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CHAPTER 22

Erik Aaes-Jørgensen

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).

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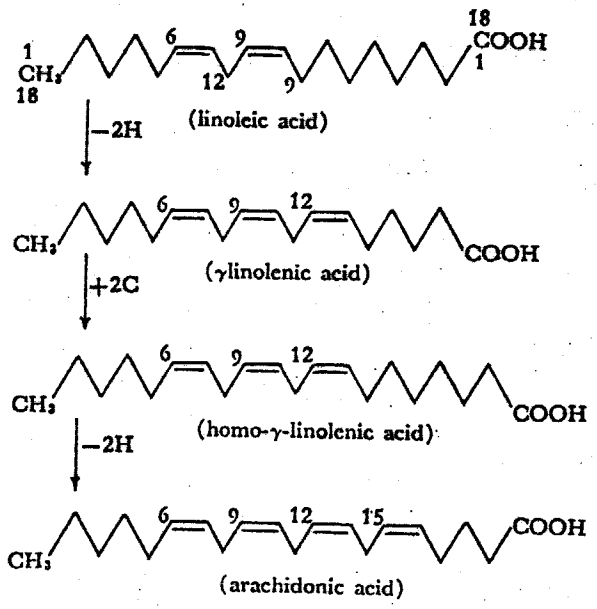

FISH OILS AS A SOURCE OF EFA

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 indicate that 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

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 growth 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 EFA deficient 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

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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 acids 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 t

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 absorbed, 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., Ruvettus 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, Aacs-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, Aacs-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 a

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

JOHN S. ANDREWS,[3] WENDELL H. GRIFFITH, JAMES F. MEAD AND ROBERT A. STEIN

Department of Physiological Chemistry, University of California Medical Center, Los Angeles

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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[3] Present address: Howe Laboratory of Ophthalmology, Harvard University Medical School, Boston 14, Mass.

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 $FeCl_3$. 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}	0.8
		$CuSO_4 \cdot 5H_2O$	0.3	*i*-Inositol	4,0
				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.

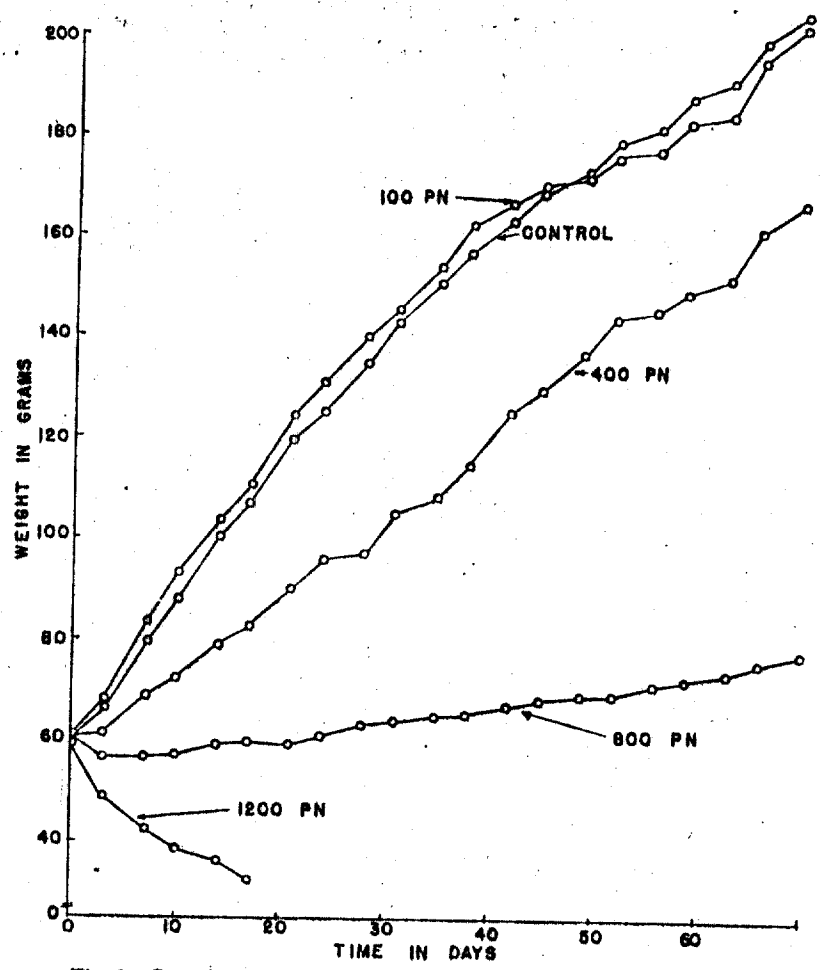

Fig. 1 Growth of female rats receiving various levels of oxidized fat.

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.

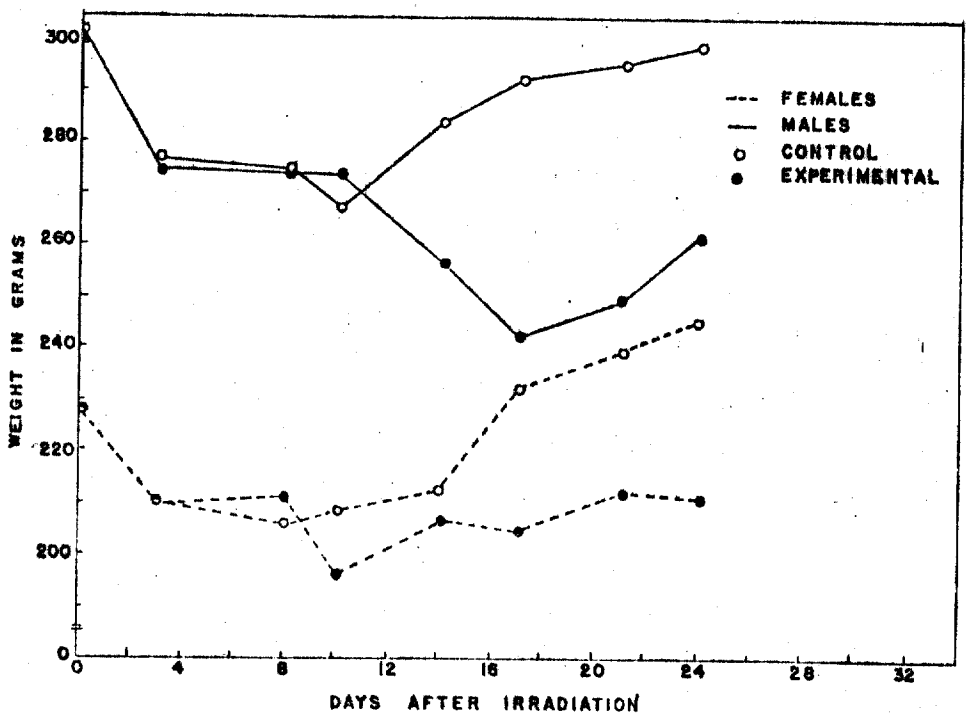

Fig. 2. Effect of diet on recovery from whole body irradiation.

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^{-3} 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

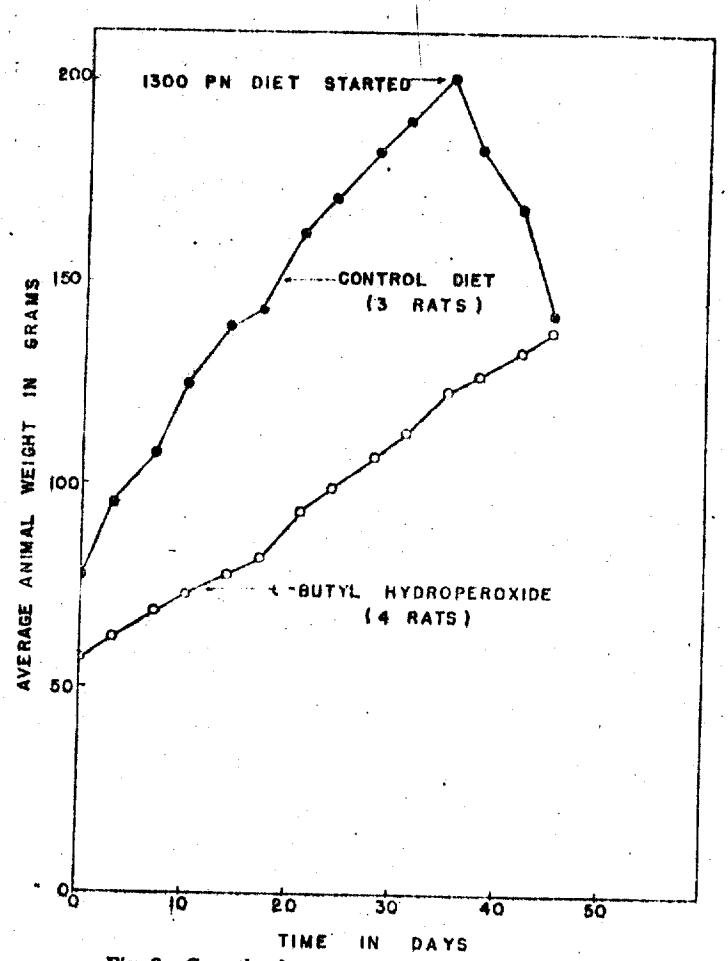

Fig. 3 Growth of rats under several dietary regimes.

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.

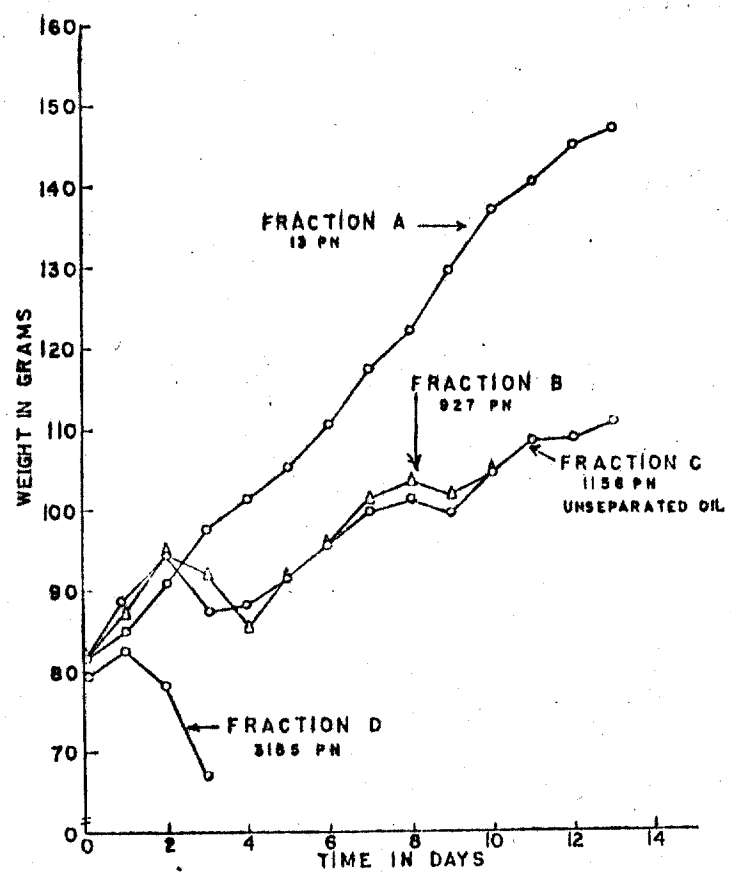

Fig. 4 Growth of rats receiving various fractions of oxidized soybean oil.

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).

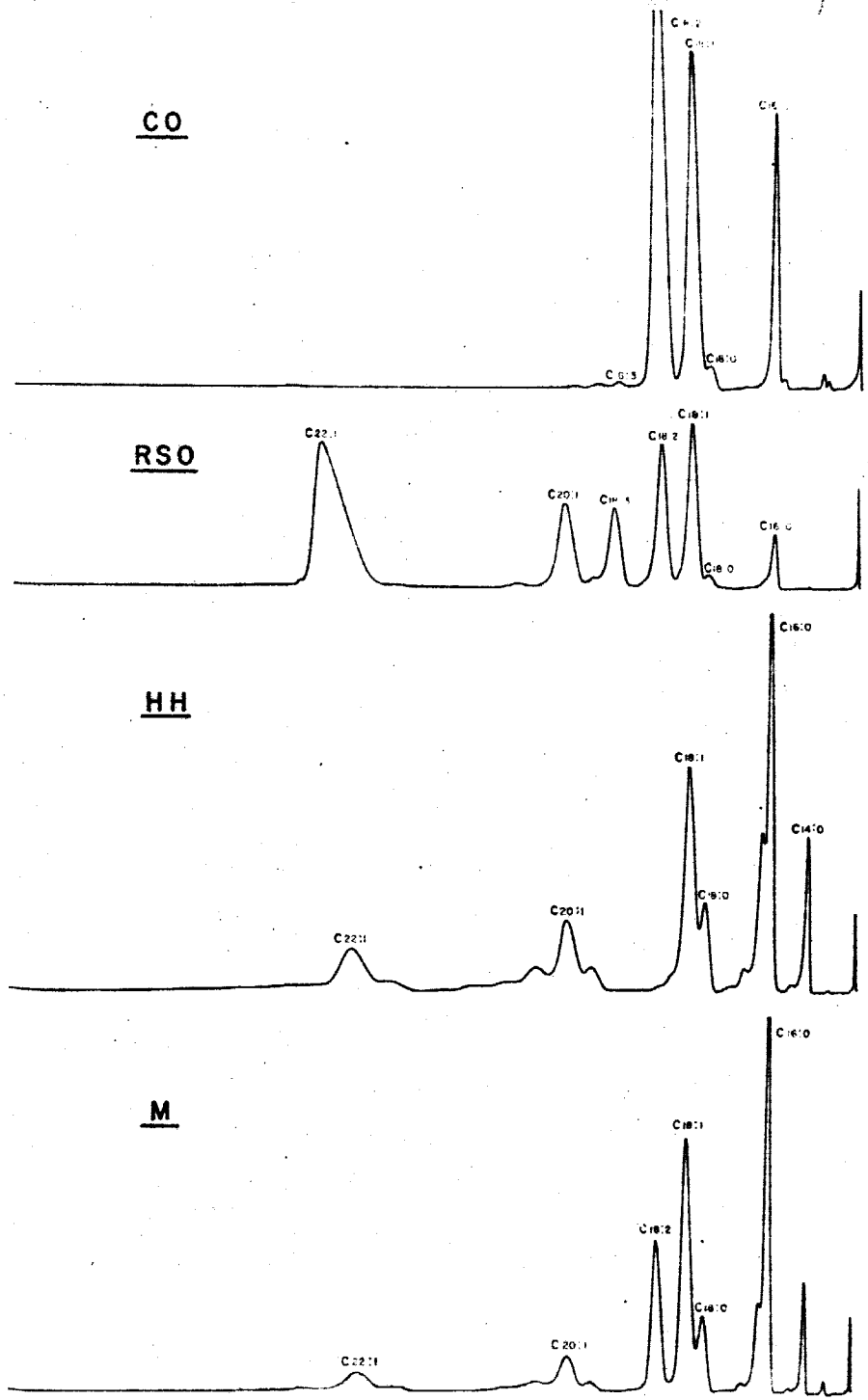

FIG. 1.

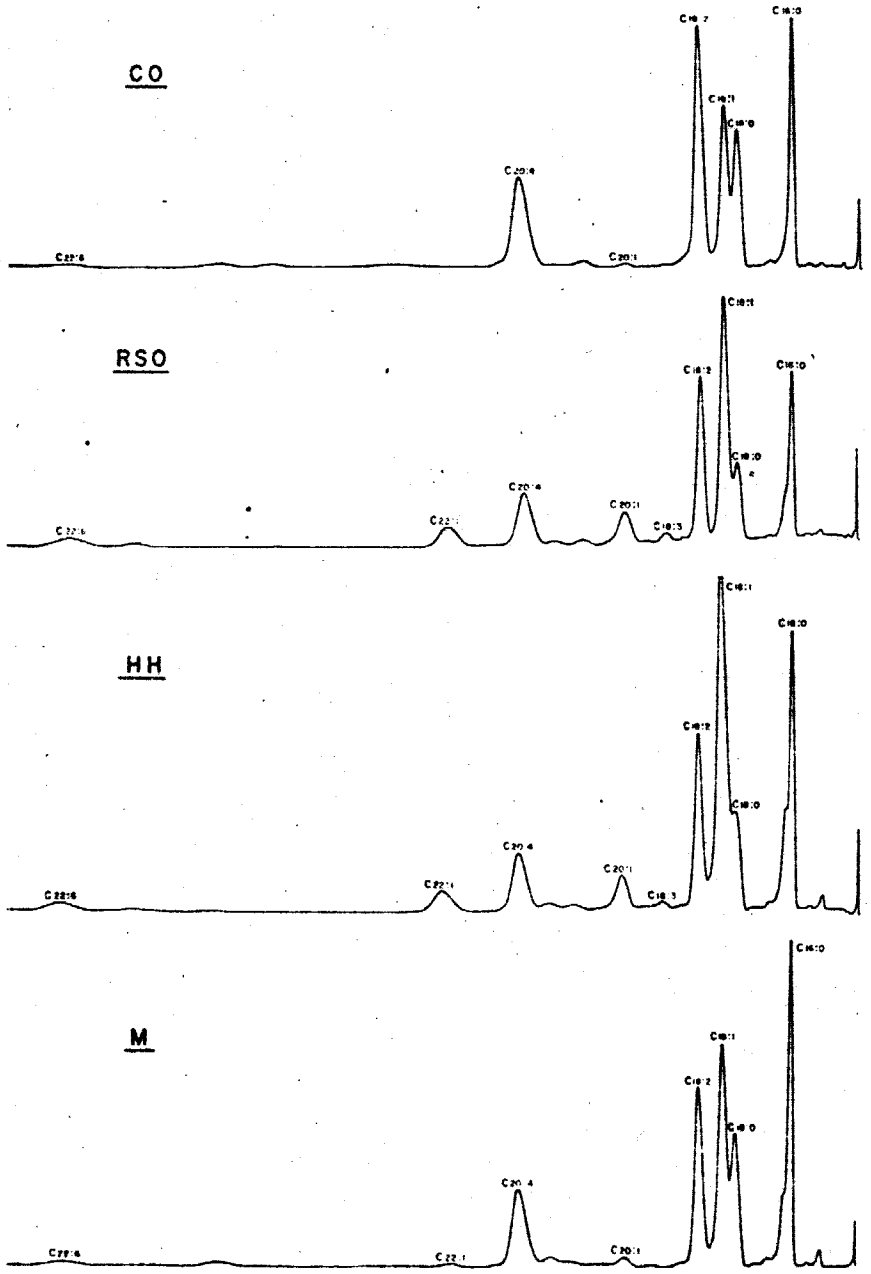

FIG. 2. Chromatograms of methyl esters of livers of rats fed corn oil, rapeseed oil, partially hydrogenated herring oil, or margarine.

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	3.4
	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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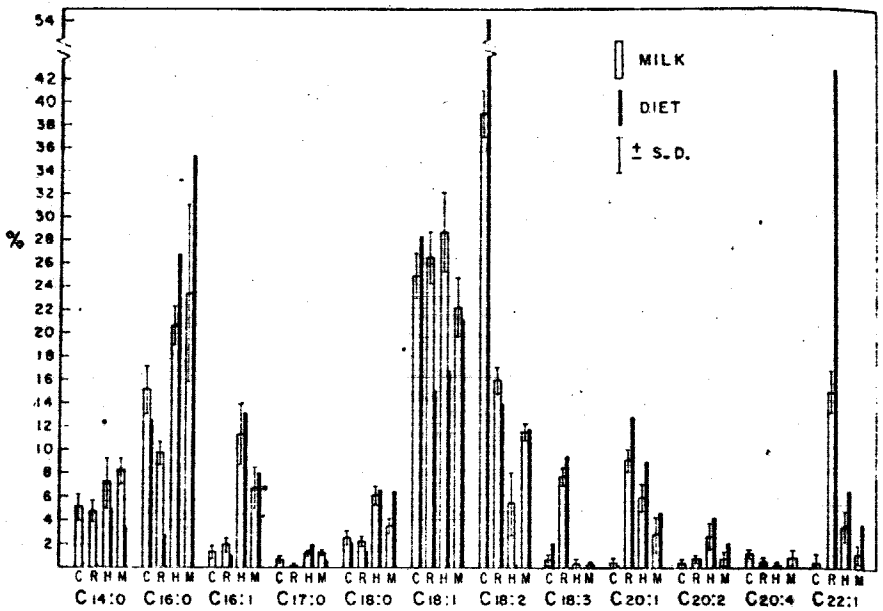

FIG. 3. The percentage of fatty acids in the total fatty acids of milk and diet of seven rats fed corn oil (C), seven rats fed rapeseed oil (R), six rats fed hydrogenated herring oil (H), and four rats fed margarine (M).

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 Nutrition of the Young Ayrshire Calf

13. The Toxicity of the Unsaturated Acids of Cod-liver Oil

By K. L. BLAXTER and F. BROWN
Hannah Dairy Research Institute, Kirkhill, Ayr
and A. M. MacDONALD
Glasgow University and Royal Hospital for Sick Children, Glasgow

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Previous work with calves has shown that when cod-liver oil is added to a ration of reconstituted dried separated milk the animals die as the result of muscular dystrophy (Blaxter, Wood & MacDonald, 1953). Ingestion of dried separated milk does not cause the disease if vitamins A and D are supplied in a concentrate instead of in cod-liver oil. The present paper deals with the toxicity of various fractions of cod-liver oil when given to calves.

In efforts to elucidate the factor or factors present in cod-liver oil concerned in the production of those diseases of laboratory animals generally ascribed to a dietary deficiency of vitamin E, fractions of cod-liver oil have been given to mice (Agduhr, 1935), guinea-pigs (Madsen, McCay & Maynard, 1935) and young rats (Dam & Granados, 1945*a*). The experiments of Agduhr (1935) and Madsen *et al.* (1935) were equivocal, since pathological changes occurred whatever fraction was used. Nevertheless it appeared probable that the saponifiable fraction of cod-liver oil produced the most severe changes. The experiments of Dam & Granados (1945*a*), however, showed that toxicity was concentrated in an unsaturated acid fraction of the oil having an iodine value of 283. This produced depigmentation of the enamel of the incisors of the rats, a marked discoloration and peroxidation of the body fat, acute anaemia and diarrhoea. The non-saponifiable fraction and two further saponifiable fractions (iodine values 13·4 and 99·4) failed to produce any pathological changes or retardation of growth.

Dam's (1944) studies with chicks receiving fractions of hog-liver fat added to a diet deficient in vitamin E showed that, although the most highly unsaturated fatty-acid fraction (iodine value 241) rapidly produced a severe encephalomalacia, both exudative diathesis and encephalomalacia occurred also when an unsaturated fraction with an iodine value of 104 was given. It appears fairly certain, therefore, that in laboratory species toxicity resides in the saponifiable fraction of these fats. It has, however, been pointed out by Moore & Wang (1945) that when cod-liver oil is given in large amounts there is a danger of hypervitaminosis A, and that in accounting for the toxicity of cod-liver oil hypervitaminosis A must be considered also. A daily dose of 28 g cod-liver oil provides approximately 28,000 i.u. vitamin A and approximately 2800 i.u. vitamin D, far more than the minimal requirements for

a 30 kg c. The presence of haemorrhagic muscles in a number of our experimen calves given cod-liver oil (Blaxter, Watts & Wood, 1952) might suggest that hype vitaminosis A was involved in the disease (Moore & Wang, 1945). Haemorrhagic lesions have, however, also been noted in field cases of muscular dystrophy, when there was no possibility of any hypervitaminosis A (Stamp & Blaxter, 1951).

EXPERIMENTAL

Animals

Twenty Ayrshire bull calves were used in two experiments. Exp. 1, with ten calves, began in early March 1952, and Exp. 2, also with ten calves, began in late May 1952.

Treatment. The housing, management and feeding routine were as previously described (Blaxter *et al.* 1952; Blaxter, Wood & MacDonald, 1953). The calves in each experiment were allocated by lot to treatment groups, details of which are given in Table 1. The individual numbers of the calves are given in Table 3.

Table 1. *Experimental treatments of the calves*

Treatment group	Daily addition to the ration	No. of calves Exp. 1	No. of calves Exp. 2
Negative controls	Two capsules of halibut-liver oil	2	2
Positive controls	28 g cod-liver oil	2	2
Non-saponifiable residue	0·28 g non-saponifiable matter of cod-liver oil	2	—
Saturated acids	3·2 g saturated acids of cod-liver oil and two capsules of halibut-liver oil	2	2
Total unsaturated acids	23·6 g unsaturated acids of cod-liver oil and two capsules of halibut-liver oil	2	—
Less unsaturated acids	15·0 g less highly unsaturated acids of cod-liver oil and two capsules of halibut-liver oil	—	2
Highly unsaturated acids	8·6 g highly unsaturated acids of cod-liver oil and two capsules of halibut-liver oil	—	2

The calves were examined twice daily for signs of abnormality, weighed onc a week and killed either when signs of dystrophy were pronounced or when, aft a long period of experimental treatment, it seemed highly unlikely that signs wou develop. The carcasses were dissected and examined for evidence of dystroph degeneration.

Diet. The basal diet was spray-dried skim milk, to which a solution of tra minerals was added twice weekly. The composition of this mineral mixture has be described (Blaxter *et al.* 1952). All animals except one negative control calf, no. 17 were given in addition 2·5 mg α-tocopheryl acetate daily. The dried milk contain approximately 0·5 mg tocopherol, so that the basal ration supplied approximat 3 mg vitamin E daily. Those calves not receiving cod-liver oil or the unsaponifial fraction of cod-liver oil were given capsules of halibut-liver oil to supply vitam A and D. The calves were given the cod-liver oil or its fractions twice daily mi with their allowance of the basal ration.

Chemical analyses

Tocopherols were determined at intervals in the serums of the calves by the method of Emmerie & Engel (1938) as modified by Blaxter, Brown & MacDonald (1953).

Dry matter, ash and creatine were determined in eight muscles of the limbs and in the heart, and total lipid was determined in samples of liver, perinephric fat and muscles of each calf. The tocopherol content of the lipid of these organs was determined by the method previously described (Blaxter, Brown & MacDonald, 1953). The peroxide content of the lipid was determined by the method used by Dam & Granados (1945*b*).

Histological examination

Histological examination was made of samples of the myocardium and the skeletal musculature of most calves and of samples of the kidneys of calves given the saturated acids of cod-liver oil.

Method of fractionating cod-liver oil

The oil, which was a sample complying with British Standard Specification no. 839/1939 was hydrolysed in batches of 100 g and the acids from 500 g were combined for fractionation. The detailed steps were as follows: 100 g of the oil were dissolved in 200 ml. of 95% ethanol containing 28 g KOH and 55 ml. ether (just sufficient to form a homogeneous solution) were added. After allowing the mixture to stand for 24 h in a stoppered bottle at room temperature, 830 ml. water were added and the solution was extracted with 800 ml. ether. The ether extract, after washing free from alkali, was evaporated to give the crystalline, non-saponifiable material. The residual soap solution was made just acid to Congo Red, by the addition of conc. HCl, and then extracted with ether. After washing the ether extract with water until it was free from mineral acid and evaporating the ether, the residual fatty acids were treated as follows:

Experiment 1. The acids from 500 g oil were dissolved in 6 l. 76% (v/v) ethanol; the solution was then held at 0° for 24 h (Ku, 1937). The crystalline acids were removed by filtration, washed with 600 ml. cold 76% ethanol and dried in a vacuum desiccator over calcium chloride. The combined filtrate and washings were evaporated at 50° under reduced pressure in an atmosphere of nitrogen, to yield the unsaturated fraction.

Experiment 2. The highly unsaturated acids were prepared by the method of Hilditch & Maddison (1942); the acids from 500 g oil were dissolved in about 1900 ml. acetone and titrated with saturated aqueous lithium hydroxide solution until just alkaline to phenolphthalein. After adjusting the acetone concentration to 95% the mixture was gently refluxed and allowed to cool slowly. The solid lithium salts were removed by filtration and washed with 95% acetone. The combined filtrate and washings containing the highly unsaturated acids were evaporated in a stream of nitrogen until most of the acetone had been removed, and acidified with conc. HCl. The highly unsaturated acids were then obtained by extraction with ether.

The solid lithium salts were heated with excess dilute HCl, and the mixed saturated and less highly unsaturated acids extracted with ether. After washing t

ether extrac water, the solvent was evaporated and the residual acids were dissolved in (v/v) ethanol and treated as described in Exp. 1 above. The yields of the fractions, their iodine values and the amounts of unsaturated material, expressed in terms of iodine value, supplied to the calves each day are summarized in Table 2. The fractions were stored in tightly stoppered bottles at 0° until required.

Table 2. *Iodine values of cod-liver oil and cod-liver oil fractions*

Material	Amount of oil accounted for by fractions (%)	Iodine value	Unsaturation of fractions given each day (as g iodine)	Iodine value of oil accounted for by fractions (%)
Cod-liver oil	100·0	158	44·2	100·0
Fractions:				
Non-saponifiable residue	1·0	144	0·4	0·9
Saturated acids (Exp. 1)	11·4	32*	1·0	2·3
Saturated acids (Exp. 2)	11·4	14*	0·4	1·0
Total unsaturated acids	84·3	164	38·7	87·5
Highly unsaturated acids	30·7	270	23·2	52·5
Less unsaturated acids	53·6	110	16·5	37·3

* Owing to the difficulty of complete separation the saturated acid fraction contained some unsaturated acids.

RESULTS

Vitamin E content of the blood serum

The initial concentration of tocopherols in the blood serum fell with continued treatment. After 28 days of experiment there was less than 50 μg/100 ml. total tocopherols in the serum of all the calves except no. 178, which received the non-saponifiable fraction. The results are presented in Table 3 and indicate clearly the

Table 3. *Tocopherol content of blood serum of experimental calves*

Calf no.	Treatment group	Days on treatment: 0	7	14	28	Last value obtained
		Tocopherol content (μg/100 ml.)				
170	Control	123	—	49	—	0 (56 days)
171		148	—	80	—	0 (35 days)
195		356	135	76	24	—
197		190	141	127	30	—
172	Whole cod-liver oil	198	—	91	—	13 (51 days)
173		73	—	22	12	—
198		144	—	105	40	—
199		144	78	26	—	—
178	Non-saponifiable residue	288	—	83	—	97 (35 days)
179		202	—	74	—	20 (57 days)
176	Saturated acids	154	—	60	—	—
177		60	—	64	—	18 (56 days)
204		95	56	50	—	—
205		85	46	52	14	—
174	Total unsaturated acids	—	—	42	—	—
175		104	—	39	—	—
202	Less unsaturated acids	—	111	30	16	—
203		260	163	88	21	—
200	Highly unsaturated acids	151	63	52	26	—
201		232	186	—	45	—

ence of any differences to be attributed to treatment. Even wi no. 170, from which the daily supplement of 2·5 mg α-tocopherol had been eld, there was little evidence of a marked acceleration in the rate of fall of serum tocopherol.

Table 4. *Clinical signs shown by calves, and fate of the calves*

Treatment group	Calf no.	Days of treatment	Predominant clinical signs	Fate	Cause of death
egative controls	170	57	No abnormality observed	Slaughtered	—
	171	36	No abnormality observed	Slaughtered	—
	195	44	No abnormality observed	Slaughtered	—
	197	73	No abnormality observed	Slaughtered	—
Whole cod-liver oil	172	52	Considerable muscular stiffness	Killed *in extremis*	Generalized muscular dystrophy
	173	29	Prostration with failure of appetite	Killed *in extremis*	Infection of umbilicus and generalized septicaemia
	198	36	Abdominal respiration and inability to rise	Killed *in extremis*	Generalized muscular dystrophy
	199	28	Muscular weakness and cardiac signs	Died	Cardiac failure
Non-saponifiable residue	178	36	Loss of appetite	Slaughtered	—
	179	58	No abnormality observed	Slaughtered	—
Saturated acids	176	33	No abnormality noted before death	Died	Severe tympanites
	177	57	No abnormality observed	Slaughtered	—
	204	36	Lumbar pain	Slaughtered	—
	205	58	No abnormality observed	Slaughtered	—
Total unsaturated acids	174	24	Erratic pulse, culminating in a series of heart attacks	Died	Cardiac failure
	175	35	Abdominal respiration, muscular weakness and cardiac abnormalities	Died	Cardiac failure and kidney infection
Less unsaturated acids	202	41	Slight signs of muscular weakness	Slaughtered	—
	203	73	Inappetence early in experiment	Slaughtered	—
Highly unsaturated acids	200	78	Slight signs of muscular weakness (days 30–50) which regressed	Slaughtered	—
	201	38	Prostration with marked weakness	Died	Cardiac failure

Observations before death

The clinical signs shown by the calves are summarized in Table 4. Abnormalities were not observed in the control calves that did not receive cod-liver oil, but the calves receiving cod-liver oil all showed some signs of muscular weakness or cardiac abnormality. In calf no. 173, however, the general weakness was not the result of a dystrophic involvement of the limbs. The body temperature was elevated, there was pneumonia with laboured breathing, and the navel was swollen, tense and hot. It being clear that the calf was suffering from an infection, it was therefore removed from the experiment and killed.

The calves receiving the non-saponifiable material of cod-liver oil both showed

normal beh[illegible] for the first 30 days. Calf no. 179 remained normal in all respects, but calf no. [illegible] tended to lose appetite and weight. Since both calves that had received the unsaturated acids of cod-liver oil had by this time succumbed, calf no. 178 was killed on the 36th day for examination, and its companion was left for a further 4 weeks. No signs of dystrophy were observed in either.

One of the calves receiving the saturated acids of cod-liver oil, no. 176, died suddenly after an acute attack of bloat. No obvious signs of dystrophy were noted during its lifetime. The cause of the tympanites was not discovered, but it was certainly unlike that previously observed in dystrophic calves (Blaxter, Wood & MacDonald, 1953). Only one, no. 204, of the remaining three calves showed any abnormality. It developed an abnormal stance with spread legs, comparable in fact to the stance adopted by calves with dystrophy of the lumbar muscles. Evident pain was felt from pressure applied to the lumbar region, and the calf was very dull for a period of several days. Its body temperature was slightly elevated for 2–3 days when this sign was present, but soon fell to normal. It was killed without having shown further signs and when its condition was quickly improving.

The calves receiving the total unsaturated acids of cod-liver oil both died. There was little doubt from the clinical signs that calf no. 175 was severely dystrophic. Its heart rate had been erratic for 8 days before death, the gait was ataxic and the respiration was laboured without any rise of body temperature being apparent. Three heart seizures and prostration occurred during the last 4 days of calf no. 174's life and its fore-legs were extremely weak. It fell suddenly during feeding on the 24th day, as the result of a cardiac seizure and died within 90 sec. During this time there was grunting, writhing, and protrusion of the tongue. The pulse was imperceptible.

Calf no. 203, which received the less unsaturated acid fraction of cod-liver oil, showed no particular abnormality other than arching of the back and signs of lumbar pain similar to those noted with calf no. 204, which had received the saturated acid fraction. These signs occurred during the 30th–40th days of treatment. This calf was given the experimental diet for a further 28 days before it was killed. It showed no further signs of abnormality. Its companion, however, calf no. 202, showed slight signs indicative of muscular dystrophy rather than the local pain in the region of the kidneys. Separation of the claws of the feet and a relaxation of the carpal-metacarpal joint took place; some slight abnormalities of the heart were noted. These signs, although not sufficient to warrant an unequivocal diagnosis, were suggestive of muscular dystrophy. It was killed for further examination.

Calf no. 201, which received the highly unsaturated acids of cod-liver oil, rapidly lost condition after the 4th week of treatment. It became weak, had to be fed forcibly and spent much time lying in an unnatural position. It was found dead on the morning of the 38th day of experiment, having kicked violently during the last hours of life. Its heart had been judged abnormal when it was examined on the previous day. Calf no. 200, which also received the highly unsaturated acids of cod-liver oil, showed slight signs of muscular weakness early in the experiment. These did not increase in severity; when the calf was slaughtered on the 78th day of experiment,

nothing, apart from a weakness in the shoulder region, was appare[illegible] had gained 328 g/day during the experimental period, which was normal.

Observations post mortem

From the observations made before death it was clear that three calves suffered from a circumscribed lumbar pain not due to the muscular weakness associated with dystrophic degeneration. These calves were no. 178, which received the non-saponifiable matter of cod-liver oil, no. 204, which was given the saturated acids, and no. 203, which received the less unsaturated acid fraction. At post-mortem examination it was found that the kidneys were covered with small white spots, a few mm in diameter, extending deep into the cortex. There was no marked inflammatory reaction, but from these areas a *Corynebacterium* was isolated. Calf no. 203 occupied the pen that had been vacated by calf no. 178, and calf no. 204 the pen immediately adjoining. Though the pen had been thoroughly scrubbed before calf no. 203 was admitted, it seems likely that infection had been brought in by calf no. 178 and transmitted to the others.

Table 5 summarizes the results of examining the calves' musculature. Muscles of those receiving the control ration, the saturated acids of the oil or its non-saponifiable residue were normal, in agreement with the clinical observations made before death. Muscles of calves receiving either whole cod-liver oil or the total unsaturated acid fraction were severely dystrophic, as had also been inferred from the clinical signs shown before death.

Muscles of the two calves receiving the less unsaturated acids were judged to be normal. A very small area of pallor was noted on the epicardium of the right ventricle of calf no. 202; on section of the heart a similar small area of pallor was found in the myocardium of the right ventricle. If it was a lesion, it was of very doubtful significance, for clearly the calf would not have died of dystrophy. The relaxation of the front limb of this calf was not associated with any lesion other than the pallor of the muscles concerned.

Examination of the two calves receiving the highly unsaturated acids revealed very mild dystrophy. The disposition of blood in the lungs of calf no. 201 suggested a cardiac failure affecting the right heart. Small areas of the myocardium of the left ventricle were dystrophic also, giving the wall a mottled appearance on section. Similar small areas were found in the muscles of the hind-limb. The heart of calf no. 200 was streaked with a whitish brown discoloration and a similar discoloration was noted in the substance of the myocardium. The skeletal muscle was very pale throughout, and the gastrocnemius of the hind-limb was found to be mildly dystrophic. This calf was slaughtered at the same time as no. 205, which had received the saturated acids and was not dystrophic. By comparison the marked pallor of the musculature of no. 200 was particularly striking.

Creatine contents of muscles

The mean creatine content of muscles and muscle groups is given in Table 6. The amount of creatine was lower in the muscles of calves given cod-liver oil than in those of controls. The muscles of both calves receiving the non-saponifiable material

Table 5. *Classification of muscles of calves given cod-liver oil or cod-liver oil fractions according to severity of dystrophy*

Treatment group	Calf no.	Fate	Forelimb and forelimb suspension	Hind-limb and hind-limb suspension	Trunk and neck excepting intercostals	Diaphragm and intercostals	Heart
Negative controls		Nos. 170, 171, 195 and 197 were slaughtered and were found to be normal					
Cod-liver oil	172	Killed *in extremis*	Severe	Severe	Very severe	Very severe	Normal
	173*	Killed *in extremis*	Normal	Normal	Normal	Normal	Very slight
	198	Killed *in extremis*	Very severe	Severe	Very severe	Very severe	Very severe
	199	Died	Slight	Slight	Slight	Very slight	Severe
Non-saponifiable residue		Nos. 178 and 179 were slaughtered and were found to be normal					
Saturated acids	176†	Died	? Normal	Normal	Normal	? Normal (pressure)	Deformed by abdominal pressure
		Nos. 177, 204 and 205 were slaughtered and were found to be normal					
Total unsaturated acids	174	Died	Very severe	Severe	Slight	Severe	Severe
	175	Died	Severe	Very severe	Very severe	Normal	Very severe
Less unsaturated acids	202	Slaughtered	Normal	Normal	Normal	Normal	? Normal
	203	Slaughtered	Normal	Normal	Normal	Normal	Normal
Highly unsaturated acids	200	Slaughtered	? Normal	Normal	Normal	? Normal	Very slight
	201	Died	Slight	Normal	Normal	Normal	Severe

* This calf died as the result of an ascending infection from the umbilicus.

† The severe tympanites which caused the death of this calf made it impossible to state whether the muscles were normal. They were certainly not severely dystrophic.

cod-liver oil contained more creatine than did those of the controls. In this respect it is of interest that this fraction of the oil contains appreciable amounts of α-tocopherol (Brown, 1953), suggesting that the negative control ration employed was indeed marginal in meeting vitamin E requirements. The same conclusion may be drawn from comparing the creatine contents of the muscles of these calves with those of calves given adequate amounts of α-tocopherol (Blaxter, Wood & MacDonald, 1953). For convenience these values have been inserted at the foot of Table 6.

Table 6. *The mean creatine content of groups of muscles of the calves*

Treatment group	No. of calves	Creatine content of fresh muscle (mg/100 g)				
		Shoulder*	Forearm†	Lower arm‡	Rectus femoris	Heart
Negative controls	4	329	341	341	412	258
Positive controls (with cod-liver oil)	3	288§	275§	300§	396	201§
Non-saponifiable residue	2	403	422	407	462	266
Saturated acids	3	345	349	344	409	237
Total unsaturated acids	2	260§	295§	324	364§	178§
Less unsaturated acids	2	326	344	372	407	223
Highly unsaturated acids	2	310	300§	294§	377§	212§
Animals given ample α-tocopherol¶	6	373	375	372	429	258

* Infraspinatus and supraspinatus.
† Long and lateral head of triceps.
‡ Coracoradialis, anterior brachial and anterior extensor of metacarpus.
§ Judged to be lower than the corresponding values for the negative-control calves.
¶ Mean values for normal calves given α-tocopheryl acetate and no cod-liver oil in previous experiments (Blaxter, Wood & MacDonald, 1953).

The amount of creatine in muscles of calves receiving the saturated acids of the oil did not differ from that of the control calves. Apart from those of the lower arm, muscles of the two calves receiving the total unsaturated acid fraction contained less creatine than did those of the controls. The marked lowering of the creatine content of the muscles of these two calves confirms the diagnosis of severe dystrophy. The calves receiving the less saturated acids had skeletal muscles that did not depart from normality, though the creatine of both hearts was slightly low. The amounts of creatine of all muscles, except those of the shoulders, of the two calves receiving the highly unsaturated acids were lower than those of the comparable control calves.

Histological examination of the tissues.

