to 60 gm. daily, resulted in further depletion (predictable by the position of its ratio, 0.08, point BD60CO in Figure 1).

In the same manner we can evaluate results obtained in the Elgin experiment with a group receiving the basal diets plus a daily supplement of d-α-tocopheryl acetate. At the beginning of the study, the diet contained 7.1 gm. of polyunsaturated fatty acid and 16.6 mg. of d-α-tocopherol (3.0 mg. in the diet and, as supplement, 13.6 mg—via 15 mg. d-α-tocopheryl acetate). Figure 1 shows the E:PUFA ratio, 16.6:7.1, or 2.3, as point 15E30L, well inside the area of relative richness. The subjects in this group had normal plasma tocopherol levels and no evidence of red cell hemolysis. These levels remained normal even when corn oil replaced lard in the diet; the new diet with a ratio of 0.8 (point 15E30CO in Figure 1) was in the area of adequacy. Upon increasing corn oil ingestion to 60 gm., the subjects began to show signs of deficiency—lowered plasma tocopherol levels and red cell hemolysis. (In Figure 1,

TABLE III

PUFA Content of Low-Fat and High-Fat Experimental Human Diets*

Dietary Ingredients	Low-Fat Diet		High-Fat Diet	
	Fat (gm./day)	PUFA (gm./day)	Fat (gm./day)	PUFA (gm./day)
Invisible fat (in daily dietary components of meat, vegetables and fruits, bread and cereals, milk, etc.)	30.0	3.96†	30.0	3.96†
Visible fat				
Lard (vitamin E-low)	30.0	3.14‡	...	
Corn oil (vitamin E-low)	...	...	60.0	31.35§
	60.0	7.10	90.0	35.31

* Calculated from Horwitt.[8]

† Value for polyunsaturated fatty acid content of invisible fat obtained from 1960 values for per capita consumption in the United States (Table II, this report). Mean daily invisible fat = 90.02 gm., of which 11.9 gm. (13.2 per cent) is polyunsaturated fatty acid; therefore the approximate polyunsaturated fatty acid supplied by 30 gm. invisible fat is 3.96 gm.

‡ Value for polyunsaturated acid content of lard: 30 gm. lard contains 95 per cent total fatty acids, or 28.5 gm., of which 11 per cent, or 3.14 gm., are polyunsaturated fatty acids.

§ Value for polyunsaturated fatty acid content of corn oil: 60 gm. corn oil contains 95 per cent total fatty acids, or 57.0 gm., of which 55 per cent, or 31.35 gm., are polyunsaturated fatty acids.

TABLE IV

Vitamin E Nutriture for Several Human Diets and E:PUFA, the Ratio of Quantity of *d*-α-Tocopherol (mg.) to Polyunsaturated Fatty Acid (gm.)

Diet*	(E) *d*-α-Tocopherol in Basal plus Supplement† (mg.)	PUFA in Invisible plus Visible Fat (gm.)	E:PUFA	Response in terms of Vitamin E Nutriture
Horwitt 15E30C.	3.0 + 13.6	4.0 + 3.1	2.3	Adequate
Horwitt 60E60CO	3.0 + 51.6	4.0 + 41.3	1.6	Adequate
Horwitt 30E60CO	3.0 + 27.3	4.0 + 31.3	0.9	Adequate
Horwitt 15E30CO	3.0 + 13.6	4.0 + 15.6	0.8	Adequate
U. S. Average diet	11.0	11.0 + 12.2	0.6	...
Horwitt 15E60CO	3.0 + 13.6	4.0 + 31.3	0.5	Inadequate
Horwitt B130E	3.0	4.0 + 3.1	0.4	Inadequate
Horwitt B130CO	3.0	4.0 + 15.6	0.15	Inadequate
Horwitt B60CO	3.0	4.0 + 31.3	0.08	Inadequate

* Details given in text.

† The supplements were 15, 30 and 60 mg. doses of *d*-α-tocopheryl acetate; these doses have been multiplied by 0.91 to give the stoichiometric values for *d*-α-tocopherol.

the E:PUFA ratio of the changed diet, 16.6:35.3 = 0.5, is shown by point 15E60CO.)

When Horwitt found that the 13.6 mg. of the vitamin E supplement was not adequate after the corn oil component of the diet was increased, he increased the daily supplement to 27.3 mg. (30 mg. of *d*-α-tocopheryl acetate). With this amount, plasma tocopherol levels returned to normal and red blood cell hemolysis was prevented. (In Figure 1, this tocopherol-supplemented diet is 30E60CO and represents an E:PUFA ratio of 30.3:35.3 = 0.9—within the relatively rich area.) However, these subjects did not appear to have, nor were they able to build up, any reserves of vitamin E, since red blood cell hemolysis developed soon (within a week) after supplementation ceased. In another experiment, daily supplementation with 60 mg. *d*-α-tocopheryl acetate to subjects with prolonged depletion caused the red blood cell hemolysis values to return to normal within three days. Horwitt concluded, therefore, that 60 mg. or more of α-tocopherol per day are apparently sufficient at this level of linoleate ingestion. The adequacy of this dose is also suggested by its high E:PUFA ratio (57.6:35.3 = 1.6, point 60E60CO in Figure 1). Details of these calculations are shown in Table IV.

Although Horwitt's experiments were not designed to determine the exact quantitative relationship between an adequate amount of *d*-α-tocopherol and the amount of polyunsaturated fatty acid ingested, the data plotted on Figure 1 show that the minimum symptom-free ratio of *d*-α-tocopherol to polyunsaturated fatty acid is between 0.5 and 0.8 for the experimental conditions he used. For subsequent discussion and evaluation of diets, we shall use 0.6 as the critical ratio, bearing in mind that, since it is the ratio for the total averaged United States diet, it is generally adequate.

EXPERIMENTS WITH ANIMALS

To see if the critical value of E:PUFA for man is the same as that for other species, we have analyzed the data in published reports of experiments with animals using regimens that produced vitamin E deficiency. In many reports the details concerning the type and amount of vitamin E administered or the kind and amount of fat fed were not complete enough for us to calculate the dietary E:PUFA ratio. However, in those that were adequate in these respects (twenty-one papers), we have shown (Table V) the lowest reported value of E:PUFA that prevented (or cured) deficiency symptoms and the highest reported E:PUFA that permitted vitamin E depletion to occur. For comparison, the appropriate

TABLE V

E:PUFA Ratios for Diets That Produced and Relieved Vitamin E Deficiency in Various Animal Species

Species and Source of PUFA	Nutrient Intake*		E:PUFA		Deficiency Symptoms Studied	Reference
	d-α-Tocopherol (mg.)†	PUFA (gm.)	Permitting Deficiency Symptoms	Permitting Normal Response		
Human	17.5	35.3	0.5	...	Erythrocyte hemolysis	Horwitt[9]
(Corn oil 12%)	17.5	19.6	...	0.9		
Monkey	0.9	4.8	0.2	...	Muscle dystrophy and anemia	Fitch and Dinning[10]
(Lard 8% CLO 3 ml.)	5.9	4.8	...	1.3		
Bovine calf	38.8	30.3	1.3	...	Muscle wasting and paralysis	Baxter et al.[11]
(Lard 28% CLO 2%)	39.0	22.5	...	1.7		
Bovine calf	10.1	16.3	0.6	...	Paralysis and death	Thomas and Okumoto[12]
(Lard 28%)	10.8	16.3	...	1.0		
Pig	9.5	25.1	0.1	...	Embryos died or young born with muscle dystrophy	Adamstone et al.[13]
(Wheat oil 1% CLO 1%)	43.0	25.1	...	1.7		
Rabbit	0.7	1.20	0.5	...	Creatinuria	Hove and Harris[14]
(Lard 5% CLO 2%)	1.4	1.20	...	1.1		
Rabbit	0.4	3.0	0.1	...	Elevated plasma aldolase and muscular weakness	Zuckerman and Marquardt[15]
(Lard 5% CLO 3%)	5.4	3.0	...	1.8		
Mouse	1.2	3.6	0.3	...	Resorption of fetus	Goettsch[16]
(Lard 20% CLO 2%)	1.7	3.6	...	0.4		
Rat	1.7	0.6	0.9	...	Insufficient lactation, uterine pigmentation	Evans and Emerson[17]
(Lard 22% CLO 2%)	5.0	0.6	...	0.8		
Rat	0.20	0.78	0.2	...	Tubular atrophy; muscular pathology	Filer et al.[18]
(Linseed oil 10%)	0.23	0.78	...	0.4		
Rat	0.4	8.4	0.05	...	Creatinuria	Century and Horwitt[19]
(Corn oil 15%)	0.7	8.8	...	0.1		
Rat	0.01	0.36	0.12	...	Resorption of fetus	Mason[20]
(Lard 18% CLO 2%)	0.09	0.36	...	0.25		
Rat	3.1	12.0	0.24	...	Testicular degeneration, tissue peroxides, anemia	Rasheed et al.[21]
(Menhaden oil 15%)	3.7	12.0	...	0.31		
Rat	0.6	3.6	0.2	...	Cardiac deposition and death	Gyorgy et al.[22]
(Lard 15% CLO 2%)	1.9	3.6	...	0.4		
Rat	2.0	16.2	0.1	...	Hemorrhagic liver necrosis and death	Dam and Granados[23]
(CLO 5%)	8.7	16.2	...	0.5		
Rat, female	0.6	1.2	0.5	...	Erythrocyte hemolysis	Ward[24]
(Lard 10%)	0.7	1.2	...	0.6		
Rat, Male	1.0	1.2	0.5	...	Erythrocyte hemolysis	Ward[24]
(Lard 10%)	1.2	1.2	...	1.0		
Chicken	2.5	7.0 (LA)	0.1	...	Low hatchability caused by embryonic deaths	Olson et al.[25]
(Safflower oil 10%)	0.0	7.0 (LA)	...	0.0		
Chicken	5.0	32.0	0.2	...	Poor hatchability	Machlin et al.[26]
(Safflower oil 10%)	80.0	32.0	...	2.5		
Chicken	8.0	22.4	0.4	...	Encephalomalacia	Century et al.[27]
(Corn oil 4%)	16.0	22.4	...	0.7		
Chicken	3.0	11.0 (LA)	0.3	...	Encephalomalacia	Machlin and Gordon[28]
(Cottonseed oil 20%)	6.0	11.0 (LA)	...	0.6		
Chicken	0.5	1.0	0.1	...	Peroxidation of adipose tissue	Dam and Granados[29]
(CLO 5%)	2.2	4.0	...	0.6		
Turkey	1.0	2.1	0.5	...	Embryonic death, low hatchability	Jensen et al.[30]
(Lard 3% Torula lipid 1%)	2.0	2.1	...	1.0		

* The intakes were calculated for whatever unit of the diet that was described in sufficient detail to enable the contents to be estimated for E and PUFA. The magnitude of the diet unit is immaterial since only the ratio, E:PUFA, is needed. "LA" used when data were available on linoleic acid content only.

† Some supplements were d-α-tocopheryl acetate; the amounts were multiplied by 0.91 to obtain the stoichiometric equivalent of d-α-tocopherol. When supplements of dl-α-tocopherol or its acetate were used, these quantities were divided by 1.26 and 1.19, respectively, to get the equivalent quantities of d-α-tocopherol.[31]

ratios for man from the Elgin studies are included.

A gross analysis of this sort cannot be expected to give a precise value for the ratios of E:PUFA because of the probability of species variation and because of differences in experimental technics. These differences include the particular deficiency symptom under examination as well as time of depletion and effect of minerals and other nutrients in the experimental diets. Nevertheless, the value of 0.6 proposed by us as the critical value for the E:PUFA ratio for man turns out to be well within the general magnitude of such ratios found in various experiments with animals.

ESTIMATION OF VITAMIN E VALUE OF FOODS AND DIETS

The acceptance of 0.6 as a reasonable value of the critical ratio of E:PUFA allows us to estimate readily the contribution of a food to vitamin E nutriture. For example, a particular sample of beef fat, although low in *d*-α-tocopherol content (2 mg. per 100 gm.), is also low in polyunsaturated fatty acid content (3 gm. per 100 gm.) and therefore with its "adequate" E:PUFA ratio of 0.7 would not have a vitamin E-depleting effect when ingested. A cod liver oil, however, with a much higher *d*-α-tocopherol content (10.2 mg. per 100 gm.) and also a higher polyunsaturated fatty acid content (84 gm. per 100 gm.) will have an E:PUFA ratio of 0.2; thus, a diet containing a few per cent of cod liver oil will produce vitamin E deficiency unless there is another source of vitamin E in the diet.[16,15]

It is probably of more practical use here to point out that some vegetable oils (considered to be rich in vitamin E) have E:PUFA ratios below 0.6. Thus, according to our working hypothesis, they should have an adverse effect on vitamin E nutriture. For example, refined oils with these reported assays[11] per 100 gm.—corn oil (7 mg. *d*-α-tocopherol:56 gm. polyunsaturated fatty acid; E:PUFA = 0.1), soybean oil (10 mg. *d*-α-tocopherol:51 gm. polyunsaturated fatty acid = 0.2), and peanut oil (11 mg. *d*-α-tocopherol:29 gm. polyunsaturated fatty acid = 0.4)—would be expected to accentuate vitamin E depletion rather than relieve it.

That these widely used food oils have been found to have a depleting effect when fed to experimental animals is recorded in the literature. When Farmer[16] and co-workers added corn oil to a vitamin E-deficient diet of guinea pigs, it intensified the symptoms of vitamin E deficiency—muscle weakness and reproductive malfunction. When Ambrose et al.[17] added soybean oil at a 2 per cent level to a diet containing sufficient vitamin E to support reproduction in rats, the addition induced reproductive failure. Griffiths[18] reported that although 0.3 mg. *dl*-α-tocopheryl acetate was adequate for young chicks on a diet containing 3 per cent arachis oil, when the diet contained 10 per cent arachis oil 1.0 mg. of *dl*-α-tocopheryl acetate per 100 gm. diet was not sufficient.

Experience has shown the impossibility of making flat rules for predicting the requirement of any nutrient without taking into account the rest of the diet, the environment, species, age or sex. Nevertheless, the use of E:PUFA = 0.6 as a guide should be of some help to the practical nutritionist trying to evaluate dietary vitamin E in the face of recorded evidence of deficiency and sufficiency at varying intakes.