Histological examination of the myocardia and certain skeletal muscles of the control calves revealed no abnormalities. Lesions were observed in the myocardia of nos. 198 and 199, which had received cod-liver oil, but, in agreement with the gross pathological examination, not in the myocardium of calf no. 172. Skeletal muscles and the myocardia were not dystrophic in calves given the non-saponifiable material or the saturated acids of cod-liver oil. The myocardium of the left ventricle of no. 204 which had received the saturated acids was, however, abnormal. A diffuse oedema

of the interstitium with slight round cell infiltration was present. This was judged to be an early inflammatory change, probable in view of the corynebacterium infection. It was not a dystrophic lesion. The muscles of the calves receiving the total unsaturated acids of cod-liver oil were clearly dystrophic. A remarkable lesion was observed in the neck muscle of calf no. 175. An extensive hyaline degeneration with necrosis had occurred, together with sarcolemmal proliferation, and this was associated with calcium deposition. We have previously commented (MacDonald, Blaxter, Watts & Wood, 1952) on the conspicuous absence of calcification of the lesion induced under our experimental conditions, whereas calcification commonly occurs under field conditions. The calf showed some evidence of kidney pyaemia; it is possible that calcification of the muscle occurs only when kidney damage has taken place. Tissue necrosis was absent, but there were several cortical foci of interstitial inflammation and casts in related tubules. The skeletal muscles of calves receiving the less unsaturated acids showed no severe hyaline degeneration. Hyaline fibres were, however, found in the skeletal muscle of calf no. 200, which had received the highly unsaturated acid fraction. These results agree with those obtained by visual examination of the carcasses and by estimation of muscle creatine.

Tocopherol content of tissues

Table 7 summarizes the tocopherol content of muscles, liver and perinephric fat.

Table 7. *Tocopherol in tissues of the calves*

Treatment group	Calf no.	Muscle (rectus femoris)		Liver		Perinephric fatty tissue	
		mg/kg	μg/g fat	mg/kg	μg/g fat	mg/kg	μg/g fat
Negative controls	170	9·1	939	6·3	227	25·9	46·6
	171	13·4	1155	6·6	206	15·9	44·7
	195	6·0	504	8·2	252	17·5	25·6
Cod-liver oil	197	7·8	988	6·6	251	11·5	34·6
	172	11·7	976	11·1	318	17·9	46·4
	173	4·8	226	2·5	61	—	—
	198	7·4	607	12·3	322	17·0	28·6
Non-saponifiable residue	178	11·3	802	9·0	254	12·8	23·5
	179	6·8	612	8·8	260	21·6	5·17
Saturated acids	176	6·6	497	8·3	268	9·3	18·9
	177	2·9	227	4·4	135	20·6	—
	204	—	—	15·7	438	15·0	41·4
	205	7·9	724	9·3	256	—	—
Total unsaturated acids	174	6·6	522	6·6	167	21·4	50·3
	175	—	—	8·3	263	15·3	35·7
Less unsaturated acids	202	—	—	10·8	318	18·7	45·8
	203	6·5	644	16·7	292	—	—
Highly unsaturated acids	200	3·2	327	8·6	236	24·3	43·0

The experimental diets had no obvious effect on the concentrations of tocopherols in fresh tissues or tissue fat. Further, dystrophic animals still contained tocopherols, often indeed more than was present in control calves that were not dystrophic. The recti femoris of nos. 172, 198 and 174 were visibly dystrophic, yet still contained

tocopherols. Our previous (unpublished) work has indeed shown that completely degenerate, white, oedematous muscle often contains more tocopherols than the contiguous normal muscle of the same animal. The fact that the tocopherol content tends to be higher in affected muscle is in agreement with previous observations that the lipids of muscle increase in concentration in dystrophy owing to a marked fall in protein content (Blaxter & Wood, 1952). Analysis of single muscles in which bands of dystrophy were present has also shown no evidence that there was less tocopherol in the dystrophic areas. Further proof that the substance present in muscle and reacting as α-tocopherol in the Emmerie-Engel test is indeed α-tocopherol was furnished by paper chromatography of the muscle extracts. The ferric-chloride reacting substance had the same R_F value as α-tocopherol. Attempts to isolate from dystrophic muscle α-tocopherol as the acid succinate were, however, unsuccessful because of the minute quantities present in the muscle. They were equally unsuccessful with normal calf tissues.

Tissue peroxides

Attempts to demonstrate the presence of peroxides in the lipids of tissues failed. If peroxidation of the body fats occurs when cod-liver oil is given, these peroxides must either have an extremely transitory existence or be present in minute concentrations.

DISCUSSION

From the above experiments it is clear that the toxicity of cod-liver oil to the calf resides in the unsaturated fatty-acid fraction. When this was given severe dystrophy occurred, recognizable clinically, chemically, on dissection and histologically as the same entity that occurs when the whole oil is given. There is thus no possibility that the disease is related to hypervitaminosis A or D.

Further fractionation of the total unsaturated acids into less highly and more highly unsaturated groups showed that mild dystrophy was produced by the latter, but, apart from slight clinical signs, a slight reduction in the creatine content of the heart and pallor of the muscles, nothing abnormal was observed in calves receiving the less unsaturated fraction, suggesting that the two fractions combined were more effective in producing the disease than either fraction alone. It has previously been emphasized that there are differences between calves in their resistance to the disease (Blaxter, Wood & MacDonald, 1953), so that a firm conclusion can hardly be drawn. It seems of interest in this connexion, however, that the total unsaturation of the lipids ingested daily by calves consuming a dystrophy-producing diet containing lard (Blaxter *et al.* 1952) was 'equivalent to 70 g iodine'; this is equivalent to about double the total unsaturation of the lipids consumed when the unsaturated acids of the cod-liver oil were given, as may be seen from Table 2. Both produced a comparable degree of dystrophy. These results tend to support those of Dam (1944), in which toxicity of hog-liver fat to chicks was found to reside in a number of unsaturated acid fractions of widely differing iodine values.

SUMMARY

1. Calves were given a basal ration of dried skim-milk powder with small supplements of α-tocopheryl acetate. Cod-liver oil or fractions of cod-liver oil were added to this diet. When vitamins A and D were not supplied by cod-liver oil or its fractions, a concentrate containing them was given.

2. Severe muscular dystrophy developed in the calves given cod-liver oil or the total unsaturated acids of the oil. Slight dystrophy occurred in calves given the highly unsaturated acids of the oil.

3. Calves receiving the non-saponifiable residue of the oil or the saturated fatty-acid fraction did not develop dystrophy. The calves receiving the less unsaturated acids of the oil did not develop dystrophy, but their musculature was pale.

4. Muscles of both dystrophic calves and normal calves contained approximately the same amount of total tocopherols. Dystrophic muscles contained the same amount as contiguous apparently normal muscles of the same animal, or even more.

5. It is concluded that the toxicity of cod-liver oil to calves is due to its content of polyunsaturated fatty acids, and not to any hypervitaminosis A or D, and it is suggested that the toxicity may be a general effect of polyunsaturation rather than of a single polyethenoid acid.

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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

By K. L. BLAXTER, F. BROWN and W. A. WOOD
Hannah Dairy Research Institute, Kirkhill, Ayr

and A. M. MacDONALD
University of Glasgow and Royal Hospital for Sick Children, Yorkhill, Glasgow

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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 chicks 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 & Søndergaard, 1951; Christensen & Dam, 1951; Aaes-Jørgensen, Dam & Granados, 1951; Dam & Granados, 1951, 1952; Dam, Prange & Søndergaard, 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 concentration of tocopherols found in their blood serum, and then the animals within

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-α-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-α-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 creatin and tocopherol contents of muscles and other tissues and the histological method employed have been described by Blaxter, Brown & MacDonald (1953*b*).

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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 of the muscle and sarcolemmal proliferation in the region where the vitamin had been injected. No other lesions were found, and chemical analysis showed that the

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

Treatment 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
α-Tocopheryl acetate by mouth	2	0	0	—	0·5	Complete prevention of muscular dystrophy
Controls	2	2	2	23	24·0	
α-Tocopheryl acetate by injection	2	0	0	—	8·5	Considerable prevention of muscular dystrophy
Controls	2	2	2	32	24·5	
Ascorbic acid	6	2	3	36	12·5	Slight prevention of muscular dystrophy
Controls	7	6	6	33	22·1	
Ethyl gallate	6	3	3	44	15·8	Ineffective in preventing muscular dystrophy
Controls	4	3	4	50	20·5	
Methylene blue	5	1	1	38	1·6	Complete prevention of muscular dystrophy
Controls	4	3	4	50	20·5	
Biotin	2	2	2	33	21·5	Ineffective in preventing muscular dystrophy
Controls	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 following numerical values were used:

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

and the values given represent the mean of the sums of scores for each of five groups of muscles. A completely normal animal would thus have a score of 0·0 and a completely affected animal a score of 40·0. Errors of differences in scores are approximately ±8·0 units.

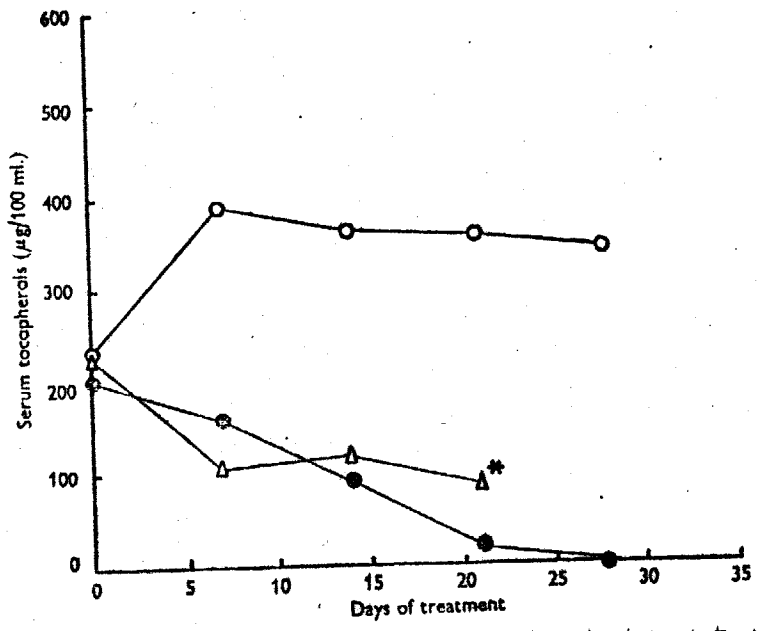

Fig. 1. Tocopherol content of blood serum of calves receiving cod-liver oil and vitamin E. △, cod-liver oil alone; ○, vitamin E by mouth and cod-liver oil; ●, vitamin E by injection and cod-liver oil.
* Both animals dead.

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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

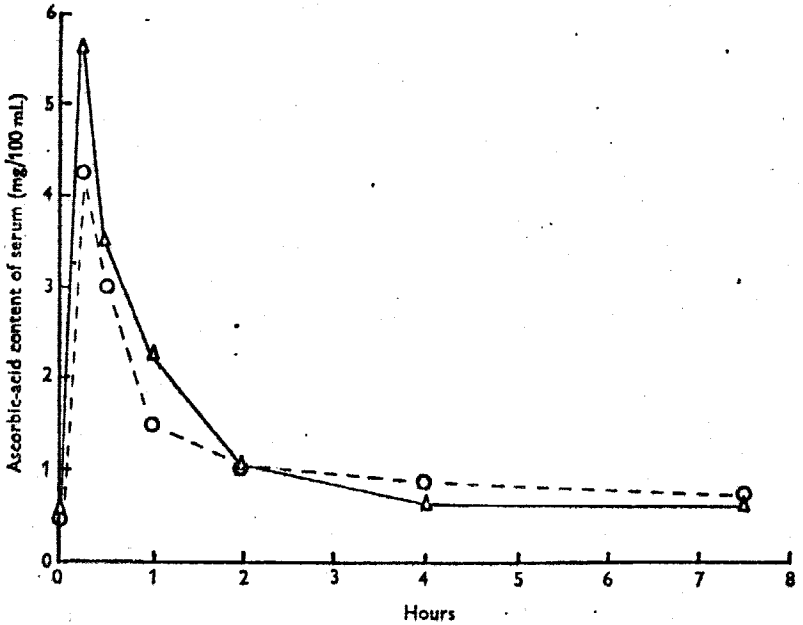

Fig. 2. Effect of an intravenous injection of 1·6 g ascorbic acid on the ascorbic-acid content of the blood serum of each of two calves.

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 the vitamin in the rumen. The present results in the calf suggest, however, that high concentrations of ascorbic acid in the blood serum, and presumably the tissues, were

not maintained in any of the experiments owing to the rapid excretion of it in the urine rather than destruction in the gut. It can therefore be assumed that the tissues of the calves were maintained at a peak of ascorbic-acid saturation throughout the experiment. One of the calves given ascorbic acid was killed after severe diarrhoea and was found to have suffered from severe enteritis. *Bacterium coli* was isolated from the heart blood. This animal has been excluded from the results given in Tables 2 and 3. It will be noted that muscular dystrophy occurred despite massive dosage

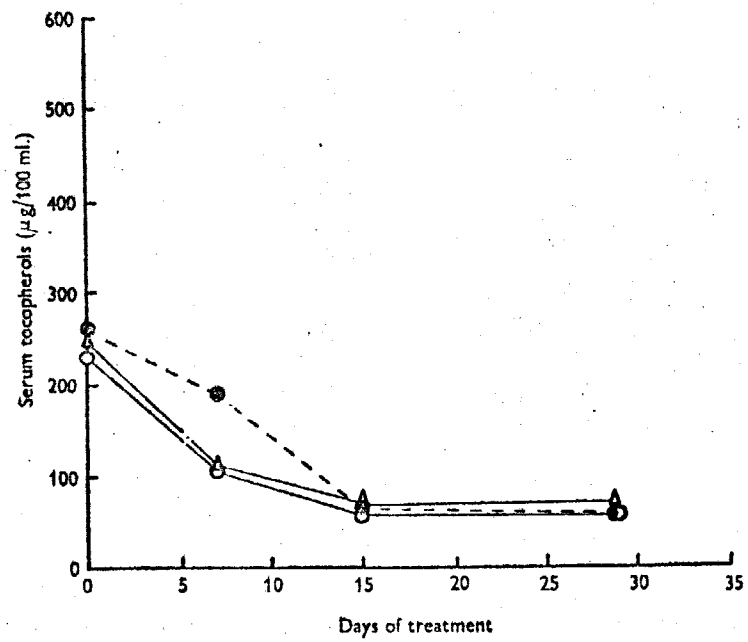

Fig. 3. Effect of ethyl gallate and methylene blue on the tocopherols of the blood serum of calves given cod-liver oil. △, cod-liver oil alone; ○, ethyl gallate and cod-liver oil; ●, methylene blue and cod-liver oil.

with ascorbic acid. In two of the calves, however, no gross lesions were observed, but the carcasses were distinguished by their marked pallor. Histological examination and determinations of creatine in the muscles confirmed that ascorbic acid had little effect in preventing the disease.

The effect of ethyl gallate. The calves given ethyl gallate did not thrive as well as calves given other supplements or even given no supplements. They developed an intense dislike for their food. Since the ethyl gallate was given by capsule and was not mixed with the basal diet, a systemic disturbance of appetite was responsible rather than a reaction to the presence of an abnormal taste in the daily allowance of food. The breath of two of the calves had an abnormal soapy, semiputrid smell, but whether or not this was related to the abnormality of appetite was not ascertained. Food refusals were few, since the calves could generally be coaxed to consume the whole of their food.

Fig. 3 shows the mean tocopherol contents of the blood serum of calves given ethyl

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gallate and those of similar control calves during the course of the experiment. 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. It will be noted there were no differences in the tocopherol contents of the tissues

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 ($P=0{\cdot}05$) 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.

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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 & 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.

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The Nutrition of the Young Ayrshire Calf

11. The Toxicity of Cod-liver Oil

By K. L. BLAXTER and W. A. WOOD
The Hannah Dairy Research Institute, Kirkhill, Ayr

and A. M. MacDONALD
Glasgow University and Royal Hospital for Sick Children, Glasgow

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Previous observations have shown that the addition of small quantities of cod-liver oil to a ration for calves that by itself produces muscular dystrophy, results in a considerable increase in the severity of the disease, and that the further addition of 50 mg α-tocopherol is not sufficient to reverse the effect (Blaxter, Watts & Wood, 1951, 1952). Cod-liver oil can be toxic when included in the rations of goats and sheep, but in cattle recent attempts to show that it is toxic have failed. The early results of Agduhr (1927), Agduhr & Stenström (1929) and of Slagsvold (1925) suggested that cod-liver oil causes muscular dystrophy in calves, but numerous experiments in the United States (Gullickson & Fitch, 1944; Davis & Maynard, 1936, 1938; Barnes, Davis & McCay, 1938) and also in Europe (Horn, 1934; Paloheimo, 1937; Schmalfuss, Werner & Esskuchen, 1938) failed to confirm them. In Britain, muscular dystrophy of calves has not been reported, as far as we are aware, and recommendations are given by various authorities (Fream, 1932; Mackintosh, 1937), regarding the use of cod-liver oil as a supplement to skim-milk or dried skim-milk rations (Woodman, 1948). Thus, in *Bulletin* no. 10 of the Ministry of Agriculture and Fisheries (Mackintosh, 1937) the following recommendation is made: 'Dried whole milk can be introduced, replacing a quarter of the new milk allowance, when the calf is 10–14 days old, and the change-over may be completed when the calf is 3–4 weeks old. When dried separated-milk powder is used, 1 oz. of good quality cod-liver oil should be added to each gallon of remade milk. These dried milks can be used as long as is considered desirable, but after 8 weeks the allowance can be gradually reduced and its place taken by a dry meal and cake mixture, hay and water.' Similarly in *Bulletin* no. 48 of the Ministry of Agriculture and Fisheries (Woodman, 1948) the recommendation is to give '1–2 oz. (cod-liver oil) per day up to a maximum of 3–4 oz. at 6 weeks of age'. These quantities are considerably larger than the ½ oz. which led to symptoms in our early experiments. It was therefore thought desirable to test whether, under practical conditions, dystrophy would follow the inclusion of cod-liver oil in the diet. For this purpose practical recommendations were followed—calves were initially reared on whole milk and then transferred to dried skim milk and cod-liver oil.

EXPERIMENTAL

Calves

Twenty-eight Ayrshire bull calves were used as experimental animals. They were bought in the open market when a few days old, and each was then confined in an individual pen, in a calf house thermostatically maintained at 60° F. On arrival each was given 20 ml. 'anti-coliform' serum by subcutaneous injection, four capsules of halibut-liver oil each containing 4500 i.u. vitamin A and 450 i.u. vitamin D, and cow's whole milk diluted with water. For the first week they were given 3, increasing to 4, l. of whole milk as the sole diet. Analyses of the milk from the cows in the Hannah herd showed the content of tocopherols to be relatively high (Blaxter, Brown & MacDonald, 1952).

Treatments

Subsequently, the calves were allocated by lot to one of the following treatments:

Positive controls. Dried skim milk 750 g, arachis oil containing 2000 i.u. vitamin A and 200 i.u. vitamin D 20 ml. and a gelatin capsule containing 50 mg DL α-tocopheryl acetate daily.

Low level cod-liver oil. Dried skim milk 750 g and cod-liver oil 1 oz. daily.

Medium level cod-liver oil. Dried skim milk 750 g and cod-liver oil 2 oz. daily.

High level cod-liver oil. Dried skim milk 750 g and cod-liver oil 4 oz. daily.

Negative controls. Dried skim-milk powder 750 g and the arachis oil solution of vitamins A and D 20 ml. daily.

Feeding arrangements

During the 2nd week after arrival of the calves, the arachis oil or cod-liver oil was added to the whole milk. The dried skim milk dissolved in tap water was gradually substituted for the whole milk and the calves were consuming the experimental diets 12–15 days after purchase. The oils were stirred into the warmed milks immediately before feeding. No emulsion was formed since the oil separated completely after standing for a few minutes. Feeding was normally twice daily but during the 1st week, and with any weakly animals, it was thrice daily. The allowance of dried skim milk was increased to 1000 g after 50 days.

Plan of experiment

In each of groups 1–4 inclusive there were six calves, in group 5 there were four. It was impossible to carry out the whole experiment at once, and so 'replications' of the experiment were carried out at different times. The first replication was begun early in September 1950 and the second in November 1950. Two further replications were begun in January 1951 and the remaining two in March 1951. For reasons given later the negative controls were all included in the replications of March 1951.

The calves were not given any roughage or solid food, but they consumed bedding on occasions. Straw was used as bedding for the first 30–40 days when it was replaced by peat moss.

Methods

Detailed observations of the posture and gait as well as of appetite, 'condition' and general wellbeing of each calf were made daily. The calves were weighed at weekly intervals, on which occasions they were allowed to run about for a few minutes under unrestricted conditions outside the calf house. When abnormalities occurred that were thought to be indicative of serious dystrophy the calf was killed. Unaffected calves and control calves were killed some 50–60 days after purchase. Three calves for which the ante-mortem evidence suggested that the cause of death was not entirely the result of muscular dystrophy were subjected to veterinary pathological examination by Mr A. L. Wilson and the staff of the Veterinary Department, West of Scotland Agricultural College.

At all post-mortem examinations the entire musculature was closely examined, sketches and notes were made of affected muscles and photographs taken. Where it was impossible to decide whether a muscle was normal or dystrophic, sections were taken for histological study. Chemical analyses were limited to the determination of dry matter, ash and creatine content of eight skeletal muscles and the heart. The methods have already been described (Blaxter & Wood, 1952). The tocopherol content of blood and tissues will be reported in a later paper (Blaxter, Brown & MacDonald, 1953).

RESULTS

General

The design of the experiment makes possible a comparison of the incidence of the disease in calves given the same diet at widely differing times of the year. In September the calves were born from cows at grass, whereas each subsequent replication was composed of calves from cows that had received winter rations for increasing periods of time. The last replication was composed of calves from cows that had been housed throughout a long and moderately severe winter. Where possible, visits or telephonic inquiries were made on the farms on which these calves had been born. In no case was any abnormality of the feeding regime of the herd cows noticed. It would seem that the calves were representative of those produced under average conditions of farm management in the west of Scotland.

The design also permits an accurate and unbiased comparison of differences between the first four treatments. Comparison of these with the negative controls is only valid for the March replication. As there is considerable evidence that the incidence of dystrophy under constant dietary and environmental conditions increases with advance of the winter (see below), this comparison is a highly critical one, since in March all animals were at their maximal sensitivity.

Observations made before death

Only one of the calves that received cod-liver oil failed to show any of those signs which previously have been ascribed to dystrophic involvement of part or parts of the skeletal or cardiac musculature. Two of the calves that received arachis oil and vitamin E were noted to be slightly splay-footed or flat-footed, but these signs could

not be categorically interpreted as indicative of any dystrophic lesions. Two of the four calves that received no additional vitamin E in the March replication showed similar slight flat-footed gaits. One animal in this group (replication no. 6*a*), however, showed quite definite signs of dystrophy of the muscles of the back. The signs when first noticed were mild and never appeared to become more pronounced, in fact, they slowly regressed as the experiment continued. Before slaughter, this calf was classified as normal.

The clinical signs observed in the most severely affected calves were broadly the same as in previous experiments—abnormal gait, flexion of the knees, winged scapulas, outward rotation of the hocks, and inability to rise. Three signs that may have occurred but had not previously been noted in dystrophic calves were also seen. These were, firstly, acute abdominal respiration, secondly, severe heart attacks with consequent prostration, and, lastly, presumed dystrophy of the lumbar muscles. Abdominal respiration occurred in calves in which post-mortem examination showed an almost complete dystrophy of both the internal and external intercostal muscles and the diaphragm. Affected calves stood in a characteristic attitude with their heads down and outstretched. No movement of the thoracic wall occurred and the abdominal wall took over the entire function of those muscles normally employed in breathing. Such respiration was accompanied by expiratory grunts. One animal thus affected died, but all the others were slaughtered to avoid unnecessary suffering. Dystrophy of the muscles of the back resulted in the extension of both forelegs and hind-legs to minimize strain on the affected muscles. The application of the hand to the back of such calves resulted in a voluntary depression of the back, whereas in normal calves this action generally results in a humping of the back. Heart attacks were in most cases precipitated by exercise. The calf would collapse after being walked outside or even after having become excited in its pen. Heavy breathing and tachycardia were predominant signs and soon the heart became irregular in its beat. For several hours after recovery the pulse was erratic and more than one attack sometimes occurred in one day.

Further clinical signs were also apparent, those of bloat. Two calves, both in the group that received 1 oz. cod-liver oil (replication nos. 1 and 5) suffered very severely from bloat. The bloating occurred every time food was given and was associated with pain. The more usual remedies had no effect, and both animals were killed, although they had shown only slight signs of dystrophy. A further calf that was given 4 oz. cod-liver oil (replication no. 4) died very suddenly when a blood sample was being taken, and post-mortem examination showed a twist of the ileum at the level of the ileo-caecal valve. Slight bloating also occurred in one calf that received 2 oz. cod-liver oil. No bloating ever occurred in the ten calves that did not receive cod-liver oil. Whether bloating and abnormality of the intestinal tract were related to dystrophy of the smooth muscular coat of the intestine could not be determined. It was, however, very suggestive that at post-mortem examination of the five remaining calves of the group that received 4 oz. cod-liver oil two showed a complete lack of the normal elasticity and tonus of the walls of the rumen.

Table 1 summarizes the signs shown by each calf, its fate, and the number of days

Table 1. *Summary of the clinical history of each calf*

Treatment	Replication no.	Fate	Signs and observations	No. of days of treatment
Positive controls: Arachis oil supplying vitamins A and D, 50 mg vitamin E daily	1	Killed	Very slight signs of muscle weakness	53
	2	Killed	No signs	42
	3	Killed	No signs	41
	4	Killed	No signs	40
	5	Killed	Very slightly flat-footed	37
	6	Killed	No signs (killed early for comparative purposes)	25
Low-level cod-liver oil, 1 oz. daily	1*	Killed *in extremis*	Continuous bloating necessitating constant surgical interference	40
	2	Killed *in extremis*	*Dystrophic*; respiratory muscles involved	43
	3	Killed	Only slight signs of dystrophy shown	41
	4	Died	*Dystrophic*; diaphragm and heart involved	35
	5*	Killed *in extremis*	Continuous bloating and poor thriving	25
	6	Killed	Only very slight signs of dystrophy shown	38
Medium-level cod-liver oil, 2 oz. daily	1	Killed	No signs observed	53
	2	Died	*Dystrophic*, heart signs, carcass haemorrhagic	35
	3	Killed	Slight signs of muscular involvement	40
	4	Killed *in extremis*	*Dystrophic*; diaphragm and chest involved	39
	5	Killed *in extremis*	*Dystrophic*; diaphragm and chest involved	22
	6	Killed *in extremis*	*Dystrophic*; severe heart attacks	25
High-level cod-liver oil, 4 oz. daily	1	Killed	*Dystrophic*; limb muscles severely involved	53
	2	Killed	Slight signs of muscular dystrophy	42
	3	Died	*Dystrophic*; heart signs and severe muscular involvement	25
	4*	Died	Only slight signs of dystrophy. A twist of the large bowel found at post-mortem examination	20
	5	Died	*Dystrophic*; diaphragm and chest involved	17
	6	Killed *in extremis*	*Dystrophic*; diaphragm and chest involved	17
Negative controls: Arachis oil alone. No vitamin E	5*a*	Killed	No signs	41
	5*b*	Killed	Very slight signs of muscle weakness	41
	6*a*	Killed	Very slight signs of dystrophy which regressed	71
	6*b*	Killed	No signs	71

* It is not known whether the dietary cod-liver oil was concerned in the death of these animals (see p. 37).

elapsing between the introduction of cod-liver oil into the ration and death. The calves that were killed *in extremis* and those that died are indicated. From the table one conclusion is quite clear; those groups that received no cod-liver oil showed no serious signs of dystrophy whereas the feeding of cod-liver oil resulted in very severe dystrophy. There was no very great difference in the severity of the disease between the three groups that received cod-liver oil. The mean numbers of days between commencement of cod-liver oil feeding and death of those calves that died or were killed when *in extremis*, however, were, with 1 oz. cod-liver oil, 36 days; with 2 oz., 30 days; and with 4 oz., 20 days. If the calves suffering from bloat, previously referred to, are excluded, the numbers of days were: 34, 30 and 21 respectively. Similarly, scoring the calves on the basis of the severity of signs, 0 representing no signs and 4 severe signs, gave the following scores out of a possible total of 36: group receiving no cod-liver oil, 2; group receiving 1 oz., 15; 2 oz., 13; and 4 oz., 19. These results do not indicate very large differences between the various doses of cod-liver oil as far as the severity of signs was concerned, but show that those calves that received the largest doses succumbed most quickly.

Table 2. *Mean gain in weight of the calves, time taken to consume their rations and loss of hair from their limbs*

Treatment	No. of calves	Gain in weight (g/day)	Time taken to consume ration (sec)	No. of calves losing hair from face and limbs
Negative controls:				
Arachis oil alone	4	308	78	2
Positive controls:				
Arachis oil with vitamin E	6	344	47	2
Low-level cod-liver oil, 1 oz. daily	6	271	109	4
Medium-level cod-liver oil, 2 oz. daily	6	365	84	5
High-level cod-liver oil, 4 oz. daily	6	267	172	5

Table 2 summarizes the mean gains in body-weight of the calves and the mean length of time they took to drink their rations. These gains and times refer only to the period before an increase was made in the quantity of diet offered. There were no statistically significant differences between the gains in body-weight of the calves. This conclusion may also be drawn from Fig. 1, in which the body-weights of the calves in the last replication of the experiment are shown. The calves that received either 2 oz. or 4 oz. cod-liver oil were both dystrophic and were killed to prevent suffering. It is remarkable that such a severe disease does not affect gain of body-weight.

Previously it had been shown (Blaxter, Watts & Wood, 1952) that muscular dystrophy was associated with a decline in the nitrogen balance and an increase in heat production. The observation that body-weight gain is not appreciably affected would thus appear at variance with these results. The explanation is to be found in the presence of slight but unmistakable oedema of the musculature (see Table 5). Protein

is lost, but water is gained. The differences between the groups in time taken to consume their rations were large. The values in Table 2 refer to those recorded at least 7 days before death or slaughter. The longer time taken by dystrophic calves to consume food is in agreement with previous results (Blaxter, Watts & Wood, 1952). Hair loss refers to depilation of the face and limbs. Bate, Espe & Cannon (1946) have

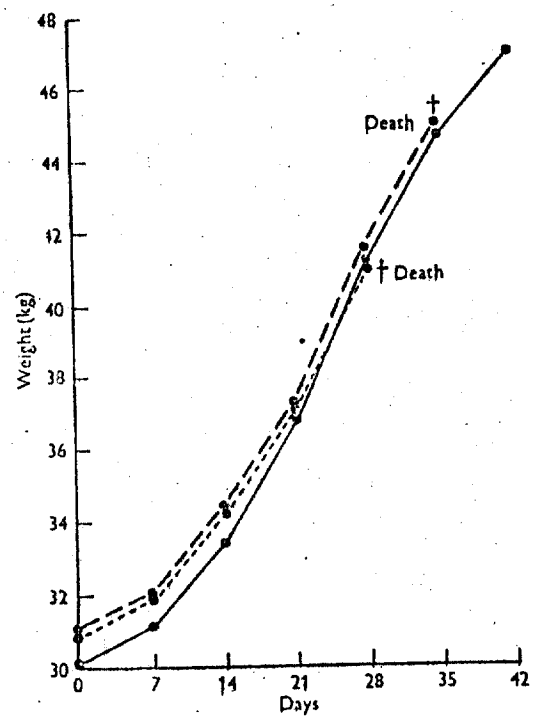

Fig. 1. Effect of dystrophy induced by cod-liver oil on the body-weight of calves. ———, positive control (arachis-oil solution of vitamins A and D with α-tocopherol); - - - - -, high-level cod-liver oil, 4 oz. daily; – – –, medium-level cod-liver oil, 2 oz. daily.

associated similar loss of hair in young calves with ingestion of non-homogenized fat. This may be the reason for the hair loss of the present calves. It was more severe with cod-liver oil than with arachis oil, and was not stopped by the addition of vitamin E.

Post-mortem examination

The appearance of the affected musculature was comparable to that already reported in our previous experiments (Blaxter, Watts & Wood, 1951, 1952) and to descriptions of the naturally occurring disease *weisses Fleisch* of cattle. The results are summarized in Table 3. No attempt has been made to classify them minutely into smaller groups than 'limbs' and 'other skeletal muscles', since dystrophy may be present to the same general extent in a carcass and yet one particular group of muscles remain apparently normal. None of the animals that were given arachis oil with or without vitamin E had abnormal heart or diaphragmatic muscles. In the

Table 3. *Summary of presence of dystrophic muscles in calves*

Treatment	Replication no.	Incidence of dystrophy (visual assessment only) in the . Heart	Diaphragm	Limbs	Other muscles
Positive controls: Arachis oil supplying vitamins A and D, 50 mg vitamin E daily	1	Normal	Normal	Normal	Normal
	2	Normal	Normal	Normal	Normal
	3	Normal	—	Normal	Part of gluteus accessorius white
	4	Normal	Normal	Normal	Normal
	5	Normal	Normal	Normal	Superficial muscles pale
	6	Normal	Normal	Normal	Normal
Low-level cod-liver oil, 1 oz. daily	1	Dystrophic	Normal	All limbs partly dystrophic	Peritonitis present—muscles partly dystrophic
	2	Pale	—	Forelimbs completely dystrophic	All skeletal muscles wet and oedematous
	3	Dystrophic and ventricle partly hardened	Pale	Dystrophic in parts only	Slight dystrophy of superficial musculature
	4	Dystrophic and ventricle partly hardened	Completely dystrophic	Dystrophic and haemorrhagic	Shoulder suspension and back muscles dystrophic (very severe)
	5	Flabby	Normal	Normal	Normal
	6	Flabby and pale	Pale	Very slightly affected	Normal
Medium-level cod-liver oil, 2 oz. daily	1	Very pale and patchy	—	Pale, otherwise normal	
	2	One pillar and wall dystrophic	Normal	Partly dystrophic	Shoulder suspensory muscles partly dystrophic
	3	Enlarged and dystrophic	Pale	Forelimb pale, hind-limb partly dystrophic	Not affected to any great extent
	4	Enlarged and dystrophic	Radially dystrophic	Severely dystrophic	All muscles inluding intercostals slightly affected, subcutaneous muscles affected
	5	Severely dystrophic	Completely dystrophic	Completely dystrophic	All muscles very severely dystrophic, external intercostals worse than internal
	6	Very severely dystrophic	Radially dystrophic	Completely dystrophic	Back muscles dystrophic, shoulder and neck muscles normal
High-level cod-liver oil, 4 oz. daily	1	Normal	—	Partly dystrophic	Partly dystrophic
	2	Very large and flabby but no lesions	Normal	Partly dystrophic	Apparently normal
	3	Very large and possibly dystrophic, very difficult to tell*	—	Shoulder muscles dystrophic, rest pale	Some obvious areas dystrophic with considerable haemorrhage
	4	Dystrophic pillars	Normal	Partly dystrophic	Scalenae and deltoids dystrophic (twist at ileo-caecal valve)
	5	Very severely dystrophic and haemorrhagic	Partly radially dystrophic	Severely dystrophic	Intercostals completely dystrophic, hind-limb suspensory completely dystrophic; back muscles normal
	6	Normal size, small pin-point lesions	Completely dystrophic	Forelimb muscles oedematous and pale; hind-limb muscles dystrophic	Intercostals partly dystrophic, back muscles dystrophic; neck and shoulder suspensory muscles completely dystrophic
Negative controls: Arachis oil alone	5*a*	Normal	Normal	Shoulder muscles very slight dystrophic	Back muscles slightly pale
	5*b*	Normal	Normal	Shoulder muscles (spinatus group) patchy in colour	Normal
	6*a*	Normal	Normal	Normal	Unilateral severe dystrophy of longissimus

group of animals receiving α-tocopherol only part of one single muscle in one calf—the gluteus accessorius of the calf in replication no. 3—was thought dystrophic. When the same ration without vitamin E was given, a patchy appearance of the supra- and infraspinatus was noted in two calves and in a third a definite dystrophic lesion of the longissimus dorsi was observed. A dystrophy involving the back muscles had been diagnosed earlier in this calf, but the signs had regressed. This lesion appeared to be in the process of involution, and is remarkable in that it was strikingly asymmetrical.

Pronounced dystrophy was present in animals of all groups that received cod-liver oil. All skeletal muscles seemed to be affected, but suspensory muscles to the limbs appeared to be slightly more affected than others. On the basis of the post-mortem reports the severity of the lesions in the groups was summarized as shown in Table 4. Scoring of the severity of dystrophy showed that there was an increase with increasing dosage of cod-liver oil. There were nevertheless animals in all these groups that showed only slight signs, and in each of the groups that received 1 oz. cod-liver oil, and 2 oz. cod-liver oil, there was one animal with apparently normal muscles. Such variation has already been noted in our previous experiments.

Dry-matter, ash and creatine contents of skeletal muscles

The mean dry-matter and ash contents of the skeletal muscles are given in Table 5.

It will be seen from the results that in every muscle group the muscles of animals that received cod-liver oil contained less dry-matter than those of comparable calves that received none, whether they had received additional α-tocopherol or not. The muscles of calves given cod-liver oil also contained more ash than did the muscles of calves given an arachis oil solution of vitamins A and D. These results are in complete agreement with our previous ones (Blaxter & Wood, 1952). Statistical analysis of these results was not made owing to the obvious non-homogeneity of the variances of the values as between control calves and those given cod-liver oil. Non-homogeneity may be inferred from Fig. 2, where it is clear that the range of variation of creatine content is far greater in the calves given cod-liver oil than it is in the control calves. This was also true of the results of determinations of dry matter and ash content. The marked asymmetry of distribution and resultant shifting of the mean is quite apparent. The graphical representation, however, shows the marked effect of even 1 oz. cod-liver oil in shifting the mean of the distribution.

The creatine content of skeletal muscles is shown further in Table 6. The muscles of animals given cod-liver oil contained less creatine than those given arachis oil and a supplement of α-tocopherol, and with three exceptions less than those given arachis oil alone. From the results it would appear that the muscles of the shoulder—the infraspinatus and the supraspinatus—tended to be the most affected, as had been inferred by inspection. In the individual calf, however, this was not always so.

From the chemical analyses it appeared that 4 oz. cod-liver oil did not cause a greater disturbance of muscle composition than either 2 oz. or 1 oz.; this at first appears anomalous, but is probably to be accounted for by the fact that animals were killed when they showed signs. Thus calves that died with severe dystrophy after

Table 4. *Classification of the calves according to severity of muscular dystrophy*

Replication no.	Positive controls: Arachis oil with vitamin E	Negative controls: Arachis oil alone	Low level cod-liver oil 1 oz. daily	Medium-level cod-liver oil 2 oz. daily	High-level cod-liver oil 4 oz. daily
1	Normal	—	Slight	Normal	Severe
2	Normal	—	Severe	Severe	Slight
3	Very slight	—	Slight	Slight	Very severe
4	Normal	—	Very severe	Severe	Severe
5	Normal	Very slight (?)	Normal	Very severe	Very severe
6	Normal	Very slight (?)	Slight	Very severe	Very severe
5*a*	—	Very slight	—	—	—
6*a*	—	Normal	—	—	—
Total score (percentage of possible)	1·7	7·5	41·7	58·3	75·0

The classification is based on an assessment of the severity of muscular dystrophy as normal, very slight, slight, severe or very severe. For numerical comparison these classes are given the values 0, 1, 3, 6, and 10 respectively.

Table 5. *Mean dry-matter content and mean ash content of the dry matter of the skeletal muscles of the calves*

Treatment	Mean dry-matter content (%)				Mean ash content of the dry matter (%)			
	Shoulder (two muscles)	Arm (three muscles)	Forearm (two muscles)	Biceps femoris	Shoulder (two muscles)	Arm (three muscles)	Forearm (two muscles)	Biceps femoris
itive control, no cod-liver oil, mg tocopherol daily (six calves)	21·4	21·7	20·9	21·8	4·9	4·9	5·1	5·3
ative control, no cod-liver oil, tocopherol (four calves)*	21·5	21·7	21·6	21·9	5·4	5·3	5·0	5·6
-level cod-liver oil, 1 oz. daily calves)	19·0	20·1	20·0	20·7	7·7	5·6	5·7	6·4
dium-level cod-liver oil, 2 oz. daily calves)	20·0	20·5	20·1	19·4	7·5	6·4	6·1	6·6
h-level cod-liver oil, 4 oz. daily calves)	19·9	20·9	20·0	21·0	6·0	5·4	5·4	5·9

* Four calves only in March replications. The values do not differ significantly from those obtained with two animals n 2-tocopherol in concomitant replications.

having received 4 oz. cod-liver oil for 17–20 days had muscles closely resembling in composition those of calves that took twice as long to succumb when given 2 oz. A further explanation may be that an acute toxicity of cod-liver oil may result in a greater disturbance of one functionally indispensable muscle and a lesser disturbance of the remainder, whereas chronic toxicity may result in a more generalized effect. The results of Pappenheimer (1940) showing that disuse of a muscle renders it immune to muscular dystrophy, and the earlier results with calves showing a voluntary redistribution of the load on affected muscles (Blaxter, Watts & Wood, 1952) would suggest that gait and stance adaptations in chronic dystrophy, which result in partial disuse of particular muscles, possibly play a part in the total severity of the disease at death.

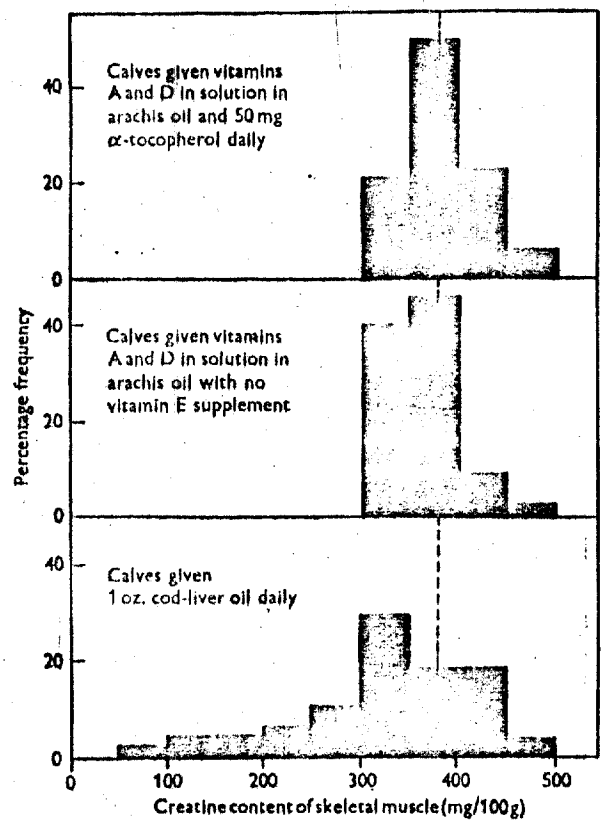

Fig. 2. Effect of 1 oz. cod-liver oil daily in the ration of the calves on the creatine content of their muscles.