We suggest that any diet having an E:PUFA ratio of less than 0.6 be considered as being probably deficient in vitamin E; however, if the total fat and polyunsaturated fatty acid are quite low, such a diet may not produce serious symptoms of deficiency for months or even years.

If a diet has an E:PUFA ratio of more than 0.6, it is quite likely to be sufficient in vitamin E. But the reserves might only be enough for a few days if the polyunsaturated fatty acid content is high—as in some high linoleate diets used by clinicians.[19] Furthermore, if for any particular reason vitamin E deficiency symptoms were to be avoided in experiments with an untested new diet, the 0.6 factor would give a "minimum" level which could be multiplied by a safety factor. If toxicity of a new diet is under study, at least two levels of vitamin E should be considered because of the protective effect of this vitamin against certain toxic agents.[7]

SUMMARY

A convenient rating for the contribution to vitamin E nutriture by diets and individual foods is "E:PUFA"—the ratio of the amount of vitamin E (milligram of *d*-α-tocopherol) to the amount of polyunsaturated fat (grams of polyunsaturated fatty acid). The E:PUFA ratio of the average 1000 diet in the United States (averaged per capita amount of foods available for consumption) is 0.6. Published data on man and on animals indicate that this ratio is not much higher than that of diets inducing definite symptoms of vitamin E

deficiency. Thus, diets with E:PUFA ratios above 0.6 are predicted generally to protect against vitamin E deficiency, but diets with ratios lower than 0.6 are expected to have a depleting effect.

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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 ± 0.5
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 ± 0.0
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 ± 1.5
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 ± [illegible]
PL relative (mg/g)	9.2 ± 0.6	9.5 ± 0.1	11.3 ± 0.0	12.3 ± 0.2	7.8 ± [illegible]

[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 Wegner 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 diacylglycerol 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

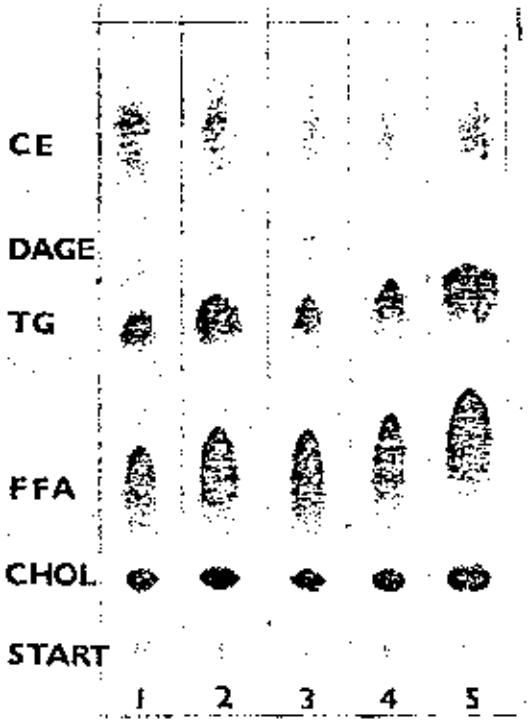

FIG. 1. Distribution of neutral lipids in testes from rats after five weeks of experimental feeding. CE, cholesterol esters; DAGE, diacylglycerol ethers; TG, triglycerides; FFA, free fatty acids; CHOL, cholesterol. The figures correspond to the various group numbers in the tables.

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

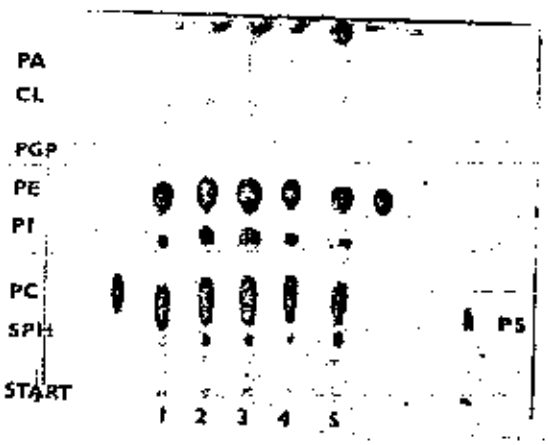

FIG. 2. Distribution of phospholipids in rat testes after five weeks of experimental feeding. CL, cardiolipin; PA, phosphatidic acid; PE, phosphatidylethanolamines; PGP, polyglycerophosphatidic acid; PI, phosphatidylinositols; PC, phosphatidylcholines; SPH, sphingomyelins; PS, phosphatidylserines. The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

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.

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Studies on the Regulation of Serum-Cholesterol Levels in Man

The Effects of Corn Oil, Ethyl Stearate, Hydrogenated Soybean Oil, and Nicotinic Acid When Added to a Very Low-Fat Basal Diet

Louis Horlick, M.D., F.R.C.P.(C)

INTRODUCTION

It is now well established that serum-cholesterol levels vary widely in different parts of the world, and are more closely related to the dietary-fat intake of the populations studied than to any other of their distinguishing characteristics.[13] The quality of the fat ingested, and particularly its degree of unsaturation, seem to be the most important factors in accounting for the cholesterol levels observed. Corn oil is the most potent of the cholesterol depressants studied to date: Its effect cannot be accounted for entirely on the basis of its content of unsaturated fatty acids.[1] Other factors are undoubtedly involved. It is possible that the plant sterols play an important role.[4] The mode of action of the unsaturated oils in lowering serum cholesterol appears to be related, in part, to the intestinal recycling of sterols. Corn oil has been shown to result in an increased excretion of labeled cholesterol[10] or bile acids[7] in the feces in amounts roughly proportional to the fall of serum levels.

It is not clear at present whether the hypocholesteremic effect of certain unsaturated fats is a positive effect or a neutral one, i.e., resulting solely from the removal of saturated fats and associated substances and their replacement by the unsaturated variety. The experiments reported below were designed to answer this question. Whereas it is generally agreed that the degree of unsaturation of fats most closely parallels the hypocholesteremic effect, this has been

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difficult to establish because of the unavailability of uniform samples of single fatty-acid esters or triglycerides. In recent studies[11] we could not observe any additional effect on the serum cholesterol when ethyl linoleate was added to a low-fat diet. The fully hydrogenated product of ethyl linoleate—ethyl stearate—resulted in steatorrhea and a further fall in the serum cholesterol. The data was interpreted as supporting a neutral role for the unsaturated oils and fats. A recent study by Kinsell *et al.*[15] with linoleic acid in 2 subjects resulted in some decline in serum cholesterol in 1 subject. We are reporting below further studies on the partial hydrogenation of vegetable oils in an effort to clarify this point.

Throughout most of the recent dietary studies, one feature seems clear: Serum-cholesterol levels may be reduced in a variety of ways—low fat, corn oil, nicotinic acid, estrogens—but nearly always to the same extent. There is, as it were, a basal level of serum cholesterol whose further depression is resisted by homeostatic mechanisms. The last part of this paper deals with our attempts to break through this basal level, using nicotinic acid, a potent hypocholesteremic agent.

MATERIAL AND METHODS

The subjects on the corn oil, ethyl stearate, and hydrogenated soya studies were all student volunteers in good health, who carried on with their regular duties during the experiment. Two of the subjects (D and F) on the nicotinic-acid experiment were postpolio paraplegics on the rehabilitation service and 1 (Fl.) was a young man recovering from a leg injury and confined to bed. The final subject on the nicotinic-acid study (Mrs. D) was a young housewife with severe xanthoma tendinosum, who was ambulatory throughout the course of the study.

During the control periods, the subjects recorded their dietary intake on diet-history forms after instruction by the dietitian. Cholesterol, total calories, and the percentage of calories taken as fat, protein, and carbohydrate were then calculated from standard tables. During the study periods—1 to 3 weeks—a low-fat diet was used to which the fats, fatty-acid esters and nicotinic acid were added. The average distribution of calories in, and the cholesterol content of, the control and low-fat diets is shown in Table 1. All diets were isocaloric for each individual, and the protein component was held constant. Efforts were made to incorporate the fats and oils into the diet in palatable, easily digestible forms.

The subjects were weighed twice a week, and the caloric intake was adjusted to keep the body weight constant during the experiments. Lipids were estimated twice a week on serum obtained in the fasting state. The feces were

TABLE 1. The Average Distribution of Calories in, and Cholesterol Content of, the Control and Low-Fat Diets

Diet	Percentage of calories yielded: Protein	Fat	Carbohydrate	Cholesterol (mg.)
Control	14	45[a]	41	512
Low-fat	16	4[a]	80	28

[a] By analysis; all other figures from tables.

TABLE 2. Changes in β-Cholesterol Per Cent with Consecutive Dietary Periods

	Control	Corn-oil	Control	Low-fat	Low-fat	Corn-oil
Subjects	8	8	14	14	6	6
Observations	23	25	53	56	19	20
Mean	72.8%	66.3%	74.5%	75.4%	71.4%	66.5%
S.d.	6.4	7.0	7.4	7.4	5.0	3.0
P	0.001		0.5		0.001	

pooled daily for fecal-fat estimation. Serum-free and total cholesterol, lipid phosphorous, lipoprotein cholesterol, and fecal fat were determined by methods previously described.[11]

RESULTS

The Addition of Corn Oil to a Low-Fat Diet

Two male subjects, S and P, received the low-fat diet for a 2-week period (Fig. 1). Subject P then received 10 Gm. increments of corn oil every 3 days up to a total of 70 Gm. (23.7 per cent of calories) at the end of the experiment. Subject S received 40 per cent of his calories as corn oil from the start of the test period. Whereas both subjects showed a drop in serum cholesterol during the low-fat period, there was no further change during the corn-oil administration. There was no appreciable change in the β lipoprotein cholesterol in S. In subject P the β lipoprotein cholesterol rose during the low-fat period and fell during the corn-oil period.

Four female subjects were observed for a 1-week control period. Subjects M and T (Fig. 2*A*) then received the low-fat diet for 1 week and increasing amounts of corn oil for another week up to a total of 40 per cent of calories. Subjects B and P (Fig. 2*B*) received the basal low-fat diet with increasing increments of corn oil from the start. All subjects showed a sharp fall in serum cholesterol during the first week of the experiment. Subjects M and T (Fig. 2*A*) then showed a slight rise in serum cholesterol when corn oil was administered. Subject P showed a sustained fall in serum cholesterol during the

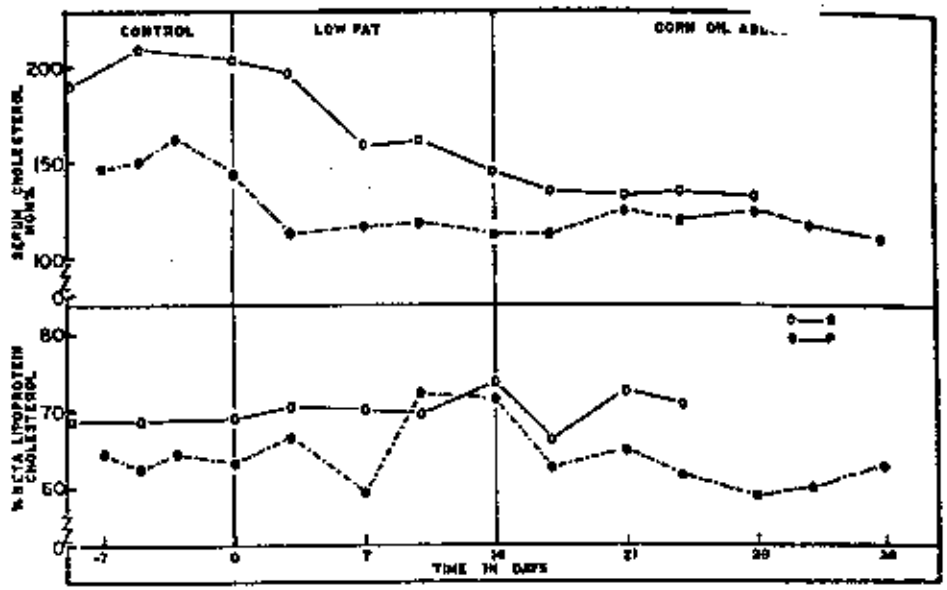

Fig. 1. The effect of corn-oil supplements after 2 weeks on a low-fat diet. Details and discussion in text.

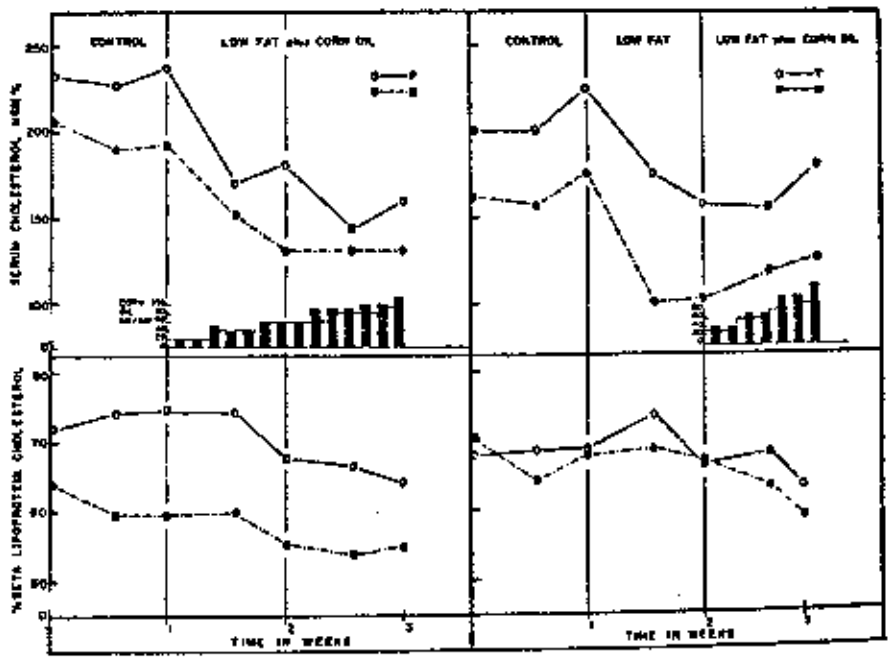

Fig. 2. (*Left-hand panel*), supplementation of low-fat diet with corn oil from the start. (*Right-hand panel*), corn oil added after 1 week on low-fat diet alone. Vertical bars indicate daily intake of corn oil in Gm. per day.

second week of corn-oil administration, while subject B showed little change. It appeared that a preceding low-fat phase altered the responsiveness of the subjects to corn oil. The β lipoprotein cholesterol showed no change during the low-fat period and a consistent decrease during the corn-oil period.