Table 6. *Mean creatine content of skeletal muscles of the calves*

(Mean values for six calves in each experimental group and for four negative control calves)

Muscle	Positive controls: No cod-liver oil + 50 mg α-tocopherol daily (mg/100 g)	Low-level cod-liver oil 1 oz. daily (mg/100 g)	Medium-level cod-liver oil 2 oz. daily (mg/100 g)	High-level cod-liver oil 4 oz. daily (mg/100 g)	Negative controls: No cod-liver oil and no α-tocopherol (mg/100 g)
Supraspinatus	380	272	282	323	346
Infraspinatus	365	272	304	303	335
Long head of triceps	401	327	340	372	417
Lateral head of triceps	348	313	296	332	356
Anterior brachial	374	369	333	362	350
Anterior extensor of metacarpus	379	345	331	376	363
Coracoradialis	363	363	351	362	354
Rectus femoris	429	400	378	413	399
Mean of all muscles	380	333	327	355	365

The muscles of the calves that received the arachis oil solution as the sole supplement to their diet were slightly lower in creatine content than those of the calves that received vitamin E. The differences were small and variation within groups excludes their significance. The apparently dystrophic muscle of the back of the calf in replication no. 6*a* had normal creatine, ash and dry-matter contents, suggesting that the hypothesis of recovery was in fact correct.

Composition of the heart muscle

The results of analyses of the hearts of individual calves are given in Table 7. Again no statistical analysis has been made since the distributions within the groups that were dystrophic were clearly asymmetrical. An indication of normality or otherwise of the hearts of the individual calves may, however, be obtained by computation of the mean values and their standard deviations of the control group, for dry matter $20{\cdot}38 \pm 0{\cdot}18\%$ and for creatine $258{\cdot}5 \pm 26{\cdot}4$ mg/100 g. Using twice the standard deviation as a criterion of the range above which the ash, or below which the dry-matter or creatine content, is unlikely to fall, the values of 5·8% for ash, 19·4% for dry-matter and 205 mg/100 g for creatine are obtained. These limits do not represent exact fiducial limits, for the numbers in the control group are small. They nevertheless allow a more critical appraisal of the results of Table 7. Values that may be judged abnormal have been marked with an asterisk. These results show that two cardiac muscles of abnormal ash content were encountered in the group that received arachis oil without additional vitamin E. Of the eighteen calves in the groups that received cod-liver oil, three were judged to have oedematous muscles, seven to have muscles with an increased ash content and twelve to have muscles with a low creatine content. It was evident that the cardiac muscle could be low in creatine content and yet be comparatively normal in ash and dry matter.

The correlation between a visual assessment of dystrophy and the assessment of dystrophy from the creatine content alone was fair. In the twenty-eight calves, only two showed disagreement. The heart of the calf that received 2 oz. cod-liver oil daily (replication no. 4), although judged to be dystrophic at post-mortem, had a normal creatine content, and the heart of the calf that received 4 oz. cod-liver oil in the same replication was normal chemically, though at post-mortem examination the pillars of the left ventricle were classified as partly dystrophic. Sampling errors may be involved in these discrepancies, though the agreement between duplicate chemical analyses was within $\pm 5\%$.

Histological examination

Broadly speaking, the lesions observed in samples of heart and skeletal muscle were the same as previously found (MacDonald, Blaxter, Watts & Wood, 1952). Agreement between the results of the histological examination and chemical analysis of muscles was good for the skeletal muscles, but only fair for the heart muscle. In no instance was cardiac muscle classified as abnormal histologically when the creatine content was normal, but the reverse was often true. This might have been due to

Table 7. *Mean content of dry matter, ash and creatine in the walls of the ventricles of the hearts of the calves*

Replication no.	Positive controls: Arachis oil with 50 mg α-tocopherol daily			Negative controls: Arachis oil alone			Low-level cod-liver oil 1 oz. daily			Medium-level cod-liver oil 2 oz. daily			High-level cod-liver oil 4 oz. daily		
	Dry matter (%)	Ash (percentage of dry weight)	Creatine (mg/100 g)	Dry matter (%)	Ash (percentage of dry weight)	Creatine (mg/100 g)	Dry matter (%)	Ash (percentage of dry weight)	Creatine (mg/100 g)	Dry matter (%)	Ash (percentage of dry weight)	Creatine (mg/100 g)	Dry matter (%)	Ash (percentage of dry weight)	Creatine (mg/100 g)
1	20·0	5·4	260	—	—	—	20·1	6·0*	188*	19·9	5·1	213	19·4	5·0	204
2	21·1	5·2	258	—	—	—	—	8·2*	189*	19·6	5·2	158*	19·9	5·5	223
3	20·1	5·5	251	—	—	—	19·6	5·8	183*	19·8	5·6	195*	19·2*	7·1*	196*
4	19·9	5·5	280	—	—	—	19·0*	5·7	194*	Lost	6·5*	214	20·1	5·8	237
5	20·6	Lost	289	19·7	5·7	231	19·7	5·5	232	19·4	6·2*	191*	18·6*	6·3*	104*
6	20·6	5·6	213	20·4	6·5*	234	20·6	5·4	196*	21·1	5·8	152*	20·4	5·9*	195*
5a	—	—	—	20·7	6·2*	275	—	—	—	—	—	—	—	—	—
6a	—	—	—	21·1	5·6	262	—	—	—	—	—	—	—	—	—
Mean	20·4	5·4	258·5	20·5	6·0	250·5	19·8	6·1	197	20·0	5·7	187	19·6	5·9	193·1

* Values that may be regarded as abnormal (see text, p. 45).

absence of the focal lesion from the tissue blocks examined, and thus represent a sampling error.

In addition to the changes in muscles previously reported, two changes were observed in early dystrophy. First, the sequence of coagulation and clumping of the sarcoplasm appeared to be preceded by a granulation leading to a total disappearance of cytoplasm, but without coagulation and necrosis. Breaking of fibres and fraying of the ends of the myofibrils were also common. The loss of staining power which was previously reported appears very characteristic of these early changes. Secondly, in several muscle sections the sarcolemmal reaction was not observed. A degeneration of the cytoplasm, generally without segmentation and necrosis, was followed by vacuole formation, but a collapse of the sarcolemma did not occur nor did the nuclei of the sarcolemma proliferate. The reason or reasons for this failure of response or, indeed, for the more usual response of the sarcolemmal nuclei is not known. It appears, however, that sarcolemmal nuclear proliferation only follows the collapse of the sarcolemmal tube.

The opportunity was taken to examine the livers, kidneys and spinal cords of affected and normal animals. Liver damage was detected in only one animal in which a fatty degeneration had taken place. No significance is attached to this observation for it may have been the result of terminal failure of appetite. No lesions other than minute changes in widely scattered glomeruli were found in the kidneys. This was unexpected since, in view of the breakdown of muscle, the presence of tubular casts was thought probable. The spinal cords were examined by sectioning at six levels, two cervical, two thoracic and two lumbar. There was no demyelination or abnormality of the pyramidal tracts, and the anterior horn cells were perfectly normal.

From these observations it is apparent that neither central nor peripheral nerves are involved in the disease, and they emphasize once again that the primary changes are in the sarcoplasm of the muscle cells.

DISCUSSION

The results of the experiment are quite clear. Administration of cod-liver oil to calves results in a high incidence of muscular dystrophy and a large number of deaths, either sudden or after a short period of prostration. The design of the experiment did not clearly distinguish between the toxicity of 1 oz. and 4 oz. of cod-liver oil as far as muscle composition was concerned, since those calves given the highest level died at an earlier age. The rations given were those recommended by a variety of authorities on calf rearing. The dried skim milk and cod-liver oil were both of high quality, and the results must be viewed with some concern. They confirm the earlier reports of Agduhr (1927), Agduhr & Stenström (1929), and Slagsvold (1925), as briefly reviewed by Blaxter, Watts & Wood (1952). These early reports were not confirmed by American or German workers. Similarly, no deaths of calves in this country as the result of muscular dystrophy have been reported from the field, though it is safe to assume either that cod-liver oil has been added to skim-milk rations or that it has been incorporated in milk substitutes which contain very high quantities of dried skim milk. If our experiments and the earlier Scandinavian experiments are unimpeachable,

dystrophy should have occurred, and the absence of reports to that effect needs explanation. One obvious explanation is that dystrophy, or death as the result of dystrophy, may have occurred without correct interpretation of the cause. Diagnosis is difficult. A dystrophic calf is a calf that is muscularly weak and lethargic, and so in fact is a calf with diarrhoea or the more common infections. Many calves have signs very easily mistaken by the farmer—and indeed by us, for calf pneumonia; still others die suddenly in convulsions that might be thought due to lead poisoning (Allcroft & Blaxter, 1950), magnesium deficiency (Duncan, Huffman & Robinson, 1935), or

Table 8. *Seasonal incidence of muscular dystrophy as judged by the performance of calves receiving cod-liver oil at different times of the year*

Month in which the experiment began	No. of calves	Deaths due to dystrophy including calves killed *in extremis* (%)	Score of all animals (see Table 4) (%)	Mean muscle* creatine of all animals (mg/100 g)
September	4	25	30	359
November	4	50	50	354
January	8	50	63	329
March†	8	62	72	318

* Mean value of seven forelimb muscles for each calf.

† Not including the calves that received vitamins A and D in arachis oil without a supplement of vitamin E.

'colic'. A further explanation is afforded by calf no. 85 which received dried skim-milk powder and an arachis-oil solution of vitamins A and D without any additional vitamin E. This animal was judged to be slightly dystrophic when 22 days of age, but the signs regressed with time and only a small but nevertheless quite definite lesion was recorded at post-mortem examination. Regression of signs in calves on farms is a possibility which needs consideration. Finally, the incidence of the disease is not 100% even at the highest level of treatment. The cause of this is not known, but further information on this aspect may supply evidence regarding the apparent absence of field cases of cod-liver oil injury in this country.

The heart failure of some of the calves is of considerable interest since a comparable 'sudden heart death' has been observed in pigs and sheep in Germany and Scandinavian countries (Karsten, 1931; Nieberle & Cohrs, 1949*a*; Seifried, 1943; Nieberle & Cohrs, 1949*b*). These diseases are characterized by sudden death upon the slightest exertion. A grey heart muscle, sometimes with a patchy appearance, is seen at post-mortem examination and on histological study a grainy vacuolar degeneration or a hyaline, scaly ('Schollig') degeneration is observed. Both appear to be followed by a nuclear proliferation of the sarcolemma. A variety of causes has been suggested for these diseases, from infection to a reflex-conditioned coronary insufficiency. Seifried (1943), however, has suggested that a vitamin deficiency may be involved in their aetiology and the present results suggest that this may be so. A similar type of disease in calves called *Transportnekrose* or transit-disease has also been described (Nieberle & Cohrs, 1949*b*). Descriptions of the heart in this disease agree well with present findings and our previous observations.

In the naturally-occurring diseases that are comparable to *weisses Fleisch* a seasonal incidence has been recorded (Hjärre & Lilleengen, 1936*a*, *b*; Nieberle & Cohrs, 1949*b*; Mahr, 1936). From the present results a similar seasonal incidence; may be inferred, as shown in Table 8. A higher number of calves died or were killed *in extremis* in March than at any other time of the year, and the scores based on the severity of the disease confirm this, as indeed does the creatine content of the muscles. This suggests a depletion of the dams of these calves of vitamin E or perhaps other protective factors, during the late winter months.

SUMMARY

1. An experiment involving twenty-eight Ayrshire bull calves was carried out to provide information on the toxicity of cod-liver oil at levels recommended for use in farm practice in Great Britain.

2. The daily addition of 1, 2 or 4 oz. cod-liver oil to a dried skim-milk ration resulted in death or severe dystrophy of a high proportion of the animals.

3. Besides severe dystrophy of limb muscles, dystrophy of diaphragmatic and intercostal muscles was observed. A high incidence of 'bloating' and a lack of tonus of the rumen walls at post-mortem were encountered. Heart lesions were particularly severe. Gain in body-weight was not affected.

4. Additional observations on the histological development of the lesions are presented. No involvement of either the central or peripheral nervous system could be detected nor was any gross abnormality present in the livers or kidneys of dystrophic calves.

5. The incidence of the disease was affected by the season of the year, a higher number of calves dying in late winter than in early autumn.

6. Four calves given the basal ration with vitamins A and D showed very mild signs of dystrophy which regressed. At post-mortem only very slight, but nevertheless definite, lesions were observed.

7. In view of the use of cod-liver oil in the rations of young calves as supplements or as components of calf starters and milk substitutes, the results are discussed in relation to the absence of reports of deaths of such a nature under field conditions in Great Britain.

Our thanks are due to Mr D. Paterson and Miss M. Hutcheson for the great care they took in looking after the experimental animals, and to Mr A. L. Wilson, M.R.C.V.S. and his staff of the Veterinary Department, West of Scotland Agricultural College, for the examination of the carcasses of three of the calves. We are also most grateful to Miss Breckenridge for technical assistance.

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Chemical and nutritional changes in stored herring meal

4*. Nutritional significance of oxidation of the oil

By K. J. CARPENTER
School of Agriculture, University of Cambridge

and C. H. LEA and L. J. PARR
Low Temperature Research Station, Cambridge

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This study was designed to investigate the nutritional significance of the interactions that may occur between oxidized fat and proteins in foods. Herring meal, because of its high content of protein and of highly unsaturated fat, was chosen as the material for study.

The first two papers in the series (Lea, Parr & Carpenter, 1958, 1960) dealt mainly with factors influencing the rate of oxidation of the fat in stored herring meal, and with the interaction between fat oxidation products and the ε-amino groups of the lysine units in the herring protein. This paper describes feeding tests designed to discover whether the nutritional value of the protein was influenced in other ways also during storage, and to ascertain whether the oxidized and polymerized fat of the stored meal could itself be influencing the outcome of the feeding tests by direct toxic or 'growth-depressant' effect. The answer to the latter question appeared to be 'no'.

During this work an unexpected growth-depressant action was encountered with meal from which the oil had been extracted by chloroform–methanol, and these findings are reported. Included also is an account of a parallel study of a particular batch of imported anchovy meal said to have shown 'rancid fat toxicity' for livestock.

EXPERIMENTAL

Materials

Herring meal 4. A sample of herring meal was prepared from fresh herring in November 1959 at the same factory and in the manner already described for meal 3 (Lea *et al.* 1960), though only a little formaldehyde, used to harden the skin of the fresh herring, was added before processing. The meal was again packed immediately in solid CO_2 for transport to the laboratory where it was gas-packed in oxygen-free nitrogen and stored at $-20°$ until required. A sample of the expressed herring oil (1) produced at the same time in the first stage of pressing the herring was stored under N_2 at $-20°$.

Herring meal 5. This meal was prepared from Whitby herring during late August

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1959, at another factory but by a generally similar procedure. It had been stored commercially in a paper sack for 10 weeks before it reached the laboratory. The addition of antioxidants is now common commercial practice in fish-meal manufacture but none was added in the manufacture of herring meals 1–5 used in this work.

Anchovy meal 6. A sample of anchovy meal, reputedly of Peruvian manufacture but otherwise of unknown history, was received in June 1960. It was despatched to us, through the courtesy of a commercial laboratory, as being part of a batch of material that had been suspected of causing feeding troubles in livestock and even of causing deaths in young pigs: it was suggested at the time that this toxicity might be attributable to the 'rancid' condition of the fat in such meals.

Storage

The meals (nos. 5 and 6) that had already been stored commercially before receipt were kept without special precautions in paper sacks at room temperature. After 2 months, however, no. 5 was gas-packed in N_2 and stored at −20° to prevent further change.

Meal no. 4 was milled to a powder and divided into two portions. A portion for use as 'control' (4) was returned to storage at −20° in an atmosphere of N_2. A second portion (4S) was packed in cans with a large head space, periodically reflushed with air and stored at 20° for 1 year; it was then returned to an atmosphere of N_2 and held at −20° until required.

'Double-fat' meal. Fresh meal no. 4 contained 17·4% of lipid extractable with chloroform–methanol (2:1, v/v) and soluble in chloroform. For preparation of a 'double-fat' meal, expressed herring oil (no. 1), in solution in diethyl ether, was mixed with herring meal 4 in the ratio of 17·4 g oil to 100 g meal, and the solvent was evaporated. Part of this double-fat meal was then packed in N_2 and held at −20° as a control (D4), and the remainder (D4S) was stored for a year in air at 20° as described above before being returned to −20°.

Preparation of the solvent-extracted and recombined meals

Extracted meal 5B. This meal was prepared by extracting herring meal 5 with boiling chloroform–methanol (9:1, v/v) for 2 h in a continuous-extraction apparatus, then filtering and removing the residual solvent from the meal by heating for 2 h at 50° in a vacuum oven with a slow 'bleed' of N_2.

Extracted meal 5C. It was prepared as was 5B, but with two additional extractions of 30 min each with boiling diethyl ether, to ensure complete removal of the chloroform, before drying in the vacuum oven for 3 h at 60°.

Extracted meal 5D. It was prepared as was 5C, but benzene–ethanol (4:1, v/v) was used as initial extraction solvent in place of chloroform–methanol.

'Recombined' meals based on meals 5 and 6. For use in a feeding test (Expt 6, see p. 155) designed to trace the cause of the poor growth on diets containing meal 6, the lipid from this meal was extracted and remixed with the fat-free solids from a

normal meal (no. 5) to give recombined meal S5L6. In the same way a recombined meal, S6L5, was prepared from the fat-free solids of meal 6 and the lipids of meal 5. Each batch of fat-free solids was also recombined with its own lipid to give meals S5L5 and S6L6, in order to detect any effect which might be introduced by the procedures of solvent extraction and evaporation. Although the proportions of lipid and fat-free solids in the original meals differed slightly, all remixing was done in the ratio of 1 part of lipid to 7 parts of fat-free solids.

Meal 5 was extracted as described above in the preparation of 5D, but the lipids of the anchovy meal (no. 6) proved resistant to extraction with benzene–ethanol so chloroform–methanol had to be used as described above in the preparation of 5C. As with 5C, the extracted lipids were carefully freed from traces of residual solvent by the addition and subsequent removal under reduced pressure of diethyl ether, the process being repeated twice.

'Recombined' meals based on meals D4 and D4S. To distinguish possible direct adverse effects of the oxidized and polymerized lipid in the stored meals from effects of the non-fat fraction, recombined meals were prepared from (*a*) the lipid of control (−20°, N_2) double-fat meal D4 and the fat-free solids of the corresponding stored meal D4S and (*b*) the lipid of the stored meal and the fat-free solids of the control meal. Extraction (with chloroform–methanol) and remixing were carried out as described above, with the same precautions to ensure the complete removal of traces of residual solvent from both oil and fat-free residue.

Meal containing peroxidized oil. To test in a direct manner for any adverse effect in the diet of fish oil of comparatively high peroxide value, a sample of expressed herring oil (no. 1) was exposed to the air in a thin layer at 25° for 5 days, by which time it had attained a peroxide content of 142 μmoles/g. This oil was then mixed with meal 4 in quantity equal to the amount of lipid already present (17·4%), to produce a peroxidized double-fat meal D4P.

It is worth recording that an attempt to develop a high peroxide value in lipid extracted from meal 4 by chloroform–methanol failed, the peroxide value after 14 days at 25° being still < 4, possibly because of an antioxidant or peroxide-decomposing action of the phospholipids present.

Chemical examination

Lipid content and properties. The analyses for lipid content of the meals and the characterization of the extracted lipids by iodine value and N content were carried out as previously described (Lea *et al.* 1958, 1960).

Solubility of the N. The method of measuring the solubility of the N of the meals after digestion with papain was that used by Olley & Watson (1961), except that the N determinations were carried out on the residual insoluble material instead of on the filtrates.

'Available' lysine. For the determination of available lysine the procedure of Carpenter (1960), based on the reactivity of the free ε-NH_2 groups of lysine units in intact protein with fluorodinitrobenzene, was used. As with other materials of low carbohydrate content, it was assumed that 8% of the dinitrophenyllysine was lost in

the acid-hydrolysis stage, and the values obtained were multiplied by 1·09 to compensate for this loss. This procedure has given results very similar to those from the procedure used previously (Lea *et al.* 1960) in which no blank deduction was used—the blank deduction compensating closely for the loss on acid hydrolysis which also was not corrected for previously.

The determination of available lysine has hitherto usually been applied to samples without preliminary extraction of the fat, but in this series all the analyses have been replicated with fat-extracted samples. The results for the latter have been calculated on the basis of the N content of the original samples and the yield of material after fat extraction, no account being taken of the small proportion of the total N taken out by the solvent extraction.

Microbiological assays

Preparation of the samples. For microbiological assay the meals were first extracted with chloroform–methanol (9:1, v/v) and then with diethyl ether as described above for the preparation of sample 5C. The dry residues were then re-ground to pass a $\frac{1}{90}$ in. sieve.

Relative nutritive value (RNV). For measurement of the RNV each sample was supplied as the sole protein source to a vigorously proteolytic strain of *Streptococcus zymogenes* (Ford, 1960). The published procedure was followed except that the test materials were treated with a buffered solution containing twice the standard concentration of papain (non-crystalline grade; British Drug Houses Ltd, Poole) so that the final concentration of the enzyme preparation was 0·09%. The growth response was measured turbidimetrically after incubation at 37° for 48 h, and expressed as a percentage of that produced by casein on an equal N basis.

Total and 'available' methionine. Similar procedures (Ford, 1962) were used for the determination of total and available methionine, *S. zymogenes* being supplied with a synthetic medium containing all the required amino acids except methionine, as well as the digest of the test protein. Values for methionine were then obtained by relating the growth response to that with pure L-methionine. For total methionine a digest prepared by autoclaving the test materials with 2N-HCl at 10 lb pressure for 5 h was used. Two sets of values for available methionine were obtained, after pre-treatment of the test materials with 0·09% or 0·36% of the papain preparation respectively, compared with 0·045% used in the original procedure. The small contribution of the enzyme itself to the final values was corrected for by subtracting values obtained in blank runs containing enzyme digests but no test material.

Feeding tests

Chick tests with semi-purified diets. The procedure in Expts 1–4 was based on the conditions used by Laksesvela (1958) for the assay with chicks of protein quality in herring meals, though it must be noted that thiamine was omitted in error from the list of vitamin supplements given in that paper. In a preliminary experiment it was found that this omission led to the cessation of growth after only 6 days of the experimental treatments. Thiamine was included in subsequent experiments as shown below.

Day-old cockerel chicks were purchased and reared for 6 days on a commercial diet containing an antibiotic supplement. At 6 days of age they were weighed, and those of very low or very high weight discarded; for this reason 50% more chicks were always obtained than were needed for the experimental treatments. Those retained were then divided into four strata by weight; from each stratum three chicks were allotted at random to each experimental treatment, and caged together as already explained (Lea *et al.* 1958). Thus twelve chicks, split up amongst four cages were allotted to each treatment. The experimental diets were then given *ad lib.* for 8–12 days (as detailed in Table 3); the chicks were weighed on alternate days. Feed consumption was also measured in all but the first two experiments.

The experimental diets were all made from the same basal diet: ground oat husk 10, arachis oil 4·15, $CaHPO_4.2H_2O$ 2, KCl 0·5, NaCl 0·3, a trace-mineral mixture, a vitamin mixture and maize starch to 100 parts. The trace-mineral mixture was designed to contribute (g/100 kg diet): $MgSO_4.7H_2O$ 600, ferrous citrate 160, $MnSO_4.4H_2O$ 30, $CuSO_4.5H_2O$ 20, KI 6·5 and $ZnCO_3$ 2·7; the vitamin mixture contributed (g/100 kg diet) choline chloride 150, Rovimix A and D (Roche Products Ltd, Welwyn Garden City; containing 8800 i.u. vitamin A and 2200 i.u. vitamin D_3/g) 100, Rovimix E (Roche Products Ltd; containing 10% α-tocopheryl acetate) 40, inositol 6·5, nicotinic acid 5, calcium pantothenate 2·7, thiamine hydrochloride 0·8, riboflavin 0·65, pyridoxine hydrochloride 0·6, menaphthone 0·26, folic acid 0·16 and biotin 0·026.

In each of the experiments the fish-meal preparations were included in the diets at a level calculated to contribute 12·5% crude protein (N × 6·25); the level of arachis oil was reduced by the amount of lipid contributed by the fish meal, but the addition was otherwise entirely at the expense of maize starch. Meals 4, 4S, 5 and 6 were therefore included at levels of 17·8, 17·8, 18·5 and 18·7% respectively and contributed approximately 3·2, 3·2, 2·5 and 1·6% chloroform-soluble lipid respectively. In Expt 1 two of the diets included 22% of soya-bean meal (contributing 10 parts of crude protein) in addition to the fish meals; this further addition was made entirely at the expense of maize starch.

Immediately after preparation, each diet was transferred to small cans, sealed in N_2 and stored at 0°. In this way the chicks could receive fresh food daily from a newly opened can. Food residues from the feed tins were discarded every 48 h.

Chick tests with natural diets. Two further tests were done with diets containing fish-meal preparations used as supplements to ingredients of the kind usual in practical diets. In Expt 5, the two experimental diets each included 20% of fish meal and the other ingredients were (%): ground wheat 25, yellow maize meal 25, wheat middlings 21·5, dehydrated grass meal 5, dried brewer's yeast 3 and Drivite A and D (Boots Pure Drug Co. Ltd, Nottingham; containing 1000 i.u. vitamin A and 200 i.u. vitamin D_3/g) 0·5. They contributed approximately a further 10% of crude protein to the diets.

In Expt 6, in which some reconstituted mixtures of 'fat-free solids' and lipids were used, the solids from meals 5 and 6 were included at the levels at which they were used in the previous experiment, 16·5 and 15·5% of diets A and B respectively. One

part of lipid was added per seven parts of 'solids' for the reconstituted mixtures, as explained above. In diets E and F 20% of meal 6 was included, so contributing again 15·5% fat-free solids. The other ingredients in the diet were grass meal, yeast and Drivite all as in Expt 5, together with ground wheat 26·5, wheat middlings 25 and yellow maize meal to 100 parts. For one treatment 0·015% of the antioxidant, 6-ethoxy-1,2-dihydro-2,2,4-trimethylquinoline (EMQ) was added to the diet, together with 0·085% arachis oil as a carrier; the other diets all included 0·1% arachis oil and no antioxidant.

For Expts 5 and 6 the day-old cockerel chicks were weighed on arrival, and after removal of those of extreme weight the selected chicks were allotted, as in the previous experiments, to the different treatments and were now offered the experimental diets immediately, without any preliminary period on a commercial diet. The experiments continued for 18–20 days.

Rat test. Five weanling albino males were taken from each of six litters. One rat from each litter was allotted to each of the five experimental treatments. The six rats allotted to each treatment were then caged in pairs, with the same arrangement for each treatment as regards litter origin of the pairs so as to simplify statistical analysis of the results. For a preliminary period of 3 days the same diet (no. 3 described below) was given to the rats in each cage; then the experimental diets were given for a further 10 days and the live-weight gain and food consumption were recorded.

The 'N-free' diet no. 1 consisted of potato starch 15, maize oil (containing 0·15% EMQ, w/w) 13, mineral mixture 4·22, vitamins and sucrose (purchased as finely ground 'icing sugar') to 100 parts. The mineral mixture was prepared so as to contribute (g/100 kg diet): NaH_2PO_4 (anhydrous) 1192, $CaCO_3$ 840, NaCl 580, $CaHPO_4$ 544, KCl 476, $FePO_4$ 240, K_2SO_4 224, $Mg(CH_3COO)_2.4H_2O$ 88, $MnCl_2.4H_2O$ 36, $CuCO_3$ 2·4, $ZnCO_3$ 1·2 and KI 0·4; vitamins were added to contribute (g/100 kg diet) choline chloride 100, Rovimix A and D 100, Rovimix E 40, calcium pantothenate 4, menaphthone 3, nicotinic acid 2, thiamine hydrochloride 0·6, riboflavin 0·6, pyridoxine hydrochloride 0·6, folic acid 0·2, biotin 0·015 and cyanocobalamin 0·002. Diets 2 and 3 were prepared by adding 14·2 parts of meals 4 and 4S respectively in place of 6·2 parts of sucrose, 5 parts of potato starch and 3 parts of maize oil in the mixture used for diet 1. Finally diets 4 and 5 were prepared by mixing 99·8 parts of diets 2 or 3 respectively with 0·2 parts of L-cystine.

RESULTS

Chemical and microbiological determinations

The results obtained with the meals as received, and with meal 4 after further storage with and without added fat, are set out in Table 1. As with herring meal 3 (Lea *et al.* 1960), the amount of lipid extractable with diethyl ether was always considerably less than the chloroform-soluble fraction of the material extractable with chloroform–methanol.

Storage of meal 4 in air for a year resulted in a fall of 48 units in the iodine value of the extractable lipid. With the double-fat meal D4 the fall was only 21 units, so

that even allowing for the double quantity of lipid, the absolute change in the number of iodine-reactive groups was slightly less. In contrast to the behaviour of meals 1, 2 and 3, which showed a fall of iodine value to well below 100 after controlled storage with a large surface exposed to the air at 10, 20 or 25° for 4 months (Lea *et al.* 1958, 1960), meal 5 still had the comparatively high iodine value of 126 after the same period

Table 1. *Results of the chemical analysis and microbiological assay for relative nutritive value and available methionine of meal 4 after various treatments and of meals 5 and 6*

	Characteristic	Meal no. 4 (fresh)	4S (stored)	D4* (fresh; double-fat)	D4S (stored; double-fat)	5 (stored)	6 (stored)
	Composition						
(i)	Moisture (%)	7·6	—	(6·5)	—	10·1	7·9
(ii)	N × 6·25 (%)	70·6	—	(60·1)	—	67·6	66·8
(iii)	Ash (%)	9·7	—	(8·3)	—	13·8	16·5
(iv)	Lipids extractable with diethyl ether (%)	13·5	—	(26·3)	—	7·0	4·2
(v)	Lipids extractable with chloroform–methanol (2:1, v/v) (%)	18·4	—	(30·5)	—	14·0	11·0
(vi)	Chloroform-soluble material present in extract v (as percentage of original sample)	17·4	—	(29·6)	—	13·3	8·5
(vii)	N in extract vi (%)	—	2·2	—	1·0	1·4	4·2
(viii)	Iodine value of extract vi	133	85	(132)	111	126	88
	Protein-quality tests†						
(ix)	N solubility after pepsin digestion (%)	97	95	—	—	—	89
(x)	Available lysine (g/16 g N):						
	(*a*) no pretreatment	7·87	7·53	(7·87)	7·27	7·47	5·78
	(*b*) after defatting	7·93	7·36	(7·93)	7·61	7·49	5·61
(xi)	Total methionine (g/16 g N)	3·35	3·22	(3·35)	3·34	3·33	3·11
(xii)	Available methionine (g/16 g N) after digestion with:						
	0·09% papain	2·60	2·20	(2·60)	2·25	2·21	1·32
	0·36% papain	3·12	2·84	(3·12)	2·97	2·99	2·31
(xiii)	PEV for *S. zymogenes* (casein = 100)	83	75	(83)	73	70	56

* Values calculated as those to be expected for a mixture of 100 g of meal 4 and 17·4 g of expressed herring oil 1 which was assumed to contribute only lipid, and was found to have an iodine value of 130.

† For tests x(*b*), xi, xii and xiii, meal 6 was pre-extracted with chloroform–methanol (2:1, v/v) and the other meals with benzene–ethanol (3:1, v/v), before being twice washed with diethyl ether.

of storage under commercial conditions, but we do not know the initial value for this sample. The anchovy meal 6 of unknown age had an iodine value of 88; the iodine values of freshly expressed samples of anchovy oil have been reported as ranging from 152 to 169 (Buttenberg & Stüber, 1906), values higher than those usually found for herring oil.

The N content of the lipid extracted from meal 6 (4·2 %) was also higher than the values of 2·2 and 1·4 % respectively for herring meals 4S and 5. Values of 4 % or over were obtained by us only for a herring meal that had been heated at 130° for 30 h (Lea *et al.* 1960).

It can be seen from Table 2 that, whereas chloroform–methanol (9:1, v/v) was slightly more efficient than benzene–ethanol (9:1, v/v) in the extraction of lipid from meal 5 and did not reduce the level of available lysine, it still gave a fat-free residue with an abnormally high chlorine content even after heating at 50° for 2 h in a vacuum oven to remove solvent.

Table 2. *Results of chemical analysis of untreated herring meal 5 and of the same meal extracted with chloroform–methanol* (9:1, *v/v*) (5*B*) *or with benzene–ethanol* (9:1, *v/v*) (5*D*)

Constituent	Meal 5	Meal 5B	Meal 5D
Moisture (%)	10·0	0·7	1·6
Crude lipid extractable with chloroform–methanol (9:1, v/v) in 2 h (%)	13·7	0·0	1·3
Fat-free solids by difference (%)	76·3	99·3	97·1
Available lysine (as percentage of fat-free solids)	6·62	6·64	6·53
Chlorine (as percentage of fat-free solids)	0·65	2·71	—

Laboratory protein-quality tests. From Table 1 it can be seen that all the values for meal 4 were reduced by storage, the biggest percentage fall being that of from 14 to 16 % in available methionine. The apparent drop in RNV was about 10 %, and the fall in available lysine was rather less. There was least change in 'N solubility after pepsin'.

Meal 5 gave values of the same order as meal 4S, the corresponding herring meal stored at normal fat content but under experimental conditions. The anchovy meal 6 gave considerably lower values than these, the value for available methionine being approximately 60 % of the corresponding values for meals 4S and 5, in contrast to the values for total methionine which showed little difference between the three meals, and little effect of storage.

The effect of defatting the meals before estimating their content of available lysine was small and not significant. On the other hand, increasing the level of papain in the pretreatment of the samples for the available methionine assay resulted in consistently higher values, the biggest proportional effect being with the poorest sample, no. 6. However, the ranking of the samples was not changed by the alteration in the strength of the papain used for digestion.

Feeding tests

Chick tests with semi-purified diets. The results for the weight gains of the chicks in Expts 1–4, with semi-purified diets, are summarized in Table 3, and the results of the statistical analyses for all the chick experiments are summarized in Table 5.

In Expt 1 the chicks receiving meal 5 grew steadily, both with and without the further

addition of soya-bean meal. Those receiving the chloroform–methanol extracted meal 5B, on the other hand, grew very much more slowly, even in the first 2 days; plotting of successive weights showed a nearly even rate of gain. At the end of the main experimental period, six of the birds on diet B were transferred to a similar diet with

Table 3. *Daily weight gain, food consumption and food conversion efficiency of chicks in four experiments with semi-purified diets*

Expt no.; strain of chick; age over trial	Treatment code	Protein source in the diet	Live-weight gain (g/head)	Food eaten (g/head)	Gain (g)/food eaten (g)
1; Rhode Island Red × Light Sussex; 6–18 days	A	Meal 5	8·4	—	—
	B	5B (extracted chloroform–methanol)	1·4	—	—
	C	5 + soya-bean meal*	12·9	—	—
	D	5B + soya-bean meal*	3·8	—	—
2; White Leghorn × Rhode Island Red; 6–14 days	A	Meal 5	7·6	—	—
	B	5C (extracted with chloroform–methanol and diethyl ether)	6·6	—	—
	C	5C + 0·3 % DL-methionine	6·5	—	—
	D	5D (extracted with benzene–ethanol and diethyl ether	7·1	—	—
	E	Soya-bean meal + casein	5·5	—	—
3; Brown Leghorn × Rhode Island Red; 6–16 days	A	Meal 4 (control)	6·8	15·1	0·45
	B	4S (stored in air)	5·9	13·5	0·44
	C	D4S (double-fat, stored in air)	5·1	12·6	0·41
	D	5	6·4	14·1	0·46
	E	6 (anchovy)	3·1	10·6	0·30
4; Brown Leghorn × Rhode Island Red; 7–17 days	A	Meal D4 (double-fat control)	7·2	15·8	0·46
	B	D4, but diet unprotected†	7·4	15·9	0·47
	C	D4P (+ oxidized oil)	7·5	16·1	0·46
	D	Solids D4 + fat D4S	7·6	16·3	0·46
	E	Meal D4S (double-fat stored in air)	5·7	14·6	0·39
	F	Solids D4S + fat D4	6·2	14·5	0·43

* The soya-bean meal contributed 10 % crude protein in these two diets in addition to the standard 12·5 % protein supplied by the fish-meal preparations in these, and all the diets used in Expts 1–4.

† This diet, after mixing, was stored with access to the air, whereas all the other diets were held under nitrogen until the day of use.

the benzene–ethanol extracted meal 5D, and in the following 8 days they had a daily live-weight gain of 6·7 g/head, compared with a gain of only 1·1 g/head made by the six chicks remaining on the diet (B) containing the chloroform–methanol extracted meal 5B.

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In Expt 2, meal 5 was compared from the beginning with both meal 5C, which had been prepared by extraction with chloroform–methanol followed by washing with diethyl ether, and meal 5D which had been prepared by benzene–ethanol extraction. The rates of weight gain of the chicks receiving 5C and 5D were respectively 88 and 94% of that of the chicks receiving the control meal 5, the differences being without statistical significance. Further, chicks receiving 5C with a supplement of methionine gained weight no faster than those given 5C alone. All the chicks gained weight faster than those receiving their protein from a mixture of soya-bean meal and casein. Food consumption was not measured in these first two experiments.

It appears from these results that most of the growth-depressing property of the chloroform–methanol extracted meal 5B was no longer present in meal 5C which had received two ether washes as well as a longer period in the vacuum oven.

For Expt 3 herring meal 4S, which had been stored for a year, was used. If the rate of live-weight gain of the chicks receiving the control meal 4 (stored in N_2 at −20°) is taken as 100, the relative rates of gain on the same meal stored in air at 20° (4S) and of the double-fat meal stored in air (D4S) were 87 and 75 respectively. The weight gain of the chicks was approximately proportional to the quantities of food eaten by them. It is possible therefore that the poorer gain in weight was the consequence of a lower acceptability of some diets. The efficiency of food conversion (i.e. g gain in weight/g food eaten) was very similar on all three diets. The gain in weight of chicks on meal 5, which had been stored in air for 4½ months, was intermediate between that of chicks receiving either the control meal 4 or the same meal stored for a full year in air (4S). Both the rate of weight gain and the efficiency of food conversion of the birds receiving the anchovy meal 6 were poor.

In Expt 4, the stored double-fat meal (D4S) was again included, this time with a fresh double-fat meal (D4) as control, and chicks receiving the former gained weight at a rate equivalent to 79% of the gain of those receiving the latter. However, the overall statistical analysis of the results of this experiment indicated a more than 5% probability of there being no difference between treatments, and comparison of individual treatments is not therefore strictly valid.

The treatments also included tests with mixtures of lipid extracted from one meal with residual solids from the other. Of the two mixtures, that containing the 'fresh' solids and 'stored' lipids supported the better weight gain in the chicks. Although differentiation of performance in individual diets is not valid, comparison of the mean gain of the chicks on the four treatments A–D receiving the solids from meal D4 (7·39 g/chick day) with that of the chicks on treatments E and F receiving the solids of meal D4S (5·94 g/chick day) showed a highly significant difference in favour of the former. The food consumption in groups A–D was also greater, so that the efficiency of food conversion was in the ratio of 100:89, whereas the ratio of weight gain for groups A–D as compared with E and F was 100:80.

It can also be seen from the results of Expt 4 that, under the conditions used, the incorporation of herring oil oxidized so as to have a comparatively high peroxide value had no depressing effect on the chicks' rate of food consumption or on live-weight gain; nor had the storage of the mixed diet in air for a week before the experiment and during it any adverse effect. Indeed, the rate of weight gain with the solids of D4S with partly oxidized fat (treatments B, C and D) was slightly, though not significantly faster than with treatment A in which the completely fresh material was used.

Table 4. *Weight gain, food consumption and food conversion efficiency of chicks in two experiments with various fish-meal preparations given as supplements to other natural feeding-stuffs*

Expt no.; strain of chick; age over trial	Treatment code	Fish-meal preparation	Daily live-weight gain (g/head)	Food eaten daily (g/head)	Gain (g)/food eaten (g)
5; Light Sussex × Rhode Island Red; 0–20 days	A	Meal 5	8·7	15·9	0·55
	B	6	5·0	11·2	0·45
6; Rhode Island Red × Light Sussex; 0–18 days	A	Solids 5 + fat 5	7·8	14·8	0·53
	B	Solids 5 + fat 6	7·3	14·7	0·49
	C	Solids 6 + fat 5	5·2	11·4	0·45
	D	Solids 6 + fat 6	5·1	12·4	0·41
	E	Meal 6	5·6*	12·2*	0·46*
	F	Meal 6 (+EMQ†)	5·5*	11·2*	0·49*

* As stated on p. 155, six of the twelve chicks used in each of these treatments received a vitamin supplement; values for them were included with those for the other chicks to give these mean values.
† 6-Ethoxy-1,2-dihydro-2,2,4-trimethylquinoline.

Table 5. *Summary of the results of statistical analysis of the live-weight gains in Expts 1–6*

Expt no.	Probability of no overall difference between treatments	Least significant differences ($P < 5\%$)* between mean gains (g/chick day) on particular treatments: Treatments compared	Value
1	< 0·1%	A, C and D	1·30
		B and A, C or D	1·44
2	< 1%	All	1·01
3	< 0·1%	All	0·95
4	> 5%	All	(1·63)†
5	< 5%	(Only one comparison)	
6	< 5%	A, C and D	1·67
		A, C or D and B or F	1·86
		A, C or D and E	2·16
		B, E and F	2·25

* Least significant differences with probabilities of < 1% and < 0·1% respectively of the differences being due to chance are approximately 1·45 and 2·1 times the corresponding values given in the table.
† Since the overall analysis showed no significant difference between treatments individual comparisons are invalid.

Chick tests with natural diets. The results of Expts 5 and 6 are summarized in Table 4. It will be seen that in Expt 5 the anchovy meal 6 again, with this different type of diet, gave a result very inferior to that obtained with herring meal 5. Feed consumption was also lower with meal 6, but not to the same extent, so that the ratio of weight gained to food eaten was also lower for meal 6 than for meal 5.