In Table 2 we have summarized all our data on β lipoprotein cholesterol during control, low-fat, and low-fat and corn-oil phases of the experiment. It is of interest that whereas the serum cholesterol invariably fell during the low-fat phase, this was not accompanied by any change in the per cent distribution of the lipoprotein-cholesterol fractions. The addition of corn oil to the low-fat diet did not result in any further decline in serum cholesterol, but was associated with a significant decline in the β lipoprotein cholesterol.

The Addition of Ethyl Stearate and Hydrogenated Soya Oils to a Low-Fat Diet

Two male subjects received ethyl stearate in increments of 20 Gms. every

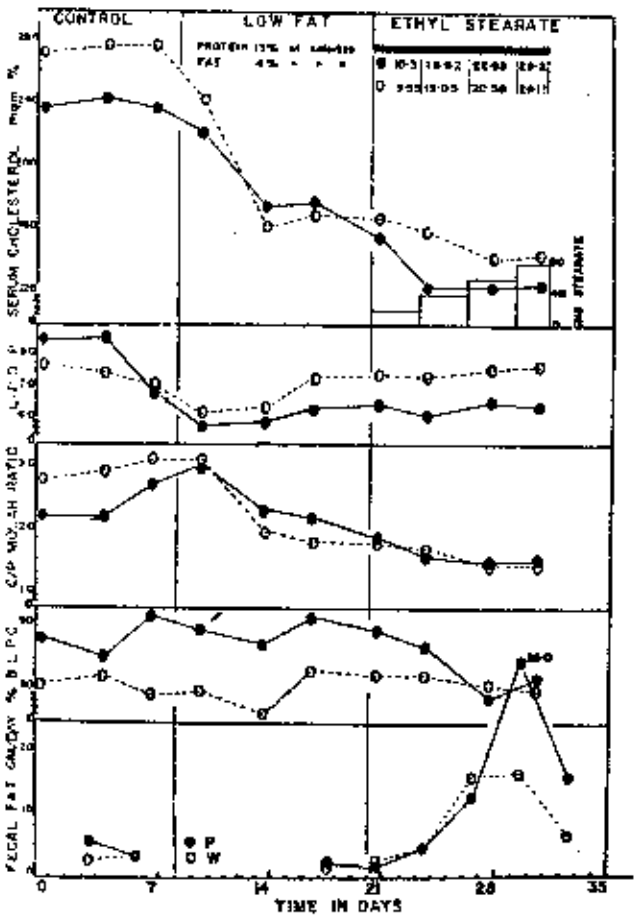

Fig. 3. Addition of ethyl stearate to a low-fat diet. Figures in upper right-hand corner under solid black line indicate per cent of total calories derived from ethyl stearate at each stage.

three days to a total of 80 Gms. per day (26.0 per cent of total calories for subject W and 29.2 per cent for subject P) (Fig. 3). During the low-fat period, the cholesterol fell considerably and then stabilized. Addition of ethyl stearate to the diet resulted in a further decline in serum cholesterol and a new plateau. In subject W, the lipid phosphorous fell during the early part of the low-fat period and quickly returned to control levels. It rose only slightly in subject P. In both, the C/P molar ration showed a slight rise early during the low-fat period and a progressive decline thereafter. The β lipoprotein–cholesterol changes were not consistent. The fecal fat rose with increasing stearate intake (the figures shown are averages for 3-day periods), and at its peak averaged 30 per cent of the intake in subject W and 58 per cent of the intake in subject P. These results are very similar to those previously reported by us in another subject.[11]

Natural soybean oil was first processed* to free it of plant sterols and then hydrogenated to one-third and two-thirds of the original iodine number: (one-third hydrogenated, 1#79; two-thirds hydrogenated, 1#40) (Fig. 4). Two male subjects received the hydrogenated soy supplements after a preliminary period on the low-fat diet. Subject A received the two-thirds hydrogenated product (1#40) at 30 per cent of calories and subject B received the one-third hydrogenated product (1#79) at 40 per cent of calories. The results obtained were very similar to those seen with ethyl stearate. Steatorrhea was observed in both subjects, but was greatest in subject A, who received the more fully saturated product. His maximal fat excretion was 109 Gms. in 1 day. He experienced nocturnal cramps and toward the end of the study period complained of numbness and tingling of the extremities.

The Addition of Nicotinic Acid to a Low-Fat Diet

Subjects C and L, healthy male volunteers, received a low-fat diet for 2 weeks (Fig. 5). During the next 2 weeks the diet was continued and nicotinic

* Soybean oil was refluxed for 2 hours with methanol, water, and potassium hydroxide to give a homogeneous solution. Most of the methyl alcohol was removed *in vacuo*, and water was added. The aqueous solution was stirred with calcium chloride solution for 1 hour. The resulting calcium soaps were centrifuged off, washed with water to neutrality, and dried *in vacuo* at 40° C. for several days. Calcium soaps were extracted 4 times with acetone to remove the unsaponifiable matter. The soaps were taken up in hexane and dilute hydrochloric acid, the aqueous phase was discarded, the hexane extract was washed several times with water, and then evaporated to dryness. The dried fatty acids were subsequently esterified by refluxing with ethanol and a small amount of hydrochloric acid; at the end of the reaction the acid was neutralized by the addition of sodium bicarbonate, the alcohol was evaporated, and the residue was taken up in hexane. The solvent extract was washed with water, and the hexane was finally evaporated to leave the fatty acid ethyl esters. The esters were then divided into 4 portions, and 3 were hydrogenated with Rufert catalyst at 400° F. and 25 lb. pressure of hydrogen. The iodine values were as follows: 79.1, 40.1, and 3.2. The unhydrogenated sample had an iodine value of 119.0. (This work was done for us by the Research Division, Canada Packers, by courtesy of Dr. L. J. Rubin, Director.)

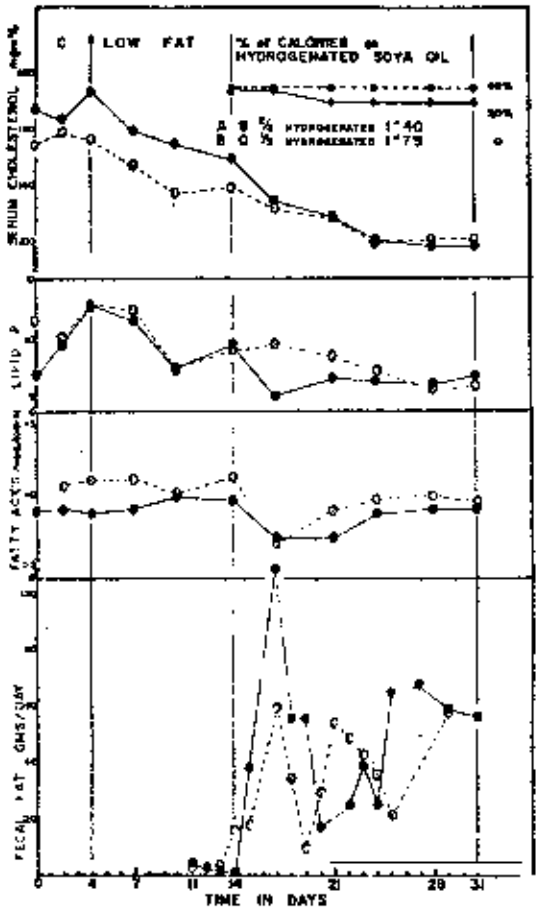

Fig. 4. Addition of hydrogenated soyabean oil to a low-fat diet. Discussion in text.

acid was added at a dose level of 3 Gm. per day during the first week and 5 Gm. per day during the second week. Subject L was unable to tolerate the 5-Gm. dose, and this was reduced to 4 Gm. per day after 24 hours. Subjects D and F were male postpolio paraplegics in a stable clinical state, who were being actively treated on the rehabilitation ward. They received 2 Gm. per day of nicotinic acid for a 1-week period. In all 4 subjects there was a substantial decline in serum cholesterol during the preliminary low-fat period and little, if any, further decline with nicotinic acid.

Subject Fl. was a young male, convalescing on the ward following a leg injury. He was permitted to consume the ward diet ad libitum, and received 3 Gm. per day of nicotinic acid. After 2 weeks he was changed to an isocaloric low-fat diet, and the nicotinic acid was continued. There was a modest fall in the serum cholesterol during the preliminary ward-diet–nicotinic-acid period and a further sharp fall when the low-fat diet was instituted.

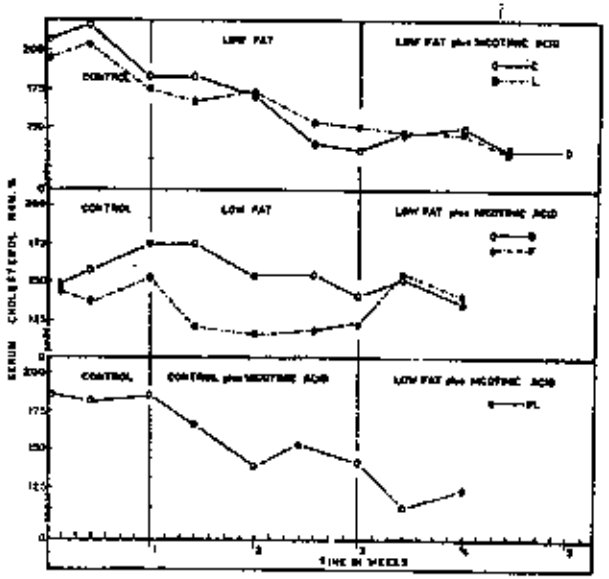

Fig. 5. (*Top panel*), subject C received 3 Gm. per day of nicotinic acid for 1 week and 5 Gm. per day the second week. Subject L received 3 Gm. per day the first week and 4 Gm. per day the second week. (*Middle panel*), subjects D and F received 2 Gm. of nicotinic acid for 1 week. (*Bottom panel*), subject Fl. received 3 Gm. of nicotinic acid per day throughout.

Subject D, a female with hypercholesterolemia and xanthoma tendinosum, was observed for 1 year on a low-fat diet with supplements of corn oil (Fig. 6). Her serum cholesterol remained stabilized for many months at approximately 600 mg./100 cc. The addition of 3 Gm. per day of nicotinic acid to her regime resulted in a sharp fall of 100 mg./cc. in her serum cholesterol in 1 month. The nicotinic acid was discontinued when she became pregnant.

DISCUSSION

Our results with corn oil following a low-fat diet may be interpreted in several ways. There was no further fall in serum cholesterol, and therefore the corn oil acted in a "neutral" capacity; on the other hand there was a significant change in the transport form of the serum lipoprotein cholesterol, suggesting a more active role.

An active depressant role for corn oil is also suggested in 2 recent studies in which the regular diet was supplemented by relatively large amounts of unsaturated fats. Gordon and Brock[8] reported on 10 men who received 50 Gm. of sunflower-seed oil per day for an average period of 23.5 weeks. Their mean serum cholesterol fell from the first control level of 247 mg./cc. to 213 mg./cc. by the end of the fat-supplement period. It is of interest that although the subjects claimed to be following instructions to take the fats

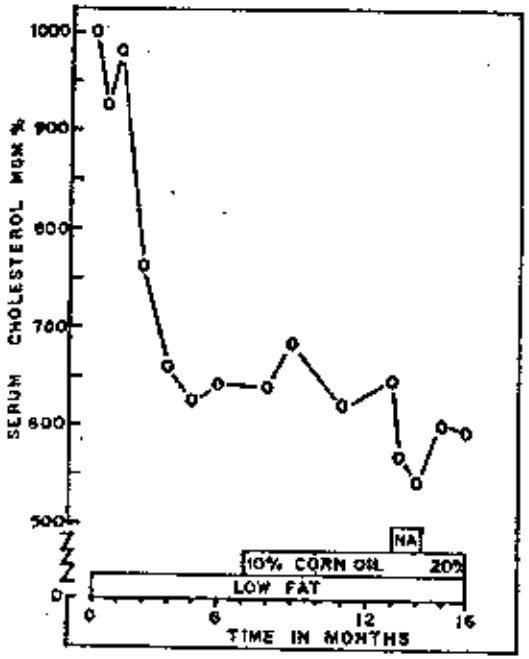

Fig. 6. Subject D, a female with xanthoma tendinosum. Low-fat basal diet throughout. Ten per cent of calories as corn oil for 3 months and 20 per cent as corn oil thereafter. Three Gm. of nicotinic acid (NA) per day for 1 month.

supplements only and not to modify their regular diet, they did not gain weight as might have been expected. Since there was no evidence of malabsorption in any of these subjects, it is likely that they must unwittingly have been restricting their food intake.

Armstrong *et al.*[3] administered daily supplements of 57 Gm. of corn oil, olive oil, sunflower oil, or butter fat to 141 young subjects who followed their usual diets during the 9-day test period. The subjects who ingested butter fat showed a slight, but statistically insignificant, rise in cholesterol, while those on the other fat showed a statistically significant fall. Each of the 4 groups of students exhibited slight increases in body weight over the 9 days of the experiment, the grand average gain being 1.37 lbs., or 0.62 kg., which was not as much weight gain as might have been expected. The authors conclude that "it is impossible to determine what ordinary food items were involved in this dietary substitution, but it must be assumed that a part of the reduction in the ordinary diet was at the expense of beef or dairy fats. . . ." Therefore, ". . . part of the reduction of serum cholesterol concentration may have been due to reduced intake of saturated fats."

From their calculations, Keys *et al.*[14] have stated that saturated fatty acids have, per gram, about twice as much effect on serum cholesterol levels as do polyenthenoids, which act in the opposite direction. Substitution of 1 oz. of sunflower-seed oil for 1 oz. of butter fat might be expected to cause a fall

of over 20 mg. per 100 ml. in 1 month, three-fourths of the fall being attributable to the reduction in butter fat. These studies indicate that while supplementation of the ordinary diet with unsaturated fats may result in a fall in serum-cholesterol levels, it is very difficult to know how much of this effect is due to subconscious restriction of saturated fats in the diet by the subjects studied.

Beveridge *et al.*[5] fed subjects a fat-free formula diet for 8 days, following which they divided the subjects into several groups. One group was continued on the fat-free diet, while 2 of the other groups received diets in which corn oil provided 20 and 60 per cent respectively of the total calories. During the initial 8-day period on the fat-free diet, the plasma cholesterol decreased 22.2 per cent, a change regarded as highly significant. Those continuing on this diet for a second 8-day period showed no further change. The group receiving 20 per cent corn oil showed little change, but that receiving 60 per cent corn oil showed a further fall in cholesterol after 8 days on corn oil.

This result is at variance with our own data reported above, and may be entirely related to the difference in experimental design. It is our conclusion that in the natural dietary situation, where mixed diets are consumed, the effects of the unsaturated fats are largely of a "neutral" character, and are related to the removal from the diet of substances which actively raise the blood-cholesterol levels. The effect of unsaturated fats in altering the transport forms of lipoprotein cholesterol may likewise be a "neutral" phenomenon, with replacement of the saturated fatty acids by unsaturated ones in the lipoprotein molecules. That this change in the transport form of cholesterol may be of equal, or even greater, significance with respect to atherosclerosis than the net fall in cholesterol itself is now well recognized. Another consequence of the low-fat–high-carbohydrate diet is an elevation of the serum triglyceride level. This was seen in some of our cases, and has been reported by Hatch *et al.*[9] Nichols *et al.*[16] noted no difference between diets high in vegetable fat and low-fat diets, insofar as the standard Sf° 0-12 and 12-20 lipoproteins were concerned. The Sf° 20-400 fraction which is high in triglyceride content was definitely elevated on the low-fat–high-carbohydrate diet.

Another area in which there is much uncertainty is the one relating to the hydrogenation of fats and oils and the effect on serum-cholesterol levels. Our own results with ethyl stearate and hydrogenated soybean oil were complicated by steatorrhea and only partial absorption of the materials fed. It is not surprising that the serum cholesterol fell under such circumstances. Indeed, the subject receiving the two-thirds hydrogenated material showed a spruelike picture with muscle cramps and tetany. It has been shown that isomerization occurs during the hydrogenation process.[20] We have postulated that suc

isomerization of the fatty acids (during preliminary removal of the soy sterols) may have played an important role in interfering with enzymatic hydrolysis and subsequent absorption of these fats.

Experiments in rats by Tsung-min Lin *et al.*[20] have shown that saturated fatty acids increase the excretion of cholesterol, whether of dietary or endogenous origin. The mechanism of action is unknown, but interference with the esterification of cholesterol, or its solution and removal in the unabsorbed residue of the fat could be a factor. The authors were unable to demonstrate a similar phenomenon in man.[12] Steatorrhea is not normally seen with commercially hydrogenated vegetable oils. Bronte-Stewart *et al.*[6] noted an increase in serum cholesterol when hydrogenated ground-nut oil was fed to a male Bantu, and a fall when the untreated oil was fed. Ahrens *et al.*[1] fed corn oil hydrogenated to iodine values of 80 and 58 and cottonseed oil hydrogenated to an iodine number of 68 to 3 subjects, with variable results. In one subject there was a progressive rise in serum cholesterol; in one, a silght rise; and in one, no rise at all.

The only conclusion that can be reached from our data is that in the presence of steatorrhea induced by eating poorly absorbed fats, a further fall in serum cholesterol from the low levels achieved on a low-fat diet may be seen. The question of moderate degrees of hydrogenation and the effect on the serum cholesterol is as yet unanswered. This important problem may be very difficult to resolve because of the isomerization by-products produced during hydrogenation procedures.

Nicotinic acid has been shown to lower the serum-cholesterol levels in rabbits and men. Altshul *et al.*[2] demonstrated this in both normocholesteremic and hypercholesteremic subjects, and showed that the response was more marked the higher the initial cholesterol levels were. This has now been confirmed by others[17, 18] who have shown that the hypocholesteremic effect is a sustained one. In all the studies reported, the diet has been uncontrolled, and the subjects were encouraged to continue whatever diets they were on at the start of the study. The decline in serum cholesterol has ranged from 8 to 20 per cent, which is much the same as that which we have observed with the low-fat diet.

Whereas "normal" young men and hypercholesteremic patients almost invariably respond with a fall in serum cholesterol, O'Reilly *et al.*[17] observed substantial rises in serum cholesterol in 3 schizophrenic patients, in 2 of whom the serum cholesterol was low initially (162.5 mg. and 130.5 mg. respectively). He felt that nicotinic acid operated as a "normalizing, regulating, homeostatic agent." The mode of action of nicotinic acid is unknown. It is of interest that Altshul *et al.*[2] first became interested in nicotinic acid as a component of the respiratory enzyme systems as a result of their success in lowering serum cholesterol with ultraviolet light and increasing concentrations of oxygen. They have suggested that the intracellular oxidation of cholesterol, mediated by nicotinic acid, might accelerate its excretion from the body. There is, however, no experimental proof for this concept.

Our own data indicates that in the normocholesteremic subject on a low-fat diet, nicotinic acid has no effect in lowering serum-cholesterol levels further. In fact, a low-fat diet resulted in a further decline in serum cholesterol in a nicotinic-acid–treated subject, probably by its additive effect on the cholesterol-control mechanisms. Therefore, in company with the low-fat diet and the unsaturated fats, nicotinic acid failed to break through the "basal cholesterol level," which in our Western society is about 20 per cent below ambient serum-cholesterol levels.

In 1 subject with xanthoma tendinosum, the addition of nicotinic acid to a low-fat–corn-oil regime resulted in a further and substantial fall in serum cholesterol. This is of interest from 2 points of view: (1) Parsons *et al.*[18] failed to note any such change in 2 patients with xanthoma tendinosum treated with nicotinic acid; and (2) it suggested that the mode of action of nicotinic acid on the serum cholesterol is different from that of either the low-fat diet or unsaturated fats.

SUMMARY

1. When corn oil was added to a very–low-fat diet, there was no further fall in the serum-cholesterol level. There was, however, a decrease in the per cent of β-lipoprotein cholesterol. This would suggest a "neutral" role for corn oil.

2. Ethyl stearate and two samples of sterol-free hydrogenated soybean oil (1#79 and 1#40) were poorly absorbed and resulted in steatorrhea. There was a further fall in the serum cholesterol from basal low-fat levels.

3. Nicotinic acid failed to lower the serum-cholesterol levels of subjects on low-fat diets. Like other hypocholesteremic agents, it does not break through the basal cholesterol level. It was, however, effective in lowering the serum cholesterol of a patient with xanthoma tendinosum, whose levels had been stable for many months on a low-fat–corn-oil regime.

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Disappearance of depot fat in rats fed extremely hydrogenated oils[1]

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HORST, ANTONI, IZYDOR KAGANOWICZ, IRWINA ZAGÓRSKA, AND DANUTA ROŻYNKOWA. *Disappearance of depot fat in rats fed extremely hydrogenated oils.* Am. J. Physiol. 201(4): 617-620, 1961.—Male adult albino rats fed ad libitum diets containing 30% extremely saturated vegetable fats (rape seed oil, iodine value 4 and soya bean oil, iodine value 91 showed marked weight reduction, with disappearance of body fat within a period of 6 weeks. The depot fat was not restored after 10 weeks of refeeding a control diet. The mechanism by which extremely saturated fats cause depletion of body fat cannot yet be clearly defined. Further investigations concerning the caloric balance, digestibility, and utilization of fats and carbohydrates are necessary. A possible explanation of the results is that extremely hydrogenated fats may inhibit lipogenesis from carbohydrates.

During studies of the effect on lipid deposition in the fat tissues and on cholesterol metabolism of rats, which resulted from a diet of saturated vegetable oils, we were struck by the complete lack of depot fat in rats that were fed extremely hydrogenated fats (1, 2). The diets used seemed to contain sufficient amounts of carbohydrates, thus making the disappearance of depot fat rather surprising.

MATERIALS AND METHODS

Adult albino rats of both sexes were used, ten animals in each group. The dietary fats were rape seed or soya bean oil. Three diets were made with each of these two fats, containing 30% by weight of oil of varying degrees of saturation. The process of hydrogenation was carried out at 150-200 C, with 0.2% nickel as catalyst. The hydrogenation products were filtered but not refined.

The basic ingredients of the control (fat free) diet and the data for diets containing rape seed or soya bean oils are given in Table 1. The animals were allowed food and water ad libitum for a period of 6 weeks. The body weights were recorded weekly.

Prior to terminal analyses the animals were fasted overnight. Blood samples were obtained by heart puncture, and all rats were sacrificed under ether anesthesia. Depot fat, i.e., mesenteric, renal, and epididymal or periovarial adipose tissue was weighed and analyzed for fatty acids by paper chromatography (3-5). For carcass fat analyses (6) the entire frozen rats were run through a hand-operated meat grinder and mixed in a homogenizer. Serum and liver total and free cholesterol was determined by the method of Zak et al. (7). Total serum lipids were estimated by the turbidimetric method (8, 9). Details of the analytical procedures used in our laboratories have been previously published (1, 2).

RESULTS

All diets were well consumed, particularly by the groups fed the diets containing extremely saturated oils. In spite of such large consumption, the rats given highly saturated oils showed a progressive loss of weight (Table 2) and a striking lack of depot fat when examined after sacrifice (Table 3). The results presented in Table 4 indicate a very low carcass fat in rats fed with extremely saturated oils.

The effects of diets consumed on the liver cholesterol was marked (Table 5). Cholesterol concentration in the liver of untreated animals fed natural oils was almost twice that of the controls. The effect of feeding moderately hydrogenated oils was smaller, but always significant. The increased accumulation of cholesterol in livers of rats fed unsaturated oils was also observed by Brown and Lewis, who used soya bean oil (10), by Grunbaum et al. with sunflower oil (11), and by Avigan and Steinberg with corn oil (12). The low liver cholesterol content in rats fed extremely saturated oils suggested poor absorbability of these oils.

The fatty acid composition of depot fat is presented in Fig. 1 (chromatogram 1) for rape oil diets and in Fig. 2 (chromatogram 2) for soya oil diets. It is evident

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TABLE 1. *Composition of diets*

Diet No.	Iodine Value of Fat Added	% Protein in Diet	% Fat in Diet	% Carbohydrates in Diet	Cal/g
1		34	4	40	3.1
2	111.3				
3	67.4				
4	4.0	16	50	20	6.1
5	130.2				
6	62.7				
7	9.0				

Diet 1 = Control (fat free), containing 34% skim milk powder; 15% eggs; 25% casein; 25% starch; 1% cholesterol; vitamin B complex (Forte). 2–7 = High fat, containing 50% by weight of different fats substituted for control diet (CD). 2, 3, and 4 = 50% CD + 50% rape seed oil. 5, 6, and 7 = 50% CD + 50% soya bean oil.

TABLE 2. *Mean body weight values (in grams) of ten male rats receiving control (fat free) and experimental (high fat) diets*

Initial Weight	Days on Diet: 1	28	29	35	42–43	Weight Gain (+) or Loss (−), % of Initial Weight
CD, fat free						
205±24	208±22	210±19	245±25	237±24	265±22	+29.1±3.61
CD + 50% soya oil, iodine value 130.2						
193±9	222±14	243±13	269±28	260±22	277±26	+41.4±6.75
CD + 50% soya oil, iodine value 62.7						
190±18	225±21	237±23	262±26	260±27	272±26	+42.4±3.97
CD + 50% soya oil, iodine value 9.0						
201±23	200±23	177±20	173±18	162±12	156±12	−22.3±3.91

TABLE 3. *Effect of control and fat diets on weight of adipose tissues deposited in abdominal cavity and in pelvis*

No. of Rats	Iodine Value	Mean Body Wt., g	Mean Adipose Tissue Wt., g	Adipose Tissue, % of Body Wt.
Male Rats				
CD, fat free				
3		279±26	5.5±1.3	2.03±0.55
CD + 50% soya oil				
4	130.2	278±33	9.2±2.7	3.28±0.46
4	62.7	275±25	8.0±1.7	2.89±0.33
5	9.0	150±5	0.3±0.1	0.19±0.08
Female Rats				
CD, fat free				
5		200±17	3.8±0.8	2.00±0.40
CD + 50% rape oil				
4	111.3	176±15	5.3±1.1	3.01±0.40
4	67.4	209±36	7.0±2.1	3.33±0.58
4	5.0	172±26	0.45±0.28	0.27±0.22

TABLE 4. *Carcass fat analyses of male rats fed experimental diets*

Rat No.	Iodine Value	Body Wt., g	Carcass Fat, %
CD, fat free			
1		250	3.8
2		280	6.9
50% CD + 50% soya oil			
3	130.2	280	3.5
4	130.2	300	5.8
50% CD + 50% soya oil			
5	62.7	290	3.6
6	62.7	255	5.2
50% CD + 50% soya oil			
7	9.0	150	0.4
8	9.0	180	

Because of extremely small quantity of fat tissue, analysis for rats 7 and 8 was performed together.

TABLE 5. *Effect of dietary fats on liver cholesterol level*

No. of Rats	Iodine Value	Mean Cholesterol Level in Liver, mg%		Free Cholesterol, %
		Total*	Free	
Female Rats				
CD, fat free				
5		915	376	41.0
CD + 50% rape oil				
4	111.3	2,102	732	34.9
4	67.4	1,597	557	34.9
4	4.0	648	297	45.8
Male Rats				
CD, fat free				
3		695±15	316±16	45.3±2.0
CD + 50% soya oil				
3	130.2	2,770±281	1,020±144	36.8±5.5
4	62.7	1,375±179	550±162	40.0±2.4
4	9.0	700±65	355±45	50.7±1.8

Cholesterol, 1%, added to all diets. Plasma cholesterol and lipid values were not altered in rape oil feeding experiments. In the soya oil iodine value 130.2 group a hypercholesterolemia of about 30% was found. Rats fed soya oil iodine value 130.2 and 62.7, showed a moderate hyperlipemia. * In the rape oil experiment, cholesterol level of pooled liver samples was duplicately determined.

that, in the groups fed rape seed oil, there are present in the depot fat all fatty acids from the diets except behenic acid. The fatty acid composition of depot fat in rats fed soya bean oil hydrogenated to iodine value 9 did not contain linoleic acid.