Table 6. *Weight gain, food intake, calculated protein efficiency ratio, protein retention efficiency and appetite quotient of rats given five different dietary supplements for 10 days*

Diet no.	Supplement to 85·8 parts of basal mix	Pair from litter nos. 1 and 2		3 and 4		5 and 6		Mean live-weight gain (g/rat day)	Mean food con-sumption (g/rat day)	PER	PRE	Appetite quotient*
		W	*F*	*W*	*F*	*W*	*F*					
1	Potato starch, 5; sucrose, 6·2 ('nitrogen-free' control)	−20·6	—	−22·6†	—	−16·8	—	−1·00	—	—	—	—
2	Meal 4, 14·2 (stored in N_2 at −20°)	46·5	174·7	50·8	180·2	72·4	216·1	2·83	9·51	2·94	64·2	0·245
3	Meal 4S, 14·2 (stored in air at 20°)	38·7	150·8	36·5	143·8	45·5	180·6	2·02	7·92	2·53	61·2	0·214
4	Meal 4, 14·2; L-cystine, 0·2	62·8	181·4	84·2	220·6	76·4	202·6	3·72	10·08	3·63	73·7	0·245
5	Meal 4S, 14·2; L-cystine, 0·2	49·1	151·9	65·6	194·6	70·7	194·8	3·09	9·02	3·35	71·5	0·225
	Standard error of the mean for each diet, 2–5 (estimates based on 6 df)							±0·22	±0·45	±0·10	±1·8	±0·0055

W, total weight gain/cage (g); *F*, total food eaten/cage (g).
* This is the term Q/C, or g food eaten daily/mean $W^{0.68}$, W being the weight of the rat in g (Carpenter, 1953).
† In this cage one rat died and the figure given is twice the weight change of the survivor.

For Expt 6, the fat-free solids and fat of meals 5 and 6 were separated and remixed in different ways. It can be seen from Table 4 that chicks receiving the solids from meal 5 gained weight faster than those receiving the solids from meal 6, regardless of the type of lipid with which the solids were mixed. It does not therefore appear to have been the extractable lipid fraction of meal 6 that was directly responsible for the slow gain in weight with this meal. Nor was the performance with meal 6 improved by addition of the antioxidant EMQ to the final mixed diet.

From the 8th day of the experiment one-half of the chicks receiving meal 6, or meal 6+EMQ, had a vitamin supplement added to their diets, in the quantities used for the purified diets in Expts 1–4. Over the remainder of the experimental period the mean rate of gain of all the chicks given vitamin supplements was 7·2 g/day, and that of the corresponding birds not given the supplements was 7·0 g/day: the small difference was not significant.

Rat test. A summary of the results is set out in Table 6. All the animals receiving the herring meals appeared to remain healthy, but one that received the 'N-free' diet died. The mean rate of weight gain of the rats receiving meal 4S, that had been allowed to oxidize, was 71 % of the value for those receiving the control meal 4, but these rats also ate less so that the protein efficiency ratio (PER), calculated as g gain in weight/g protein eaten, was relatively less reduced—86 % that of those receiving meal 4. Another value, protein retention efficiency (PRE), takes into account not only the weight gain on the test diet, but also the sparing of the weight loss that occurred on the N-free diet, and was calculated as 0·16 × (g gain in weight on test diet + g loss in weight on diet 1)/(100 g protein eaten on test diet) (Bender & Doell, 1957). The value of PRE for the rats receiving meal 4S was 95 % of that for the rats receiving meal 4, the difference being without statistical significance.

The rats receiving the same two meals with a further supplement of 0·2 % cystine showed a better performance by all three criteria. The relative performance with '4S+cystine' was 83, 92 and 97 % of that with '4+cystine' as judged by rate of weight gain, PER and PRE respectively.

Presumably the greater difference observed in weight gain than in the other measures is to be explained in terms of a lower appetite of the rats for the stored material. The 'appetite quotient' (Carpenter, 1953), a value derived in an attempt to measure such an effect, was in fact significantly lower for the two diets (with or without cystine) containing the oxidized meal than for the two diets containing the fresh meal. We cannot use this value to obtain figures in terms of metabolizable calories, but since the oxidized meal was probably slightly less well digested than the fresh meal, such conversion would only tend to widen the difference.

DISCUSSION

Possible toxicity of the oxidized oil

There has been considerable discussion in the past decade of the possible dangers associated with the presence of oxidized fats in both human and animal diets (cf. Colborn, 1953; Greenberg & Frazer, 1953; Kaunitz, 1960; Perkins, 1960) and analysts

are sometimes expected to condemn or approve particular diets or edients on the basis of chemical analysis for such easily determined factors as peroxide value or even free fatty acids.

Several ways can be suggested in which autoxidation of the fat in foods might be harmful. The oxidized fat itself might be toxic through its content of peroxide or of polymerized or cyclized fatty acids, which it certainly is if included at very high levels in the diet. Fat oxidation may also impair appetite by its adverse effect on palatability. Finally, the nutritive value of fat-containing foods or diets can be reduced by protein damage such as we have been considering, or by the induction of particular vitamin deficiency states, either as a result of oxidative destruction or through increased vitamin requirements.

The experiments reported in this paper were all short-term ones using basal diets rich in all the known vitamins, and were not designed to provide evidence on possible effects on vitamins. However, the animals used, whether chicks or rats, were young and rapidly growing, so that any short-term toxicity effect or check to appetite should readily have become apparent. Moreover, the herring meal used in the tests was allowed to oxidize extensively so that the iodine value of its oil had fallen by nearly 50 units or, in one experiment, herring oil oxidized separately to a peroxide value of 142 μmoles/g was added to it. The oxidized meals were given at twice the level that could be considered practical, and with the 'double-fat' meals this level meant a total addition of over 6 % fat to the diet, or four times the practical level. These conditions can therefore be considered as extreme in relation to what might occur in practice, though they do not approach the severity of conditions adopted by some workers to demonstrate the toxic effects obtainable with oxidized fat. Diets containing as much as 10, 15 or even 20 % of strongly heated and oxidized fat have been given to laboratory animals, often causing depression of growth, or sometimes even loss of weight, and death. In a less extreme experiment 7 % of fat oxidized to a peroxide value of 600 μmoles/g and given to young rats depressed their rate of weight gain to about four-fifths of normal (Andrews, Griffith, Mead & Stein, 1960).

The conclusion from our own experiments was that, although both the rats and the chicks gained weight more slowly on the oxidized meal (4S) than on the control meal (4), the difference was not to be explained by any growth-depressant effect of the extractable lipid itself. If it had been so one would have expected that when the extractable lipid from the oxidized meal (4S) was mixed with the extracted solids of the fresh meal (4) the chicks would have performed less well with the mixture than with the entire fresh meal. In fact, in Expt 4, in which the comparison was made, they did just as well. This finding is in agreement with other recent findings that the lipids extractable from stored herring meals did not depress the rate of weight gain of chicks (Laksesvela, 1961; March, Biely, Goudie, Claggett & Tarr, 1961) and that solvent extraction of commercial herring meals had little or no effect on their value in chick diets (Biely, March & Tarr, 1955; March, Biely & Tarr, 1957).

Kraybill & Nilson (1947) also found no growth-depressant effect in rats over a 6-week period of a diet containing 3 % of lipid extracted from a commercially store sample of menhaden meal (the period of storage was not stated); but rats receivin

a 15 % supplement grew at only 75 % the rate of controls receiving the same level of lard. Bender & Haizelden (1957) could find no consistent effect of defatting a variety of fish meals on their biological values for rats.

Since it might be suspected that, under laboratory conditions, the particular set of circumstances needed for the development of fat toxicity had not been reproduced, we asked for the co-operation of field workers in obtaining a sample of fish meal (no. 6) that had been reported to show 'toxic rancidity' with farm animals. It was clear from Expts 3 and 5 that this meal was very much inferior nutritionally to meal 5, but again it is apparent from Expt 6, in which the extractable lipids from meals 5 and 6 were interchanged, that this inferiority was associated with the residual solids rather than with the extractable lipid.

The results presented in this paper have therefore provided no evidence for the toxicity of oxidized fish lipids under the experimental conditions chosen. It must be repeated, however, that these conditions were not such as to bring out any vitamin deficiency effects; the need for precautions, such as the use of protected vitamin concentrates and of antioxidants to prevent the occurrence of vitamin A or vitamin E deficiency with diets rich in unsaturated fat, is already understood and non-controversial (cf. Singsen, Potter, Matterson, Bunnell, Kozeff & Jungherr, 1955).

Laksesvela (1959) has shown that the level of free fatty acids in the lipid of herring meals is not a good indicator of their feeding value, and Branion, Dawson, Cavers & Motzok (1938) had earlier come to the same conclusions for cod-liver oils. Our finding of normal results with a meal of artificially high peroxide value suggests that this criterion too is unlikely to be a useful indicator of the suitability of a sample of herring meal for feeding, probably because it is a measure only of a labile intermediate oxidation product.

Feeding tests of protein quality in stored meals

Our previous work (Lea *et al.* 1960) showed a fall of only 8–9 % in the available lysine content of herring meals stored at 20–25° for 1 year. This result, although supported by a biological assay, appeared to be somewhat in conflict with American reports indicating much greater declines in the proportional growth rates of chicks receiving as their sole sources of protein fish meals stored for varying periods (Grau, Kerrick, Lundholm & Barnes, 1959; Rand, Collins, Varner & Mosser, 1960; L. E. Ousterhout & D. G. Snyder, 1962, unpublished results).

We can now compare results obtained with fresh and stored meals in feeding tests of the same type with both chicks and rats. As was to be expected from the results of Miller (1956) and Njaa (1961), the sulphur-containing amino acids were the limiting factor in the rat test. Expressing the results for the 12-month stored meal 4S (and in parentheses those for the double-fat stored meal D4S also) as a percentage of the corresponding results with the control meal 4, we have

	Chick Expt 3	Rat Expt
Gain in weight	87 (75)	71
Food intake	89 (84)	84
PER	98 (90)	86
PRE	— —	95

If it is conceded that differences in growth rate, and even differences in PER, exaggerate apparent differences in protein quality when less is eaten of the inferior diet (Carpenter, 1953; Bender & Doell, 1957) then a closer approach to a measure unaffected by appetite differences is that of PRE. This value is an indirect measure of net protein utilization (or g N retained/100 g N eaten) on the assumption that both the weight lost in the negative control group and that gained in the test groups contain 16 % protein (N × 6·25) (Bender & Doell, 1957). PRE thus takes into account the diversion of some of the dietary protein for maintenance needs rather than growth. We cannot calculate PRE values for the chick experiments, but they would certainly be higher than the corresponding figures found for PER.

It would seem, therefore, that neither in the chick nor in the rat experiment was there a fall in protein quality during storage of more than 5–10 %, the larger differences in weight gain being explained by the additional factor of a drop of about 10 % in the appetite of the experimental animals.

Whether or not this appetite-depressant effect exists, even to a smaller degree, when stored meals are included at a level of approximately 5 % together with natural ingredients is not known for certain. It was not observed in two published experiments of this type comparing fresh and stored material (Evans, Carver & Hamm, 1944; Lea *et al.* 1958).

Laboratory tests of protein quality on stored meals

Expressing the chemical values found for the stored meals (and in parentheses those for the double-fat stored meal D4S also) as a percentage of those for the control meal 4, as above, we have

Solubility of the N after digestion with pepsin	98
Available lysine	94 (94)
Available methionine	88 (91)
RNV	90 (88)

The figures quoted for available lysine and for available methionine are the means for each amino acid of the results obtained by the two variations of procedure described (pp. 153, 154).

We know from other work (e.g. that of Dreosti & Rowan, 1959; Boyne, Carpenter & Woodham, 1961) that 'N solubility after pepsin' can very much underestimate nutritional damage. Moreover, the content of available methionine has been shown to fall proportionally more than that of available lysine during controlled heat treatment (Carpenter, Morgan, Lea & Parr, 1962) and the same has now been demonstrated as a result of storage. However, all the changes are small and of the same order as the experimental errors of the procedures, and we can only conclude that neither has given a grossly erroneous indication of the extent of the nutritional damage.

No certain explanation can at present be offered for the consistently lower nutritive value of the stored as compared with the control meals. It cannot be due to a physical barrier of oxidized and perhaps partly polymerized oil round the protein since the effect persists even after solvent extraction, and previous experiments have shown that chloroform–methanol is an efficient solvent for the lipids in oxidized herrin

meal (Lea *et al.* 1958). Nor can we hypothesize for methionine any binding mechanisms analogous to the reaction of lysine ϵ-NH_2 groups with aldehydes formed during the oxidation of fat. We have not shown directly that the fall in nutritive value of the material is a consequence of the oxidation of the fat, but circumstantial evidence that it is so is provided by our earlier observation that the fall in available lysine content did not occur (*a*) in herring meal stored in N_2 or (*b*) in defatted herring meal stored in air. If the nutritive value of proteins *per se* deteriorated when the meals were in contact with the air one would expect to find evidence for it in the literature.

The allegedly toxic meal

Meal 6, the poorest sample in the series, was also the only anchovy meal. However, meal made from this fish can be of as high a quality as any other type of meal (Olley & Watson, 1961; Carpenter & Woodham, unpublished results). It is known that anchovy meal may overheat during storage, presumably as a consequence of the autoxidation of its highly unsaturated oil, with resultant damage to the quality of the protein. The observation that the level of total methionine in meal 6 was nearly equal to that for the other meals tested, whereas the level of available methionine was much lower, supports the suggestion that the low quality was due to subsequent heat damage rather than to any deficiency in the raw material. This suggestion is also in harmony with our failure to find any growth-depressing effect when the extracted lipid was given, and also with the low available lysine value of the meal and the high N content of the lipid—both of which have been produced experimentally in herring meal by deliberate heat damage (Lea *et al.* 1960).

In another small test (Carpenter, unpublished results), the same meal was given to a newly weaned piglet at increasing levels up to 15% of the diet without any harmful effect becoming obvious during the 10 days' test. The meal was also assayed for histamine by a procedure using guinea-pig gut (Gaddum, 1935–6) and showed an apparent content of 0·01%: this is well below the lowest level of 0·12% found in meal made from spoiled tunny which caused depression of growth when given to chicks (Shifrine, Ousterhout, Grau & Vaughn, 1959).

These observations combine to give the impression that, whereas meal 6 was undoubtedly of low protein quality, there was no evidence of its being directly toxic. It might be thought that perhaps the meal had gone through a toxic stage of oxidation. However, our experiment in which an oil meal specially prepared to have a considerable peroxide value was given to chicks failed to demonstrate any toxicity under the conditions used.

Deleterious effect of extraction with chloroform

Solvent extraction of meal 5 with chloroform–methanol (9:1, v/v) followed by 2 h in a vacuum oven at 50° to yield meal 5B caused a severe fall in its nutritive value, as shown in Table 3. The same solvent-extraction procedure, but followed by washing with ether before vacuum treatment, to give meal 5D, had only a small and statistically insignificant effect (Expt 2). On analysis, meal 5 showed an increased chlorine content (Table 2), and it seems probable, therefore, that a small amount of chloroform

was tenaciously held by the herring protein, and that this retained chloroform was responsible for reducing the performance of chicks on diets containing meals defatted by chloroform–methanol extract unless special precautions were taken to remove the solvent completely. Morrison, Sabry & Middleton (1962) have recently made a similar observation with fish flours from which the bones had been separated by flotation in chloroform.

SUMMARY

1. Part of a sample of herring meal (no. 4) containing 17·4% lipids was held in nitrogen at −20° as control; another part (no. 4S) was stored in air at 20° for 12 months during which the iodine value of its lipid fell from 133 to 85. A 'double-fat' meal, prepared by blending expressed herring oil with the control meal, was similarly stored to give meals D4 and D4S respectively.

2. Weanling rats receiving meal 4 as their sole source of protein (at a 10% level) gained 2·8 g/day; those receiving meal 4S gained 2·0 g/day. With each diet there was a significant response to a supplement of cystine. The protein retention efficiencies (PRE) on the two diets were 64 and 60 respectively. The 'appetite quotient' of the rats receiving meal 4S was 90% of that of those receiving meal 4.

3. Chicks receiving meal 4 for 10 days as their sole source of protein (at a 12·5% level) gained 6·8 g/day; those receiving meals 4S and D4S gained 5·9 and 5·1 g/day respectively.

4. The lipids of meals D4 and D4S were extracted and re-mixed, each with the fat-free solids of the other. When these mixtures were given to chicks, growth depression was observed with the diets containing the non-fat solids from the air-stored meals (D4S), but not with those containing the oxidized lipids. The addition to the diet of 2·5% herring oil pre-oxidized to a peroxide value of 142 μmoles/g also failed to depress growth.

5. In laboratory tests meal 4 showed 97% nitrogen solubility after pepsin digestion, 7·9 g/16 g N of available lysine (fluorodinitrobenzene method), 2·9 g/16 g N of available methionine (mean of results by two procedures with *Streptococcus zymogenes*) and a relative nutritive value (RNV) of 83. The corresponding figures for meal 4S were respectively 98, 94, 88 and 90% of these values. The figures for meal D4S did not differ significantly from those for meal 4S.

6. An allegedly toxic sample of anchovy meal (no. 6) supported a growth rate of only 3·1 g/day in chicks, but the extractable lipids were again found not to be growth-depressant. The meal gave low values in the laboratory protein-quality tests and may well have become overheated (scorched) during storage as a result of lipid oxidation.

7. In a preliminary experiment a herring meal (no. 5) extracted with chloroform–methanol to remove the lipid and then heated in a vacuum oven to remove the solvent, supported only poor growth. Since further washing of the extracted meal with diethyl ether largely restored its value for chicks it was concluded that the effect was probably due to the toxicity of firmly held residual chloroform.

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A STUDY OF THE POTENTIAL APPLICATIONS OF OLFACTORY RESEARCH IN MAN

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FOREWORD

The Life Sciences Research Office (LSRO), Federation of American Societies for Experimental Biology (FASEB), provides scientific assessments of topics in the biomedical sciences. These reports are based upon comprehensive literature reviews and the perceptive observations of knowledgeable scientists engaged in work in the field. Although LSRO reports are recognized by FASEB as contributions to societal needs and most LSRO consultants are members of FASEB constituent societies, the reports do not necessarily reflect the views of the members of its six constituent societies. However, the report has been reviewed for policy matters by the LSRO Advisory Committee, which includes representatives of each constituent society.

This technical report was prepared for the Life Sciences Office, Office of the Chief of Research and Development, Department of the Army, by the staff of the Life Sciences Research Office, FASEB, in accordance with the provisions of U.S. Army Contract No. DAHC19-72-C-0010.

We acknowledge the contributions of the numerous investigators and consultants who have assisted with this study. The report reflects the opinions expressed by participants in an *ad hoc* study group that met at Beaumont House, FASEB, on September 19-20, 1972, and other consultants. The report has been reviewed by these scientists and a judicious attempt has been made to incorporate the different viewpoints and opinions.

The listing of the consultants' names in Section XII must not be construed as indicating that they are authors of the report or that they endorse the conclusions or recommendations. The authors accept full responsibility for the contents of this report.

C. Jelleff Carr, Ph.D.
Director
Life Sciences Research Office

Humphrey F. Sassoon, Ph.D.
Research Associate

Kenneth D. Fisher, Ph.D.
Research Associate

SUMMARY

This study provides a comprehensive review of the feasibility of developing and utilizing the chemical senses of the soldier. While biosensors and odor-sensing devices can supplement, they cannot replace the potential performance of the normal human olfactory system. There is no current program in the Army to train soldiers to use their chemical senses. Experience in other countries suggests that a soldier trained to recognize olfactory cues is more effective in the field, more responsive to hazards, and less likely to give olfactory information to the enemy than one whose chemical senses are underutilized.

Practical testing and training methods suited to the Army's needs have been lacking in the past, and basic knowledge of the mechanisms of olfaction and taste is incomplete. However, elements of a suitable technology have been identified in agencies such as the National Bureau of Standards and in certain industries. This technology appears suitable for further development within the Army research and development community. This report concludes that it is feasible for the Army to use these methods in determining classifications of personnel that require normal acuity of the chemical senses and to institute training programs.

In addition, regular testing for normal acuity of the chemical senses may uncover early cases of some illnesses, indicate extensive exposures to ionizing radiation, and categorize personnel as either suitable or unfit for certain Army duties. If administered at recruitment, these tests can be used later to document disability claims for service-related losses of olfaction and taste.

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I. THE PROBLEM

Visual and auditory proficiency are generally recognized as important requirements for the soldier, and are not only related to efficient performance in the military environment but also may be essential for survival. The military significance of these sensory modalities has been reviewed recently (Carr and Fisher, 1970, 1971, Carr *et al.*, 1969, Fisher *et al.*, 1969). On the other hand, acuity of the "chemical senses," olfaction and taste, has been less widely recognized as important, and the only available review of the subject related to Army interests was concerned mainly with animal biosensors (Smith and Coates, 1965). Another trend has been to substitute instruments for odor detection in the field without considering the performance potential of man.

Although some of the basic mechanisms of the neurophysiology of olfaction and taste are unknown, human skill in the recognition of odors and tastes can be enhanced by training and may be used efficiently by the soldier (Woods, 1968). The Army has sponsored major work related to olfaction and taste on nutrition, diet, food components, and acceptability of rations, but there has been little in-house military research on the potential contribution of the chemical senses to military performance.

When men are incapable of detecting odors of fire, toxic gases, or spoiled foods, this must obviously be a handicap to performance and even to survival. Most men are capable of detection, yet are often untrained in odor recognition. Failure to identify odors that characterize machine malfunctions, enemy operations, states of the environment or other situations could mean the difference between success or failure of a military mission. In general, any underutilization of sensory responses to environmental stimuli detracts from the quality of human performance. Woods (1968) has indicated that olfactory acuity is required for certain military duties in the British and Commonwealth armies. In contrast, in the United States Army, no military duties are specified as requiring a normal level of acuity and the chemical senses are not even tested.

A major requirement of this study was to identify aspects of the military environment in which normal acuity of olfaction and taste are important and factors that contribute to losses of these senses. In the past, a major difficulty has been inadequate testing methods to evaluate

acuity of olfaction and taste. Although many testing methods continue to be directed toward elucidation of basic mechanisms rather than evaluation of human capabilities, recent technical advances indicate that the latter objective should now be attainable.

II. SCOPE OF THE STUDY

Preliminary discussions with research investigators in universities and Army laboratories revealed many facets of the problems of understanding the mechanisms of human olfactory and taste systems, the nature of loss of acuity, recognition and detection levels, environmental and genetic influences, and analytical techniques employed in assessment of acuity in man.

From this background an agenda was developed for an *ad hoc* review group meeting convened at Beaumont House, Federation of American Societies for Experimental Biology, on 19-20 September 1972 (see Section XII-A). These reviews of the subject provided insight into the basic and applied aspects of olfactory and taste research and the Army programs related to these areas. The scope of this report emerged from these reviews.

The study includes a synopsis of the current limits of scientific and technical knowledge, especially of olfaction, and a more detailed assessment of opinions on how this information may be applied to Army needs. It was not deemed necessary to review current in-house Army research programs on food acceptability, biosensors, or odor-sensing devices. The study stresses the practical applications of human sensory acuity in olfaction and taste, rather than fundamental concepts and theories that are available in the literature.

III. COMPONENTS OF THE HUMAN OLFACTORY AND TASTE SYSTEMS

A. OLFACTION

1. Air Passages

The nose is a paired organ, like the eyes but unlike the mouth (Figure 1). The olfactory system is exquisitely sensitive; in nose-breathing, only 3 percent of the inspired air passes over the olfactory epithelium (Stuiver, 1958). When the nasal epithelium is either dry or congested the airflow is abnormal and olfactory function is further diminished. The rate of flow and the temperature of the air are important (Schneider and Wolf, 1960). When exhaled air passes through the mouth it may pick up odorants that affect responses of the olfactory epithelium to inhaled odorants.

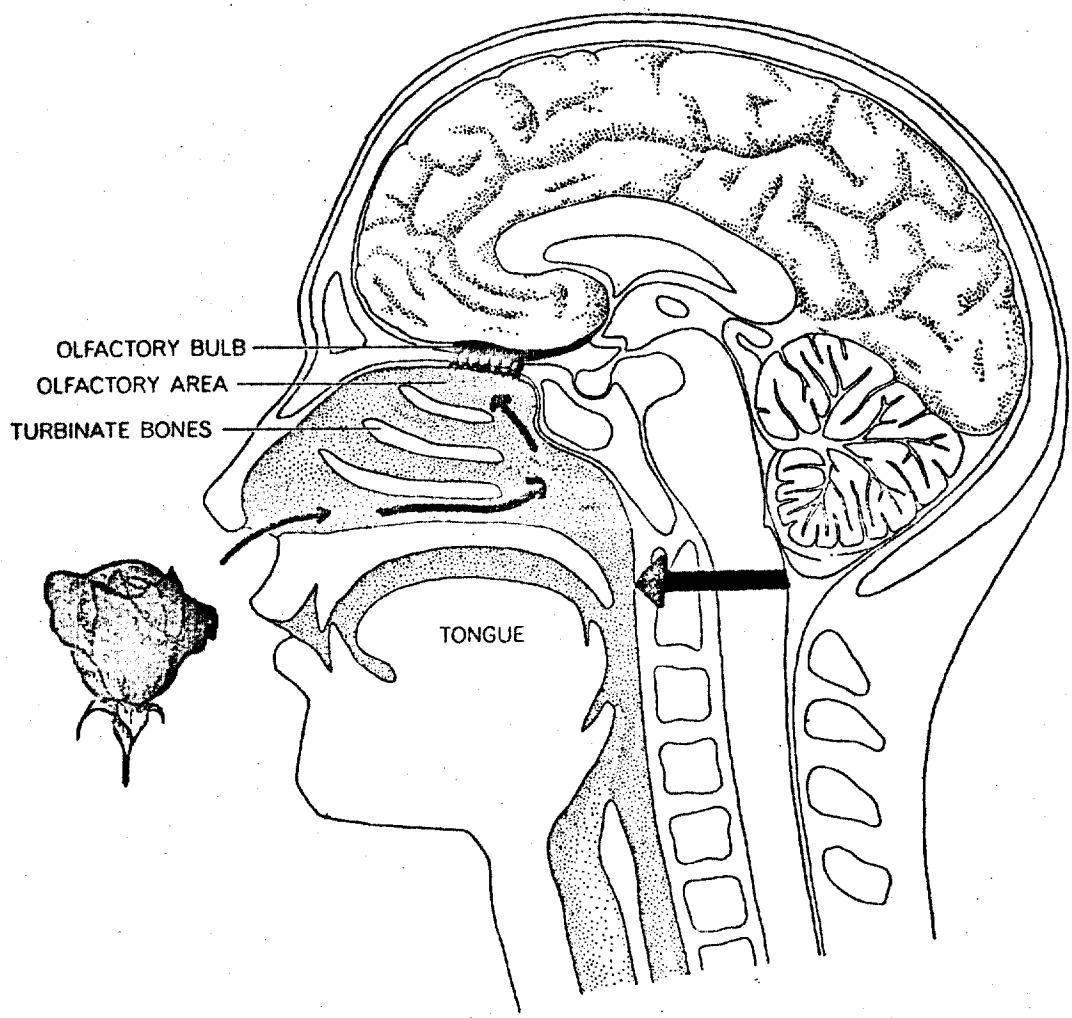
2. Olfactory Epithelium

A small area of the mucous membrane at the apex of the nose contains odor-sensitive cells (Figures 1 and 2). This epithelium contains pigments (Takagi, 1969, Moulton, 1971). In animals, some of these pigments are carotenoids. Carotenoids are precursors of vitamin A and losses of smell occur in vitamin A deficiency. But since vitamin A deficiency affects virtually all membranes, and carotenoids are absent from the pigments in human olfactory epithelium, Moulton and Beidler (1967) have concluded that these pigments probably have little to do with olfaction in man.

3. Olfactory Receptor Cells

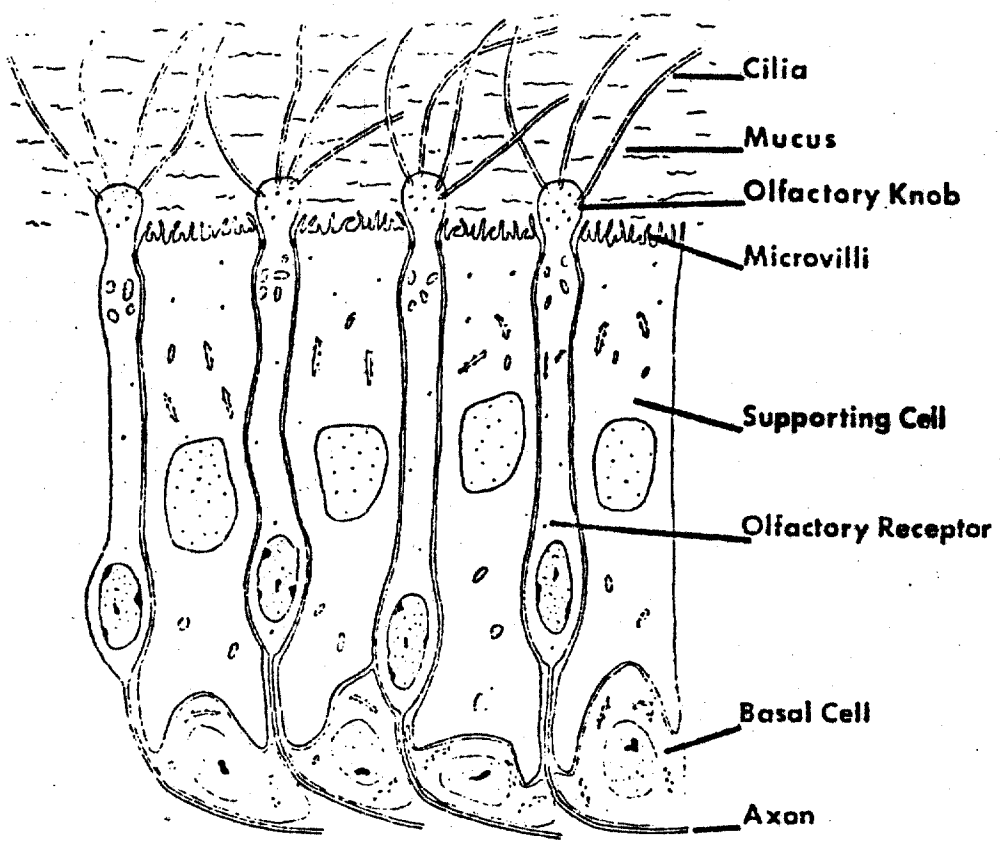
Estimates of cell numbers vary. Allison and Warwick (1949) estimated that the olfactory nerves of the rabbit contain millions of primary fibers, each presumably connected to a cell body. From electrophysiological responses, Beets (1971) has estimated 10 million cells, each able to conduct 10 impulses/sec, giving a system capacity of 100 million inputs/sec, comparable to the input capacity from the retina of the eye.

FIGURE 1



Human anatomy of olfaction, showing the right nasal air passage with olfactory area (epithelium) and bulb. Similar structures on the left are not shown. Left and right air passages enter the single air duct at the throat (large arrow). Reproduced from Amoore, Johnston, and Rubin (1964) with permission of the copyright holder, Scientific American, Inc.

FIGURE 2



Human anatomy of olfaction, showing the structure of the olfactory epithelium. The olfactory receptor cells are nerve cells distributed among the supporting, mucous-producing cells. Not shown are trigeminal nerve endings which occur between the supporting cells and, like other tactile nerve endings, do not reach the surface of the epithelium. Reproduced from Moulton and Beidler (1967) with permission of the American Physiological Society and the authors. For further anatomical detail see Schneider, 1967.

The cilia of the olfactory nerve cells protrude above the epithelial surface (Figure 2). Receptor molecules are believed to be proteins in the outer membranes of the olfactory cells including the cilia. The number of cilia per cell is known for several species (Moulton and Beidler, 1967), but the number of receptor molecules per cell is unknown. Most authorities agree that the receptor cells including their axons are continually replaced; thus, each cell is functional for only part of its life. This turnover might explain the diversity of the estimates of input capacity from the olfactory epithelium.

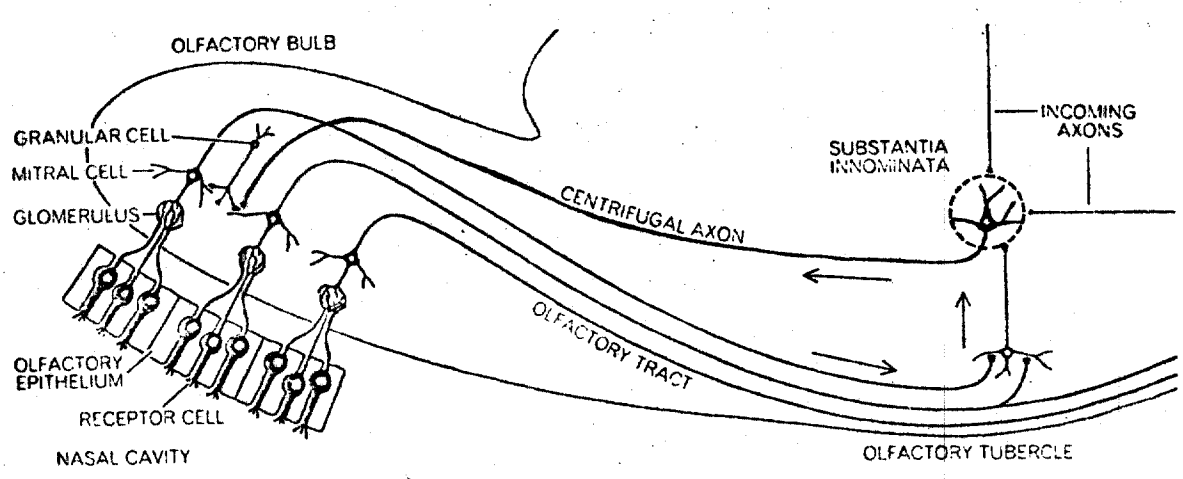
4. Olfactory Nerve Connections

Fibers from the receptor cells track back to left and right olfactory bulbs (Figures 2 and 3). "Accessory" olfactory bulbs and vomeronasal organs are found in lower animals but are absent or vestigial in man. The role of the bulb may be analogous to that of the horizontal cell layer of the retina. Each bulb contains a large number of glomeruli. Allison and Warwick (1949) have estimated that each glomerulus collects about 26,000 primary nerve fibers, and provides one nerve fiber to the higher brain centers; this is a large input reduction. From each bulb about 2,000 channels go back to the higher centers, some to the same side, others to the opposite, as with the optic nerves (Stone, 1969). Responses evoked by odorants have been detected electrophysiologically in the 1st, 5th, 7th, 9th and 10th cranial nerves.

Trigeminal nerve endings, usually associated with pain or irritation, also are stimulated by impulses arising in the olfactory epithelium; some of these fibers enter the glomeruli, where they interconnect with the olfactory nerves (Amoore *et al.*, 1964).

Channels from the olfactory bulbs are distributed in the general area of the prepyriform cortex (Figure 3). The interconnecting pathways in the brain have not been fully delineated (Stone, 1969), but from psychophysical data there is little doubt that they extend to the reticular cortex, thalamus, hypothalamus (MacLeod, 1971), and other areas in the brainstem and midbrain. These observations suggest that there are interconnections between brainstem pathways and the endocrine system.

FIGURE 3



Nerve pathways of the rat olfactory system. Primary nerves from the epithelium enter glomeruli in the olfactory bulb, where they synapse with fewer nerves that go deeper into the brain (right side of diagram). The brain sends other nerves ("centrifugal axon") back to the olfactory bulb, and it is thought that by this pathway the brain can modify sensory input from the olfactory bulb. Reproduced from Heimer (1971) with permission of the copyright holder, Scientific American, Inc.

B. TASTE

1. Anatomy of Taste Receptors

The primary organs of taste are the taste buds. The salivary glands contribute to taste by secreting fluid in which solid tastants are dissolved; undissolved molecules cannot be tasted. Locations that exhibit differential sensitivity to specific tastants (e.g., Figure 4) have been demonstrated by experiments in which sensations in parts of the mouth were blocked by local anesthetics (Henkin, 1971, Murray and Murray, 1970). Taste buds contain receptor cells associated with nerve endings (Figure 5); unlike olfactory receptors, these are not nerve cells and do not have fully developed cilia. Taste buds are renewed every 10-10.5 days if innervated; if the nerve supply is severed, the taste bud dies and does not regenerate until the nerve grows back (Beidler, 1970). The number of taste buds varies widely between animal species (Table 1), and cannot be correlated with taste acuity in animals or man (Kare, 1971).

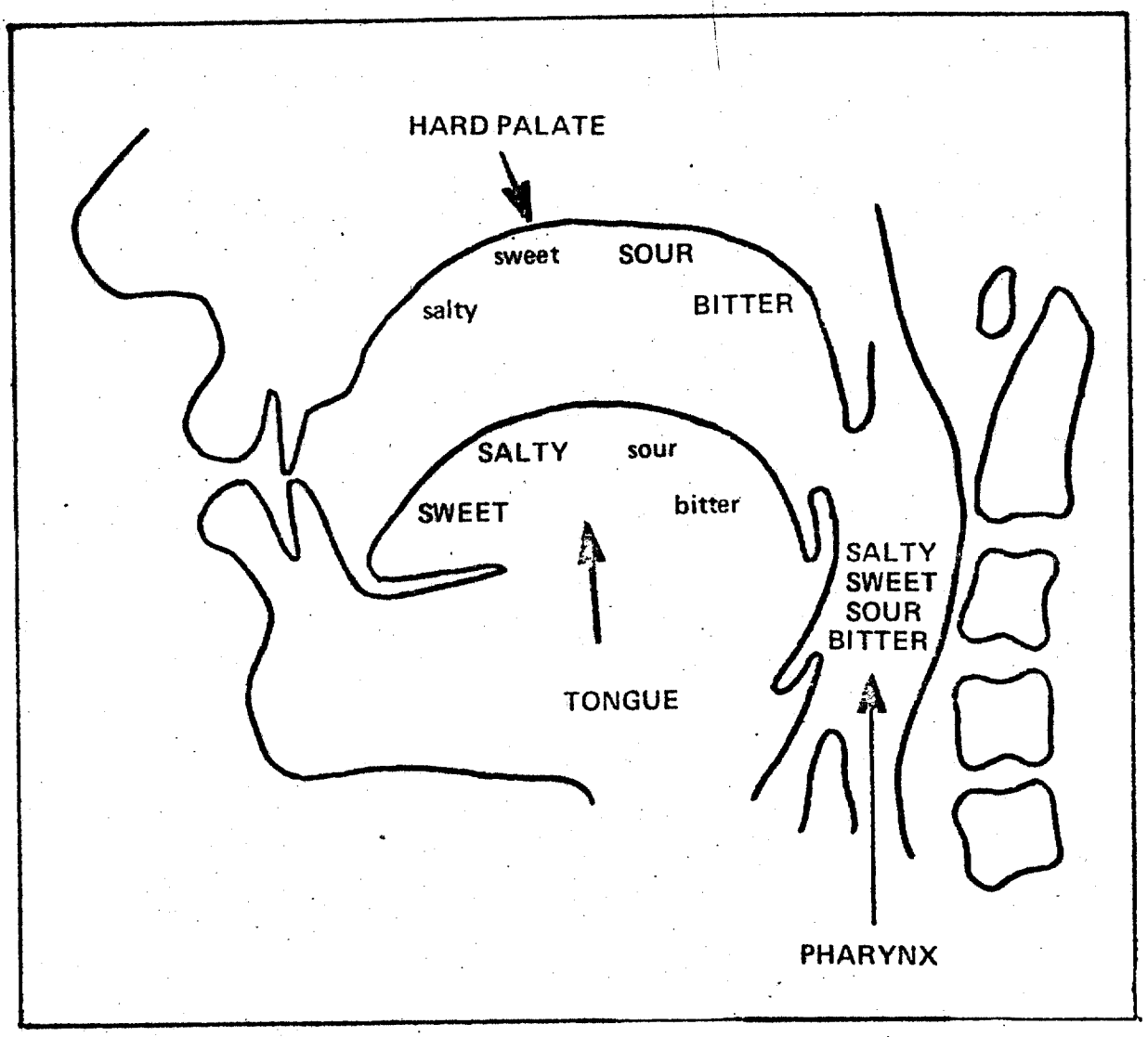
Receptor molecules are thought to be proteins of the receptor cell membranes, because all taste sensations diminish after proteolytic enzymes are held in the mouth (Giroux and Henkin, 1971). It has been suggested that they might be lipoproteins with weak binding capacities. Others believe that the shape of the binding site is more important than the identity of the protein (Amoore *et al.*, 1964, Mazur *et al.*, 1969). However, proteins that bind dissolved tastant molecules and initiate taste sensations have not been identified. Dastoli and Price (1966) have isolated a protein from the sweet-sensitive areas of the tongue. Price and Hogan (1969) reported this protein had dehydrogenase activity, but its structure is not known.

2. Taste Nerve Connections

The taste bud of a rat may contain about 200 nerve fibers, which make contact with most of its cells, but only about 50 fibers enter the base of the bud (Beidler, 1969, 1970). The reduction factor is 4:1; in man the cell numbers may be smaller, but the reduction factor is thought to be of the same order (Pfaffmann, 1959).

The afferent fibers from the taste buds in the anterior two-thirds of the tongue join the lingual nerve, and then are included in the chorda tympani nerve, which leaves the lingual nerve, passes through

FIGURE 4



Taste sensitivity of the normal human tongue, palate and pharynx in terms of the "primary" tastes. Each area perceives all four tastes, and differences are relative; capital letters indicate high sensitivity, lower-case letters lower sensitivity. (Redrawn from Henkin, 1970a).