As it appeared quite probable that the extremely saturated fats were poorly digested, the rectal content of the respective animals was inspected. The feces analysis showed 70% fat and, chromatographically, the presence of all fatty acids from the administered lipids.

The preceding experiments suggested the possibility that the dietary fats with the highest degree of saturation were not absorbed; this evidently induced caloric imbalance, with subsequent depot fat mobilization. The following observation, however, failed to confirm this conclusion:

A section of small intestine was removed at the phase of alimentary resorption after feeding soya bean oil (iodine value, 9) and examined microscopically for lipids (Fig. 3). There was evidence that lipids had passed into the intestinal wall. Quantitative chemical resorption analyses were not made.

Moreover, in the subsequent experiment, when some of the animals were transferred from all high fat diets

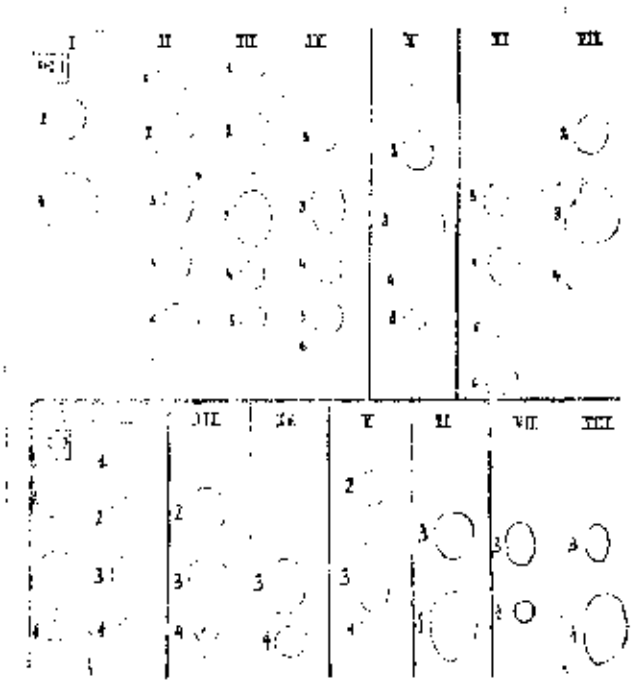

FIG. 1. Paper chromatographic patterns of fatty acids obtained for *I*: depot fat of control rats; *II*: rape oil, iodine value 111.3; *III*: depot fat of rats fed rape oil, iodine value 111.3; *IV*: rape oil, saturated to iodine value 67.4; *V*: depot fat of rats fed rape oil, iodine value 67.4; *VI*: rape oil, saturated to iodine value 4.0; *VII*: depot fat of rats fed rape oil, iodine value 4.0. Chromatographic spots represent following fatty acids: *1*, linolenic; *2*, linoleic; *3*, oleic, palmitic; *4*, stearic, arachidic; *5*, erucic; *6*, behenic. For the chromatographic analyses pooled fat tissue samples taken from 3-7 rats (Table 3) were used. Each analysis was made in duplicate.

FIG. 2. Paper chromatographic patterns of fatty acids obtained for *I*: depot fat of control rats; *II*: soya bean oil, iodine value 130.2; *III*: depot fat of rats fed soya bean oil, iodine value 130.2; *IV*: soya bean oil saturated to iodine value 62.7; *V*: depot fat of rats fed soya bean oil, iodine value 62.7; *VI*: soya bean oil saturated to iodine value 9.0; *VII*: depot fat of rats fed soya bean oil, iodine value 9.0; *VIII*: feces of rats fed soya bean oil, iodine value 9.0. Chromatographic spots represent following fatty acids: *1*, oleic; *2*, linoleic; *3*, oleic, palmitic; *4*, stearic, arachidic. For the chromatographic analyses pooled fat tissue samples taken from 3-7 rats (Table 3) were used. Each analysis was made in duplicate.

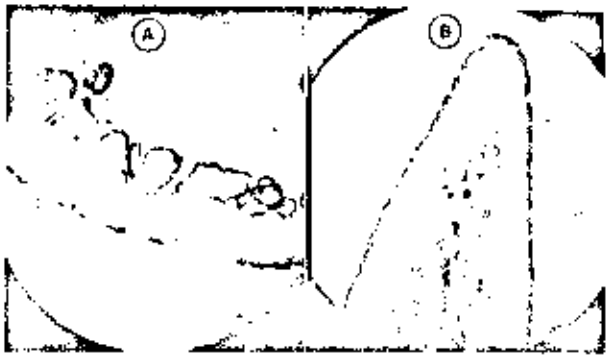

FIG. 3. *A*: Photomicrograph of frozen section of intestinal wall from a rat that had been given soya bean oil, iodine value 9.0, 4 hr before fixation (after a fast of 40 hr). Fixation in formal-calcium, staining with Oil Red O in propylene alcohol. Intestinal epithelium is packed with sudanophilic masses of fat (× 145). *B*: apical part of intestinal villus from a rat that had been fed on diet containing rape oil, iodine value 4.0. Fixation in formal-calcium, staining frozen sections with Oil Red O and hematoxylin. Epithelial cells contain dark masses of lipid positive material (× 580).

TABLE 6. *Weight of adipose tissue deposited in abdominal cavity and in pelvis after 10 weeks refeeding control diet without cholesterol*

No. of Male Rats	Diet Consumed Prior to Fat Free Control Diet	Iodine Value	Mean Body Wt., g	Depot Fat, Mean Wt., g	Depot Fat, % of body Wt.
3	CD, fat free		241±17	3.5±0.4	1.39±0.18
2	CD + 50% soya oil	130.2	260±35	4.9±1.1	1.74±0.15
2	CD + 50% soya oil	62.7	255±5	5.1±0.8	2.08±0.41
3	CD + 50% soya oil	9.0	236±17	11.9±0.8	5.31±0.31

to control diets free from fat and cholesterol, which they consumed *ad libitum*, the depot fat in the group fed rape oil (iodine value, 4) and soya bean oil (iodine value, 9) was still very poor after 10 weeks. The recovery findings are presented in Table 6.

DISCUSSION

Rats fed diets containing highly saturated oils from rape seeds or soya beans and receiving satisfactory amounts of carbohydrates showed a very significant decrease in their body fat. This feature may be associated with the following possibilities:

a) There was a loss of calories due to incomplete digestion and excretion of fat in the feces, leading subsequently to body fat mobilization for energy supply.

b) Administration of fats was responsible for the incomplete digestion and utilization of carbohydrates which also induced the caloric imbalance.

c) Extremely hydrogenated fats may contain toxic products as a result of heating and polymerization

processes, causing deleterious effects on adipose tissue (increased catabolism?).

d) Hydrogenated fats of very low iodine value or some polymerization products may replace the physiological fatty acids and, in this way, block the fatty acid assimilation and synthesis in the body.

The first explanation may be rejected, because the caloric supply from carbohydrates and proteins was essentially sufficient for body energy requirements. Even if we disregard the fat calories in our calculation we may assume that the other ingredients of experimental diets, consumed in excess, should yield the required calories. Food intake was not restricted. The animals in the groups fed rape oil saturated to iodine value 4, or soya oil to iodine value 9, could and actually did eat more. It should be emphasized that the control diet did not exert significant effect on adipose tissue and that the control animals on fat free diets retained their normal body fat.

Much more difficult to explain is the second hypothesis, that the intestinal resorption of food was inhibited by the presence of saturated fats. This does not appear to be the case, as the content of the small intestine was liquid, i.e., digested.

The possible toxicity of fats, especially when heated to 275 C, is well known (13). A temperature of 200 C was used in the hydrogenation process for the fats in our experiments. Otherwise, hydrogenation treatment is known to produce various isomers of fatty acids with toxic properties. Toxic effect was not found in our rats. They seemed to be well and had a normal and healthy appearance despite their progressive loss of body weight.

The last alternative merits special attention. It seems very probable that, in our experiments, the synthesis of endogenic fatty acids from dicarbon fragments in the citric acid cycle was blocked. The following observations tends to support our last hypothesis. Despite sufficient calorie intake in recovery experiments, depot fats were hardly restored. As is known from investigations with isotopes, the fat tissues of the body are quickly metabolized, contrary to former views; for this reason, only continued lipogenesis through fatty acid synthesis can prevent their disappearance. In our experiments this mechanism of fat exchange seemed to have been altered and therefore restoration of body fat did not take place. Some unphysiological fatty acids, not present in the normal diet, could be responsible for this block. The enzymatic system is not adapted to the hydrolysis of these fatty acids and therefore it also can be blocked for the normal physiological hydrolysis. This is, of course, only a hypothesis, which will require further investigation to establish its validity.

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THE EFFECT OF HARDENING OF VEGETABLE OILS ON THE METABOLISM OF FAT AND CHOLESTEROL IN WHITE RATS

PART II: SOY OIL

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In the first part of the experiments devoted to research on the metabolism of fat and cholesterol in white rats which had been fed hardened vegetable fat we used rapeseed oil with various degrees of hydrogenation (7). In the group of animals given maximally hydrogenated rapeseed oil with iodine number 4 in their diet we determined that with that there was atrophy of the fatty tissue deposit. Transferring these rats to a carbohydrate-protein diet for a period of ten weeks did not bring about a restoration of the fatty tissue.

Liquid rapeseed oil in addition to oleic, stearic, palmitic, linoleic and linolenic acids includes about 40% long-chain (22c) erucic acid which, as it is known, has a smaller coefficient of digestability in the rat (2) and which in the process of hydrogenation is converted into behenic acid not encountered in the fatty tissue of animals (4).

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In view of this the question arose whether the observed disturbance in the metabolism of fat is to be attributed to the degree of metabolism alone or also to the specific composition of rapeseed oil. We therefore decided to conduct research again with the use of vegetable oil with a different composition of fatty acids. We used soy oil.

Techniques for the Experiments

The experimental material consisted of 40 adult white rats, males weighing about 200 g, divided into four dietetic groups. Three groups of rats received fatty diets made up of a fat-free basic diet and the experimental fat in weight relation 1:1 and the fourth group included the control rats fed exclusively a basic fat-poor diet. One percent of pharmaceutical cholesterol was added to all the diets. The composition of the experimental diets is shown in Table 1.

For feed we used liquid soy oil, refined, with iodine number 130 2, acid number 0.28 and saponification number 190.0 and hydrogenated soy oils: to Iodine number: 52.7 and to Iodine number 9. The hardening process was conducted under the same conditions as for rapeseed oil.

The animals were given the food at will so that through the consumption of a greater quantity of the corresponding diet it was possible to adjust the differences in the calorific value of the fatty diets and the control diet.

After the expiration of six weeks of feeding a determination of the percentage content of the total fat was made in all the groups of rats, an analysis was made of the composition of the fatty acids by the method of blotting-paper chromatography in the

collected fatty tissue deposit, determination of the level of cholesterol in the blood and in the liver, determination of the level of lipids in the blood, and in the group S_9 an analysis of the feces for fat content and the presence of fatty acids was made. The methodology for the majority of the tests has been cited in the previous report.

For the purpose of determination of the percentage content of total fat two rats from each group, after being killed by putting to sleep with ether, after a thorough cleaning of the fur, were opened up in the stomach area, the entire alimentary canal was rinsed with water to remove the remnants of food and placed for a period of 24 hours in the freezer at a temperature of minus 30° C. While in a totally frozen state the rat was cut up and put through a meat machine and then placed in a homogenizer to obtain a uniform mass. From the material prepared in such a way a sample was taken for a quantitative determination of total fat (7) and from the remaining part a chloroform extract was prepared (6) to determine the composition of the fatty acids. In view of the small content of fat in the rats in the group with maximally hardened oil (S_9), both rats being studied were included in one mass and the determination was made jointly.

In addition a microscopic examination was made of the wall of the small intestine during the period of absorption of the diet containing soy oil hardened with Iodine number 9 using a dye - oily red.

Results

The results of the tests on the rats fed with soy oil were basically in agreement with the results obtained in the case of

diets with rapeseed oil. The animals in the group with soy oil maximally hardened (I. number 9) definitely lost weight in spite of the increased consumption of the diet. During the collection of the fatty tissue designated for chromatographic tests it was found that they were practically devoid of the perinephric, mesenteric, omental fatty tissue and in the area of the sexual organs and the subcutaneous layer of fat. Significant differences in the weight of the animals and in the quantity of fatty tissue ascertained between the various groups are shown in Tables 2 and 3.

Table 4 shows the percentage content of total organic fat confirming the considerable loss of fat in rats fed completely with hardened soy oil.

Chromatographic studies for the presence of fatty acids made on the collected fatty tissue and on the total fat extracted from the organism are shown in Illustration 1. It is evident from these chromatograms that neither in the total fat nor in the fatty tissue deposit was linoleic acid, characteristic for the composition of fat in the control animals, found in the rats in group S_9 (maximally hardened fat). In the animals fed with partially hydrogenated soy oil (group S_{62}), linoleic acid continues to show up in the fats subjected to analysis although it also did not appear in the consumed fat.

The analyses completed on the composition of the fatty tissue deposit are shown in Table 5 and the analyses of the composition of the total organic fat are shown in Table 6. In the group with soy oil with I. number 9 corresponding analyses were not made because of an insufficient quantity of fat in the extract. The effect of

the consumed fat on the organic fats was noted very explicitly in the group with the liquid soy oil (S_{130}) causing an increase in the content of unsaturated acids.

The results of the analysis of fat in the feces of rats fed soy oil hardened to Iodine number 9 were identical with the group with rapeseed oil with Iodine number 4. These feces included 70% fat in the dry mass and the presence of all fatty acids included in the consumed fat was shown chromatographically.

The results of the analysis of the level of cholesterol in the liver were also in agreement with those arrived at in a previous study of rapeseed oil pointing to the low absorption of the maximally hardened fats (Table 7).

In Table 8 we show the results of determination of the level of cholesterol and lipids in the blood serum. In the groups of rats fed liquid soy oil and partially hardened oil we verified at this time, in contrast to the experiments with rapeseed oil, an increase in the level of cholesterol in the group fed liquid soy oil.