FIGURE 5

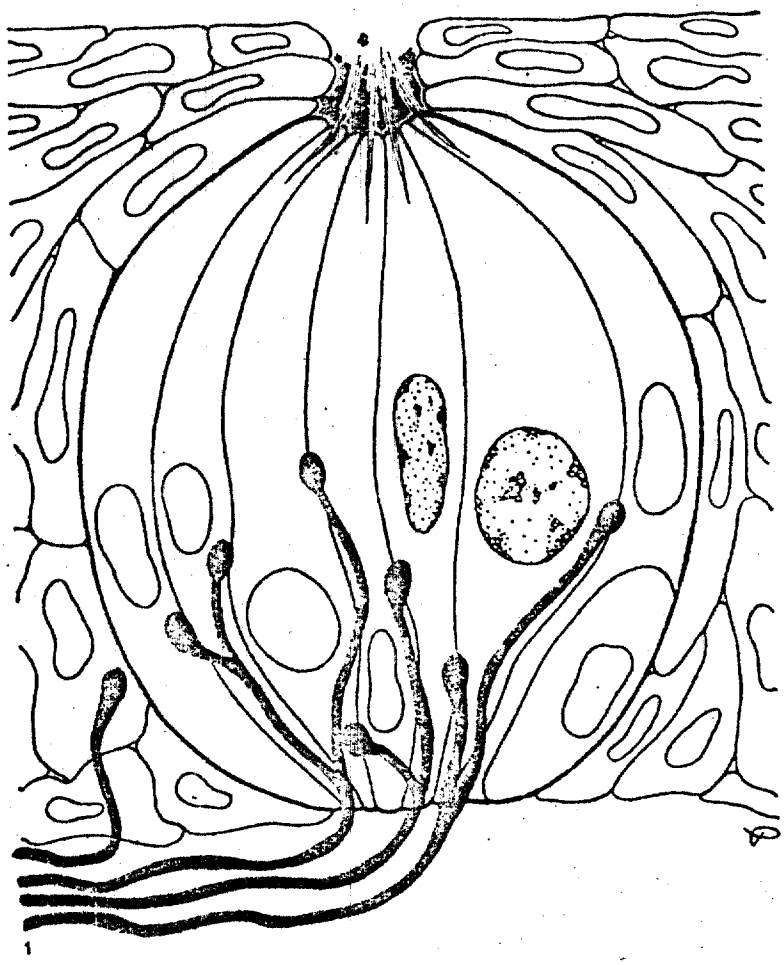


Diagram of a taste bud. The nerves are outside of the cells. Some cells are slender, and others broad, as illustrated in the two cells with detailed nuclei; this difference may or may not be meaningful. Reproduced from Murray and Murray (1970) with permission from the copyright holder, Longman Group, Ltd.

TABLE 1

NUMBERS OF TASTE BUDS IN VARIOUS ANIMALS AND MAN

Snake	0	Kitten	473
Chicken	24	Bat	800
Pigeon	37	Human	9,000
Bullfinch	46	Pig and Goat	15,000
Starling	200	Rabbit	17,000
Duck	200	Calf	25,000
Parrot	350	Catfish	100,000

Estimates by many investigators published between 1906 and 1961. (Adapted from Kare, 1971).

the middle ear, and enters the brain as part of the 7th cranial nerve (Pfaffmann, 1959, Zotterman, 1971, Sato, 1971). Taste nerves from other parts of the mouth and palate join the 7th, 9th and 10th cranial nerves, which enter the medulla (Pfaffmann, 1959).

Taste areas in the brain are believed to be diffuse, and closely correlated with the somatosensory system (Pfaffmann, 1959). The hindbrain, thalamus and hypothalamus are probably involved (Halpern and Nelson, 1965, Halpern, 1967), but the pathways remain obscure (Burton and Benjamin, 1971).

C. OTHER RELATED SENSORY INPUT

The neuroanatomy of olfaction may be analogous to that of vision; however, the neuroanatomy of taste appears more analogous to that of touch. Both the olfactory and taste-sensitive areas are innervated for transmission of pain sensations, but the innervations differ. Irritation (leading to sneezing) is integrated into the trigeminal system. Touch, leading to texture recognition, is separate from taste but is important in the identification of foods. Although taste fibers pass through the middle ear, there is no anatomical evidence that auditory stimuli interact with taste sensations within the chorda tympani nerve. However, middle ear damage or other closed head injuries often lead to chorda tympani destruction and subsequent abnormal taste responses. Also middle ear surgery often affects taste responses (Benjamin *et al.*, 1965).

It is evident that all senses interact, but the pathways are largely unknown. Brush and Halpern (1970) have noted that efferent nerve impulses are involved. In addition, the associating functions of memory and vivid recall are factors in these interactions.

IV. BASIC MECHANISMS OF OLFACTION AND TASTE

A. OLFACTION

1. Odorants - Substances Perceived by Olfaction

All odorants are volatile, are thought to have molecular weights of 300 or less, and with a few exceptions, contain 1 to 2 functional atomic groups. Aggregates of small molecules or macromolecules may be odorless. Beets (1971) considered that much published information on compounds under 140 molecular weight was unreliable. It is likely that these issues are related to the purity of the compounds tested.

Nonmolecular stimuli such as X-rays and ionizing radiation can be "smelled." The nature of this effect is unknown. Passage of X-rays through air is known to generate ozone and peroxides; several investigators (Smith and Tucker, 1969, Garcia and Koelling, 1971) have suggested that these substances are generated by interactions at the surface of the olfactory epithelium. Cooper (1968) has shown that the surgically exposed nasal epithelium of anesthetized rabbits can be stimulated by soft β-rays. Olfactory bulb neurons responded only when the rays were directed at the olfactory mucosa.

2. Odor Receptor Function

Current theories about how substances interact with chemoreceptors have been reviewed by Moulton and Beidler (1967), and Dravnieks (1967). These theories are based on the principle of a three dimensional "fit" between molecules (Laffort, 1969). A number of pure substances has been found to be consistent with this concept (Table 2). Amoore (1971) has proposed that an odorant initiates impulses when it alights on a specific protein of the receptor cell membrane.

While there is considerable evidence that a single molecule of some odorants can excite one receptor, the probability of a single inhaled molecule reaching a receptor site is quite low. There are other theories of impulse initiation.

TABLE 2

FIFTY PURE SUBSTANCES WITH MOLECULAR CHARACTERISTICS THAT HAVE BEEN CORRELATED WITH RESPONSES OF THE OLFACTORY SYSTEMS

Ethane	Formic acid
Butane	Acetic acid
Pentane	Propionic acid
Heptane	Butyric acid
Octane	Pentanoic acid
Nonane	Hexanoic acid
Hendecane	Heptanoic acid
	Octanoic acid
Methanol	Nonanoic acid
Ethanol	Decanoic acid
Propanol	Dodecanoic acid
Butanol	
Pentanol	Acetaldehyde
Hexanol	Acetone
Heptanol	Allylic alcohol
Octanol	Ammonia
Decanol	Bromine
Dodecanol	Carbon disulfide
	Chloroform
Ethyl acetate	Dichloro-1-2-ethane
Ethyl butyrate	Isopropyl alcohol
Ethyl hexanoate	Propenal
Ethyl octanoate	Pyradine
Ethyl nonanoate	Hydrogen sulfide
Ethyl decanoate	Ethane thiol
Ethyl hendecylate	Ethyl sulfide
	Benzene

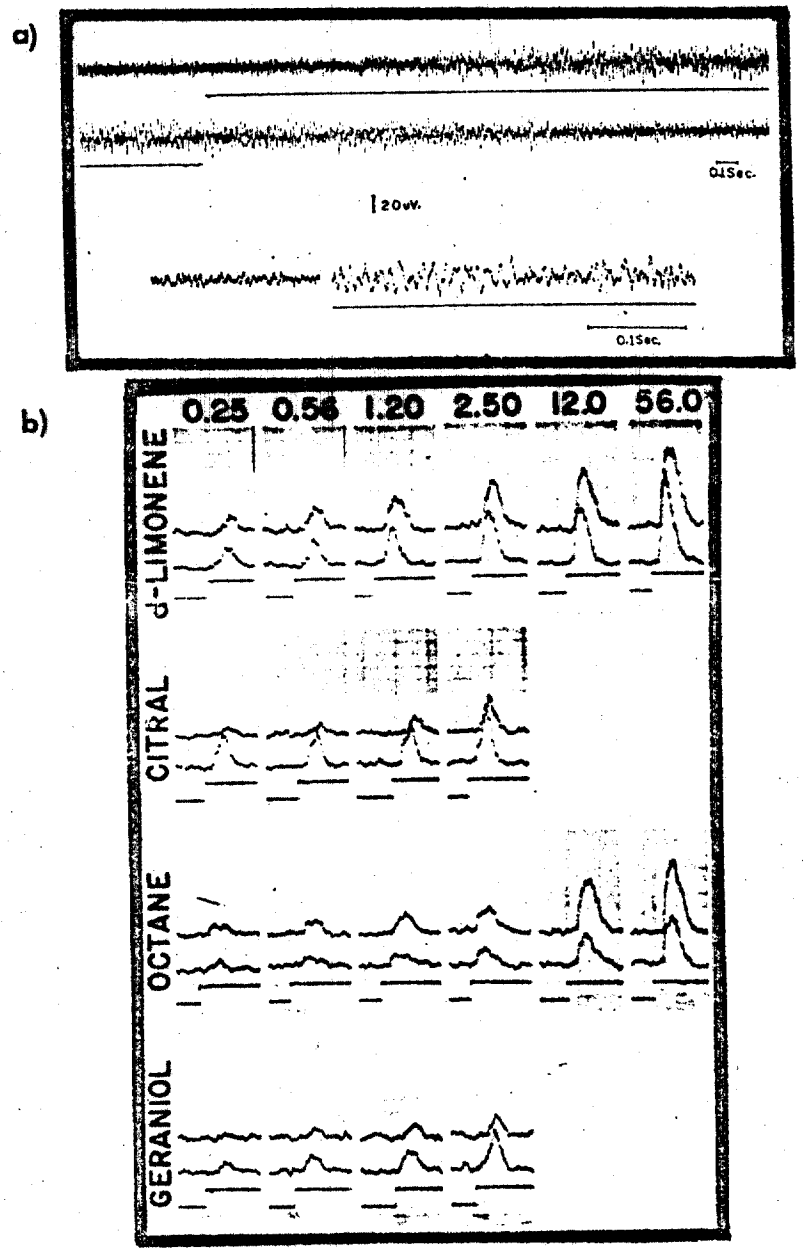
(Modified from Laffort, 1969).

The "chromatographic" theory was proposed first by Adrian (Moulton and Tucker, 1964) and is being studied by Mozell (1971). Mozell has shown that odorant molecules are repeatedly adsorbed onto the olfactory mucosa and revolatilized, a process that is mimicked by gas-chromatography. The tenacity of the mucosa for an odorant is a major, but not the only determinant of migration rate across the entire mucosa, which can vary from less than 2 to more than 270 seconds. Because most odorants are mixtures, each shows typical "spatial and temporal" migration spectra which, from electrophysiological measurements, are reversed when the air flows in the opposite direction (Figure 6). The absence of quality differences in records of nerve impulses taken from single receptors was a major reason for originally hypothesizing that odorants were somehow separated spatially and temporally.

The arrest mechanism for odorant molecules by receptors is unknown. Beets (1971) suggested that an odorant molecule is held by the receptor molecule until the former is degraded; on the other hand, Davies (1969, 1970) believed that the odorant "penetrates or punctures" the receptor cell, allowing sodium to enter and potassium to escape. The biochemical mechanism by which the nerve impulses are initiated is also unknown, but a recent finding that cyclic AMP (adenosine 3', 5'-monophosphate) is involved (Kurihara and Kayama, 1972) suggests that the mechanism is the same as that by which hormones and other circulatory solutes trigger nerve impulses. Receptor responses have been observed following intravenous injection of some odorants, suggesting also that receptor sites may not be limited to the exposed, ciliated parts of the receptor cells. However, there is evidence that these effects may be due to volatilization of the substances into the alveoli of the lungs and then exhalation through the nose.

To account for the logical inference that specificity resides in the receptor cell membrane, and also for the degrees of specificity observed subjectively, limitations on specificity have been proposed (Cain, 1969, Dravnieks and Laffort, 1972). Thus, theoretically a binding site may attract a range of different odorant molecules, an odorant molecule may bind at a range of different sites, and a receptor cell may contain many different sites and may bind more than one molecule at a time.

FIGURE 6



Electrophysiological tracings from a frog olfactory nerve: a) Examples of raw data; b) Electronically summated raw data of neural discharges recorded from the lateral (upper tracing of each pair) and medial (lower tracing) branches of the olfactory nerve of the frog. The time differences and relative sizes of the peaks are part of the evidence for the "chromatographic" theory of odor reception. (Supplied by Mozell).

3. Olfactory Nerve Responses

The transmission of olfactory nerve impulses is better understood from both the anatomy and electrophysiological recordings when they are interpreted by electronic summations (Figure 6). Such recordings may be made in animals from single neurons, nerve branches, or the olfactory bulb. Thus it is known that the bulb either summates or to some extent analyzes receptor impulses before transmitting them centrally. The chromatographic theory was inferred from electronic summations of responses recorded simultaneously in the lateral and medial branches of the primary olfactory nerve. Trigeminal nerve responses to odorants differ from olfactory responses and in some cases are more sensitive (e.g., to phenyl ethyl alcohol), but the sensations are difficult to separate subjectively (Moulton and Beidler, 1967).

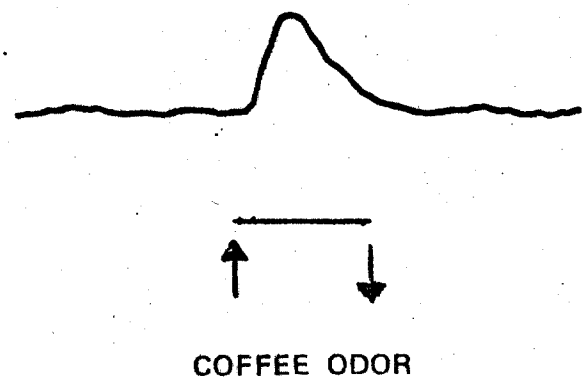
Although the details of the trigeminal inputs into the olfactory bulb (Smith and Tucker, 1969) and the efferent inputs from the cortex that evidently influence transmissions by the bulb (Moulton and Beidler, 1967) have not been fully elucidated, it is clear that the bulb has selective functions and that bulbar lesions may diminish, distort or prevent the perception of odors.

4. Detection Thresholds

The presence of most odorants is detected at concentrations lower than are needed for their identification. The latter is called the *recognition threshold* and the former is the *detection threshold.*

Detection thresholds can be established in animals and man by the electro-olfactogram (EOG) (Figure 7), as revealed by Ottoson in 1956. He defined the EOG as "*the monophasic negative potential evoked by odors in the sensory region of the nasal mucosa* " as measured by simultaneous recordings from the mucosa under well-controlled conditions of stimulus (Ottoson, 1971). EOG responses vary in amplitude, while "unit" responses (all-or-nothing) are found in the nerves that leave the bulb (Takagi, 1969). Increases of odorant concentrations above certain levels are not detected olfactorily, although some increases may be signaled by the trigeminal nerves; these are perceived as changes in odor quality with increasing concentrations. However, the significance of EOG data in relation to the existence of true thresholds in man is controversial.

FIGURE 7



Typical human EOG (electro-olfactogram), from the olfactory epithelium. The peak response is very brief. Coffee contains at least 500 different volatile substances, all of which may contribute to the odor (Wick, 1965). Redrawn from Osterhammel *et al.*, 1969 .

In man, detection thresholds, estimated by sniffing tests, differ among people and fluctuate over time, so much so that the range of normality is usually expressed in log units of odorant concentration. (Amoore, 1968). In a population, the highest normal threshold may be 10^3 of the lowest, and the range in 95 percent can be 10-fold above and below the mean (Amoore, 1968, Berglund *et al.*, 1971). The direction of an odor source can sometimes be detected within 7-10 degrees (Moulton and Beidler, 1967), especially if the subject is free to move his head or if the odorant stimulates the trigeminal nerves (Schneider, 1967).

5. Odor Recognition Thresholds

The inferred location of recognition mechanisms is the prepyriform cortex (Adey, 1970). Recognition is studied indirectly, by its behavioral effects, which currently are better understood than biochemical or electroencephalographic data. The involvement of memory is implicit (Engen, 1972); however, other sensory input and psychomotor responses are "unwanted variables" that cannot be excluded. Engen (1970) has proposed three forms of mental judgement: discrimination, scaling, and recognition itself. Discrimination, or perception that odors differ, appears to be described by the psychophysical power function (quantum steps) proposed by Stevens (1972). Scaling, or categorization, includes a quantitative element. To the subject, recognition is identification beyond doubt.

Tests comparing odorants are usually more reliable than tests of single odorants alone. Observations suggest that memory generally becomes confused after identification of about 16 single odorants in sequence. Furthermore, behaviorally obtained thresholds were elevated by the addition of thresholds for psychomotor responses, and complicated by "training" effects of repeated test performance. Thus observed recognition thresholds tend to vary more than do detection thresholds. Beets (1971) suggested that, all factors considered, human acuity for odor recognition may be, at its best, no less than canine acuity.

As odorant concentrations increase, there comes a point above which either increases will not be recognized or the odor quality will appear to change. These upper thresholds have been relatively little studied but could be relevant to recognition of dangers, especially when dangerous concentrations are above the upper recognition threshold.

Most odors are generated by mixtures of odorants, and most odorants are hard to obtain in chemically pure form. When this is done, the recognized odor can sometimes be attributed to some impurity or contaminant. Thus, the question of whether all pure odorants are "primary" remains unanswered, and for all of the above reasons it is difficult to utilize any current listing of "primary" odors (Table 3) (Schneider, 1967). The concept of primary odors is derived by analogy from primary tastes and colors. The analogy is in doubt owing to the differences in anatomy and physiology among these three sensory systems.

B. TASTE

1. Taste Receptor Function

All tastants are soluble (Beidler, 1954); even quinine ethylcarbonate has been demonstrated experimentally to be tastable (Faull *et al.*, 1971). This material, originally believed tasteless and insoluble has been shown to be very slightly soluble in saliva. Vastly more fluid bathes the taste areas than the olfactory epithelium, and there is no evidence of "chromatographic" migration of tastants (Davies 1971). Larger molecules can be tasted than can be smelled and there seems to be no grounds for hypothesizing penetration of taste receptor cells. It is emphasized that, unlike olfactory receptors, taste receptor cells are not nerve cells. Support is growing for the proposal that taste receptor molecules are proteins with one or more sites that bind and react with tastant molecules so as to generate nerve impulses (Beidler, 1954, 1971).

Stereochemistry is a guide to taste with some but not all tastants. Sugars and some amino acids can be ranked for sweetness (Tables 4 and 5), and the "glucophore" appears to be some functional group of atoms within the molecule (Kier, 1972).

2. The Primary Tastes

Most workers provisionally accept the theory, formulated by Fick in 1864 (Andersen, 1970), of 4 species of receptors corresponding to 4 "primary" sensations: sweet, bitter, salty, and sour (Figure 8). In 1817, Park had proposed "warm" and "cool" as primary taste sensations; however, these additions are not universally accepted although these 2 sensations are compatible with the 4 primary sensations (Dzendolet, 1969, Sato *et al.*, 1969). The 4 primary sensations are interrelated

TABLE 4

RELATIVE SWEETNESS OF VARIOUS SUGARS IN SOLUTION*

β-D-Fructose	100-175
Sucrose	100
α-D-Glucose	40-79
β-D-Glucose	<α-D-Glucose
α-D-Galactose	27-67
α-D-Mannose	59
β-D-Lactose	48
β-D-Maltose	32-46
β-D-Galactose	**
α-D-Lactose	16-38
Raffinose	23
Stachyose	**
β-D-Mannose	Bitter

*Compared with arbitrary value of 100 for sucrose.
**Value undetermined.

Table adapted from Shallenberger and Acree (1971).

TA. E 3

SCHEMA PROPOSED FOR CLASSIFICATION OF PRIMARY ODORS

Linnaeus 1756 (7)	Zwaardmaker 1895 (30)	Henning 1915 (6)	Crocker & Henderson 1927 (4)	Amoore 1952 (7)	Schultz 1964 (9)	Wright & Michels 1964 (8)	Harper et al. 1968 (44)	Amoore 1969 (added)
	Almond				Spicy		Almond	
	Amber						Animal	
	Aniseed					Benzo-thiazole		
	Bed-Bug							
	Bromine							
	Burnt	Burnt	Burnt		Burnt	Affective	Burnt	
	Camphor			Camphor			Camphor-Mothballs	
Hircine	Caproic		Caprylic				Sweaty	
Nauseous	Carrion						Sickly	
	Cat-Urine							
	Cinnamon	Spicy				Spice	Spicy	
	Citrous	Fruity				Citral	Citrous	
Aromatic	Clove						Aromatic	
	Ethereal			Ethereal	Etherish		Etherish-Solvent	
	Fecal						Fecal	
	Fishy						Ammonia-Fishy	
	Fruity					Hexyl-acetate	Fruity	
	Jasmine	Flowery		Floral			Floral	
Alliaceous	Leek						Garlic	
	Lily							

	Minty			Minty			Minty	
Ambrosial	Musky			Musky			Musk	
Repulsive	Narcotic							
Fragrant	Orange-Blossom		Fragrant		Fragrant		Fragrant	
	Phenolic						Carbolic	
	Rosy							
	Thyme				Sweet		Vanilla-Sweet	
	Vanilla						Soapy	
	Violet						Soapy	
	Waxy					Resinous	Resinous-Paint	
		Resinous						
		Foul		Putrid	Sulfurous	Unpleasant	Putrid-Sulfurous	
			Acid				Acid	
					Oily		Oily	
					Rancid		Rancid	
					Metallic		Metallic	
							Meaty	
							Moldy	
							Grassy	
							Bloody	
							Cooked-Vegetable	
								Sandal
								Watery
								Urinous

Modified from Amoore (1969), who has reduced the 118 proposed odor classifications to 44 "primary" odor descriptions. See Amoore (1969) for a more complete discussion and the original references.

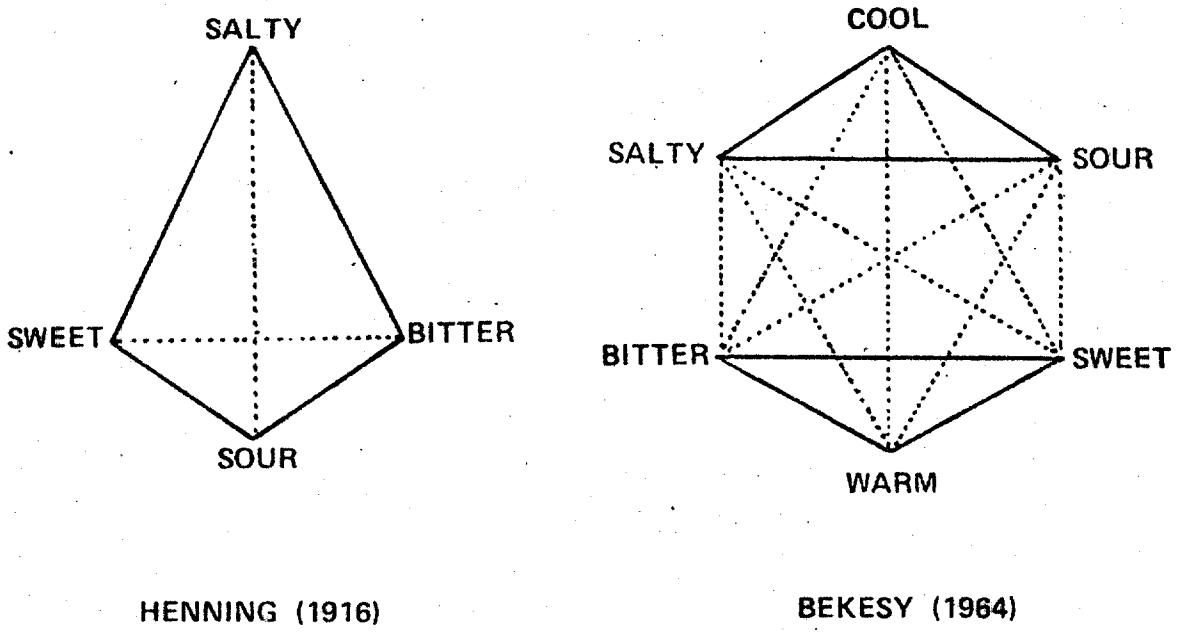
TABLE 5

TASTES OF SOME AMINO ACIDS*

Amino Acid	Form of Structure D-	L-
Alanine	sweet	sweet
Asparagine	sweet	tasteless
Glutamic acid	mixed	tasteless
Glycine	sweet	sweet
Histidine	sweet	tasteless
Hydroxyproline	-	sweet
Isoleucine	sweet	bitter
Leucine	sweet	bitter
Methionine	sweet	tasteless
Phenylalanine	sweet	bitter
Proline	-	sweet
Serine	sweet	sweet
Threonine	sweet	sweet, bitter
Tryptophane	sweet	tasteless
Tyrosine	sweet	bitter
Valine	sweet	sweet, bitter

*Data from Shallenberger and Acree (1971), who note that according to Mazur *et al.* (1969) the dipeptide, or joined pair of amino acids, L-aspartyl-L-phenylalanine methylester is 100-200 times sweeter than sucrose although singly, both amino acids taste bitter.

FIGURE 8



Two proposed relationships between the primary tastes in man. Left: the 4-taste system proposed by Henning in 1916, cited by Pfaffmann *et al.* (1971). Right: the 6-taste system proposed by von Békésy in 1964, cited by Sato (1971), who found nerve responses to all 6 "tastes" in hamsters but somewhat different relationships between them, interpreted as interspecies differences.

(Figure 8), either at the receptor level or, more likely, at a cognitive level in the brain (Frank and Pfaffmann, 1969, Pfaffmann *et al.*, 1971). The opposed pairs, sweet-bitter, salty-sour, warm-cool, were inferred from after-tastes that could not be attributed to breakdown products in the mouth (Pfaffmann *et al.*, 1971). Most workers accept the 4-taste classification as convenient and recognize that the underlying biological systems are more complex.

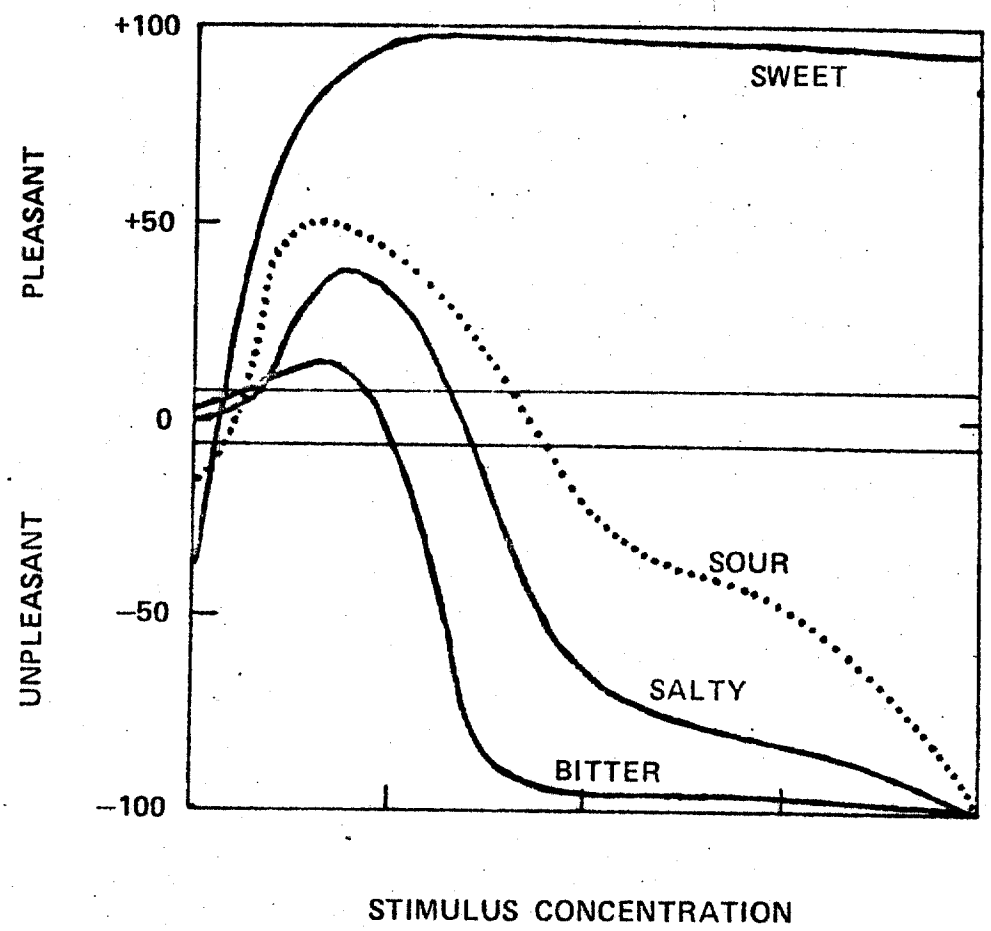
This theory is supported by some electrophysiological data, but supporting stereochemical data are at best equivocal. Some amino acids taste sweet, but others do not; and not all acids taste sour. Very weak sodium chloride solutions taste sweet. There are electrophysiological indications that some taste buds contain more than one species of receptor, while others do not. Perhaps such tastants are bound at more than one type of site. Some shifts of taste quality indicate merely that a tastant is a mixture. The "taste" of electrical current has been debated since 1754; whether it reflects nerve stimulation, electrolysis of saliva components, or both, is still undecided (Bujas, 1971).

3. Taste Nerve Responses

Chemical mediators are probably involved in the generation of responses, as in other sensory modalities (Kurihara and Kayama, 1972). Responses are monitored electrophysiologically in animals, from single fibers or nerve trunks, and thus detection thresholds are estimated (Doetsch *et al.*, 1969).

Unlike the olfactory system and its paired organ, the olfactory bulb, the innervation of taste appears to have few peripheral neural ganglia for sensory analysis. The taste bud does not seem comparable with the olfactory glomerulus; this suggests two problems for future study. The first is whether the primary taste sensations result from local dominance patterns in the transmission of taste information by the many nerve fibers, rather than from receptor cell differences. The second concerns the possible evolution of taste sense by a localized development of chemoreceptivity in epithelial cells associated with tactile nerve endings. Compared with olfaction, the perception of taste obviously is more restricted by the receptors' microenvironment, cell type, cell turnover, and the need to transfer responses to an associated nerve instead of transmitting them directly.

FIGURE 9



Human subjective judgments of "pleasant" or "unpleasant" sensations in response to increasing concentrations of cane sugar ("sweet"), tartaric acid ("sour"), sodium chloride ("salty"), and quinine sulfate ("bitter"). The maximum concentrations were: sugar 40%, acid 1.12%, NaCl 10%, quinine 0.004% (weight/volume). The minima were all zero. "Pleasant" and "unpleasant" are separated by a neutral zone. Redrawn from Pfaffmann *et al.* (1971), who reproduced the figure from Engel.

4. Taste Detection and Recognition Thresholds

Detection thresholds are measured electrophysiologically in animals and there is limited work on their subjective assessment in man. Thresholds in man involve some degree of identification, first in terms of the primary taste, and with increasing intensity, in terms of recognition of the tastant, involving memory and usually other factors as well. It seems likely that chemically pure tastants that stimulate only taste receptors can evoke only recognition of primary tastes (Figure 8). Tactile input, indicating texture, accompanies taste input, but is not integrated peripherally as is trigeminal input.

Recognition is enhanced by practice, and the amount of practice is largely determined by the hedonic effects of tastants (Figure 9). For example, people tend to repeat "pleasant experiences." The mechanism of hedonic effects is important but obscure (Pfaffmann *et al.*, 1971). Enhanced sucrose preference is inducible, and is evident in a vast majority of the U.S. population. The role of taste mechanisms in sugar-seeking appears limited to detection and recognition; thus, sugar preference may have its basis in physiological mechanisms other than taste *per se* (Gentile, 1969, Nachman and Cole, 1971). Contrary opinions have been expressed (Bartoshuk *et al.*, 1971). This is an area in which basic research is urgently needed.

V. FACTORS INFLUENCING THE CHEMICAL SENSES

A. OLFACTION

The variability of sensory responses to olfactory stimuli reflects a large number of independently varying factors, such as stimulus, receptor, and subjective factors. On the other hand, this variability may have been exaggerated by tests designed for basic research rather than for screening, because observation of the different stages of a response requires the elimination of stabilizing factors normally present, e.g., efferent impulses from the brain to the olfactory bulb.

Olfaction can be altered in various ways, including anosmia (absence), hyposmia (decrease), hyperosmia (enhancement), parosmia (distortion), dysosmia (unpleasant distortion), and phantosmia (hallucination.) Such states may be transient or permanent, and may arise within the subject or be related to his environment.

1. Stimulus Factors

Seldom if ever is the air so pure that only one odorant is present. Odorant concentrations vary, and different odorants have different potencies. An odor may be masked by others present either at the same time or shortly beforehand. If a stimulus is too brief, the odorant is not recognized; if too prolonged, adaptation occurs. The time and intensity factors in recognition of repeated or inconstant stimuli are complex and poorly understood. Odorants, or concentrations of an odorant, that stimulate the trigeminal nerves, add further complications. Similarity between odors may hinder identification, especially when odorants are presented singly or under conditions precluding comparison.

Some odorants and odorless gases may actively distort the perception of others, an effect that is better known in taste and requires study in olfaction. A face mask may affect inhaled air by adding or removing odorants. Intense input from other senses can mask olfactory input (Stone and Pangborn, 1968). Sensory responses are affected by the temperature, pressure, and humidity of the inhaled air, and by its flow rate and turbulence; these factors are partly under the subject's control.

2. Receptor Factors

Other determinants include the condition of the olfactory epithelium, and the amount and pH of the mucus. Factors such as blood endocrine levels and the degree of vasodilation may affect receptor sensitivity. The functional activity of the nasal mucous membranes has an important influence on acuity. Best acuity is seen when the membrane is moderately active (moderately swollen and red), poor acuity is associated with low membrane activity (shrunken and pale) and acuity is lost when the membrane is in a hyperfunctioning state (marked swelling, redness, and wetness) (Schneider and Wolf, 1960).

3. Subjective Factors

A normal, healthy person may associate an odor with particular visual, auditory or tactile sensations, absence of which will modify his detection or recognition threshold for that odor. The presence of other sensations may induce phantosmia, the hallucination that an odor is also present. Other sensations can also distract a person from recognizing an actual odor (Stone and Pangborn, 1968). On the other hand, odor may confirm or deny suspicions raised by other sensory input, or may raise suspicions for other sensory input to confirm or dismiss. Engen (1972) has emphasized that expectation strongly influences judgments of odors.

Odor discrimination can be taught; thus acuity can be enhanced by practice, and self-training can be directed by appetitive or aversive effects of odorants. However, these effects are not uniformly predictable and their mechanisms are obscure. Children are very tolerant of smells (Engen and Moskowitz, 1972), and thus aversive effects may be unique to adult humans. Examples of such aversive odors are onions, smoke and mercaptan. The food and perfume industries capitalize on supposed hedonic effects of odors. Human pheromones (sex attractants) are thought to exist, probably in the apocrine system, though none have yet been identified (Comfort, 1971).

A number of physiological variables influence olfactory acuity. Olfactory response is thought to vary with age and may vary by sex according to some workers (Moulton and Beidler, 1967, Venstrom and Amoore, 1968). Schneider (1967) has reported that females are generally more sensitive, and are most sensitive when ovulating. This has been attributed to cyclical effects of certain hormones, including

an androgen, 5-α-androst-16-en-3-one (Comfort, 1971) and estrogens (Moulton and Beidler, 1967). Circadian fluctuations of acuity can be ten-fold (Stone and Pryor, 1967). Acuity is influenced by exercise (Stone *et al.*, 1967), but may not be affected by smoking (Venstrom and Amoore, 1968) nor by alcohol consumed in moderation. Woods (1968) holds that smoking, alcohol, and diet markedly affect olfactory acuity.

Variations of acuity in naive subjects during preliminary tests should be expected, but the processes of long-term and short-term adaptation differ, and the variations are large. All persons adapt to the constant presence of an odorant (Moncrieff, 1968). Schneider and Costiloe (1969) have shown that the same subject whose pattern of adaptation is determined in the laboratory at intervals, may vary from rapid adaptation to no adaptation, and most often the latter occurs in a setting of psychosocial stress. Food deprivation may be one such stress, but reports that people are more sensitive when hungry are not unanimous (Moulton and Beidler, 1967). Henkin *et al.* (1972) have reported that olfactory acuity is diminished when tissues are depleted of zinc, copper, or vitamin A. The steroid hormones secreted by the gonads and adrenal cortex are influential. Women who are estrogen deficient have relatively low olfactory acuity which increases when they are given estrogens and decreases when they are given androgens (Schneider *et al.*, 1958). Hyperosmia is associated with Addison's disease (adrenocortical insufficiency) and with cystic fibrosis of the pancreas, and hyposmia with gonadal dysgenesis in females. Many stresses that alter the output of these hormones are believed to affect olfactory acuity directly.

Connective tissue disorders, e.g., Sjogren's syndrome (associated often with rheumatoid arthritis), diminish olfactory acuity (Henkin *et al.*, 1972), as do atrophic rhinitis, and any injuries in the 1st, 5th, 9th, or 10th cranial nerves, such as may result from trauma or surgery to the head (Henkin *et al.*, 1968). Several causes of secondary anosmia or hyposmia have been briefly reviewed by Schneider (1972). Males *et al.*, (1972) have noted evidence for a relationship between olfactory performance and pituitary function. When sensitivity to particular odorants is lost, the symptom may be parosmia (Amoore, 1969, 1971), which is usually noticed when it affects a subject's scale of hedonic values.

General anosmias of various origins are estimated to occur in about 0.2 percent of the U.S. population, and it is believed that they are seldom reported (Amoore, 1968). The view that all albinos are

anosmic has been proved false (Moulton and Beidler, 1967). Anosmias to specific substances (Table 6) are thought to arise by mutations of specific receptor proteins; however, very few such anosmias have been studied in detail. Anosmias or hyposmias for the musky odor typical of urine or sweat are relatively common (Amoore, 1969, Brown *et al.*, 1968).

B. TASTE

1. Stimulus Factors

Temperature influences the potency of tastants, which tends to be greatest at 30-35° C (Pfaffmann *et al.*, 1971). Refrigeration of foods diminishes the potency of most tastants. Dentures that cover the hard palate diminish the acuities for sour and bitter tastes (Henkin and Christiansen, 1967).

A number of substances distort the tastes of other substances, because they do not affect all the receptor types equally. For instance, anesthetics applied to the tongue block taste sensations in the order: bitter>salty>sour>sweet (Kare and Henkin, 1969). Some antibiotics reportedly cause dysgeusia (Henkin, 1970a). Two vegetable extracts have recently attracted attention: "miracle-fruit" and gymnemic acid.

Miracle-fruit *(Synsepalum dulcificum)* contains a basic glycoprotein of about 44,000 *M* with L-arabinose and D-xylose. When applied to the mouth, it may cause acids to taste sweet for over three hours without altering thresholds for sour, salty or bitter tastes. It is hypothesized that the glycoprotein binds to the epithelium near the sweet receptors so that acids act on these as well as on their "own" receptors (Kurihara, 1971).

The leaves of *Gymnema sylvestre* contain gymnemic acids of which one, known as A_1, contains a molecule each of gymnemagenin and glucuronic acid. When gymnemic acid A_1 is applied to the tongue it suppresses sweet taste for up to 30 minutes. Other taste judgments are also modified (Meiselman and Halpern, 1970). The nerve responses are blocked, which could mean merely that the receptors are blocked. However, there are additional biological effects, so that the mechanism of action must be described as unknown at present (Kurihara *et al.*, 1969, Kurihara, 1971).

TABLE 6

REPUTED SPECIFIC ANOSMIAS

Acetic acid	Hibiscolide
Adamantane	Hydrogen cyanide
Allicin	Indole
Allyl isothiocyanate	Idoform
$\Delta^{4,16}$-Androstadien-3-one	Ionone
Δ^{16}-Androsten-3-ol	Isobutyraldehyde
Anisic aldehyde	Isobutyric acid
Anisole	Isovaleric acid
Benzene	Menthol
Benzyl alcohol	d, l-Menthone
Benzyl salicylate	Methional
t-Butyl carbinol	Methyl cyclopropyl ketone
n-Butyl mercaptan	Methyl mercaptan
t-Butyl mercaptan	Musk xylol
Caproic acid	Naphthalene
Cedryl acetate	PEME carbinol
l, 8-Cineole	Pentadecanolactone
Cinnamaldehyde	Phenylacetic acid
Cyclotene	Phenylethyl alcohol
p-Dichlorobenzene	Phenyl isocyanide
Dimethyl disulfide	Phenyl isothiocyanate
Ethylene brassylate	l-Propenylsulfenic acid
Ethylenedichloride	Putrescine
Eugenol	Salicylaldehyde
Farnesol	Skatole
Formic acid	Thiophane
Geranial	Thymol
Geraniol	Trichloroethylene
2-Heptanone	γ-Undecalactone
Δ^{9}-Hexadecenolactone	Vanillin
Hexylamine	Versalide

Substances to which some people are reputedly anosmic as listed by Amoore, 1969.

Other vegetable extracts, e.g., from *Discoreophyllum cumminsii* and *Thaumatococcus danielli*, have been found to contain proteins that taste very sweet but do not actively distort the reception of other tastes (Morris and Cagan, 1972).

General enhancement of taste acuity is claimed for substances such as monosodium glutamate, the 5'-nucleotides, and maltol (Kermode, 1972). Their mechanisms of action are obscure.

2. Subjective Factors

Carbohydrate-active adrenal corticosteroid hormones inhibit taste acuity; when more of them are secreted, acuity diminishes, and *vice versa*. Thus, a progressive taste loss may signify an adrenocortical tumor, or a progressive gain may signify a case of Addison's disease (Henkin, 1970b). By contrast, estrogens promote taste acuity. Stresses that affect secretion of these hormones will influence taste acuity accordingly. It has been noted that acuity increased with rising levels of plasma ascorbic acid, which were influenced by corticosteroid secretion and, in females, by the estrous cycle. Furthermore, the capacity of taste receptors to initiate nerve impulses appears to be governed by blood pressure (Hellekant, 1970).

Dependence of taste acuity on adequate tissue levels of zinc and copper was reported after examination and manipulation of these levels in patients presenting with ageusia (Henkin and Bradley, 1969). However, other workers have found few abnormalities of taste acuity in patients presenting with zinc deficiency. Further studies are needed in which the selection of subjects will be a controlled variable.

Taste thresholds are thought to be influenced by hunger and satiety, but the mechanisms are obscure; nevertheless, taste recognition can directly stimulate processes of digestion (Kare, 1969). Both detection and recognition of tastes are influenced by other sensory input. For instance, the sight and smell of a meal elicits salivation as well as changes in insulin levels not related to modifications in the level of blood glucose (Engen, 1972, Goic *et al.*, 1969).

On the other hand, other sensory input may have masking or distracting effects that diminish taste acuity (Stone and Pangborn, 1968). Many observations are difficult to interpret, because behavioral responses of animals differ between species and tend not to correspond either with electrophysiological responses or with human subjective

judgments of taste. Nevertheless, taste preferences are thought to result from diet selection in man and animals, and to reinforce that selection rather than to determine it in the first place.