The results of the above studies showed that maximally hardened fats in practice did not become absorbed causing in turn a caloric deficiency in connection with the atrophy of the fatty tissue in the organism.

However, subsequent experiments do not entirely confirm this conclusion. Additional microscopic analysis made of the wall of the small intestine during the stage of alimentary resorption after maximally hydrogenated soy oil was given testifies to the passing of these fats into the wall of the small intestine. (Quantitative analyses of absorption were not made).

Moreover, when we exposed some of the rats from all the fatty

groups to a basic carbohydrate-protein diet ad lib. during the following ten weeks even after this time the rats previously fed maximally hardened soy oil continued to show a deficiency in the fatty tissue (Table 9) confirming the pertinent observations in the previous study.

Discussion

Considerable atrophy of fatty tissue occurred in rats fed maximally hydrogenated vegetable oils - rapeseed and soy oils, with a sufficient supply of hydrocarbons in the diet. Analyzing the reason for this phenomenon we must consider the following possibilities:

1. The quantity of assimilable calories given in the diet was so low that a caloric deficiency occurred and in connection with this there was also atrophy of the fatty tissue.

2. The fats given in the diet prevented the regular utilization of the remaining feed components also bringing about a caloric deficiency.

3. The substances given in the diet could have a toxic action and cause an increased disintegration of the fatty tissue.

4. Maximally hydrogenated fats added to a fat-poor diet brought about a consumption of the organic, indispensable, unsaturated fatty acids and in this way prevented the regular storing of fat.

5. Hardened fats given in the diet, especially products formed in the course of hardening, can become combined with the regular fatty acids in the organism and in this manner block the assimilation or creation of endogenic fatty acids.

Ad 1. The first possibility can be discarded because the quantity of calories arising from the given carbohydrates and proteins

was basically sufficient to sustain the organic reserves. Even if the usefulness of fatty calories was discarded then the consumed remaining surplus components of the diet should, in our opinion, ensure the additional caloric balance. We remind that the control animals fed the fat-free basic diet preserved the regular content of fat in the tissues.

Ad 2. More difficult to explain is the possibility of blocking by the hardened fats absorptions in the alimentary canal. From the point of view of good macroscopic digestion (fluxing of feed in the small intestine) it does not seem possible that there would be a blocking by these fats of absorption of carbohydrates.

Ad 3. The possibility of a toxic action of fats heated to a temperature of 275°C is known (2). In our research the fats were hydrogenated at a temperature of 160-200°C. During the process of hydrogenation however certain products can arise as a result of side reactions which can perhaps be of a toxic nature. Contrary to this hypothesis our research shows that rats on the experimental diets did not show effects of disease except for a maximal loss of weight in the animals in the group with fats with the lowest Iodine number.

Ad 4. The possibility of a deficiency of indispensable, unsaturated fatty acids is shown by the lack of linoleic acid both in the fatty deposit tissue and in the fatty extract of the entire animal in the groups of rats fed maximally hydrogenated rapeseed or soy fat. It is known that in the adult rats the assembly of deficiency of indispensable unsaturated fatty acids does not appear as a result of the same fat-free diet, but it can appear with an addition-

al encumbrance (cholesterol, choleic acid, saturated fat, previous withholding of food (6,7)). These authors also observed in these cases loss of weight in the animals. The mechanism of separating the indispensable unsaturated fatty acids for the storing of organic fat is not explained.

Ad 5. Quite probable is the possibility of blockage of the synthesis of fatty endogenic acids from the bicarbon fragments. As is known, endogenic fats arise chiefly in the course of carbohydrate metabolism. In favor of this hypothesis there is the fact that in spite of the sufficient supply of carbohydrates in the diet the fat deposit tissue was not subject to restoration. From the isotopic analyses of the metabolism of the fatty tissue we know that it is subject to rapid metabolism and from here only continuous production of new fatty acids can prevent its atrophy. We must admit that in our case this mechanism remained disturbed and because of the non-restoration of the fatty tissue it was atrophied. This is naturally only a hypothesis which requires further research.

Table 1. Composition of experimental diets

Diet symbol	Content of the diet	I.No. of the fat	Protein %	Fats %	Carbohydrates %	Cal/g
K	Fat-free control (34% powdered dry milk, 15% eggs, 25% casein, 25% starch, 1% cholesterol) + vit. B Compl. Forte	--	32	2	40	3.1
S 130	50% control diet + 50% liquid soy oil	130.2				
S 62	50% control diet + 50% hardened soy oil	62.7	16	50	20	6.1
S 9	50% control diet - 50% hardened soy oil	9.0				

Table 2. Combination of the average values of the weights of the rats in the various groups

Tabela 2. Zestawienie średnich wartości wag szczurów w poszczególnych grupach

1 Ilość szczurów	2 Symbol diety	4 Średnie wagi grupowe szczurów w g						7 Średni przyrost (lub ubytek) wagi wyrażony w % wagi wyjściowej
		5 wyjściowa	6 dni spożywania diety					
			7	16	29	35	42-49	
10	3 Control Kontrolna	205 ±24	208 ±22	210 ±20	243 ±25	237 ±24	265 ±22	+29,2 ±3,61
10	S 130	194 ±9	222 ±14	243 ±23	267 ±28	260 ±22	277 ±26	+41,4 ±6,75
10	S 62	190 ±18	225 ±21	237 ±23	262 ±26	260 ±27	272 ±26	+42,4 ±3,97
10	S 9	201 ±23	200 ±23	177 ±29	173 ±8	162 ±12	156 ±12	−22,3 ±3,19

1. Number of rats, 2. Diet symbol, 3. Control, 4. Average group weights of rats in g, 5. initial, 6. days of use of diet, 7. Average increase (or decrease) of weight expressed in % of initial weight

Table 3. Effect of experimental diets on the storing away of fatty tissue

Tabela 3. Wpływ diet doświadczalnych na odkładanie tkanki tłuszczowej

1 Liczba zwierz.	2 Rodzaj diety	7 Tłuszcz depozytowy w g średnio		8 Waga tłuszczu dep. w % wagi ciała średnio	
3	3 Kontrolna „beztłuszcz."	5,5	±1,3	2,03	±0,55
3	4 Olej sojowy, L. J. 130,2	9,2	±2,7	3,28	±0,46
4	5 „ „ L. J. 62,7	8,0	±1,7	2,89	±0,33
4	6 „ „ L. J. 9,0	0,3	±0,1	0,19	±0,08

1. Number of animals, 2. Type of diet, 3. Fat-free control, 4. Soy oil, I.No. 130.2, 5. Soy oil, I. No. 62.7, 6. Soy oil, I. No. 9.0, 7. Fat deposit in g on the average, 8. Weight of fat deposit in % of body weight on the average

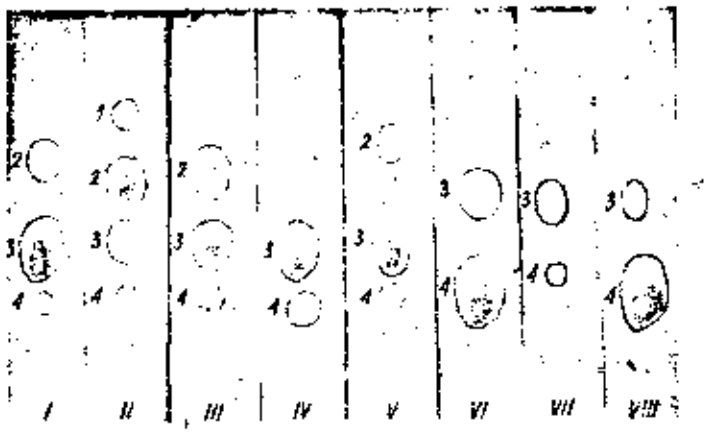

Illustration 1. Chromatographic analyses of fatty acids in the following samples:

I. Fatty tissue of rats fed the control diet
II. Liquid soy oil, Iodine number 130.2
III. Fatty tissue of rats on a control diet + 50% of the weight of liquid soy oil, Iodine number 130.2
IV. Hydrogenated soy oil to Iodine number 62.7
V. Fatty tissue of rats on a control diet + 50% of the weight of hydrogenated soy oil, Iodine number 62.7
VI. Hydrogenated soy oil to Iodine number 9
VII. Fatty tissue of rats on a control diet + 50% of the weight of hydrogenated soy oil, Iodine number 9
VIII. Feces of rats fed 50% supplement of soy oil, Iodine number 9

The zones on the chromatograms represent the following fatty acids: 1 --- linolenic acid, 2 --- linoleic acid, 3 ---oleic and palmitic acid, 4 --- stearic and eicosanoic acid.

In all the experiments binary chromatographic analyses from the combined samples of deposit fat within the compass of one dietetic group.

Table 4. Analysis of the percentage value of total fat in rats fed the experimental diets

Type of diet	Total fat in %	
	rat 1	rat 2
Fat-free	3.3	6.9
Soy oil, I.#130.2	3.5	5.8
" " I.# 62.7	3.5	5.2
" " I.# 9.0	average 0.4	

Table 5. Composition of deposit fat of rats calculated from I.# and R#

Type of diet	I. No. glic.	R. No. glic.	Composition of acids in %		
			dieth.	monoeth.	saturated
Fat-free control	61.2	53.0	9.6	51.3	39.1
Soy oil, I.#130.2	109.6	71.3	46.6	33.6	19.8
" " I.# 62.7	68.3	62.8	6.4	66.5	27.1

Table 6. Composition of total fat of rats calculated from I.# and R.#

Type of diet	I.No.glic.	R.No.glic.	Composition of acids in %		
			dieth.	monoeth.	saturated
Fat-free control	66.5	56.8	11.7	53.8	34.5
Soy oil, I.#130.2	112.0	77.9	42.0	45.7	12.3
" " I.# 62.7	74.1	64.2	11.9	62.2	25.9

Table 7. Level of cholesterol in the liver

Tabela 7. Poziom cholesterolu w wątrobie

1 Liczba zwierząt	2 Rodzaj diety (+1% cholesterolu)	7 Cholesterol mg %		% wolnego cholesterolu
		8 całkowity	9 wolny	
3	3 Kontrolna „beztłuszczowa"	695± 15	316± 16	45,3±2,0
3	4 Olej sojowy, L. J. 130,2	2770±281	1020±144	36,8±5,5
4	5 „ „ L. J. 62,7	1375±179	550± 62	40,0±2,4
4	6 „ „ L. J. 9	700± 65	355± 45	50,7±1,8

(+ 1% cholesterol)

1. Number of animals, 2. Type of diet,/3. Fat-free control, 4. Soy oil, I.No. 130.2, 5. Soy oil, I. No. 62.7, 6. Soy oil, I.No. 9, 7. Cholesterol mg %, 8. total, 9. free, 9. % of free cholesterol

Table 8. Level of cholesterol and lipids in blood serum

Tabela 8. Poziom cholesterolu i lipidów w surowicy krwi

1 Liczba zwierz.	2 Rodzaj diety	7 Cholesterol mg %		10 % wolnego cholesterolu	11 Lipidy mg %
		8 całkowity	9 wolny		
3	3 Kontrolna „beztłuszczowa"	69 ± 3,6	17±2,2	24,1±1,4	385±50
3	4 Olej sojowy, L. J. 130,2	119±15,2	33±5,8	27,6±3,8	575±59
4	5 „ „ L. J. 62,7	86±16,1	29±8,7	33,4±6,9	570±34
4	6 „ „ L. J. 9	61±14,4	12±2,5	20,4±1,2	325±49

1. Number of animals, 2. Type of diet, 3. Fat-free control, 4. Soy oil, I. No. 130.2, 5. Soy oil, I. No. 62.7, 6. Soy oil, I. No. 9, 7. Cholesterol mg %, 8. total, 9. free, 10. % of free cholesterol, 11. Lipids mg %

Table 9. Weight of collected fatty tissue in rats after transferring the animals of all dietetic groups to the basic diet for a period of 10 weeks

Number of animals	Previous diet	Average of body weight g	Average of weight of fatty tissue deposit g	Fatty tissue as % of body weight
3	Fat-free control (C.D.)	241±37	3,5±0,4	1,39±0,08
2	C.D. + 50% soy oil I.#130.2	280±35	4,9±1,3	1,74±0,15
2	C.D. + 50% soy oil I.#62.7	245± 5	5,1±0,8	2,08±0,41
3	C.D. + 50% soy oil I.#9	256±17	0,9±0,8	0,35±0,34

Illustration 2. Microphotograph of a segment of the wall of the small intestine of a rat. The segment was taken within four hours after the rat consumed soy oil with I.#9. Consumption was preceded by 40 hours of fasting. The epithelium of the mucous membrane of the intestine shows the presence of graininess colored an intensive red. (Baker's formalin-calcium fixer, cuttings frozen, dyed Oil.Red.O. in isopropyl alcohol).

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WPŁYW UTWARDZANIA OLEJÓW ROŚLINNYCH NA PRZEMIANĘ TŁUSZCZU I CHOLESTEROLU U BIAŁEGO SZCZURA

CZĘŚĆ II: OLEJ SOJOWY

Z Zakładu Patologii Ogólnej i Doświadczalnej AM w Poznaniu
Kierownik: prof. dr A. Horst
Z Instytutu Przemysłu Tłuszczowego w Warszawie
Dyrektor: dr A. Bereźniak

W pierwszej części doświadczeń poświęconych badaniu przemiany tłuszczu i cholesterolu u białych szczurów pod wpływem żywienia utwardzonym tłuszczem roślinnym stosowaliśmy olej rzepakowy o różnym stopniu uwodornienia [7]. W grupie zwierząt otrzymujących w diecie olej rzepakowy uwodorniony maksymalnie o liczbie jodowej 4 stwierdziliśmy przy tym zanik tkanki tłuszczowej depozytowej. Przestawienie tych szczurów na dietę węglowodanowo-białkową przez okres 10 tygodni nie doprowadziło do odnowy tkanki tłuszczowej.

Olej rzepakowy ciekły oprócz kwasów olejowego, stearynowego, palmitynowego, linolowego i linolenowego zawiera około 40% długołańcuchowego (22 c) kwasu erukowego, który jak wiadomo, ma mniejszy współczynnik strawności u szczura [2], a który w procesie uwodornienia przechodzi w niespotykany w tkance tłuszczowej zwierząt kwas behenowy [4].