A recent preliminary report (Lum *et al.*, 1971) suggests that taste acuities may vary slightly with age and between the sexes, and do vary consistently with the amino acid composition and pH of the saliva. Inherited ageusias have been little studied except for insensitivity to bitter compounds with an N-C=S group, such as phenylthiocarbamide (PTC). The incidence of this is correlated with that of goiter (Sunderland and Cartwright, 1968); nevertheless, a Mendelian genetic pattern is accepted (Fischer and Griffin, 1964, Henkin, 1970b, Kalmus, 1971).

Traumatic head injuries, in which some of the taste nerves lose their function, can result in distortions of taste sensations (parageusia, dysgeusia) (Henkin *et al.*, 1971, 1972).

VI. TECHNIQUES FOR STUDIES IN MAN

Both olfaction and taste are tested by methods developed for basic research into the mechanisms of those senses (Engen, 1971). Electrophysiological observations describe stimuli transmitted by the nerves to which electrodes are applied but do not indicate perception of those stimuli by the conscious brain. Perception is estimated subjectively by volunteers. An indirect measure of perception, behavior, includes responses to the sense tested and to other sensory input, as well as variables and thresholds pertaining to the mechanisms of the behavioral response. A subject responds differently before and after he has learned the study techniques (Cain and Engen, 1969).

However, this LSRO study is concerned only with techniques available for practical evaluation of human olfactory and taste acuities by screening populations who are not volunteers, not with tests that are used only in basic research. Methods that can be used for setting and maintaining standards and for interpretation of results of screening tests are of prime importance.

A. OLFACTION

1. Standards and Criteria

Perhaps the most important difficulty has been simultaneous standardization of both background and test stimuli, because it is the difference between these that must be detected and recognized. Odorant-free air probably cannot be achieved or maintained; however, the composition of air can be monitored by analysis.

Methods available for analyzing air for odorants include gas chromatography (GC), mass spectrometry (MS), and plasma chromatography (PC). Each method gives its own pattern of results, that is, it senses some odorants and some odorless gases, fails to sense others, and senses some more acutely than others. Some workers use combinations of these methods, and the relative merits appear to depend on the situation and purposes of the analyses. Many odors are complex; e.g., Wick (1965) showed that coffee contained over 500 volatiles,

and Young *et al.* (1970) demonstrated the analytical problems by synthesizing a "bourbon" with a gas-liquid chromatogram similar to that of real bourbon but with dissimilar odor and taste.

Analysis of air samples for dilute vapors can sometimes be facilitated by preconcentration methods (Dravnieks *et al.*, 1971). The GC-MS combination is sensitive for detection of traces of contaminants in larger samples of gaseous, liquid, or solid materials. Such techniques would have contributed greatly to a recent study on the social and economic impact of odors in the community (Copley Corp., 1971), where not a single air sample was analyzed. However, the preparation of air samples for analysis by GC or MS may be a problem outside the well-equipped laboratory. Here the PC method has the advantage that air samples do not require preparation.

The PC method is relatively new, and its instrumentation (Franklin GNO Corp., 1972) is still under development. Recent research at the National Bureau of Standards suggests that air from different sources may be simultaneously and continuously monitored by one instrument, and this could be useful for screening situations in the military environment. The degrees of analytical specificity and sensitivity (Cram and Chesler, 1973) seem comparable with those obtainable by GC or MS, and combinations of PC with these methods are currently being explored.

Evaluation of human olfactory acuities by these methods will require the assembly of many standards: identity, quality and intensity of test odors; controlled test conditions; information about subjects; and interpretation of results. Knowledge from basic research will need to be reassessed before application to evaluation of human olfactory acuities (Engen, 1971).

2. Choice of Odors

In selecting odorants for tests, preference should be given to those that are chemically pure and with known olfactory effects (Table 2). When study goals require the use of less well-defined odorants, the limits of the information to be obtained should be recognized. A balance will be struck between the number of odorants and the number of concentrations used. At the present time, approximately 20-30 subjects can be screened per day by double-blind techniques.

3. Threshold Determinations

The utility of threshold studies is highly controversial. One common definition of "threshold" is the lowest concentration detected in 50 percent of responses. There are always many false positive and negative responses; the same applies to recognition thresholds, and to upper thresholds above which concentration increases are not recognized. It may be that studies on lower thresholds are more useful in a training situation than for simple evaluations.

4. Testing of Humans

Techniques rely primarily on subjective responses to odorants delivered to the nasal passages under relatively standard conditions of temperature, pressure, flow rate, and humidity of the air carrying the odorant (Schneider *et al.*, 1963, Amoore, 1968). Though it is difficult to standardize the airflow over the olfactory epithelium, this is desirable for determining differences in olfactory acuity of the two sides of the nose (Schneider and Schmidt, 1966, Schneider, 1972). In practice, subjects tend to determine for themselves the airflow rate that gives them the greatest response. To minimize self-adaptation, some investigators use intervals between successive stimuli of at least 7 seconds, while others find 5 to 10 minutes necessary.

The importance of routinely examining subjects for gross unilateral or bilateral intranasal conditions, such as atrophic rhinitis, or polyps, has been emphasized (Schneider, 1972). Furthermore, it is obvious that valid test results will not be obtained during or immediately after respiratory tract infections, especially influenza.

Behavioral responses and subjective judgments contain extraneous variables that often detract from the validity of olfactory test results (Mozell, 1969). Much of the early work was conducted without emphasis on standardized test conditions. Standardized psychophysical testing techniques are being developed that are said to interfere minimally with the assessment of odor detection and recognition (Cain and Engen, 1969). These include statistical procedures such as factor analysis and "frequency component hypothesis" (Hughes *et al.*, 1969), that may be expected to assist in evaluating the responses of screened subjects who are not research volunteers.

B. TASTE

Acuity in the primary tastes is tested with standard substances: sucrose (sweet), quinine (bitter), sodium chloride (salty), dilute hydrochloric acid (sour) (Sato *et al.*, 1969). Temperature of the tastant is important (Stone *et al.*, 1965). To test the detection and recognition thresholds, prepared solutions are applied to the tongue or are held in the mouth for a set time and then swallowed or ejected (McBurney, 1969). Tongue application avoids unevenness of dilution in saliva, but also neglects the taste areas of the hard palate and pharynx. The perceived intensity of taste is compared with the concentration of tastant. However, perception includes many subjective variables (Powers *et al.*, 1971) and some workers prefer not to use primary taste judgments to evaluate human acuities.

Foods seldom if ever contain only single tastants and their analysis by taste seldom corresponds with instrumental or chemical analysis (Powers, 1970, 1972). Consistent with the relatively minor role of taste in diet selection, panelists in the food industry who judge the potential acceptability of foods before marketing are not screened for acuity in the primary tastes (Hirsh, 1973). Food processing techniques have diminished the usefulness of taste as an indicator of spoilage or contamination, but awareness may still be valuable; often there is an intensity change in only one of the primary sensations. For sensory evaluation of taste acuities, behavioral controls can minimize many errors in volunteer research subjects, and may be effective in the comprehensive screening of nonvolunteers. Such studies are needed; little is known about standardization of screening methodologies for taste acuity.

Electrophysiological studies of taste acuity in man are seldom practical, although in a few cases the results have corresponded well with subjective test data (Zotterman, 1971). The methods tend to interfere with responses, and the results need complex statistical treatment in order to be interpreted (Beets, 1971).

VII. SIGNIFICANT ASPECTS OF HUMAN OLFACTION AND TASTE IN THE MILITARY ENVIRONMENT

A. OLFACTION

The need for acuity in perception of odors is evident from the emphasis that the military has placed on developing sensory devices and biosensors. Yet, the greater part of this need can probably be fulfilled more adequately, reliably, and economically by man. In a recent report to the U.S. Air Force, Gestetland (1972) stated:

> "No broad-spectrum detection and identification system for air born chemical substances exists which begins to approach the performance of the vertebrate nose with respect to discriminability, sensitivity, dynamic range and variability of substances detectable."

Instruments do give objective information. However, they require operators and servicing personnel. They have poor directional sensitivity and the ranges of their responses are different from those of man. In addition, there are relatively few substances for which instrumental response spectra have been determined.

Therefore, while instruments and biosensors, such as dogs, will continue to have specialized roles in detection of explosives, illicit drugs, and intruders, and while instruments should become useful in standardization of personnel tests and testing situations, these roles do not substitute for human olfactory performance.

Olfactory acuity seems potentially important in soldiers selected for certain duties, e.g., point duty on patrol. This criterion for selection is seldom used and it seems to be generally unrecognized. Olfactory training is known to enhance aspects of performance and survival of military personnel (Woods, 1968). Enemy weapons, personnel, bivouacs, propellants, arson and chemical agents are examples of odor sources that personnel can be trained to recognize. This training could provide a "vocabulary" of odors and a repertoire of behavioral responses.

It should be noted that man has the ability to mask out many unacceptable odors, particularly those unfamiliar to him and encountered for the first time in foreign lands. Unfortunately, these are the very odors that he should be trained to recognize as they are often peculiar to the enemy. Failure of timely recognition may result in needless casualties.

Though machine-operation odors may mask odors from other sources, machine dysfunctions often give off other odors that can be recognized by the operator before breakdown occurs, e.g., an electrical short circuit or overheating of a power unit. Because sight, hearing, and touch often are fully employed in the operation of military machines, it has been suggested that specific olfactory cues (e.g., amyl acetate) might be built into some machines to give early warning of certain critical dysfunctions.

Personnel who are untrained in the use of their olfactory capabilities will tend to give olfactory information to the enemy and to be unaware that the enemy is using it. Anecdotal information suggests that indigenous military forces may be able to map the deployment of troops by the odors of their personal deodorants and insect repellants. Odors emanating from a work area or a specific military operation may give critical intelligence to a trained enemy agent. The element of surprise in military field operations can be squandered through lack of utilization of the olfactory sense.

Tactical uses of odorants have not been explored to any great extent. Odors typical of certain military preparations might be used to deceive enemy intelligence. An enemy who is trained or experienced might do likewise, and untrained personnel would have little immediate defense.

An improved knowledge of the effects of odors on behavior could be useful to the military. For instance, appetitive or aversive effects of odors on the responses of soldiers to food in mess-halls should be recognized. Specific odor signals could be used judiciously to alert personnel to the presence of hazards, e.g., damage to packaged foods. Personnel with defective olfactory acuity could be allocated to jobs in unavoidably malodorous environments. If personnel were regularly screened for olfactory acuity, options such as these would be available.

Sudden or progressive alterations of olfactory acuity (gains, losses or distortions) can signify hidden trauma or early cases of certain diseases. Examples include the endocrine disorders, anterior fossa tumors, head trauma, the phantosmia of incipient schizophrenia, and exposure to high-intensity X-rays or ionizing radiation. Olfactory testing could result in early referral of such cases for specific medical diagnosis and thus permit early treatment. In contrast, the present practice is to refer a soldier complaining of olfactory or taste loss to either a neurologist or a psychiatrist without ever verifying the original complaint.

A permanent loss of olfaction is rated as a 10% disability for compensation purposes (Veterans Administration, 1964). Claims of this type are being prepared in increasing numbers, especially by personnel who may have had service-connected head trauma. Ideally, claims for service-related olfactory losses should be based on the degree of olfactory competence established at the time of induction.

B. TASTE

Taste is an important guide to recognition of spoiled foods. The use of sauces is thought to have originated in order to mask food spoilage. Kermode (1972) has noted that numerous substances are added to foods to diminish the rate of spoilage and suggested that additives may also diminish the role of taste in recognizing food spoilage. Military personnel abroad have problems of discrimination with unfamiliar foods, in which spoilage may be present. Spoiled foods often result in gastrointestinal casualties which could be minimized by appropriate training in taste recognition and by screening to identify ageusic and hypogeusic individuals.

Taste enhancers, aversants, or distorters might be introduced into an enemy's food supply to induce, respectively, inordinate appetite, rejection, or suspicion. The purpose would be to disrupt the planned use of rations. However, the technical problems may be formidable. Because an enemy might adopt this approach, training of personnel in detection and recognition of tastes may be a desirable protective measure.

Service-related loss of taste is rated like loss of olfaction, as a 10% disability (Veterans Administration, 1964). Claims that such losses are service-related are being prepared in increasing numbers and will be difficult to evaluate in personnel who were not previously tested.

VIII. TESTING AND TRAINING OF ARMY PERSONNEL IN THE CHEMICAL SENSES

The current techniques of sensory evaluation in olfaction and taste, other than for basic research on their mechanisms, were developed about 35 years ago, and there have been few recent advances (Hirsh, 1973). The methods of personnel training that have evolved from basic research are non-uniform. Industrial corporations with products identified by aroma and taste have of necessity developed techniques of testing and training. Because much of this information is empirical, it is not readily available in the published literature. However, this body of knowledge and experience is applicable to the Army's needs.

In a pilot program for personnel evaluation and training, the following appear to be the initial steps and priorities.

A. OLFACTION

1. Requirements

A preliminary list of odors that ought to be recognized by trained personnel would be compiled, together with the estimated concentrations at which recognition should become easy. Critical concentrations above which increases should be recognized, will be important in some cases. Situations requiring normal olfactory acuity, e.g., tasks involving "fieldcraft" or machine operation, should be surveyed and subcategorized according to (a) whether or not parallel input would be available from other senses such as vision and audition, and (b) whether recognition of the odors would contribute to assessment of a situation or would demand a specific behavioral response, e.g., to take cover. At this stage feasibility in terms of human olfactory acuity would not be considered.

2. Standards and Criteria

The next step would be to estimate the extent to which these requirements could be met by both instruments and trained human subjects. These estimates would set provisional standards and

criteria for a pilot program to evaluate and train personnel in odor recognition and discrimination. At this stage the effects of different diets, stresses, and fatigue on odor recognition in the military environment could be assessed.

3. Evaluation of Personnel

Personnel would be screened according to these standards and methods, both before and after training. Primarily screening data would be used to assist in future selection of personnel for specific military duties. In addition these data would be useful: (a) as part of the recruiting process, to assist in detecting disease conditions that might disqualify a recruit, (b) as a component of a soldier's continuing medical record, and (c) for reference in case of a future claim for service-related loss of olfaction. During the pilot program, screening data could be used to evaluate the feasibility of the provisional standards and criteria, and to evaluate the design and the execution of the training program.

B. TASTE

Initially personnel could be screened for acuity in the four accepted primary tastes and for discrimination between compound tastes. As with suggested olfactory studies, screening and training methodologies developed for industrial use might be adopted or adapted to meet Army needs. Screening would be carried out before and after training, and the uses of the screening data would be analogous.

IX. SUGGESTIONS FOR FUTURE EMPHASIS

The following suggestions for future emphasis have been developed during the course of this study. The order of presentation does not imply an order of priority.

1. The olfactory and taste acuities of Army personnel should be assessed and aspects of the military environment in which these acuities are significant should be catalogued. Although techniques for these purposes have been inadequate in the past, this no longer seems to be the case (See Section VI & VII).

2. The usefulness of the chemical senses can be developed in regular personnel training programs. The olfactory component of this training could include recognition of the presence of personnel, recognition by operations and maintenance personnel of various aspects of machine operation and malfunction, and training of combat personnel in detection and security related to field missions (See Section VIII).

3. Self-protection of personnel against environmental hazards from volatile substances can be improved by training in olfactory detection and discrimination. Similarly, hazards from eating unfamiliar or spoiled foods can be minimized by training in taste detection and discrimination. In addition, specific odor producing substances might be used in packaging or in foods to provide olfactory or taste clues to food spoilage (See Section VI & VII).

4. The standards and criteria used for evaluating human sensory performance in civilian environments may not always be appropriate to military situations. Thus the Army will need to determine its own general and special requirements in terms of odors and tastes, their critical

concentrations, and significant thresholds for recognition by personnel. These determinations and the preparation of the standards and criteria could be conducted by the Army utilizing existing research personnel and facilities. (See Section VI).

5. A number of resources are available for the determination of standards and criteria that the Army will need. These include Army laboratories and other government agencies in which instruments or biosensors are being developed to detect military explosives or personnel. For example, the National Bureau of Standards is studying plasma chromatography instrumentation for the chemical analysis of air. Plasma chromatography avoids the need for sample preparation that is inherent in other analyses and has potential for multiple sampling that should be useful for simultaneous monitoring of background and stimulus during Army personnel testing programs (See Section VI, A).

6. In developing techniques for training and evaluation of personnel in the use of their chemical senses, the potential contributions of industrial laboratories should be explored. Industries whose products are identified by aroma and taste have, of necessity, developed evaluation techniques that appear to be directly relevant to many of the Army's needs. Relatively little of this information has been reported compared to the quantity of literature published on basic research (See Section VI, B).

7. There is a need to explore the effects of odors on behavior and the modification of human behavior in the military environment. The Army has an opportunity to investigate and utilize the behavioral consequences of individual variability in detection, discrimination, and responses to odorants and tastants (See Section IV & V).

8. There is a need to explore alterations of sensory performance in personnel exposed to X-rays or

ionizing radiation. Alterations of olfactory and taste sensations may be useful early signs of exposure or of inadequate radiation protection. Human patients undergoing radiation therapy or X-ray diagnosis would be suitable subjects for such observations (See Section IV & VII).

9. There is a need to study the effects of drugs of abuse and therapeutic drugs on the chemical senses. This may be of particular concern to the Army if drug use compromises a man's olfactory or taste acuity and subsequently his performance.

10. The relationships of hyposmias and hypogeusias to nutritional deficiencies of zinc and other trace elements are controversial. Because zinc deficiency might be found in nutritionally deficient inductees and may follow regular heavy consumption of alcohol, these uncertainties should be resolved (See Section V).

11. Because service-related losses of the senses of olfaction and taste are rated as disabilities, testing procedures are required to check the validity of complaints. Evaluation of olfactory and taste acuities at induction would assist in documentation of subsequent claims for service-related losses (See Section VII).

12. While instruments and biosensors have specialized roles in odor detection, these do not substitute for human olfactory capabilities. At present, detection devices and biosensor programs are being developed without reference to the performance potentialities of the man. Increased emphasis on the use of the human olfactory system is required (See Section VII & VIII).

13. When the soldier knows that his full performance capabilities are recognized and used, morale tends to be high. On the other hand, when a soldier's personal capabilities are not fully used, he has less pride in himself. Substitution of detection devices and

biosensors for the chemical senses of man underestimates his capabilities. A program to develop the full capabilities of soldiers to exercise their senses of olfaction and taste in the military environment would tend to enhance their morale and potentially, their performance.

14. This study suggests that there is a need to designate some office or laboratory within the Army research and development community as a center for collation of projects involving the chemical senses and odor-sensing devices.

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XI. GLOSSARY

Ageusia	Lack of a sense of taste, for all tastants ("general") or for particular tastants ("specific").
Anosmia	Lack of a sense of smell, for all odorants ("general") or for particular odorants ("specific").
Cilia	Hair-like projections of some cells.
Biosensor.	An animal trained to signal its responses to sensory stimuli, e.g., a dog trained to communicate to man the recognition of certain odors.
Dysgeusia.	Unpleasantly distorted taste sense; some workers prefer the term "cacogeusia."
Dysosmia	Unpleasantly distorted sense of smell; some workers prefer the term "cacosmia."
Glomerulus	A cluster of small structures, tubules or, in the present context, the nerve fibers in the olfactory glomerulus.
Hypergeusia.	Overly sensitive taste sense; can be general or specific.
Hyperosmia.	Overly sensitive sense of smell; can be general or specific.
Hypogeusia	Abnormally diminished sense of taste, general or specific.
Hyposmia	Abnormally diminished sense of smell, general or specific.

Odorant	Substance perceived by olfaction.
Olfaction	The sense of smell.
Olfactory bulb.	A nerve center intermediate between the receptors for olfaction in the nose, and the brain itself.
Parosmia	Altered sense of smell, without implying pleasant or unpleasant. See dysosmia.
Parageusia	Altered sense of taste, without implying pleasant or unpleasant. See dysgeusia.
Phantosmia	Hallucination that an odor is present when in fact it is not. Phantogeusia means the same for taste.
Tastant	Substance perceived by taste.

XII. SCIENTIFIC CONSULTANTS

ON

A STUDY OF THE POTENTIAL APPLICATIONS
OF OLFACTORY RESEARCH
IN MAN

A. ATTENDEES, *AD HOC* STUDY GROUP MEETING
September 19 and 20, 1972

CHAIRMAN

C. Jelleff Carr, Ph. D.
Director
Life Sciences Research Office
Federation of American Societies
for Experimental Biology
Bethesda, Maryland 20014

CO-CHAIRMAN

Lloyd M. Beidler, Ph. D.
Professor of Biophysics
Florida State University
Tallahassee, Florida 32306

CONSULTANTS

Preston Abbott, Ph. D.
Director
Kensington Office
American Institute for Research
10605 Concord Street
Kensington, Maryland 20795

John E. Amoore, Ph. D.
Chemist, Vegetable Laboratory
Western Regional Research Laboratory
Agricultural Research Service
Department of Agriculture
Berkeley, California 94710

Andrew Dravnieks, Ph. D.
Technical Director
Odor Sciences Center
IIT Research Institute
10 West 35th Street
Chicago, Illinois 60616

Trygg Engen, Ph. D.
Professor of Psychology
Brown University
Providence, Rhode Island 02912

John Garcia, Ph. D.
Professor of Psychology
University of Utah
Salt Lake City, Utah 84112

Bruce P. Halpern, Ph. D.
Associate Professor of Psychology
and Biology
Social Sciences Building
Cornell University
Ithaca, New York 14850

Jimmy L. Hatfield, Ph. D.
Visual Sciences Division
Optical Sciences Group
1301 Earnestine Street
McLean, Virginia 22101

Robert I. Henkin, M. D., Ph. D.
Chief
Section of Neuroendocrinology
Experimental Therapeutics Branch
National Heart and Lung Institute
Bethesda, Maryland 20014

H. L. Jacobs, Ph. D.
Associate Director for Behavioral
Sciences
Pioneering Research Laboratory
Department of the Army
Natick, Massachusetts 01760

Charles J. Long, Ph. D.
Associate Professor
Department of Psychology
Memphis State University
Memphis, Tennessee 38152

Herbert M. Meiselman, Ph. D.
Head, Food Acceptance Group
Behavioral Sciences Division
Pioneering Research Laboratory
Department of the Army
Natick, Massachusetts 01760

Howard R. Moskowitz, Ph. D.
Head, Measurement and
Methodology Unit
Food Acceptance Group
Behavioral Sciences Division
Pioneering Research Laboratory
Department of the Army
Natick, Massachusetts 01760

David G. Moulton, Ph. D.
Associate Professor of Physiology
Monell Chemical Senses Center
University of Pennsylvania
Philadelphia, Pennsylvania 19104

Maxwell M. Mozell, Ph. D.
Professor of Physiology and
Associate Dean for Academic
Program Development
Upstate Medical Center
State University of New York
Syracuse, New York 13210

Colonel Thomas G. Murnane, VC
Chief, Veterinary Research Division
U.S. Medical Research and
Development Command
Department of the Army
Washington, D.C. 20315

Carl Pfaffmann, Ph.D.
Rockefeller University
New York, New York 10021

Marvin Nachman, Ph.D.
Professor
Department of Psychology
University of California
Riverside, California 92502

Robert A. Schneider, M.D.
University of Oklahoma Health
Sciences Center
P.O. Box 26901
Oklahoma City, Oklahoma 73190

Herbert Stone, Ph.D.
Director
Food and Plant Sciences
Department
Stanford Research Institute
Menlo Park, California 94025

ARO STAFF

Mr. J.L. Barber
Chief, Human Factors Branch
Behavioral Sciences Division
Office of the Chief of Research
and Development
3045 Columbia Pike
Arlington, Virginia 22204

Carl Lamanna, Ph.D.
Deputy Chief and Scientific
Advisor
Life Sciences Office
Office of the Chief of Research
and Development
3045 Columbia Pike
Arlington, Virginia 22204

Eugene Sporn, Ph.D.*
Chief, Special Projects Branch
Life Sciences Office
Office of the Chief of Research
and Development
3045 Columbia Pike
Arlington, Virginia 22204

* Contractor's Technical Representative.

LSRO STAFF

Kenneth D. Fisher, Ph. D.
Life Sciences Research Office
Federation of American Societies
for Experimental Biology
Bethesda, Maryland 20014

Humphrey F. Sassoon, Ph. D.
Life Sciences Research Office
Federation of American Societies
for Experimental Biology
Bethesda, Maryland 20014

George W. Irving, Jr., Ph. D.
Life Sciences Research Office
Federation of American Societies
for Experimental Biology
Bethesda, Maryland 20014

Mrs. Evelyn Volkman
Assistant to the Director
Life Sciences Research Office
Federation of American Societies
for Experimental Biology
Bethesda, Maryland 20014

FASEB STAFF

Eugene L. Hess, Ph. D.
Executive Director
Federation of American Societies
for Experimental Biology
Bethesda, Maryland 20014

Mrs. Ann Nixon
Executive Assistant
Federation of American Societies
for Experimental Biology
Bethesda, Maryland 20014

B. OTHER CONSULTANTS

Stephen N. Chesler, Ph. D.
Analytical Chemistry Division
National Bureau of Standards
Washington, D. C. 20234

Donald F. Miller, M. S.
Acting Chief
Nutrition & Food Composition Section
Division of Nutrition (BF-125)
Bureau of Foods
200 "C" Street, S. W.
Washington, D. C. 20204

Stuart P. Cram, Ph. D.
Chief, Separation and Purification Section
National Bureau of Standards
Analytical Chemistry Division
Bldg. 222, Room A105
Washington, D. C. 20234

Joseph T. Roberts, M. D., Ph. D.
Medical Member, Disability Policy Board
Compensation and Pension Services
Department of Veterans Benefits
VA Central Office
810 Vermont Avenue
Washington, D. C. 20420

Frank R. Dastoli, Ph. D.
Director of Research
Mirlin Corporation
8 Kane Industrial Drive
Hudson, Massachusetts 01749

Glenn Spangler, Ph. D.
Commanding Officer
USA, MERDC
Attention SMEFB-XR
Ft. Belvoir, Virginia 22060

Ms. Naomi L. Hirsh, Manager
Sensory Evaluation Department
The Coca-Cola Company
Foods Division
Houston, Texas 77001

Major F. H. Woods
1 Bn. Depot
Burnham Military Camp
Burnham, Christchurch
New Zealand

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13. ABSTRACT
This study provides a comprehensive review of the feasibility of developing and utilizing the chemical senses of the soldier. While biosensors and odor-sensing devices can supplement, they cannot replace the potential performance of the normal human olfactory system. Experience in other countries suggests that a soldier trained to recognize olfactory cues is more effective in the field, more responsive to hazards, and less likely to give olfactory information to the enemy than one whose chemical senses are underutilized. Practical testing and training methods suited to that Army's needs have been lacking in the past, and basic knowledge of the mechanisms of olfaction and taste is incomplete. However, elements of a suitable technology have been identified. This technology appears suitable for further development within the Army research and development community. This report concludes that it is feasible for the Army to use these methods in determining classifications of personnel that require normal acuity of the chemical senses and to institute training programs. Regular testing for normal acuity of the chemical senses may uncover cases of some illnesses, indicate extensive exposures to ionizing radiation, and gorize personnel as either suitable or unfit for certain Army duties. If inistered at recruitment, these tests can be used later to document disability claims for service-related losses of olfaction and taste.

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Chemical Senses						
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Detection						
Recognition						
Standards and Criteria						
Olfaction						
Taste						
Detection						
Recognition						
Standards and Criteria						

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RECOMMENDED INTERNATIONAL STANDARD FOR MARGARINE

FOOD AND AGRICULTURE ORGANIZATION
OF THE UNITED NATIONS
WORLD HEALTH ORGANIZATION

Issued by the Secretariat of the
Joint FAO/WHO Food Standards Programme, FAO, Rome

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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 Margarine. 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 General Principles of the Codex Alimentarius. An extract from the General Principles containing this procedure is included in the Appendix to this publication. The full text of the General Principles of the Codex Alimentarius is contained in the Second Edition of the Commission's Procedural Manual (Ref. No. CX 8/7 - 1969). Member Nations and Associate Members of FAO and/or WHO which are not Members of the Commission are 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 practice.

RECOMMENDED INTERNATIONAL STANDARD FOR MARGARINE

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1. SCOPE

This standard will not apply to any product which contains less than 80% fat and is not labelled in any manner which implies, either directly or indirectly, that the product is margarine.

2. DESCRIPTION

2.1 Product Definition

Margarine is a food in the form of a plastic or fluid emulsion, which is mainly of the type water/oil, produced principally from edible fats and oils, which are not or are not mainly derived from milk.

2.2 Other Definitions

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

2.2.2 Pre-packed means packed or made up in advance, ready for retail sale in a container.

3. ESSENTIAL COMPOSITION AND QUALITY FACTORS

3.1 Raw Materials

3.1.1 Edible fats and/or oils, or mixtures of these, whether or not they have been subjected to a process of modification.

3.1.2 Water and/or milk and/or milk products.

3.2 Minimum Fat Content — 80% m/m of the product

3.3 Maximum Water Content — 16% m/m of the product

3.4 Additions

The following substances may be added to margarine :

3.4.1 Vitamins: Vitamin A and its esters
Vitamin D
Vitamin E and its esters
Other vitamins

Maximum and minimum levels for Vitamins A, D and E and other Vitamins should be laid down by national legislation in accordance with the needs of each individual country including, where appropriate, the prohibition of the use of particular Vitamins.

3.4.2 Sodium chloride

3.4.3 Sugars 1/

3.4.4 Suitable edible proteins

4. FOOD ADDITIVES	Maximum level of use
4.1 Colours	
4.1.1 Beta-carotene	Not limited
4.1.2 Annatto (*)	Not limited
4.1.3 Curcumin (*)	Not limited

(*) Temporarily endorsed.

1/ 'Sugars' means any carbohydrate sweetening matter.

	Maximum level of use
4.1.4 Canthaxanthine	Not limited
4.1.5 Beta-apo-8'-carotenal	Not limited
4.1.6 Methyl and ethyl esters of Beta-apo-8'-carotenoic acid	Not limited
4.2 Flavours	
4.2.1 Natural flavours and their identical synthetic equivalents, except those which are known to represent a toxic hazard (*)	Not limited
4.2.2 Other synthetic flavours approved by the Codex Alimentarius Commission (*)	Not limited
4.3 Emulsifiers	
4.3.1 Mono- and diglycerides of fatty acids	Not limited
4.3.2 Mono- and diglycerides of fatty acids esterified with the following acids: acetic, acetyltartaric, citric, lactic, tartaric and their sodium and calcium salts	10 g/kg
4.3.3 Lecithins and components of commercial lecithin	Not limited
4.3.4 Polyglycerol esters of fatty acids	5 g/kg
4.3.5 1,2-propylene glycol esters of fatty acids	20 g/kg

(*) Temporarily endorsed.

	Maximum level of use
4.3.6 Esters of fatty acids with polyalcohols other than glycerol:	10 g/kg
Sorbitan monopalmitate Sorbitan monostearate Sorbitan tristearate	
4.3.7 Sucrose esters of fatty acids (including sucroglycerides) (*)	10 g/kg
4.4 Preservatives	
4.4.1 Sorbic acid and its sodium, potassium and calcium salts 4.4.2 Benzoic acid and its sodium and potassium salts	1000 mg/kg individually or in combination, expressed as the acids
4.5 Antioxidants	
4.5.1 Propyl, octyl, and dodecyl gallates 4.5.2 Butylated hydroxytoluene (BHT) Butylated hydroxyanisole (BHA)	100 mg/kg individually or in combination
4.5.3 Natural and synthetic tocopherols	Not limited
4.5.4 Ascorbyl palmitate 4.5.5 Ascorbyl stearate	200 mg/kg individually or in combination
4.6 Antioxidant Synergists	
4.6.1 Isopropyl citrate mixture	100 mg/kg
4.7 Other Additives	
4.7.1 Citric and lactic acids and their potassium and sodium salts	Not limited

(*) Temporarily endorsed.

	Maximum level of use
4.7.2 L-tartaric acid and its sodium and sodium-potassium salts	Not limited
4.7.3 Sodium hydrogen carbonate, sodium carbonate, sodium hydroxide	Not limited
5. CONTAMINANTS	Maximum level
5.1 Iron (Fe)	1.5 mg/kg
5.2 Copper (Cu)	0.1 mg/kg
5.3 Lead (Pb)	0.1 mg/kg
5.4 Arsenic (As)	0.1 mg/kg

6. HYGIENE

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

7. PACKAGING

Margarine when sold by retail shall be pre-packed and may be sold in a pack of any shape.

8. 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:

8.1 The Name of the Food

The product shall be designated margarine and all products designated as margarine shall conform to this standard.

8.2 List of Ingredients

A complete list of ingredients shall be declared on the label in descending order of proportion in accordance with sub-sections 3.2 (c) of the General Standard for the Labelling of Pre-packaged Foods.

8.3 Net Contents

The net contents shall be declared by weight in either the metric ("Système International" units) or avoirdupois or both systems as required by the country in which the product is sold.

8.4 Name and Address

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

8.5 Country of Origin

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

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

8.6 Labelling Prohibitions

8.6.1 No reference shall be made to the presence of milk fat or butter in margarine except in a complete list of ingredients.

8.6.2 No reference shall be made, other than in a complete list of ingredients, to the presence of any vitamin in margarine unless the name and quantity of the vitamin is stated on the label.

9. METHODS OF ANALYSIS AND SAMPLING

The methods of analysis and sampling described hereunder are international referee methods.

9.1 Estimation of Milk Fat Content (*) CAC/RM 15-1969

Milk fat content in margarine is estimated by the determination of the following values which are obtained by specifically defined empirical methods:

Reichert-Meissl-Wollny value (R)
Polenske value (P)
Kirschner value (K)

9.1.1 Determination of (R) and (P) values

According to the IUPAC (1964) method (IUPAC Standards Methods for the Analysis of Oils, Fats and Soaps, 5th Edition, 1966, II.D.9, Determination of Soluble and Insoluble Volatile Acids) and the AOAC (1965) method (Official Methods of Analysis of the AOAC, 1965, 26.032 - 26.033).

Note I: Both the methods of IUPAC and AOAC are necessary because of the extremely empirical nature of the methods depending on regional deviations.

Note II: Determination of (R) and (P) values are not sufficient for the evaluation of milk fat content in margarine, which also involves the determination of (K) values as in 9.1.2.

9.1.2 Determination of (K) value (Based on British Standards Institution Method).

9.1.2.1 Principle of the method

The Reichert Value (R) is the number of ml of 0.1 N aqueous alkali solution required to neutralize the water-soluble volatile fatty acids distilled from 5 g of the oil or fat under the precise conditions specified in the method.

The Polenske Value (P) is the number of ml of 0.1 N aqueous alkali solution required to neutralize the water-insoluble volatile fatty acids distilled from 5 g of the oil or fat under the precise conditions specified in the method.

(*) It is intended to replace the methods described below by gas chromatographic methods in the near future.

The Kirschner Value (K) is the number of ml of 0.1 N aqueous alkali solution required to neutralize the water-soluble volatile fatty acids which form water-soluble silver salts distilled from 5 g of the oil or fat under the precise conditions specified in the method.

Margarine fat is saponified. The fatty acids are then liberated from the soap and distilled in a standard apparatus under specified conditions. The water-insoluble fatty acids are filtered off, dissolved in ethanol and titrated. (P) is calculated from the amount of alkali required. The water-soluble fatty acids are then titrated with barium hydroxide solution, and (R) is calculated from the amount of the solution required in the titration. The neutralized solution is then treated with silver sulphate. After separation of the insoluble silver salts by filtration, the filtrate containing the water-soluble silver salts is acidified, distilled in the standard apparatus under the same specified conditions and titrated to determine (K). The percentage of milk fat in margarine fat is then calculated according to a formula based on (K) and (P). This process does not determine the total quantities of volatile fatty acids, soluble and insoluble in water, present in combination in the fat. The amounts of these acids actually determined by the process are dependent on strict adherence to the dimensions of the apparatus and the details of procedure.

9.1.2.2 Reagents

The reagents used shall be of recognized analytical reagent quality.

9.1.2.2.1 Glycerol

9.1.2.2.2 Sodium hydroxide, 50% m/m solution. Dissolve sodium hydroxide in an equal mass of water and store the solution in a bottle protected from carbon dioxide. Use the clear portion free from deposit.

9.1.2.2.3 Sulphuric acid, dilute. Dilute approximately 25 ml of concentrated sulphuric acid to 1 litre and adjust until 40 ml neutralize 2 ml of the 50% sodium hydroxide solution.,

9.1.2.4 Pumice powder, ground, passing through a 60 mesh British Standard (B.S.) test sieve and remaining on a 120 mesh B.S. test sieve (*) or equivalent (**).

9.1.2.2.5 Phenolphthalein indicator, 0.5% solution in 95% (v/v) ethanol.

9.1.2.2.6 Ethanol, 95% (v/v), neutralized to phenolphthalein immediately before use.

9.1.2.2.7 Sodium hydroxide, approximately 0.1 N solution, accurately standardized.

9.1.2.2.8 Barium hydroxide, approximately 0.1 N solution, accurately standardized.

9.1.2.2.9 Silver sulphate, powdered.

9.1.2.3 Apparatus

9.1.2.3.1 Graduated cylinder of 100 ml capacity, complying with B.S. 604, or equivalent.

9.1.2.3.2 Pipette of 50 ml capacity, complying with B.S. 1583 ('one-mark bulb pipettes') Class B, or equivalent.

9.1.2.3.3 Distillation apparatus, as shown in Fig. 1. The constituent parts are as follows:

- Flat-bottomed boiling flask (Polenske) of borosilicate glass. The volume contained to the bottom of the neck is 310 ± 10 ml.

(*) British Standard 410, 'Test sieves'.
(**) These sieves correspond respectively to ISO sieves, the meshes of which are made by so weaving wire as to form square openings of 250 μm x 250 μm and of 125 μm x 125 μm (ISO Recommendation R 565).

- Still-head, of glass tubing of wall thickness 1.25 ± 0.25 mm and conforming to the shape and dimensions shown in Fig. 2. A rubber stopper, fitted below the bulb of the longer arm of the still-head, and used for connecting it to the flask, shall have its lower surface 10 mm above the centre of the side hole of the still-head

- Condenser of glass tubing. The wall thickness of the inner tube shall be 1.0 ± 0.2 mm and that of the outer jacket and of the wider part at the top 1.25 ± 0.25 mm.

- Receiver, consisting of a flask complying with B.S. 675 ('Sugar flasks') or equivalent carrying two graduation marks on the neck, one at 100 ml and the other at 110 ml.

- Asbestos board. An asbestos board, 120 mm in diameter and 6 mm in thickness, with a circular hole about 65 mm in diameter, used to support the flask over the burner. During distillation the Polenske flask shall fit snugly into the hole in the board so as to prevent the flame from impinging on the surface of the flask above the hole. A new asbestos board may be conveniently prepared by levelling the edge of the hole, soaking in water, moulding the edge with a flask, and drying.

- Bunsen burner. The burner should be sufficiently large to allow the distillation to be completed in the time specified. The apparatus is supported on a retort stand, not shown in the diagramme.

9.1.2.4 Procedure

9.1.2.4.1 Hydrolysis and distillation

Weigh 5 ± 0.01 g of the fat into a Polenske flask. Add 20 g of glycerol and 2 ml of the 50% sodium hydroxide solution. Protect the burette containing the latter from carbon dioxide, and wipe its nozzle clean from carbonate deposit before withdrawing solution for the tests; reject the first few drops withdrawn from the burette. Heat the flask over a naked flame, with continuous mixing, until the fat, including any drops adhering to the upper parts of the flask, is saponified, and the liquid becomes perfectly clear; avoid overheating during this saponification. Cover the flask with a watch glass.

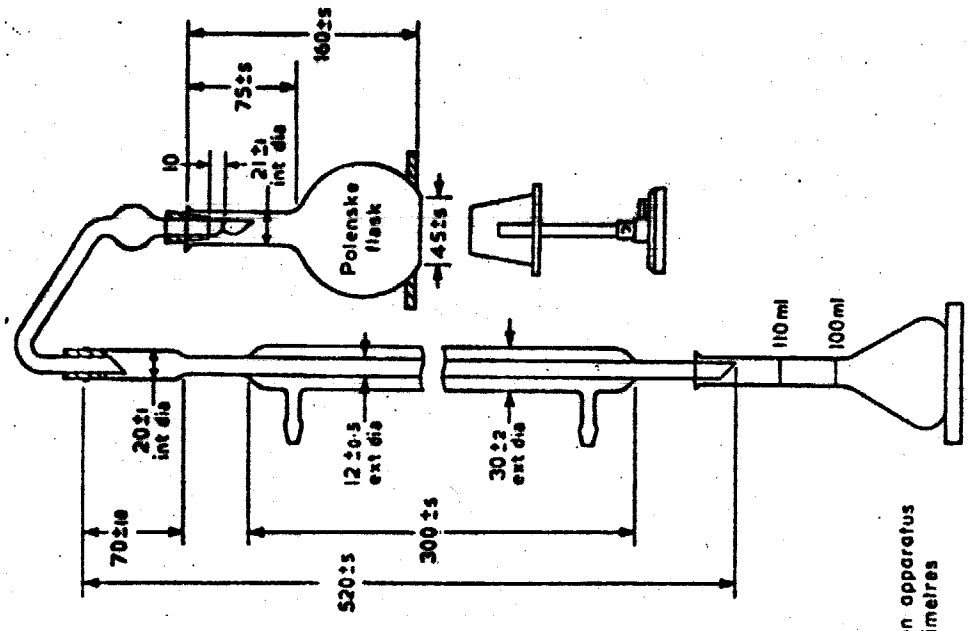

Fig. 1 - Polenske distillation apparatus
Dimensions in millimetres

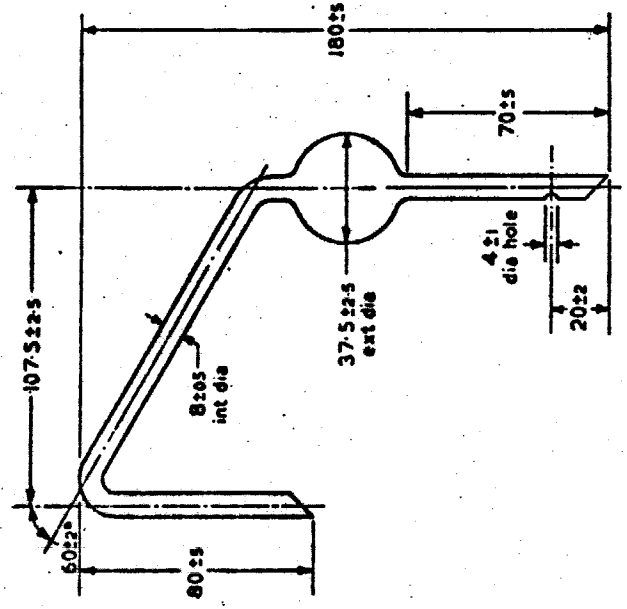

Fig. 2 - Still-head for Polenske distillation apparatus
Dimensions in millimetres

Make a blank test without fat, but using the same quantities of reagents and following the same procedure, again avoiding over-heating during the heating with sodium hydroxide; such over-heating would be indicated by darkening of the solution.

Measure 93 ml of boiling distilled water, which has been vigorously boiled for 15 minutes, into a 100 ml graduated cylinder. When the soap is sufficiently cool to permit addition of the water without loss, but before the soap has solidified, add the water, draining the cylinder for 5 seconds, and dissolve the soap. If the solution is not clear (indicating incomplete saponification) or is darker than light yellow (indicating over-heating) repeat the saponification with a fresh sample of the fat.

Add 0.1 g of powdered pumice, followed by 50 ml of the dilute sulphuric acid, and connect the flask at once with the distillation apparatus. Heat the flask without boiling its contents, until the insoluble acids are completely melted, then increase the flame and distil 110 ml in between 19 and 21 minutes. Keep the water flowing in the condenser at a sufficient speed to maintain the temperature of the issuing distillate between 18°C and 21°C.

When the distillate reaches the 110 ml mark, remove the flame and replace the 110 ml flask by a cylinder of about 25 ml capacity, to catch drainings. Close the 110 ml flask with its stopper, and without mixing the contents place it in water at 15°C for 10 minutes so as to immerse the 110 ml mark. Remove the flask from the water, dry the outside and invert the flask carefully, avoiding wetting the stopper with the insoluble acids. Mix the distillate by four or five double inversions, without violent shaking. Filter through a dry 9 cm open-texture filter paper (a Whatman No. 4 or 41 or a Barcham Green No. 801 paper or equivalent is suitable) which fits snugly into a funnel. Reject the first runnings and collect 100 ml in a dry flask, cork the flask and retain the filtrate for titration as under 9.1.2.4.2 (a). The filtrate should be free from insoluble fatty acids.

Where liquid, insoluble fatty acids pass through the filter, receive the filtrate in a separating funnel, and after separation draw off the lower (aqueous) layer, leaving behind insoluble acids which have risen to the surface. Add these to the main bulk of the insoluble acids.

Detach the still-head and wash the condenser with three successive 15 ml portions of cold distilled water, passing each washing separately through the cylinder, the 110 ml flask, the filter and the separating funnel, nearly filling the paper each time and draining each washing before filtering the next. Discard the washings.

Dissolve the insoluble acids by three similar washings of the condenser, the cylinder, and the filter with 15 ml of neutralized ethanol, collecting the solution in the 110 ml flask and draining the ethanol after each washing. Cork the flask, and retain the solution for titration, as at 9.1.2.4.2 (b).

9.1.2.4.2 Determinations (*)

(a) Reichert, or soluble volatile acid, value (R)

Pour 100 ml of the filtrate containing the soluble volatile acids into a previously dried titration flask, add 0.1 ml of phenolphthalein indicator and titrate with the barium hydroxide solution until the liquid becomes pink, rinsing the 100 ml flask with the nearly neutralized liquid towards the end of the titration. Note the actual volume of barium hydroxide solution used; drain the 100 ml flask into the titration flask, close with a cork and continue as at 9.1.2.4.2 (c).

(b) Polenske, or insoluble volatile acid, value (P)

Titrate the alcoholic solution of the insoluble volatile acids after addition of 0.25 ml of phenolphthalein indicator with the 0.1 N barium hydroxide solution until the solution becomes pink.

(c) Kirschner value (K)

Add 0.5 g of finely powdered silver sulphate to the neutralized solution from (a) above. Allow the flask to stand in the dark for one hour with occasional shaking and filter the contents in the dark through a dry filter. Transfer 100 ml of the filtrate to a dry Polenske flask, add 35 ml of cold distilled water, recently boiled for 15 minutes, 10 ml of the dilute sulphuric acid and a loosely-wound 5 mm coil of 30 cm of aluminium wire (about 1 mm thick or 18 to 20 Standard Wire Gauge) or 0.1 g of pumice powder.

(*) See Note under 9.1.1.

Connect the flask with the standard apparatus and repeat the process as described above, i.e. the distillation of 110 ml in from 19 to 21 minutes, the mixing (but without the cooling for 10 minutes), and the filtration and the titration of 100 ml of the filtrate with the barium hydroxide solution.

9.1.2.5 Calculation and expression of results

Reichert value (R) = $1.10\,(T_1 - T_2)$

Polenske value (P) = $T_3 - T_4$

$$\text{Kirschner value (K)} = \frac{121\,(100 + T_1)\,(T_5 - T_6)}{10\,000}$$

where

T_1 = ml of 0.1 N barium hydroxide solution used for sample under 9.1.2.4.2 (a).

T_2 = ml of 0.1 N barium hydroxide solution used for blank under 9.1.2.4.2 (a).

T_3 = ml of 0.1 N barium hydroxide solution used for sample under 9.1.2.4.2 (b).

T_4 = ml of 0.1 N barium hydroxide solution used for blank under 9.1.2.4.2 (b).

T_5 = ml of 0.1 N barium hydroxide solution used for sample under 9.1.2.4.2 (c).

T_6 = ml of 0.1 N barium hydroxide solution used for blank under 9.1.2.4.2 (c).

Milk fat content in margarine is estimated according to the following formula:

$$\text{Percentage m/m milk fat in margarine fat} = \frac{K - 0.1P - 0.24}{0.244}$$

where K = Kirschner value
P = Polenske value

Note I: Correction factors are to be used, if necessary, for the Reichert and Polenske values due to the fact that both values have been found to be low when determined at low barometric pressures of ρ mm Hg, such as may occur at high altitudes. The following factors may be applied to values determined at a barometric pressure of ρ mm Hg, to convert them to the values determined at normal pressure (2).

$$\text{Corrected Reichert value} = \frac{(\text{observed value} - 10)\log 760}{\log \rho} + 10$$

$$\text{Corrected Polenske value} = \text{observed value} \times \frac{760 - 45}{\rho - 45}$$

Note II: The referee methods of determining (P) values are those indicated under 9.1.1 In the case of discrepancies between (P) values determined according to 9.1.2.5 and those determined according to 9.1.1, these latter values are to be used in the calculation of butter fat content.

Note III: Tables and graphs relating to Polenske values with palm kernel oil and coconut oil contents and Kirschner values with butter fat contents were reported by K.A. Williams (2).

9.1.2.7 Literature references

(1) British Standard 684: 1958, Methods of Analysis of Oils and Fats, pages 65-71.

(2) Kirkham (1920) Analyst, 44, 293

(3) Williams, K.A. (1949) Analyst, 74, 508

9.2 Determination of Fat Content CAC/RM 16-1969

(Direct method of determination of total fat content in margarine. Based on the Official Netherlands Food Analysis Method).

9.2.1 Principle of Method

A preliminary treatment will dilute hydrochloric acid is followed by extraction with light petroleum.

9.2.2 Reagents and Materials

9.2.2.1 Hydrochloric acid, 25% d = 1.126

9.2.2.2 Light petroleum, freshly distilled, boiling range 40° to 60°

9.2.2.3 Pumice, pieces of, diameter 0.9 to 1.0 mm

9.2.3 Apparatus

9.2.3.1 Liquid/liquid continuous 'ether' extractor as illustrated in Fig. 1 or equivalent, with fat determination flask.

9.2.3.2 Electrically heated drying oven adjusted to operate at 102° ± 1°C.

9.2.3.3 Conical flask (type Philipps beaker or equivalent) of 100 ml, with spout and perfect rim.

9.2.4 Procedure

Melt about 50 g of the product in a wide-necked bottle of suitable size with a close-fitting stopper at about 40°C and cool whilst shaking vigorously until the mass is homogeneous and viscous. Introduce about 10 g accurately weighed, into the pre-weighed conical flask. Add 20 ml of 25% hydrochloric acid and five pieces of pumice and boil very gently while swirling continuously until all non-fatty substances are dissolved. Allow to cool to about 30°C.

Introduce five pieces of pumice into the fat-determination flask and dry the flask for 1 hour in the drying oven at 102° ± 1°C. Allow the flask to cool to room temperature in a dessicator, weigh the flask to the nearest 0.1 mg and fit it to the extraction apparatus (9.2.3.1).

Transfer the contents of the conical flask into the extractor's bulb and rinse the flask alternatively with tepid water and light petroleum, transferring each time the liquid into the extractor's bulb until the bulb is filled with the aqueous liquid to half level. Rinse again the conical flask with light petroleum until enough light petroleum running through the bulb of the extraction apparatus makes the fat-determination flask filled to about 2/5, and heat the flask for 4 hours.

Check the completion of the extraction using a second fat-determination flask and extracting during a further period of 1 hour with a fresh portion of solvent. The increase in mass should not exceed 0.01% of the sample mass.

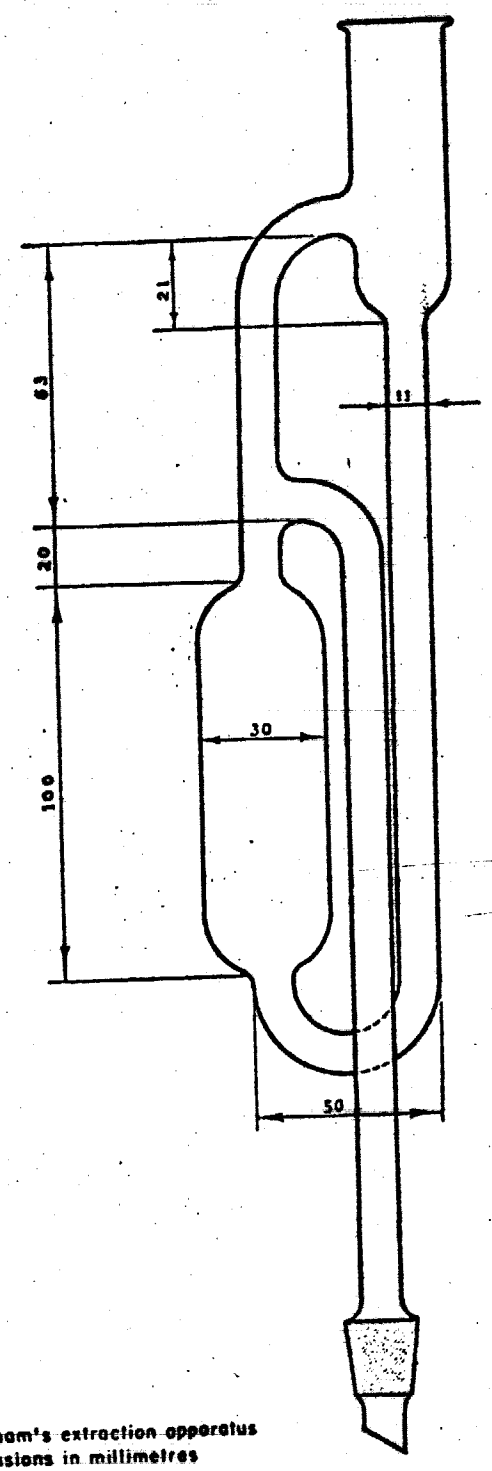

Fig. 1 - Smetham's extraction apparatus
Dimensions in millimetres

After extraction, remove the fat-determination flask from the extraction apparatus and distill off the solvent on a water-bath. Evaporate the last traces of the solvent (when desired) on the water-bath, using a current of nitrogen. Dry the flask for 1 hour in drying oven at $102^{\circ} \pm 1^{\circ}C$, allow to cool to room temperature in a dessicator and weigh to the nearest 0.1 mg.

Repeat drying and weighing every half hour until two successive weighings do not differ by more than 1 mg.

9.2.5 Calculation and Expression of Results

Results are expressed as % m/m fat content, calculated as follows:

$$\% \text{ m/m fat content} = \frac{\text{mass of light petroleum extract (g)}}{\text{original sample mass (g)}} \times 100$$

9.2.5.1 Repeatability

The difference between duplicate determinations (obtained simultaneously or in rapid succession by the same analyst) does not exceed 0.2% of the fat content.

9.2.6 Literature References

Smetham, A; Analyst, 17 (1892) 44-45
Van Ginkel, J.G. and Roos, J.B.; the Analysis of Butter and Cheese in H.A. Bockenogen "The Analysis and characterisation of Oils, Fat and Fat products", Vol. 1, pp. 191-193, Interscience Publishers.

9.3 Determination of Water Content CAC/RM 17-1969

(To be developed)

9.4 Determination of Vitamin A Content

According to the AOAC (1965) method (Official Methods of Analysis of the AOAC, 1965, 39.001 - 39.007, Chemical Methods, Vitamin A in Margarine).

Results are expressed as microgrammes retinol (Vitamin A - alcohol) per kg.

9.5 Determination of Vitamin D Content

According to the AOAC (1965) method (Official Methods of Analysis of the AOAC, 1965, 39.116 - 39.129 Vitamin D).

Results are expressed as IU Vitamin D per kg.

9.6 Determination of Vitamin E (Tocopherols) Content CAC/RM 18-1969

9.6.1 Scope: Tocopherols which are present in foods are known for their two main properties:

(a) in nutrition (Vitamin E activity)

(b) in preventing oxidization of foods (antioxidant activity)

The determination of Vitamin E, by chemical method, involves the determination of tocopherols, either natural or synthetic. Results are expressed as the quantity of each (α, β, γ, etc.) tocopherol and may also be expressed as the quantity of total tocopherols. The method is applicable to oils and fats and is suitable for determining tocopherols in micro-amounts. It is also applicable to fat-soluble extracts of other biological materials. The tocopherols are sufficiently stable to involve no special hazards or difficulties when one of the several normal methods of fat extraction is used. Several such methods, applied particularly to tocopherol determination have been described (2) (4) (8).

9.6.2 Principle of Method: The method is based on the Emmerie-Engel method (5). It involves the determination of all seven tocopherols present in the material. After removal of interfering substances, the tocopherols are treated with a mixture of ferric chloride and 2:2'-dipyridyl in ethanol solution. Tocopherols reduce the iron to the ferrous state; it then combines with the dipyridyl to form a red colour, which is measured in a spectrophotometer or electric colorimeter. The method may involve all, or most of, the seven stages enumerated below with their purposes:

(a) Solvent extraction of a representative sample with a lipid solvent for quantitative seperation of all tocopherols present, along with other fatty material

(b) Saponification of the lipoid extract and seperation from it of the unsaponifiable matter. The method is based on that of Tosic and Moore (9)

(c) Seperation of most of the steroid material by freezing from solution in methanol

(d) Column chromatography on floridin earth to remove carotenoids and residual steroids (6)

(e) Two dimensional paper chromatography (7) to separate the tocopherols into five zones, and individual elution

(f) Treatment of the eluates with the Emmerie-Engel reagent, the reading of the extinctions at 520 nm in a suitable instrument (4). The concentration of reagents is such that all the tocopherols give maximum or almost maximum colour in the short time (2 minutes) before measurement is made

(g) Calculation of tocopherol equivalents from extinctions by using the established factor for each tocopherol.

The seven stages enumerated above may not all be necessary. The most obvious omission is of stage (a) when the sample to be examined is a clean oil or fat. Stage (c) is only necessary for materials of low potency. Stage (d) can be omitted during the analysis of samples containing more than 5 to 10 mg of tocopherols per g of oil.

9.6.3 Reagents

The reagents used shall be of recognized analytical reagent quality.

9.6.3.1 Pyrogallol solution, 5% m/v in ethanol - Prepare this solution daily for each assay.

9.6.3.2 Potassium hydroxide solution - Dissolve 160 g of potassium hydroxide in 100 ml of distilled water.

9.6.3.3 Diethyl ether - Note: Normally, ether is stabilized by adding to it a small amount of a reducing substance that prevents peroxide formation. The reducing substance does not affect tocopherol analysis; it is removed during washing after saponification or during floridin earth purification. If, however, the ether has been in the laboratory for a long time, it may contain peroxides. These may be removed by the method of Werner (11). Shake 500 ml of ether for 6 minutes with a mixture of 4 g of silver nitrate in 30 ml of water and 2 g of sodium hydroxide in 50 ml of water, allow the phases to separate, decant and use the ether without distillation.

9.6.3.4 Ethanol, absolute - Purify by distillation in an all-glass apparatus over potassium permanganate and potassium hydroxide (1 and 2 g, respectively, per litre of ethanol).

9.6.3.5 Ethanol, diluted - Add 75 parts by volume of absolute ethanol to 25 parts by volume of distilled water.

9.6.3.6 Floridin earth XS - Specially selected for use in tocopherol determination. "Floridin earth XS for the determination of Vitamin E", (made by British Drug Houses Ltd.) or equivalent.

9.6.3.7 Stannous chloride

9.6.3.8 Hydrochloric acid, d = 1.18

9.6.3.9 Benzene

9.6.3.10 Cyclohexane

9.6.3.11 Zinc oxide

9.6.3.12 Ammonium hydroxide, d = 0.880

9.6.3.13 Ammonium carbonate

9.6.3.14 Sodium fluorescein - 0.1% m/v solution in water

9.6.3.15 Papers - Whatman No. 1 grade "for chromatography", or equivalent, in sheets 51.2 cm x 46.4 cm (22 1/2 inches x 18 1/4 inches).

9.6.3.16 Liquid paraffin solution - 3% m/v solution of liquid paraffin, British Pharmacopoeia, or equivalent, in light petroleum, boiling range 60° to 80°C.

9.6.3.17 Ferric chloride solution - Ferric chloride hexahydrate, 0.2% m/v in ethanol. Being extremely liable to photochemical reduction, this solution must be prepared directly in the amber bottle in which it is to be stored. The bottle should not be exposed even to moderate daylight and is best masked by a covering of black paper or black paint.

9.6.3.18 Dipyridyl solution - 0.5% m/v solution of 2:2'-dipyridyl in ethanol.

9.6.3.19 Phenolphthalein, ethanolic solution, 1% m/v, neutralized before use.

9.6.4 Apparatus

9.6.4.1 Columns for chromatography

As described under 9.6.5.3.1

9.6.4.2 Bi-dimensional chromatography apparatus

As described under 9.6.5.4.3.

9.6.4.3 Special glassware: 3.5 ml automatic pipette, Trenner pipettes, Agla micrometer syringes, chromatographic tubes for floridin earth columns and glass atomizers for spraying chromatograms (e.g. from V.C. Ward, Church Street, Little Shelford Cambridge), or equivalent may be used.

9.6.4.4 Spectrophotometer or photoelectric colorimeter suitable for reading extinctions at 520 nm.

9.6.5 Procedure

9.6.5.1 Saponification of the oil or the fat

Weigh accurately approximately 1 g of the oil or the fat into a round-bottomed flask (50 to 150 ml is a suitable size). Add 4 ml of the pyrogallol solution, and heat in a water bath, using a cold-finger condenser or a small reflux condenser fitted with a ground-glass joint. When the mixture boils, remove the condenser, and add 1 ml of the potassium hydroxide solution. Replace the condenser, immerse the flask in the boiling-water bath, and heat under vigorous reflux for 3 minutes, with occasional shaking.

Remove the flask from the water bath, cool the contents, and add 20 ml of distilled water. Extract the unsaponifiable matter with diethyl ether three times, using about 25 ml for each extraction. If the phases fail to separate quickly and sharply, add 1 or 2 ml of ethanol. If emulsions persist, consult Notes on Procedure 9.6.7.1. Wash the combined ether extracts with 20 ml portions of distilled water until neutral to phenolphthalein, avoiding vigorous shaking at first. Three or four washes are usually necessary. Transfer the ether solution to a round-bottomed flask, and remove the ether by evaporation in a

current of nitrogen, or under reduced pressure, while warming the solution on the water bath. Dry the residue, if necessary, by adding a little absolute ethanol and benzene and re-evaporating. Do not dry the solution with magnesium sulphate or sodium sulphate, because these chemicals may contain traces of iron. Dissolve the dry residue either in 5 ml of benzene for step 9.6.5.3 (or in methanol for step 9.6.5.2 if it is required to remove sterols).

9.6.5.2 Removal of Sterols

It is rarely necessary to remove sterols before chromatography. However, with extracts of certain materials, poor separation on paper may be due to the presence of sterols.

Dissolve the unsaponifiable residue in boiling methanol and transfer the solution to a 15 ml centrifuge tube, using, in all, 12 ml of solvent. Cool to about -10°C in a suitable bath, remove the precipitate by centrifugation in a previously cooled centrifuge cup, and pour the supernatant liquid into a flask. Redissolve the sterol precipitate in 5 ml of hot methanol, and repeat the process twice. Evaporate the combined methanol solutions to dryness under reduced pressure. Dissolve the residue in 5 ml of benzene.

9.6.5.3 Floridin earth separation

9.6.5.3.1 Apparatus - Columns are prepared in the usual type of chromatographic absorption tube, 12 to 14 mm internal diameter and about 160 to 200 mm long. Tubes must not have smaller dimensions than those indicated.

9.6.5.3.2 Preparation of column - Mix 5 g of floridin earth and 0.5 g of stannous chloride with 20 ml of hydrochloric acid, and bring to the boil. With rapid stirring, pour the slurry into a chromatographic tube in one continuous operation, so that the earth settles with an even gradation. After the liquid has passed through, wash the column successively with five portions, each of 5 ml, of ethanol, and then with five portions, each of 5 ml, of benzene.

9.6.5.3.3 Column chromatography - Pass the benzene solution from step 9.6.5.1 (or step 9.6.5.2) through the prepared floridin earth column. Elute the column with seven portions, each of 5 ml, of benzene, the complete elution taking not less than 45 minutes. Nitrogen under pressure or gentle suction may be used to hasten both the preparation of the columns and the chromatography, provided the latter process takes not less than 45 minutes. If total reducing substances are to be determined, an aliquot may be taken at this stage. It is safe to store the benzene solution overnight in a refrigerator.

9.6.5.4 Paper chromatography

9.6.5.4.1 Preparation of zinc-ammine solution - Dissolve 16 g of zinc oxide and 25 g of ammonium carbonate in a mixture of 150 ml of ammonium hydroxide and 600 ml of distilled water. Add 5 ml of sodium fluorescein solution. This zinc-ammine solution is indefinitely stable in a stoppered bottle.

9.6.5.4.2 Preparation of zinc carbonate impregnated paper - Because solvents move faster along the machine direction of filter-paper than across it, all the papers should be similarly orientated. The procedure described below ensures the most evenly coated papers with the minimum handling of the working portion; the position of the written data gives a clue to the machine direction of the paper. Cut the large sheets of paper into four equal parts, as indicated in Fig. 1. Pour the zinc-ammine solution into a large dish. Immerse each sheet separately in the solution to within about 5 cm of one short edge, and hang all the sheets to drain at room temperature for about an hour. The dry margin allows the use of clips for supporting the papers. Dry the papers, hanging vertically, at 95° to 100° for 3 hours in an oven with or without forced air circulation. Remove the dried papers, cut a 20-cm square from the coated portion of each sheet, and write the date close to the edge GH. The prepared papers may be stored for a few weeks in a thoroughly dry place, but they are soon spoiled by humidity.

Rule each paper lightly in pencil, as shown by the thin lines in Fig. 1. Punch holes in the corners, if necessary, for use on a frame.

Figure 1

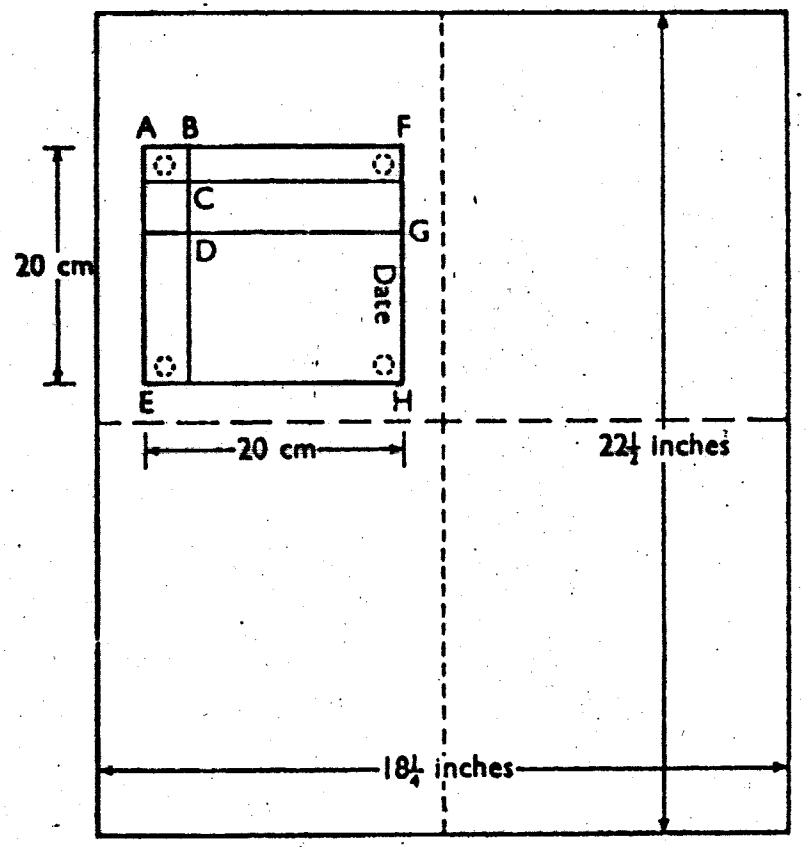

Fig. 1. Dimensions of zinc carbonate impregnated paper. Care should be taken to preserve the machine direction of the paper (as indicated on the package). AB = BC = 2 cm. CD = 3 cm. The letters need not be copied; they are inserted here only for explanatory purposes

Note I: Some batches of chromatographic paper have contained reducing material that raised the blank value. This may be removed by continuous extraction of the paper with hot methanol in a Soxhlet apparatus or by extraction and decantation in a measuring cylinder.

Note II: Zinc carbonate paper of sufficient activity can be prepared by drying in the atmosphere overnight (providing the relative humidity is not too high) instead of in an oven, but oven drying gives the more active paper.

9.6.5.4.3 Apparatus - Chromatography in both dimensions is done by the ascending-solvent method, and should be carried out in a glass tank large enough to hold the 20-cm x 20-cm papers in a vertical position. It is advisable to use a separate tank for each dimension. Alternatively, both runs may be carried out in the same tank; the necessary change of solvent must then be made before running the second dimension. The inside walls of each tank must be lined with a sheet of filter-paper, saturated with the mobile phase. The tanks must have gas-tight lids, and they should be shielded from strong light.

When less than four papers are run together, and provided that the chromatograms are to be examined later under ultra-violet light (method (a) of section 9.6.5.4.6 below), they may be supported in each tank by glass rods, arranged so that the papers hang evenly, with their edges dipping into the solvent. It is, however, more convenient to use some sort of two-dimensional chromatographic frame for carrying the papers, and this is recommended. If spot location is to be by spraying (method (b) of section 9.6.5.4.6 below), or if four or more papers are to be run together, such a frame is essential. Its use enables the papers to be manipulated easily and simultaneously during the various stages of the chromatographic separation, and ensures that the chromatograms are uniform and reproducible. Such frames have been described (3) and (10) and are suitably made of aluminium. Commercial frames are also available.

9.6.5.4.4 First dimension chromatography - Evaporate the solution from step 9.6.5.3.3 to dryness under reduced pressure or in nitrogen. Dissolve the residue in 0.5 to 3 ml of benzene in a stoppered flask, to give a solution containing 0.5 to 2.0 mg of tocopherol per ml, if possible (polar solvents

must not be used for preparing this solution). Set the tocopherol solution, by means of discrete additions of 10 to 20 μl as a narrow band across the starting line CD (Fig. 1), so that, in all, 5 to 30 μg of each tocopherol are present. The total solution set should not contain more than 100 μg of total tocopherols. Use three replicate papers for each test sample and a fourth paper as a blank. The additions should be made from a fine-tipped glass capillary, calibrated by weight, from a 10-μl blood pipette, from a Treuner pipette with a curved tip or from an Agla micrometer syringe (see 9.6.4.3). It is important to apply the solution as a regular band.

Into the first chromatographic tank pour enough 30% v/v benzene in cyclohexane to produce a depth of about 1/2 to 1 cm. Support test and blank papers with the dates at the top and their edges AE just immersed in the solvent. Leave the papers in the tank until the solvent front has migrated about 15 cm. This usually takes about 1 hour.

9.6.5.4.5 Second-dimension chromatography - If no frame is being used, remove the papers individually from the first tank and dip each one in the liquid paraffin solution, with edge EH at the bottom, to within 1/2 cm of the line DG. If a frame is used, remove it with the papers from the first tank, and, with edges EH at the bottom, immerse the assembly in a bath of the liquid paraffin solution to within 1/2 cm of the line DG. Remove the papers from the paraffin solution, drain, and allow the light petroleum to evaporate in the air. Then place the papers in the second tank with edge AF just immersed in the 75% ethanol as the mobile phase. Run the papers for 2 to 3 hours or until the front has migrated to within 4 or 5 cm from the top of the papers.

Remove the papers from the tank, and dry them by means of a current of nitrogen or air.

9.6.5.4.6 Removal of spots from chromatograms - Remove the papers from the frame, if one is used. Use either of two methods for spot location and removal for analysis. Method (a) is much to be preferred, but method (b) can be used when no ultra-violet lamp is available. Even when method (a) is used, it is still desirable to spray one of the papers as in the first part of method (b) in order to confirm the position of reducing spots.

(a) Examine the dry papers in a dark room under ultra-violet light; the lamp should not be nearer to the paper than 10 cm. Tocopherol spots are visible as dark patches on a fluorescing background, less than 1μg being easily detected (See Fig. 2). Mark the spots by ringing with a hard pencil as quickly as possible with a slight margin round each spot. Remove the paper, and cut out the marked spots with sharp scissors, cutting within the pencilled lines. With the minimum of handling, roll the cut-out pieces of paper into cylinders and drop each one separately into a numbered test-tube (measuring about 10 cm x 1.5 cm and fitted with a ground-glass stopper) containing 3 ml of ethanol and 0.5 ml of the dipyridyl solution. Mark the blank chromatogram correspondingly, and cut out the blank areas similarly.

Note I: A suitable lamp for this purpose is one emitting light with a waveband around 254 nm. A new lamp should be run for 2 or 3 hours before being put into use for ultra-violet location of tocopherols on paper. Other sources of ultra-violet illumination have been found suitable.

Note II: When large numbers of tubes are to be filled, it is convenient to use 3.5 ml of 0.07% m/v 2:2'-dipyridyl solution instead of 0.5 ml of 0.5% m/v dipyridyl solution plus 3 ml of ethanol. This solution is conveniently dispensed from a 3.5-ml automatic pipette.

(b) Spray one of the test papers heavily from a fine glass atomizer with a mixed reagent (equal parts of the ferric chloride solution and the dipyridyl solution). Tocopherols appear as red spots, which should be ringed with a hard pencil. If no ultra-violet lamp is available use these spots to locate tocopherols on the assay papers. Remove the sprayed spots and a generous margin by cutting outside the pencilled rings, and use the resulting series of holes as a template for marking another test paper, and a blank paper. Alternatively, trace the sprayed paper on to a clean sheet of thin paper, and use the latter as a template. Having marked the tocopherol positions, cut out the areas from both test and blank papers, and drop them into tubes containing dipyridyl solution, as in (a) above.

Figure 2

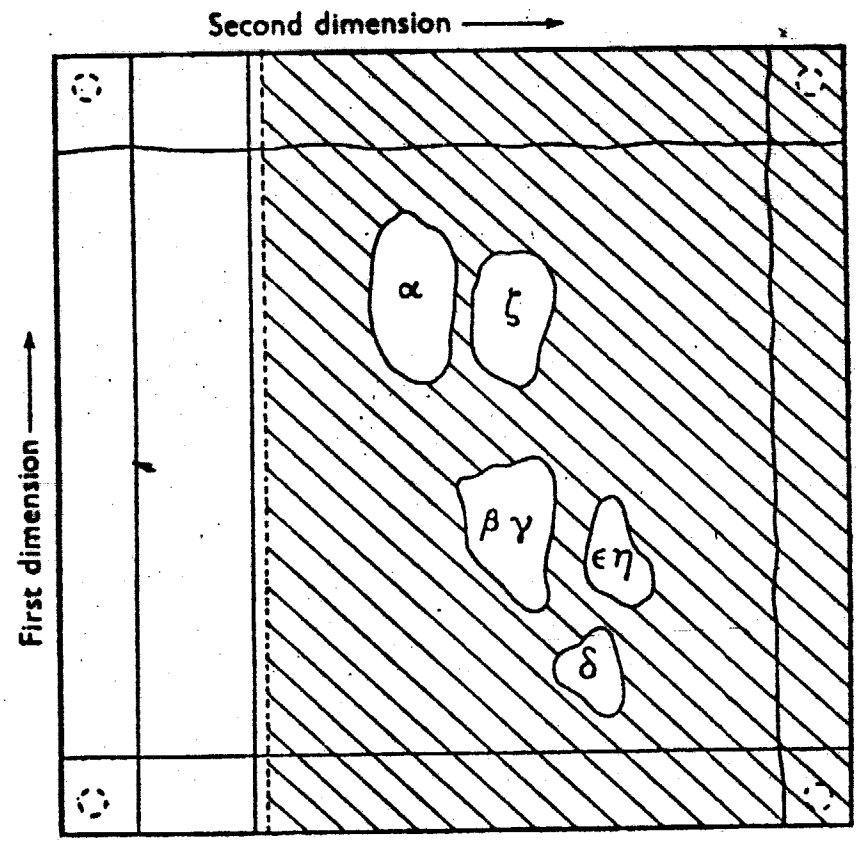

Fig. 2. Reproduction, one-third full size, from a paper, marked to show the positions of tocopherol zones after two-dimensional chromatography. The shaded area shows the part of the paper impregnated with paraffin after running in the first dimension. The wavy lines show approximate levels reached by the solvent fronts

9.6.5.5 Positions of the spots on the chromatogram

The seven tocopherols separate as shown in Fig. 2. Depending on the material being analysed, one spot or several spots may be visible. Although by this method, β - γ tocopherol are inseparable, as are ε - and η - tocopherol, instructions for their distinction are given under Note below. Other spots appearing on the papers are normally distinguished from the tocopherols by their positions. It must be emphasized that, depending on the degree of purification of the sample and the relative proportions of the different tocopherols present, some distortion of the movements of the spots may occur. Absolute R and R_F values may be disregarded, however; provided separation occurs according to the general pattern shown in Fig. 2, the next stage may be carried out. If separation is unsatisfactory, or recognition difficult, consult Notes on procedure (9.6.7).

Note: Detection of γ -, η -and δ -tocopherol by diazotised o-dianisidine

Sodium carbonate - a 5% m/v solution in water.

Diazotised o-dianisidine - Dissolve 0.5 g of o-dianisidine dihydrochloride in 60 ml of water. Add 6 ml of hydrochloric acid, d = 1.18, and then 12 ml of 5% m/v aqueous sodium nitrite solution. Mix thoroughly. After 5 minutes add 12 ml of 5% m/v aqueous urea solution.

Spray one of the test papers (it is advisable to run an extra paper if the presence of γ -, η - or δ -tocopherol is suspected) heavily with the sodium carbonate and then with the diazotised o-dianisidine from a fine glass atomiser. The tocopherols may be identified by the resultant colours: γ -, blue-green changing to indigo; η -, brownish purple changing to purple; δ -, reddish brown changing to purple. The colours may be stabilised by washing the papers in water after spraying.

The presence of colour in either the γ- or the η - position does not exclude the presence of β - or ε -tocopherol, respectively.

9.6.5.6 Colorimetric determination

Gently swirl the stoppered test-tubes, each containing 3.5 ml of ethanolic dipyridyl solution (9.6.3.18) and the slip of paper, for a few seconds. Transfer to a darkened room, and carry out the rest of the determination under dim artificial light. To one of the solutions add 0.5 ml of the ferric chloride solution (9.6.3.17) from a fast-delivery pipette, shake the stoppered tube for a moment, and then transfer the contents to a glass spectrophotometer cell 1 cm in path length. Exactly two minutes after adding the ferric chloride, measure the extinction at 520 nm against a similar cell containing ethanol. Repeat this procedure with each tube. Subtract the reading of each "blank" tube from the reading obtained from the corresponding "test" tube. This gives the "net extinction" reading. Multiplication of the net extinction reading by the spectrophotometric factor for the tocopherol being measured gives directly the number of micrograms of tocopherol in the cut-out spot.

9.6.5.7 Blanks

The tocopherol-containing solution has been indirectly measured against a corresponding blank solution. The highest accuracy can only be obtained, therefore, if it can be safely assumed that the blank reaction of the mixed reagents is identical in both "blank" and "test" tubes. The "blank" has two components, namely (i) that derived from the residual reducing material in the paper, and (ii) the reaction between the dipyridyl and traces of ferrous iron in the ferric chloride. Component (i) amounts to an extinction of about 0.010; component (ii) can be reduced to about 0.050 by suitable precautions, such as preparing and keeping the ferric chloride solution in the dark and the observance of scrupulous cleanliness in the use of the pipettes for ferric chloride. Ferric chloride is converted to the ferrous state photochemically; hence the solution should not be exposed to daylight in transparent glass. The total blank value can be reduced to about 0.060 to 0.080. Blanks that give extinctions of over 0.100 are likely to be unstable and should be suspect. If high blank values continue, and other forms of contamination have been eliminated, it is best to prepare a new ferric chloride solution in a dark bottle previously cleaned with chromic acid solution, thoroughly rinsed with distilled water and dried.

9.6.6 Calculation and expression of results

The amount of each tocopherol, in microgramme per gramme of product is calculated as follows:

$$\mu g \text{ tocopherol} = \frac{D \times F \times V \times 1000}{v \times M}$$

where:

M = mass in grammes of sample taken
V = volume in millilitres of benzene used to prepare solution for paper chromatography
v = volume in microlitres spotted on paper
D = net extinction
F = spectrophotometric factor for 4 ml

The recommended factors F are:

α -tocopherol	98
β -tocopherol	96
γ -tocopherol	90
δ -tocopherol	75
ε -tocopherol	96
ζ -tocopherol	94
η -tocopherol	88

The β - and ε -tocopherol are determinable when spots in their respective positions give no colour on spraying with the dianisidine reagent (See Note under 9.6.5.5). However, if the dianisidine reaction shows the presence of γ - or η - tocopherol the analyst may either assume that β - and ε - tocopherol are absent or attempt a differential analysis of the four substances by chromatographic separation of nitroso-derivatives, such as has been described by Marcinkiewicz and Green (7). Factors for γ - and η -tocopherol should be used even though β - or ε -tocopherol, or both, may be present but masked.

Results are expressed as milligrammes of each tocopherol per kilogramme of the product.

9.6.7 Notes on Procedure

Some difficulties may be encountered in the carrying out of the various phases of the analysis. They are covered in the following descriptions and suggestions thereto are given:

9.6.7.1 Saponification and extraction

After the saponification has been completed, the extraction of the tocopherols into ether must be carried out as rapidly as possible, the aim being to achieve clean, fast separation of the two phases at every stage. Sometimes emulsions are formed; they can usually be dispersed by adding a few millilitres of ethanol. If they persist, it may be because the directions have not been followed closely enough; the proportions of reagents are somewhat critical, and too much ethanol may even encourage the formation of emulsions. Vigorous shaking at any stage is unnecessary and should be avoided, especially as some products are rich in emulsifying agents.

9.6.7.2 Floridin earth chromatography

Occasionally a batch of floridin earth may be found with too slow a percolation rate for column chromatography. This fault is difficult to remedy; in practice it is simplest to procure another batch.

Carotenoids, retinol and its derivatives, sterols and some other substances are adsorbed on floridin, producing a green, blue or red band. Sometimes, when the eluate is yellow or the coloured band reaches the bottom of the column, subsequent paper chromatography may be unsatisfactory. This may be because the column was developed too fast; or the ethanol used in its preparation contained water; or possibly the column was overloaded, too large a sample of oil having been saponified. The "activity" or adsorptive power of the column depends chiefly on the fineness of the particles and the extent of dehydration produced by washing with ethanol. The conditions recommended in the method described here give an active column, suitable for removing most normal amounts of interfering substances. It may occasionally be found if an extremely active batch of floridin is used, that even 35 ml of benzene is insufficient to elute all the δ -tocopherol, which is the most strongly adsorbed tocopherol; if so, increase the amount of benzene to 40 or even 45 ml.

9.6.7.3 Paper chromatography

Exploratory techniques - An analyst first practising the method will find it a good plan to chromatograph first a solution of α - tocopherol and then a solution containing a known mixture of tocopherols. Reference solutions are best prepared in benzene and, for convenience, should contain about 1 mg of each tocopherol per ml. They may be kept in a refrigerator without appreciable deterioration.

In analysing a specimen of oil whose tocopherol content cannot be approximately guessed it is often advisable in step 9.6.5.4.4 to apply a different amount of the benzene solution to each of two or three papers. When the developed chromatograms are scanned, the one with the optimum amount of each tocopherol can be recognised easily after a little practice. It is sometimes useful to survey an unknown solution by a preliminary run on a single-dimensional zinc carbonate paper strip or, more rarely, to run a preliminary paraffin-coated strip. These preliminary runs give information about the tocopherols present and their separations. It must be emphasized that either single-dimensional procedure is for guidance only.

The use of tracers - Sometimes a lipid is encountered containing uncommon proportions of tocopherols or excessive amounts of unusual impurities. Such material may produce a distorted paper chromatogram, on which the spots are sufficiently displaced to make difficult the identification of tocopherols by position alone. If this occurs, a duplicate paper, to which small amounts of α -, γ - or δ -tocopherol have been added to act as tracers, may be of use. It should be prepared as follows: Add from a micro-pipette, along the starting line CD of chromatographic paper, about 10 μg of each tocopherol being investigated. The solutions of tocopherols in benzene, described above, are convenient for this purpose, 10 μl being a satisfactory amount to add to the paper. Then add the test solution in the usual fashion. It is important that the tracers be added first, to ensure their even solubility in the small amount of test solution. Prepare a test paper, omitting the tracers, and run both papers together. The tracer tocopherols will migrate to the same positions as those in the test solution when they are identical with them.

Unsatisfactory chromatograms - If the developed papers are still damp with aqueous solvent, observation of the spots under ultra-violet light is unsatisfactory.