Wobec tego wynikło pytanie, czy zaobserwowane zaburzenie w przemianie tłuszczu należy odnieść do samego stopnia utwardzenia, czy też do swoistego składu oleju rzepakowego. Postanowiliśmy więc przeprowadzić ponownie badania przy użyciu oleju roślinnego o innym składzie kwasów tłuszczowych. Zastosowaliśmy olej sojowy.

TECHNIKA DOŚWIADCZEŃ

Materiał doświadczalny stanowiło 40 dorosłych szczurów białych, samców wagi ok. 200 g, podzielonych na 4 grupy dietetyczne. Trzy grupy szczurów otrzymywały diety tłuszczowe, sporządzane z beztłuszczowej

* Praca subsydiowana przez Polską Akademię Nauk, Wydział VI Nauk Medycznych oraz przez Zjednoczenie Przemysłu Olejowego, Warszawa.

diety podstawowej i tłuszczu badanego w stosunku wagowym 1 : 1, grupa czwarta zawierała szczury kontrolne żywione, jak w poprzednich badaniach, wyłącznie podstawową dietą ubogotłuszczową. Do wszystkich diet dodano cholesterol farmaceutyczny w ilości 1%. Skład diet doświadczalnych zawiera tabela 1.

Do żywienia używaliśmy oleju sojowego ciekłego, rafinowanego, o liczbie jodowej 130,2, liczbie kwasowej 0,28 i liczbie zmydlenia 190,0 oraz uwodornionych olejów sojowych: do L. J. 62,7 i do L. J. 9. Proces utwardzania był przeprowadzany w tych samych warunkach jak dla oleju rzepakowego.

Tabela 1. Skład diet doświadczalnych

Symbol diety	Zawartość diety	L. J. tłuszczu	Białko %	Tłuszcze %	Węglowodany %	Kal/g
K	Kontrolna „beztłuszczowa" (34% chudego mleka w proszku, 15% jaj, 25% kazeiny, 25% krochmalu, 1% cholesterolu) + vit. B Kompl. Forte	—	32	2	40	3,1
S 130	50% diety kontrolnej + 50% oleju sojowego ciekłego	130,2	16	50	20	6,1
S 62	50% diety kontrolnej + 50% oleju sojowego utwardzonego	62,7				
S 9	50% diety kontrolnej + 50% oleju sojowego utwardzonego	9,0				

Zwierzęta otrzymywały pokarm do woli, tak że drogą spożycia większej ilości odpowiedniej diety było możliwe wyrównanie różnic w kaloryczności diet tłuszczowych i kontrolnej.

Po upływie 6 tygodni żywienia wykonano we wszystkich grupach szczurów oznaczenie odsetkowej zawartości tłuszczu całkowitego, badanie składu kwasów tłuszczowych metodą chromatografii bibułowej w zebranej tkance tłuszczowej depozytowej, oznaczenia poziomu cholesterolu we krwi i w wątrobie, oznaczenia poziomu lipidów we krwi, oraz w grupie S_9 badanie kału na zawartość tłuszczu i obecność kwasów tłuszczowych. Metodyka większości badań została przytoczona w poprzednim doniesieniu.

Celem oznaczenia procentowej zawartości tłuszczu całkowitego, po 2 szczury z każdej grupy, po zabiciu przez uśpienie eterem, dokładnym oczyszczeniu futerka, otwierano w części brzusznej, przepłukiwano wodą cały przewód pokarmowy celem usunięcia resztek pokarmu i umieszczano na przeciąg 24 godzin w zamrażarce w temperaturze minus 30°C. W sta-

nie całkowitego zamrożenia szczura pocięto i przepuszczono przez maszynkę do mięsa, a następnie umieszczano w homogenizatorze dla uzyskania jednolitej masy. Z tak przygotowanego materiału pobierano próbkę do ilościowego oznaczenia tłuszczu całkowitego [7] a z pozostałej części przygotowano wyciąg chloroformowy [6] dla oznaczenia składu kwasów tłuszczowych. Ze względu na małą zawartość tłuszczu u szczurów w grupie maksymalnie utwardzonego oleju (S_9), obydwa badane szczury połączono w jedną masę i oznaczenie wykonano łącznie.

Dodatkowo wykonano badanie mikroskopowe ściany jelita cienkiego w okresie wchłaniania diety zawierającej olej sojowy utwardzony do liczby jodowej 9, posługując się barwnikiem — czerwień olejowa.

WYNIKI

Wyniki badań na szczurach żywionych olejem sojowym, były w zasadzie zgodne z wynikami uzyskanymi w przypadku diet z olejem rzepakowym. Zwierzęta w grupie oleju sojowego maksymalnie utwardzonego (L. J. 9) wyraźnie chudły mimo wzmożonego spożywania diety. Podczas

Tabela 2. Zestawienie średnich wartości wag szczurów w poszczególnych grupach

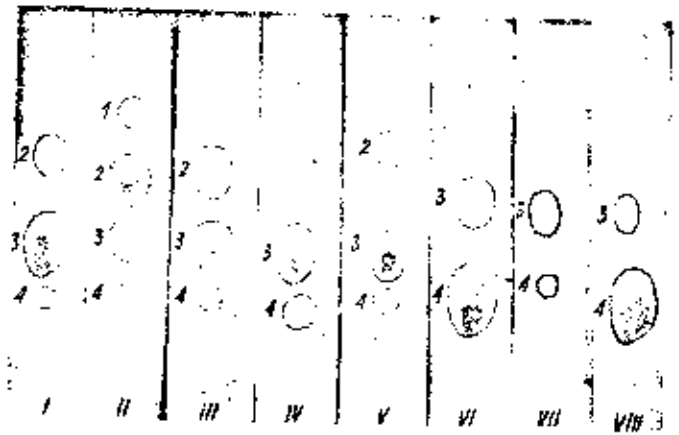
Ilość szczurów	Symbol diety	Średnie wagi grupowe szczurów w g						Średni przyrost (lub ubytek) wagi wyrażony w % wagi wyjściowej
		wyjściowa	dni spożywania diety					
			7	16	29	35	42–49	
10	Kontrolna	205 ± 24	208 ± 22	210 ± 20	243 ± 25	237 ± 24	265 ± 22	+29,2 ± 3,61
10	S 130	194 ± 9	222 ± 14	243 ± 23	267 ± 28	260 ± 22	277 ± 26	+41,4 ± 6,75
10	S 62	190 ± 18	225 ± 21	237 ± 23	262 ± 26	260 ± 27	272 ± 26	+42,4 ± 3,97
10	S 9	201 ± 23	200 ± 23	177 ± 29	173 ± 8	162 ± 12	156 ± 12	—22,3 ± 3,19

zbiórki tkanki tłuszczowej przeznaczonej do badań chromatograficznych okazało się, że praktycznie zostały one pozbawione tkanki tłuszczowej okołonerkowej, krezkowej, sieciowej i okolicy narządów płciowych oraz podściółki tłuszczowej skóry. Znamienne różnice w wadze zwierząt, oraz w ilości tkanki tłuszczowej, stwierdzone między poszczególnymi grupami, przedstawiają tabele 2 i 3.

Tabela 4 przedstawia odsetkową zawartość całkowitego tłuszczu ustrojowego, potwierdzając znaczną utratę tłuszczu u szczurów żywionych maksymalnie utwardzonym olejem sojowym.

Tabela 3. Wpływ diet doświadczalnych na odkładanie tkanki tłuszczowej

Liczba zwierz.	Rodzaj diety	Tłuszcz depozytowy w g średnio		Waga tłuszczu dep. w % wagi ciała średnio	
3	Kontrolna „beztłuszcz."	5,5	± 1,3	2,03	± 0,55
3	Olej sojowy, L. J. 130,2	9,2	± 2,7	3,28	± 0,46
4	„ „ L. J. 62,7	8,0	± 1,7	2,89	± 0,33
4	„ „ L. J. 9,0	0,3	± 0,1	0,19	± 0,08



Ryc. 1. Badania chromatograficzne kwasów tłuszczowych w następujących próbkach:
I. Tkanka tłuszczowa szczurów żywionych dietą kontrolną
II. Olej sojowy ciekły, L. J 130,2
III. Tkanka tłuszczowa szczurów na diecie kontrolnej + 50% wag. oleju sojowego ciekł. L. J 130,2
IV. Olej sojowy uwodorniony do liczby jodowej 62,7
V. Tkanka tłuszczowa szczurów na diecie kontrolnej + 50% wag. oleju sojowego uwodorn. L. J 62,7
VI. Olej sojowy uwodorniony do liczby jodowej 9
VII. Tkanka tłuszczowa szczurów na diecie kontrolnej + 50% wag. oleju sojowego uwodorn. L. J 9
VIII. Kał szczurów żywionych 50% dodatkiem oleju sojowego, L. J 9.
Strzały na chromatogramach przedstawiają następujące kwasy tłuszczowe: 1 — kwas linolenowy, 2 — kwas linolowy, 3 — kwas olejowy i palmitynowy, 4 — kwas stearynowy i eikozenowy.
We wszystkich doświadczeniach wykonano podwójne badania chromatograficzne ze złączonych próbek tłuszczu depozytowego w obrębie jednej grupy dietetycznej.

Badania chromatograficzne na obecność kwasów tłuszczowych, wykonane w zebranej tkance tłuszczowej jak i w wyekstrahowanym z ustroju tłuszczu całkowitym przedstawiamy na ryc. 1. Z chromatogramów tych

wynika, że ani w tłuszczu całkowitym, ani w depozytowej tkance tłuszczowej nie znaleziono u szczurów grupy S_0 (maksymalnie utwardzony tłuszcz) kwasu linolowego, charakterystycznego dla składu tłuszczu zwierząt kontrolnych. U zwierząt żywionych częściowo uwodornionym olejem sojowym (grupa S_{62}), kwas linolowy występuje nadal w tłuszczach poddanych analizie, jakkolwiek również nie występował w skarmianym tłuszczu.

Tabela 4. Badanie procentowej zawartości tłuszczu całkowitego u szczurów żywionych dietami doświadczalnymi

Rodzaj diety	Tłuszcz całkowity w %	
	szczur 1	szczur 2
„Beztłuszczowa"	3,8	6,9
Olej sojowy, L. J. 130,2	3,5	5,8
„ „ L. J. 62,7	3,5	5,2
„ „ L. J. 9,0	średnia 0,4	

Wykonane badania składu depozytowej tkanki tłuszczowej są zestawione w tabeli 5, a badania składu całkowitego tłuszczu ustrojowego w tabeli 6. W grupie oleju sojowego o L. J. 9 nie wykonano odpowiednich badań z powodu niewystarczającej ilości tłuszczu w ekstrakcie. Wpływ spożywanego tłuszczu na tłuszcze ustrojowe, zaznaczył się szczególnie wyraźnie w grupie ciekłego oleju sojowego (S_{130}), powodując zwiększenie zawartości kwasów dwu-nienasyconych.

Tabela 5. Skład tłuszczu depozytowego szczurów obliczony z L. J. i L. R.

Rodzaj diety	L. J. glic.	L. R. glic.	Skład kwasów w %		
			dwuet.	monoet.	nasyc.
Kontrolna „beztłuszczowa"	61,2	53,0	9,6	51,3	39,1
Olej sojowy, L. J. 130,2	109,6	71,8	46,6	33,6	19,8
„ „ L. J. 62,7	68,3	62,8	6,4	66,5	27,1

Wyniki badania tłuszczu w kale szczurów żywionych olejem sojowym utwardzonym do liczby jodowej 9, były identyczne jak w grupie oleju rzepakowego o liczbie jodowej 4. Kał ten zawierał 70% tłuszczu w suchej masie a chromatograficznie wykazano w nim obecność wszystkich kwasów tłuszczowych zawartych w skarmianym tłuszczu.

Tabela 6. Skład tłuszczu całkowitego szczurów obliczony z L. J. i L. R.

Rodzaj diety	L. J. glic.	L. R. glic.	Skład kwasów w %		
			dwuet.	monoet.	nasyc.
Kontrolna „beztłuszczowa"	66,5	56,8	11,7	53,8	34,5
Olej sojowy, L. J. 130,2	112,0	77,9	42,0	45,7	12,3
„ „ L. J. 62,7	74,1	64,2	11,9	62,2	25,9

Wyniki badania poziomu cholesterolu w wątrobie były również zgodne z uzyskanymi w poprzedniej pracy dla oleju rzepakowego, wskazując na małą wchłanialność maksymalnie utwardzonych tłuszczów (tabela 7).

Tabela 7. Poziom cholesterolu w wątrobie

Liczba zwierząt	Rodzaj diety (+1% cholesterolu)	Cholesterol mg %		% wolnego cholesterolu
		całkowity	wolny	
3	Kontrolna „beztłuszczowa"	695± 15	316± 16	45,3±2,0
3	Olej sojowy, L. J. 130,2	2770±281	1020±144	36,8±5,5
4	„ „ L. J. 62,7	1375±179	550± 62	40,0±2,4
4	„ „ L. J. 9	700± 65	355± 45	50,7±1,8

W tabeli 8, przedstawiamy wyniki oznaczeń poziomu cholesterolu i lipidów w surowicy krwi. W grupach szczurów żywionych ciekłym olejem sojowym jak i olejem częściowo utwardzonym stwierdziliśmy tym razem, w odróżnieniu od doświadczeń z olejem rzepakowym, podwyższenie poziomu cholesterolu w grupie ciekłego oleju sojowego.

Tabela 8. Poziom cholesterolu i lipidów w surowicy krwi

Liczba zwierz.	Rodzaj diety	Cholesterol mg %		% wolnego cholesterolu	Lipidy mg %
		całkowity	wolny		
3	Kontrolna „beztłuszczowa"	69± 3,6	17±2,2	24,1±1,4	385±50
3	Olej sojowy, L. J. 130,2	119±15,2	33±5,8	27,6±3,8	575±59
4	„ „ L. J. 62,7	86± 16,1	29±8,7	33,4±6,9	570±34
4	„ „ L. J. 9	61±14,4	12±2,5	20,4±1,2	325±49

Wyniki powyższych badań wskazywały, że tłuszcze maksymalnie utwardzone praktycznie raczej nie wchłaniały się, powodując z kolei niedobór kaloryczny i w związku z tym zanik tkanki tłuszczowej w ustroju.

Jednak następne doświadczenia nie potwierdzają w zupełności tego wniosku: Dodatkowo wykonane badanie mikroskopowe ściany jelita cienkiego w okresie resorpcji pokarmowej po podaniu oleju sojowego maksymalnie uwodornionego, świadczy o przechodzeniu tych tłuszczów do ściany jelita cienkiego. (Ilościowych badań wchłaniania nie wykonywano).

Tabela 9. Waga zebranej tkanki tłuszczowej u szczurów po przestawieniu zwierząt wszystkich grup dietetycznych na żywienie podstawowe przez okres 10 tygodni

Liczba zwierząt	Poprzednia dieta	Średnia wagi ciała g	Średnia wagi tkanki tłuszczowej depozyt. g	Tkanka tłuszczowa jako % wagi ciała
3	Kontrolna „bez-tłuszczowa" (D. K.)	241 ± 37	3.5 ± 0.4	1,39 ± 0,08
2	D. K. + 50% oleju sojowego L. J. 130,2	280 ± 35	4,9 ± 1,3	1,74 ± 0,15
2	D. K. + 50% oleju sojowego L. J. 62,7	245 ± 5	5,1 ± 0,8	2,08 ± 0,41
3	D. K. + 50% oleju sojowego L. J. 9	256 ± 17	0,9 ± 0,8	0,35 ± 0,34

Ryc. 2. Mikrofotografia wycinka ściany jelita cienkiego szczura. Wycinek pobrano w 4 godziny po spożyciu przez szczura oleju sojowego o L. J. 9. Spożycie poprzedzono 40 godz. głodzeniem. Nabłonek śluzówki jelita wykazuje obecność ziarnistości barwiących się intensywnie czerwono. (Utrwalacz formalinowo-wapniowy Bakera, skrawki mrożone, barwione Oil. Red. O. w alkoholu izopropylowym).

Co więcej, gdy przestawiliśmy część szczurów z wszystkich grup tłuszczowych na dietę podstawową węglowodanowo-białkową ad lib. przez następne 10 tygodni, to nawet po tym czasie szczury żywione poprzednio maksymalnie utwardzonym olejem sojowym, wykazały w dalszym ciągu niedobór tkanki tłuszczowej (tabela 9) potwierdzając odnośne spostrzeżenia w poprzedniej pracy.

DYSKUSJA

U szczurów żywionych maksymalnie utwardzonymi olejami roślinnymi — rzepakowym i sojowym, przy dostatecznej podaży węglowodanów w diecie, doszło do bardzo znacznego zaniku tkanki tłuszczowej. Analizując przyczynę tego zjawiska musimy rozważyć następujące możliwości:

1. Ilość podanych kalorii przyswajalnych w diecie była tak niska, że doszło do niedoboru kalorycznego i w związku z tym do zaniku tkanki tłuszczowej.

2. Podane w diecie tłuszcze uniemożliwiły prawidłowe wykorzystanie pozostałych składników pokarmowych, wywołując również niedobór kaloryczny.

3. Podane w diecie substancje mogły mieć działanie toksyczne i wywołać wzmożony rozpad tkanki tłuszczowej.

4. Dodane do diety ubogotłuszczowej tłuszcze maksymalnie uwodornione, spowodowały zużycie ustrojowych niezbędnych nienasyconych kwasów tłuszczowych i na tej drodze uniemożliwiły prawidłowe odkładanie tłuszczu.

5. Podane w diecie tłuszcze utwardzone, względnie produkty powstające w trakcie utwardzania, mogą wchodzić w połączenia prawidłowych kwasów tłuszczowych w ustroju i w ten sposób blokować przyswajanie lub wytwarzanie endogennych kwasów tłuszczowych.

Ad 1. Możliwość pierwszą można odrzucić, ponieważ ilość kalorii pochodząca z podanych węglowodanów i białek była zasadniczo wystarczająca dla podtrzymania zasobów ustrojowych. Gdyby nawet odrzucić użyteczność kalorii tłuszczowych to spożywane w nadmiarze pozostałe składniki diety powinny, naszym zdaniem, zapewnić dodatni bilans kaloryczny. Przypominamy, że zwierzęta kontrolne, żywione podstawową dietą beztłuszczową, zachowały prawidłową zawartość tłuszczu w tkankach.

Ad 2. Trudniejszą do wytłumaczenia jest możliwość zahamowania przez tłuszcze utwardzone wchłaniania w przewodzie pokarmowym. Ze względu na dobrą makroskopową trawienność (upłynnienie pokarmu w jelicie cienkim) nie wydaje się, aby było możliwe zahamowanie przez te tłuszcze wchłaniania węglowodanów.

Ad 3. Możliwość toksycznego działania tłuszczów ogrzewanych do temperatury 275°C jest znana [2]. W naszych badaniach tłuszcze były uwodornione w temperaturze 160—200°C. Podczas procesu uwodorniania, mogą jednak powstać w wyniku ubocznych reakcji pewne produkty, być może o działaniu toksycznym. W naszych badaniach przeciwko tej hipotezie przemawia fakt, że szczury na dietach doświadczalnych nie sprawiały wrażenia chorych, poza maksymalnym wychudzeniem zwierząt w grupie tłuszczów o najniższej liczbie jodowej.

Ad 4. Na możliwość niedoboru niezbędnych nienasyconych kwasów tłuszczowych, wskazuje brak kwasu linolowego tak w tkance tłuszczowej depozytowej jak w ekstrakcie tłuszczowym całego zwierzęcia, w grupach szczurów żywionych maksymalnie uwodornionym tłuszczem rzepakowym czy sojowym. Wiadomo, że u dorosłych szczurów zespół niedoboru niezbędnych nienasyconych kwasów tłuszczowych nie występuje z powodu samej diety beztłuszczowej, lecz może się pojawić przy dodatkowym obciążeniu (cholesterol, kwas cholowy, tłuszcz nasycony, uprzednie głodzenie [6, 7]). Autorzy ci również obserwowali w tych przypadkach chudnięcie zwierząt. Mechanizm oddziaływania niezbędnych nienasyconych kwasów tłuszczowych na magazynowanie tłuszczu ustrojowego nie jest wyjaśniony.

Ad 5. Dosyć prawdopodobną wydaje się możliwość zablokowania syntezy kwasów tłuszczowych endogennych z fragmentów dwuwęglowych. Jak wiadomo, tłuszcze endogenne powstają głównie w toku przemiany węglowodanowej. Za tą hipotezą przemawia fakt, że mimo dostatecznej podaży węglowodanów w diecie, tkanka tłuszczowa depozytowa nie uległa odnowieniu. Z badań izotopowych przemiany tkanki tłuszczowej wiemy, że ulega ona szybkiej przemianie, stąd jedynie ciągłe wytwarzanie nowych kwasów tłuszczowych może zapobiec jej zanikowi. Należy przypuszczać, że w naszym przypadku ten mechanizm został zakłócony i wskutek nieodnawiania się tkanki tłuszczowej doszło do jej zaniku. Jest to naturalnie tylko hipoteza, która wymaga dalszych badań.

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ВЛИЯНИЕ ОТВЕРЖДЕНИЯ РАСТИТЕЛЬНЫХ МАСЕЛ НА ОБМЕН ЖИРА И ХОЛЕСТЕРОЛА У БЕЛОЙ КРЫСЫ

Часть II. Соевое масло

Содержание

В течение 5 недель вскармливали крысы диетой, содержащей 1% холестерола с добавлением 50% (по весу) соевого масла с различной степенью гидрогенизации жирных кислот (Л. J 130 62 и 9). Изучали влияние указанных диет на уровень холестерола и липидов в сыворотке крови и в печени, а также на количество и состав общего жира в организме и в депонированной жировой ткани.

Обнаружили повышение уровня липидов сыворотки крови у крыс вскармливаемых жидким соевым маслом (Л. J 130) и маслом частично отверждённым (Л. J 62). Уровень холестерола в сыворотке крови был повышен в группе животных, получавших жидкое соевое масло. Эта группа обнаружила также очень высокие показатели содержания холестерола в печени.

Хроматографический анализ жирных кислот в общем жире организма и в жировой ткани у крыс вскармливавшихся максимально гидрогенизированным соевым маслом обнаружил отсутствие линолевой кислоты, характерной для жировой ткани животных, вскармливаемых диетой бедной жиром.

Исчезновение депонированной жировой ткани, возникающее в связи с вскармливанием максимально отверждённым маслом замеченное в экспериментах с сурепным маслом — выступило также в группе, получавшей соевое масло. Это было подтверждено очень низким процентным содержанием общего жира в организме крыс этой группы. После перевода части подопытных животных этой группы обычной основной диеты в течение 10 недель, количество жировой ткани не увеличивалось.

В конце обсудили возможные пути влияния максимально гидрогенизированного масла на жировой обмен организма.

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THE EFFECT OF HARDENING OF VEGETABLE OILS ON THE FAT AND CHOLESTEROL METABOLISM IN WHITE RATS

II. Soya oil

Summary

Laboratory rats were given a diet containing 1 per cent of cholesterol and 50 per cent (weight) soya oil with various degrees of hydrogenation of the fatty acids (iodine numbers 130, 62 and 9). The effect of the diets on the levels of cholesterol and lipids in the blood serum and in the liver were studied, as well as on the amount and composition of total fat in the body and deposited fatty tissue.

Raised blood serum lipid levels were observed in rats fed with liquid soya oil (iodine number 130), and partially hydrogenated oil (iodine number 62). The blood serum cholesterol level was elevated only in the group of rats receiving liquid soya oil, which also had very high cholesterol content in the livers.

Chromatographic analysis of the fatty acids in total body fat and in adipose tissue in rats receiving maximally hydrogenated soya oil showed absence of linoleic acid, which is characteristic of fatty tissues in animals fed on a basic fat-poor diet.

Disappearance of deposited fat tissue under the influence of feeding with maximally hardened fat, which was observed in experiments with rapeseed oil, occurred also with soya oil, and was confirmed by the very low percentual content of body fat in the rats of this group. After transfer of part of the animals to the basic diet for 10 weeks, the low content of adipose tissue continued.

The possible effect of maximally hydrogenated oils on the fat metabolism of the body is discussed.

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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

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 1/2% 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

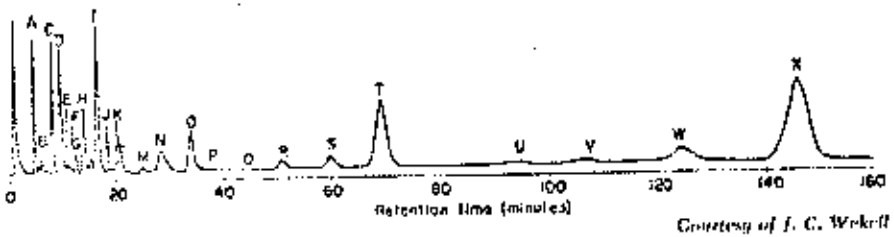
The identification of the components of an unknown mixture must be correlated with standard reference compounds and reference mixtures of known composition. Some of the fatty acid methyl esters common to fish oils are listed in Table 9. All of these are available from the Hormel Institute, Austin, Minnesota. 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, unsaturated, 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.

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 lignoceratе
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	—

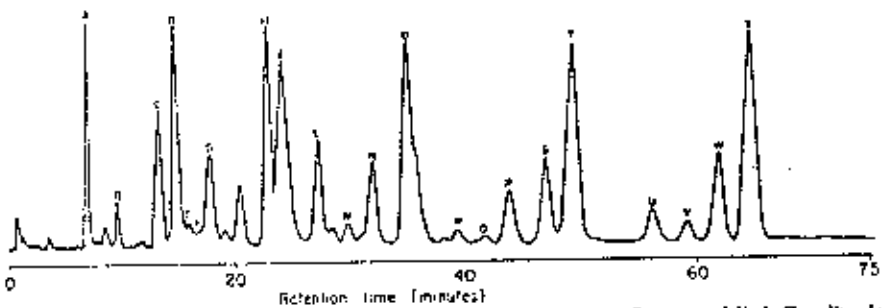
There are several techniques for verifying the structural determinations other than those based upon retention time of peaks on liquid phases of differing polarity and the use of equivalent chain length data as described above. For example, the sample may be hydrogenated so that only saturated compounds 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. INSTITUTIONAL 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

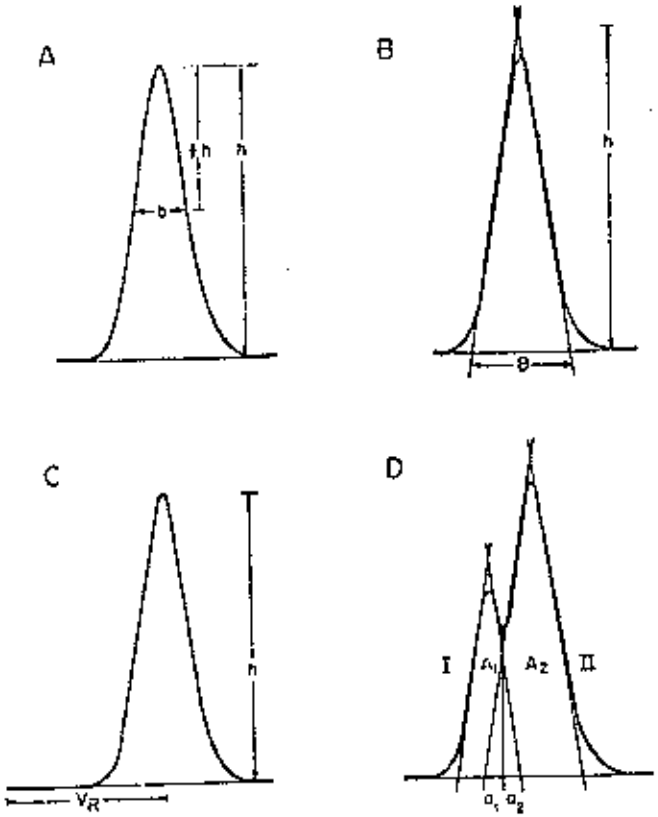


FIG. 4. COMMON METHODS OF MEASUREMENT OF PEAK AREAS

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, 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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