If the tocopherols seem to have migrated too far in the first dimension or if they are poorly separated, it may be because the papers are stale; the zinc adsorbent deteriorates in time, especially in a humid atmosphere. If the tanks are shallow and are not lined with filter paper, excessive evaporation of solvent from the front may occur; this causes fast running and poor separation of bands. Alternatively, the solvent in the first tank may need modifying. It has been found that, under the conditions that obtain in some laboratories, 25% of benzene in cyclohexane is sufficient. If the tocopherols do not appear to have migrated enough, it may be necessary to increase the benzene to 35%. It was considered that 0.5% of ethanol in cyclohexane produces as good chromatograms as do the benzene-cyclohexane mixtures, but this percentage is somewhat critical and difficult to maintain in the tank. If the migration of the tocopherols in the second dimension is grossly abnormal, the solvent in the second tank may be at fault; it should be changed.

If papers show ill-defined spots of unexpected shapes or in the "wrong" places, it may well be that the papers have been overloaded, possibly with tocopherol, but more probably with other lipid materials. Spots that absorb ultra-violet light and do not reduce ferric chloride, as well as spots that fluoresce, are not tocopherols. If large amounts of interfering substances are observed on the paper, saponification may have been incomplete or the treatment with floridin earth ineffectual or the oil one requiring the "removal of sterols" step.

When lipids of very low potency (say, less than 0.05 mg of total tocopherols per g) are being analysed, two further difficulties in the paper chromatography are occassionally encountered, particularly if the lipids have been extracted from certain animal tissues. Even when purification according to the recommended method (including the "separation of sterols" step) has been performed, the residual tocopherol fraction may contain substantial amounts of hydrocarbons and other inert material. Because of the low tocopherol content of the sample, the fraction has to be added to the starting line of the paper in such an amount that not only does the first dimension become clogged for several centimetres along its length, but also an

immiscible barrier may form at the paraffin boundary along the line DG. Tocopherols may be trapped in the barrier; if this happens, no separation will take place. Sometimes the phenomenon leads to streaking over a large area of the paper. One device that has proved successful in dealing with many of these difficult materials is a modification of the floridin earth purification. After normal preparation of the column, including the wash with five portions of 5 ml benzene, pass 25 ml of light petroleum (40° to 60°C) through the column. Dissolve the tocopherol residue from step 9.6.5.1 (or, more usually with these difficult materials, step 9.6.5.2) in 5 ml of light petroleum, and add the solution to the top of the column. Develop the column with 25 ml of light petroleum. Discard the filtrate, which contains many of the interfering substances, and elute the tocopherols with the normal 30 ml of benzene.

9.6.7.4 Spectrophotometric factors

The factors given in the recommended method have been obtained as a result of careful study in several laboratories, and it is unlikely that the individual analyst will want to determine them for himself. The determination of tocopherols by the ferric chloride-dipyridyl oxidation method is affected slightly by conditions that vary from laboratory to laboratory; the recommended procedure has eliminated most of these effects as causes of variation. Those that remain - due to variations in temperature, humidity and purity of reagents - are not normally under control; in any event, the factors are not likely to vary by more than 2% between different laboratories. If the analyst should wish, for the most accurate work, to determine any factor in his own laboratory, he should procure a sufficiently pure specimen of the tocopherol concerned and carry out some determinations daily for a while at two or three different levels of tocopherol. In practice, he is most likely to be interested in the accurate determination of α-tocopherol.

9.6.7.5 General Precautions

The tocopherols are reasonably stable substances. They are most vulnerable to oxidation in the presence of traces of alkali, and the necessity for care and speed during the saponification stage has already been stressed. Strong light, abnormally high temperatures and the presence of ozone or ammonia in the air have all been found to affect the tocopherols

during paper chromatography. Chromatograms should not be left in the dry state for longer than necessary. For useful information on paper chromatography, including hints on cleanliness in handling papers, consult Mitchell (12).

If an analysis cannot be completed in a day, it is advisable to interrupt the procedure after saponification and extraction, or after floridin treatment, and to store the benzene solution in a refrigerator.

9.6.8 Literature references

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9.7 Determination of sodium chloride content CAC/RM 19-1969

9.7.1 Principle of method

After melting the margarine by adding boiling water, the chlorides in the mixture are titrated with a standard solution of silver nitrate, using potassium chromate as indicator, according to the Mohr procedure.

9.7.2 Reagents

The reagents used shall be of recognized analytical reagent quality.

9.7.2.1 Silver nitrate solution, 0.1 N, standardized.

9.7.2.2 Potassium chromate solution 5% (m/v) in distilled water.

9.7.3 Apparatus

9.7.3.1 Analytical balance

9.7.3.2 Conical flasks, capacity 250 ml

9.7.3.3 Burette, graduated to 0.1 ml

9.7.4 Procedure

9.7.4.1 Preparation of the sample

Soften sample in closed sample container by warming in water bath kept at as low a temperature as practicable in order not to break the emulsion. Shake sample container at frequent intervals during the softening process to thoroughly mix sample. Remove sample container from water bath and shake vigorously at frequent intervals until sample has cooled to a thick, creamy consistency. A mechanical shaker may be used.

9.7.4.2 Blank test

Do a blank determination using the same reagents in the same amounts and according to the same procedure as described under 9.7.4.3, excluding the sample.

9.7.4.3 Determination

Weigh, to the nearest 0.01 g approximately 5 g of the sample into the conical flask. Carefully add 100 ml of boiling distilled water. Allow to stand for 5-10 min., swirling occasionally, while cooling to 50-55°C (titration temperature). Add 2 ml of the potassium chromate solution (9.7.2.2). Mix by swirling. While swirling continuously, titrate with the silver nitrate solution (9.7.2.1) until the colour change to orange-brown persists for 30 sec.

9.7.5 Calculation and expression of results

9.7.5.1 The sodium chloride content (expressed as percentage m/m NaCl) is:

$$\frac{5.85\ N\ (V_1 - V_0)}{m}$$

where:

N = normality of the silver nitrate solution.

V_1 = number of ml of silver nitrate solution used in 9.7.4.3.

V_0 = number of ml of silver nitrate solution used in 9.7.4.2.

m = mass, in grammes, of sample used.

Round off the result to the nearest 0.01%.

9.7.5.2 Repeatability

The difference between results of duplicate determinations (results obtained simultaneously or in rapid succession by the same analyst) should not exceed 0.02 sodium chloride for 100 g of the product.

9.7.6 Literature references

Report of the Tenth Session of the Joint FAO/WHO Committee of Government Experts on the Code of Principles concerning Milk and Milk Products (Ref. No. SP-10/105, 10th Session, September 1967, Appendix IV-D).

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ACCEPTANCE PROCEDURE FOR CODEX COMMODITY STANDARDS

EXTRACT OF THE GENERAL PRINCIPLES OF THE CODEX ALIMENTARIUS

ACCEPTANCE OF CODEX COMMODITY STANDARDS

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

i) Full acceptance

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

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

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

ii) Target acceptance

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

iii) Acceptance with minor deviations

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

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

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

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

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

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

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

ii) Where, in an importing country, a product claimed to be in compliance with a Codex standard is found not to be in compliance with that standard, whether in respect of the label accompanying the product or otherwise, the importing country should inform the competent authorities in the exporting country of all the relevant facts and in particular the details of the origin of the product in question (name and address of the exporter), if it is thought that a person in the exporting country is responsible for such non-compliance."

J. Nutr. 27: 193-211, 1943

STUDIES ON VITAMIN E DEFICIENCY IN CHICKS[1]

HENRIK DAM

Departments of Anatomy and Biochemistry, University of Rochester School of Medicine and Dentistry, Rochester, N. Y.

FIVE FIGURES

(Received for publication August 23, 1943)

The two main symptoms of vitamin E deficiency in chicks are exudative diathesis and encephalomalacia. They may appear separately or concomitantly depending upon the composition of the diet. The experiments reported here were designed to examine the manner in which modification of various components of vitamin E deficient diets may influence these symptoms, in the hope that such observations might throw additional light upon the physiological rôle of vitamin E.

In order to evaluate the results of these studies it seems necessary to outline the chief characteristics of these two symptoms and the existing state of our knowledge concerning them.

Exudative diathesis, a condition in which plasma exudes from the capillaries, appears as massive accumulations of fluid in the subcutaneous tissue or as edema of muscles and connective tissue more generally (Dam and Glavind, '38).[2] The affected tissue, especially the adipose tissue, is usually reddish due to fine diffuse hemorrhage. Microscopically there is edema, slight extravasation of red cells and migration of white cells in the affected tissue, and a mild eosinophilia (Dam and Glavind, '39b). The extravasated fluid, which is often greenish from decomposition products of hemoglobin, has approximately the same composition as that of normal plasma. An augmented capacity of the tissue to take up trypan blue intravenously injected (Dam and Glavind, '40) suggests an increased permeability of the capillaries. Since the specific

[1] Aided by a grant from the Josiah Macy, Jr. Foundation.

[2] The fact that vitamin E is the factor which protects against the exudative diathesis has been well established (Dam and Glavind, '39a, '39b; Bird and Culton, '40; Dam, Glavind, Prange and Ottesen, '41). A level of 10 mg. of d, l-α-tocopherol acetate mixed with 100 gm. of the diet always affords sufficient protection. β-tocopherol is much less active against this disease than α-tocopherol. During the study of the distribution of the anti-exudate factor in foods it was found that the lipid of rose hips (Rosa rugosa) is approximately ten times as rich in vitamin E as wheat germ oil (Dam, Glavind, Prange and Ottesen, '41).

gravity of the serum is normal[3] it seems unlikely that a decrease in serum proteins contributes to the appearance of the exudates. Plasma prothrombin and the thromoplastin in the affected tissue are normal. There is no decrease in osmotic resistance of the red cells, no increase in sedimentation rate, and no elevation of body temperature (Dam, unpublished studies).

Although individual chicks of an experimental group as well as different shipments of chicks may vary somewhat with respect to the appearance and severity of the symptoms, the basal diet 182 (table 1) used in most of the present studies usually produces exudates within 14 to 30 days in chicks 1 day old at the beginning of feeding. The exudates generally appear first in adipose tissue, later affect muscles, and are rare in the pericardial and peritoneal cavities. They may disappear spontaneously and recur later in the same chick with no change of the dietary procedure. Absorbed exudates usually leave a buff coloration in the adipose tissue, and sometimes leave a necrotic degeneration of breast and leg muscles leading to permanent invalidism. Severe exudates may be associated with deep and forced respiration. Encephalomalacia occurs occasionally. Chicks grow somewhat subnormally on this diet, attaining weights of 120 to 160 gm. in 4 weeks.

Chicks showing spontaneous disappearance of the symptoms and continued for longer periods on the diet often die with a perforating ulcer of the gizzard, which is unrelated to vitamin E and can be prevented by other modification of the diet.

Using a somewhat different type of diet (table 1), Bird and Culton ('40) report the exudative diathesis to be associated with a considerable accumulation of fluid in the pericardium and peritoneal cavity, indicating that the degree and localization of the exudation may be influenced by the makeup of the diet. The addition of 5% cod liver oil to the Bird and Culton diet has produced, in the author's laboratory, essentially the same symptoms as observed when chicks were fed the exudate-producing diet 182.

Encephalomalacia, the other symptom, was first observed by Pappenheimer and Goettsch ('31).[4] Chicks reared on their diet 108 (table 1)

[3] Fasting values of 1.0154, with a standard deviation of 0.0006, for five 40-day-old chicks receiving the exudate-producing diet, compared with 1.0157, with a standard deviation of 0.00015, for ten 34-day-old chicks on a commercial chicken diet.

[4] The evidence for the relation of encephalomalacia to vitamin E deficiency rests on the finding of a protective factor in the non-saponifiable fraction of various lipids of vegetable origin (Goettsch and Pappenheimer, '36). When synthetic d, l-α-tocopherol became available this substance was found to protect (Dam, Glavind, Bernth and Hagens, '38; Pappenheimer, Goettsch and Jungherr, '39).

become atactic, may fall when trying to walk, get cramps and carry out uncoordinated movements with legs, wings and neck. Frequently they lie on the side with legs stretched or assume a sitting position with the back supported against the wall of the cage. Upon touching, violent movements with legs ("bicycling") and wings occur. In milder cases the birds are only slightly atactic or sit with the body raised and slightly

TABLE 1

	BASAL DIET NO. 32[1]	BASAL DIET NO. 182[1]	BIRD AND CULTON'S DIET[7]	PAPPENHEIMER AND GOETSCH DIET 108[8]	BASAL DIET NO. 190
Casein, unextracted	...	...	...	22.8	...
Casein, alcohol-extracted	15	15	...	...	15
Skimmed milk powder	...	...	54	16.7	...
Dried yeast, unextracted[2]	...	...	...	5.6	...
Dried yeast, ether-extracted	10[2]	10[2]	...	...	10[2]
Corn starch, unextracted	...	...	...	22.2	...
Corn starch, alcohol-extracted	...	...	...	...	24.6
Sucrose	59.8	54.6	44	...	...
Gelatine	8	8	...	...	...
Gum arabic	5	5	...	...	5
Salt mixture	2[3]	7.2[4]	1.1332[5]	7.2[10]	7.2[6]
Ground limestone	...	...	1	...	...
l-cystine	0.1	0.1	...	...	0.1
Choline chloride	0.1	0.1	...	...	0.1
Lard	...	...	...	23.3[4]	30[4]
Cod liver oil	5[4]	5[4]	fed 3 × weekly	2.2[4]	...
Vitamin K substitute	1 mg.[5]	1 mg.[5]	...	...	1 mg.[5]
Vitamin A, D concentrate	...	...	...	...	1 drop 2 × weekly[11]

[1] This diet is deficient in the peptic ulcer factor (cf. text).

[2] Fleischmann type 2019. Ether-extraction hastens the onset of the symptoms considerably when this type of yeast is used. The Carlsberg yeast used in Copenhagen apparently needed no ether-extraction.

[3] Salt mixture no. 3. 2 gm. consists of: $CaCO_3$ 1457.8 mg., $MgCO_3$ 72.89 mg., NaCl 320.5 mg., Ferric citrate 116.5 mg., Diiodotyrosine 0.018 mg., $CuSO_4$, $5H_2O$ 10.92 mg., $MnSO_4$, $4H_2O$ 21.84 mg.

[4] Added fresh to the diet every day.

[5] 2-methyl-1, 4-naphthohydroquinone diacetate or the tetra-sodium-salt of 2-methyl-1, 4-naphthohydroquinone-diphosphoric acid ("Synkavite, Roche").

[6] Consists of 7.16 gm. of McCollum's salt mixture no. 185, 0.966 mg. KI, 9.93 mg. $CuSO_4$, $5H_2O$ and 39.8 mg. $MnSO_4$, $4H_2O$.

[7] This diet should be supplemented with vitamin K.

[8] Consists of NaCl 1 gm., $MnSO_4$, $4H_2O$ 0.012 gm. and $CuSO_4$, $5H_2O$ 0.0012 gm.

[9] This diet should be supplemented with vitamin K and $MnSO_4$. It gives a marked perosis when fed without added manganese. In the formula given in table 1 the roughage (10% paper pulp) has been omitted, because it has no influence on the experiments.

[10] McCollum's salt mixture no. 185.

[11] Consists of: vitamin A concentrate from fish liver oil (containing 10^6 units per gram) 0.200 gm., 0.300 gm., vitamin D concentrate (Delsterol, containing 200,000 chick units per gram) 0.200 gm., oleic acid 24,500 gm. The concentrates were obtained from Distillation Products, Inc., Rochester, N. Y. 1 drop (29 mg.) represents 350 units A and 46 chick units D.

swaying. Forced breathing may be apparent. Localized or diffuse areas of edema, fine hemorrhages, and necrosis are usually seen in the cerebellum or the cerebrum. The extent or magnitude of these lesions is not always related to the severity of the symptoms observed. According to Pappenheimer and Goettsch ('31) the cerebellar lesions are characterized histologically by edema, with disorganization of the fibrillar and cellular elements, degeneration of the Purkinje cells and those of the granular layer, small hemorrhages scattered in both white and gray matter, and hyaline thrombosis of capillaries in and around the necrotic areas. The authors stress the capillary thrombosis as the primary cause of the necrotic lesions.

As in the case of exudates, spontaneous recovery with or without recurrent attacks, a buff coloration persisting for some time at the site of earlier lesions, and subnormal body growth are common phenomena in chicks fed the encephalomalacia-producing diet 108; while exudative diathesis rarely appears and no peptic ulcers occur. The water content of the affected parts of the brain is increased (Pappenheimer, Goettsch and Jungherr, '39). A reported decrease in cholesterol content (Adamstone, '41) must be taken with some reservation since the values given for normal chick brain are considerably lower than those generally recorded for brain tissue.

METHODS OF RECORDING SYMPTOMS

One-day-old white leghorn chicks, ten in each experimental and in each control group, were reared in ordinary brooders with raised screen floors and inspected daily, including Sunday, for the appearance of symptoms. The latter are recorded in the graphs as follows. Abscissae indicate age in days, ordinates the number of animals showing symptoms within a given number of days; giving one stair-case curve for exudate (full line) and another for encephalomalacia (broken line), each beginning from the bottom line. In order to give a more accurate representation of the development of the symptoms, exudates are graded by recording a reddishness of the fat tissue which is visible through the skin but not yet accompanied by a palpable exudate as "½ exudate", and adding another "½ exudate" for the same chick when a palpable exudate appears. One animal can be represented by only 1 unit on the ordinate since it is only the beginning of the symptom (eventually divided in two stages) and not its duration or recurrence which is recorded. Animals dying without showing exudate or encephalomalacia are recorded by full lines and broken lines, respectively, moved

down 1 unit from the top (or ½ unit if the animal has had "½ exudate"). The end of each experiment is indicated by a black triangle. In only a few instances were symptoms found at autopsy which could not be observed in vivo. Experiments carried out simultaneously are grouped together in one series. Weight charts are not given but in cases where changes of the diet caused marked changes in body weight specific mention of this effect is made in the text, since one might expect that slow growth would retard the symptoms and rapid growth accelerate them.

FACTORS AFFECTING THE APPEARANCE OF EXUDATES

The salt mixture

The earlier experiments on exudative diathesis (Dam and Glavind, '38, '39a, '39b) were carried out with a lower salt content than that indicated in diet 182; namely, 2% of salt mixture no. 3 (cf. diet 32, table 1), which only approximately supplements the inorganic constituents of 10% yeast and 15% casein. As shown in series 1, the symptoms came much later with such a diet (exp. 3) than with the basal diet 182 containing 7.2% of McCollum's salt mixture no. 185 supplemented with Cu and Mn (exp. 1) or after the addition of sufficient NaCl to diet 32 to give the mineral constituents about the same osmotic concentration as in diet 182 (exp. 4; see also Dam and Glavind, '42).[5] The accelerating effect of NaCl, also observed by Bird ('43), is in agreement with the common clinical experience that salts which tend to enter the extracellular fluids enhance a tendency to edema. On the other hand, the addition of KCl (a salt which does not accumulate in the extracellular fluid) in the same molecular concentration had a slight retarding effect (exps. 8 and 9, series 2), which may or may not be of significance.

In experiments not recorded in the graphs, addition of 5% NaCl to the low salt diet 32 at the fifty-second day of feeding regularly precipitated (within 3 days) pericardial exudates not observed in those members of the group given no additional NaCl. A few instances of such exudates were induced by 5% NaCl even when the diet contained amounts of d, l-α tocopherol (10 mg. %) usually sufficient to prevent exudative diathesis. The pericardial type of exudate appearing rapidly after the feeding of high NaCl is definitely unlike the exudative symptoms developing more slowly on diets containing a more normal salt content.

[5] It will be noted that the experiments recorded in series I took a much longer time than all the following experiments. The reason for this is that in the first series of experiments the yeast was not extracted with ether.

Effect of histamine, etc.

Addition of traces of histamine (25 mg. per kilogram) to the exudate-producing diet with low salt content accelerated the symptom (exp. 5), yet single intravenous injections of shock-producing but sublethal doses of histamine (1 ml. of a 1.25% solution in 0.9% saline) or peptone (5.5 ml. of a 20% solution in 0.9% saline) per 300 gm. body weight failed, even though a transient edema was produced by peptone injection. Addition of histamine (100 mg. per kilogram of diet) did not accelerate the symptoms when there was no fat in the diet.

Symptoms in birds receiving the low salt diet were not accelerated by pinching wings or legs, or by injecting (subcutaneously, intramuscularly or intravenously) 1–2 ml. of the exudate, or by repeatedly injecting 1 ml. of a solution of 1% casein in alkali adjusted to pH 7.3.

Type of carbohydrate

In other experiments not recorded in the graphs the 54.6% sucrose in diet 182 was replaced by either fructose, glucose, cornstarch, alcohol-extracted cornstarch, lactose or galactose. Sucrose and fructose and perhaps glucose gave somewhat more severe symptoms than cornstarch. The effect of the latter was apparently unaffected by alcohol extraction, which removes about 0.6% of partly fat-soluble material. Neither exudates nor encephalomalacia were observed when the diet contained 54.6% lactose, a sugar which is poorly absorbed from the intestine of chicks (cf. Lenkert and Becker, '39), gives poor growth (average weight 69 gm. at 4 weeks) and causes profuse diarrhea when given in such high amounts. Galactose at the same level induces severe cramps and death within a week, associated with high blood sugar (but without any significant change in fermentable blood sugar or muscle glycogen) and almost complete absence of liver glycogen. However, a moderate amount of galactose (10% for instance) can be incorporated in diet 182 without harm and without influencing the symptoms. During the feeding hours sucrose, fructose, lactose and galactose gave reducing sugars in the urine, collected after ligation of the large intestine just superior to the cloaca and rinsing of the latter.

Type of protein

Alcohol-extracted meat meal has been used instead of alcohol-extracted casein with the same effect (Dam and Glavind, '39b). Although extraction of the casein is not absolutely necessary, it increases somewhat the incidence of exudates. Commercial desiccated

chicken eggwhite, non-extracted or extracted with ether or alcohol, and supplemented with biotin[6] proved unsuitable for the development of exudative diathesis, the reason for which is not clear. Since addition of casein (2%) to the eggwhite diet did not increase the incidence of the symptoms, the role of a foreign protein in their production seems unlikely.

The protein-carbohydrate ratio definitely influences the tendency for the exudates to appear. With cornstarch replacing sucrose in the basal diet 182, a casein-cornstarch ratio of 15% : 54.6% (exp. 10) caused an earlier onset of exudates than a ratio of 7.5% : 62.1% (exp. 11). It is possible that retarded growth on the low protein diet, giving an average weight of 74 gm. at 4 weeks compared with 158 gm. for the diet with 15% casein, contributed to the slower development of the symptom, but a casein-starch ratio of 35% : 34.6% likewise retarded the exudates (exp. 12) even though growth was slightly improved (to 166 gm. at 4 weeks). There is apparently a certain protein-carbohydrate ratio which is optimal for the development of the symptom. As was the case in the experiments with the salt mixture, this finding may also have its parallel in clinical medicine where it is common experience that a low carbohydrate diet tends to counteract a tendency to edema.

How far the yeast or other natural source of the B-complex such as skimmed milk powder can be replaced by a mixture of the known B-vitamins without influence on the symptom remains to be investigated.

Type of fat

The fat content of the diet exerts a marked influence on the appearance of both types of symptoms. Their reported acceleration by fat (Dam, Glavind, Prange and Ottesen, '41), attributed to the highly unsaturated fatty acids present (Dam, '43), is amply confirmed by the present studies (series 5). Replacing cod liver oil in diet 182 with a concentrate of vitamins A and D[7] caused no symptoms (exp. 15), whereas with 5% cod liver oil (exp. 16) and the fatty acid fraction of 5% cod liver oil (exp. 17) the symptoms came as usual; the non-saponifiable fraction having no such effect (exp. 18). Also ineffective was 5%

[6] Smaco no. 200, from S.M.A. Corporation, Chagrin Falls, Ohio.

[7] In the experiments recorded in series 4 all groups of chicks received concentrates of vitamins A and D by pipette twice weekly, the amount corresponding to 68 units of A and 5 chicken units of D dissolved in 7 mg. cod liver oil per animal per day. In all other series which included diets without cod liver oil a similar or a higher amount of A and D was given in the same manner, the solvent being either cod liver oil or oleic acid in the amount mentioned. It appeared to be of no importance which solvent was used as long as the amount was very low

commercial oleic acid freed from traces of non-saponifiable matter (exp. 20), whereas fatty acids from linseed oil similarly purified resembled cod liver oil fatty acids in their effect (exp. 19). The observation, not recorded in the graphs, that the addition of 5% cod liver oil to the diet of chicks reared for 4 weeks on diet 182 minus this oil precipitated exudates 4 to 7 days later indicates that a certain time is required to induce the capillary damage. The symptoms produced by 5% lard were slower in their appearance but no less severe than those induced by 5% cod liver oil (exps. 13 and 14). It has further been observed that 5% commercial C_{20} unsaturated fatty acids[8] (iodine value 235) also accelerate exudates even though they retard body growth (to 83 gm. at 4 weeks). There seems to be little doubt, therefore, that it is the highly-unsaturated fatty acids which accelerate the exudative diathesis.[9]

It is well known that cod liver oil exerts a muscular dystrophy-inducing action in rabbits which is accentuated by rancidity changes in the oil and reduced or abolished by hydrogenation (McCay, Paul and Maynard, '38; Mattill and Golumbic, '42; Mackenzie, Mackenzie and McCollum, '41), leading to the hypothesis that auto-oxidation of the fatty acids in cod liver oil destroys traces of vitamin E in the diet (and perhaps also in the body). That cod liver oil exerts a direct damage on tissues seemed unlikely, since muscular dystrophy occurs in rabbits fed an E-deficient diet exceedingly low (0.05%) in animal fats (Mackenzie, et al., '41). Since fat in the diet is prerequisite to the marked development of E-deficiency symptoms in chicks, and since previous studies (Dam, '43) indicate that the exudate-inducing action of fresh cod liver oil is unchanged by slight rancidity but abolished by thorough rancidity, the effect of fats and of rancidity upon dystrophy in rabbits appears to have no parallel in the exudative diathesis in chicks.

Additional studies reported here, carried out under slightly different conditions, further confirm these findings. Without cod liver oil no symptoms occurred (exp. 23). With fresh cod liver oil (iodine value 173) exudates developed as usual (exp. 24). With cod liver oil made thoroughly rancid in the diet by exposing the latter in thin layers for 1 week at 50°C., reducing the iodine value to 62, no symptoms occurred (exp. 25). Separate daily addition of the yeast to the diet after rancidity was developed gave the same result (exp. 26). Likewise, no symptoms appeared when the diet contained cod liver oil which had been heated in a rapid current of air at 100°C. for 1 week, reducing the iodine value

[8] Obtained from Armour and Company, Chicago.

[9] The basal diet 182 minus cod liver oil, which contains only traces of fat in the extracted yeast and casein (Mackenzie et al., '39), has produced exudates in only 3 out of 100 chicks. Two of these exudates were not accompanied by reddening of adipose or muscle tissue.

to 86 (exp. 27). Although in the latter experiment polymerisation of the oil may have occurred and interfered with its intestinal absorption, this series of experiments clearly demonstrates that thorough rancidity of the cod liver oil developing in the diet abolishes its accelerating effect on the symptoms.[10] This effect of cod liver oil (or highly unsaturated fatty acids) on exudative diathesis must therefore be due to changes independent of destruction of traces of vitamin E in the diet through rancidity of the oil. The possibility that rancidity of the oil leads to the formation of a factor which protects against the symptoms is excluded because the addition of 5% fresh cod liver oil to the rancid diet resulted in appearance of exudates just as when no rancidity was present (exps. 28 and 24). The general trend of these experiments is in agreement with the observations that various hydroquinones which may act as antioxidants do not prevent the exudative diathesis in chicks (Bird, '43) and that the antioxygenic activity of a given compound is not a measure of its vitamin E activity (Golumbic, '41). It is also in accord with the fact that cod liver oil has been used as solvent for free tocopherol in experiments with chicks (Bird and Culton, '40) and with rats (Bacharach et al., '37; K. E. Mason, personal communication) without destroying the protective effect of the tocopherol.[11]

Cholesterol and lipotropic substances may influence the effect of cod liver oil on the exudates (Dam and Glavind, '42). Thus the addition of 1% cholesterol to diet 32, which in itself gives a slow development of the symptoms, will cause the symptoms to appear more rapidly, whereas addition of 1.5% inositol or 2% lipocaic[12] to diet 182 resulted in marked retardation. The experiments on which the preliminary report was based are presented in series 1 (exps. 2 and 6), and series 7 (exps. 29 to 31). It was found later that cholesterol had no accelerating effect when cod liver oil was omitted from the diet. It has also been found that mannitol, sorbitol, and dulcitol do not possess the exudate-retarding property of inositol.

[10] On an earlier occasion Dam and Glavind ('39) thought that rancidity might favor exudates because, in a period where the diet unintentionally had become rancid due to lack of cleanness, chicks which received a supposedly vitamin K free diet showed exudates before hemorrhages were observed. However, this must have been due to the fact that the rancidity was not complete and that simultaneous putrefaction of the casein delayed the decline of prothrombin so that the exudates appeared before the hemorrhages.

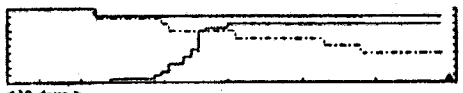
[11] That tocopherol acetate can be incorporated in diets with 5% cod liver oil without losing its protective effect is not surprising because the acetate is less easily oxidized than the free compound.

[12] From Eli Lilly and Co., Indianapolis. The absence of tocopherol from the lipocaic preparation was ascertained by hydrolysis of 2 gm. with 60% KOH in absence of air extraction washing and evaporating under the same conditions and carrying out the Emmerie and Engel test as described by Dam, Glavind, Prange and Ottesen ('41).

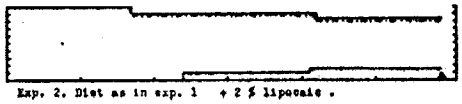
FACTORS AFFECTING THE APPEARANCE OF ENCEPHALOMALACIA

The Pappenheimer and Goettsch diet 108, hitherto used extensively for the study of encephalomalacia, contains large amounts of lard and moderate amounts of carbohydrate in the form of cornstarch. It also contains lactose from skimmed milk powder but, as previously mentioned, this carbohydrate is poorly utilized by chicks and, therefore, can be left out of consideration. With this in view it is possible to modify the exudate-producing diet 182 so that encephalomalacia appears as the chief symptom. Series 9 shows experiments of this kind.

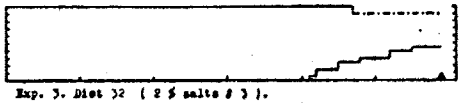
SERIES 1.



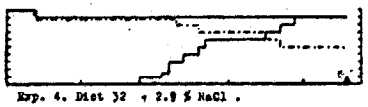
<10 days>
Exp. 1. Diet 182 with unextracted yeast.



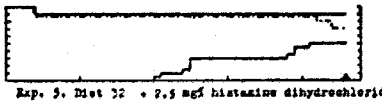
Exp. 2. Diet as in exp. 1 + 2 % lipocaic.



Exp. 3. Diet 32 (2 % salts # 3).

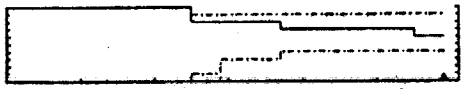


Exp. 4. Diet 32 + 2.9 % NaCl.



Exp. 5. Diet 32 + 2.5 mg% histamine dihydrochloride

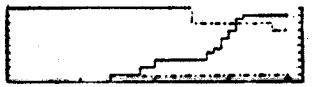
Exp. 6. Diet 32 + 1 % cholesterol.



Exp. 7. Diet 32 with dried hog liver instead of casein.

iver oil was omitted in order to make the experiments simpler ne variations of the diet consisted in changing the lard and carbo- te as indicated in the legend, the other constituents being un- d.

SERIES 2

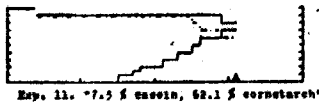


<10 days>
Exp. 8. Diet 32 (2 % salts # 3).

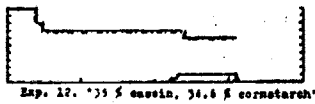
Exp. 9. Diet 32 + 3.4 % KCl.

SERIES 3.

Exp.10. " 15 % casein, 54.6 % cornstarch "

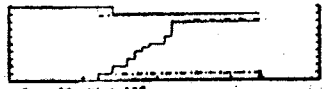


Exp. 11. "7.5 % casein, 62.1 % cornstarch"

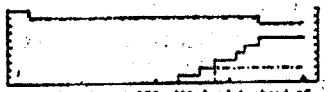


Exp. 12. "35 % casein, 34.6 % cornstarch"

SERIES 4.

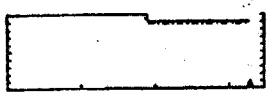


Exp. 13. Diet 182.



Exp. 14. " Diet 182 with lard instead of cod liver oil"

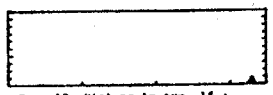
SERIES 5



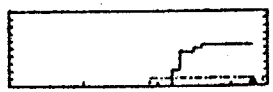
<10 days>
Exp. 15. Diet 182 without cod liver oil.

Exp. 16. Diet 182 (5 % cod liver oil).

Exp. 17. Diet as in exp. 15 + 4.8 % fatty acids from cod liver oil.



Exp. 18. Diet as in exp. 15 + non-saponifiable fraction from 5 % cod liver oil.



Exp. 19. Diet as in exp. 15 + 4.8 % fatty acids from linseed oil.

Exp. 20. Diet as in exp. 15 + 5 % oleic acid.

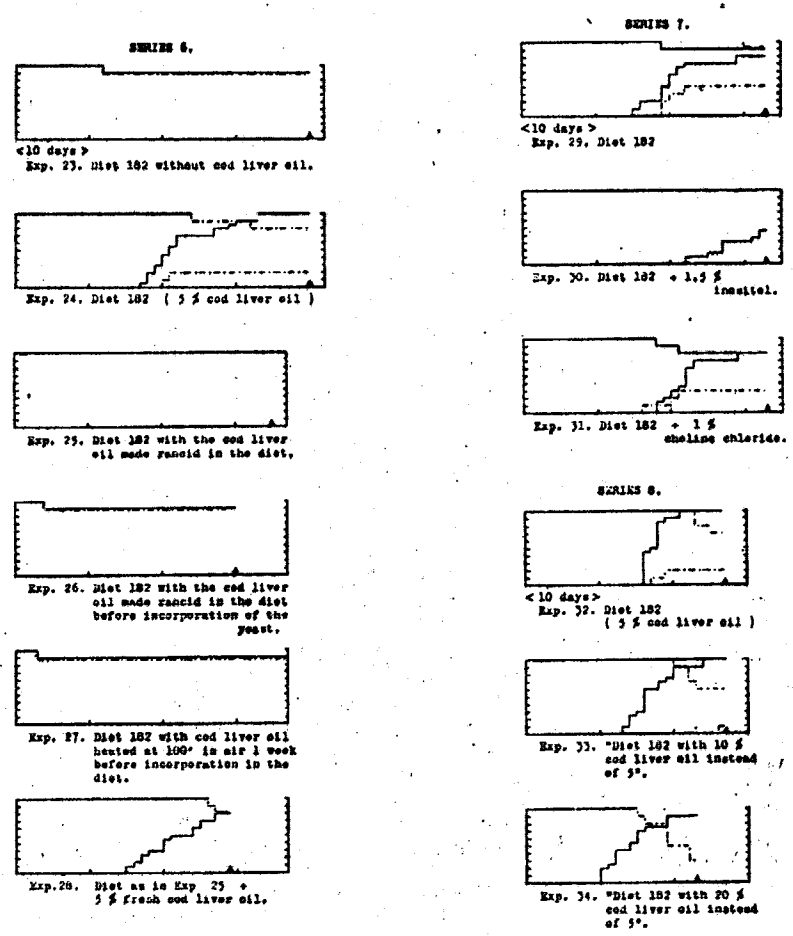
Exp. 21. Diet as in exp. 15 + 6.5 % hog liver fat.

Exp. 22. Diet as in exp. 15 + 4.8 % fatty acids from hog liver fat.
(.... = intropericardial exudates)

Fat, protein and carbohydrate

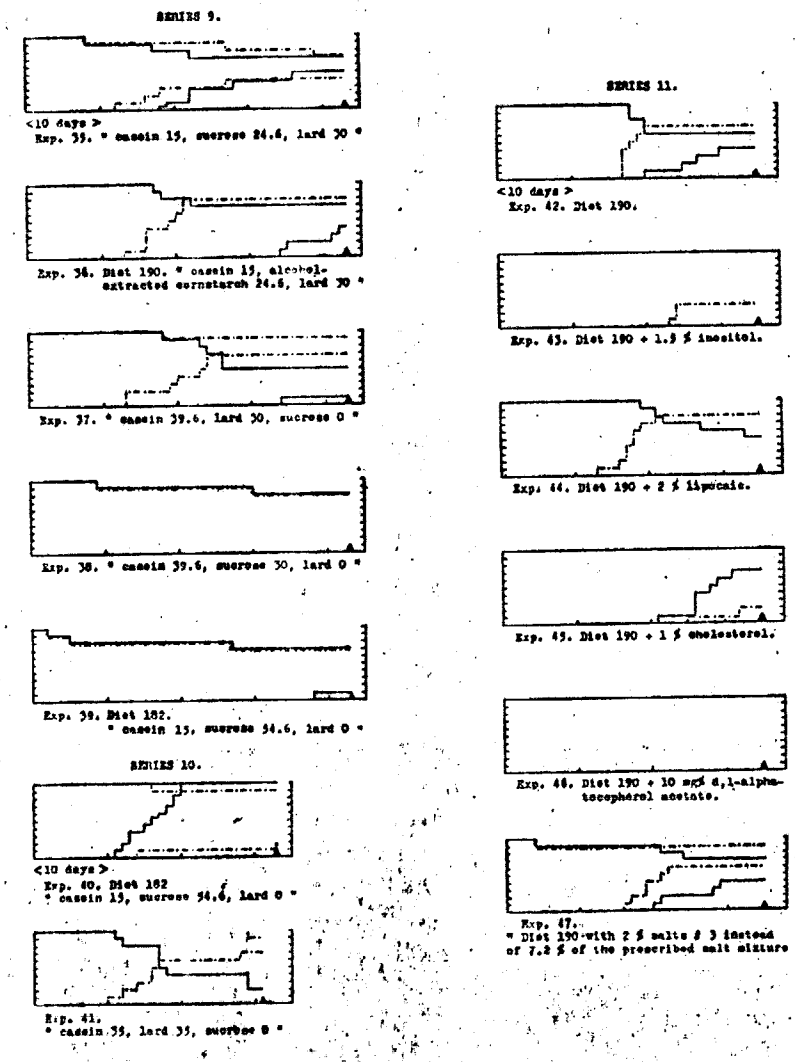
With 30% lard replacing the same amount of sugar, whereby the sugar was reduced to 24.6%, both exudates and encephalomalacia developed about equally; encephalomalacia appeared slightly in advance of the exudates (exp. 35). With 30% lard and 24.6% cornstarch (diet 190, table 1), encephalomalacia was by far the dominating symptom, exudates coming decidedly later (exp. 36). Encephalomalacia was still more favored when all the carbohydrate of the diet containing 30% lard was replaced by casein, the total casein content being 39.6% (exp. 37).

Without lard (exps. 38 and 39) no encephalomalacia appeared but one instance of exudate, rarely seen on "fat-free" diets, did occur (exp. 39). It is interesting to compare the latter with experiments 40 and 41,



series 10, in which the diets contained 5% cod liver oil. When in diet 182 the sugar was removed and the casein increased to 35%, with the simultaneous introduction of 35% lard, the symptom shifted from exudate to encephalomalacia, the body weight increasing to 265 gm. in 4 weeks.

A similar shift occurred when cod liver oil or its fatty acids in diet 182 (exps. 16 and 17) were replaced by hog liver fat or its fatty acids (exps. 21 and 22), and when dried hog liver replaced casein as the source of protein in diet 32 (exp. 7). Hog liver fatty acids produced intrapericardial exudates in two chicks (exp. 22).



Not only cod liver oil (exp. 16) but also linseed fatty acids (exp. 19) and lard (exp. 14) caused some encephalomalacia when constituting about 5% of the diet, while commercial oleic acid (exp. 20) or thoroughly rancid cod liver oil (exps. 25–27, series 6) did not. Furthermore, increasing the cod liver oil in diet 182 from 5% to 20% (exps. 32–34, series 8) did not increase the incidence of encephalomalacia but may have reduced it.

The data thus far collected show that certain fats will favor exudates whereas others will favor encephalomalacia. Whether this is due to the fatty acids themselves or to trace substances which go into the fatty acid fraction remains to be definitely settled. Encephalomalacia has never been observed, and exudates rarely seen, when fat was not added to the diet. Furthermore, low carbohydrate or the use of a moderate amount of starch as the source of carbohydrate instead of sucrose will depress the tendency for the exudates to appear and favor encephalomalacia, provided sufficient fat such as 30% lard is present.

Cholesterol and lipotropic substances

The experiments included in series 11 demonstrate that both encephalomalacia and exudates were counteracted by 1.5% inositol (exp. 43), only exudates by 2% lipocaic (exp. 44), and only encephalomalacia by 1% cholesterol (exp. 45); whereas 10 mg. % of α-tocopherol prevented both symptoms (exp. 46). Changing the salt mixture to 2% of salts no. 3 (exp. 47) had no significant influence except that growth was retarded (average weight of 126 gm. at 4 weeks, compared to 175 gm. with diet 190 and 200 gm. with diet 190 plus inositol). It is of interest to note that with diet 182 containing 5% cod liver oil, 2% lipocaic likewise suppressed exudates (exp. 2) whereas 1% cholesterol added to diet 32 exerted an accelerating effect upon exudates (exp. 6) which did not occur with diet 190 (cf. exps. 45 and 42). From the foregoing it is apparent that substances influencing fat metabolism will also influence the nature and incidence of the symptoms. Obviously the problems raised by these observations require further investigation with varying amounts of the substances tested.

The failure of the Pappenheimer and Goettsch diet 108 to induce exudates has been explained on the assumption that certain ingredients of the diet provide traces of vitamin E sufficient to prevent this symptom but not adequate to meet a supposedly higher requirement for the prevention of encephalomalacia (Dam and Glavind, '39b). The results of the present studies indicate that the low carbohydrate content of this diet in itself tends to delay or minimize the tendency to exudates.

DISCUSSION

One might ask whether encephalomalacia is not simply due to exudation in brain tissue. Several of the above mentioned facts obviously speak for this view. However, the question is complicated by the fact that there is no complete parallelism between the factors which favor or counteract the two symptoms.

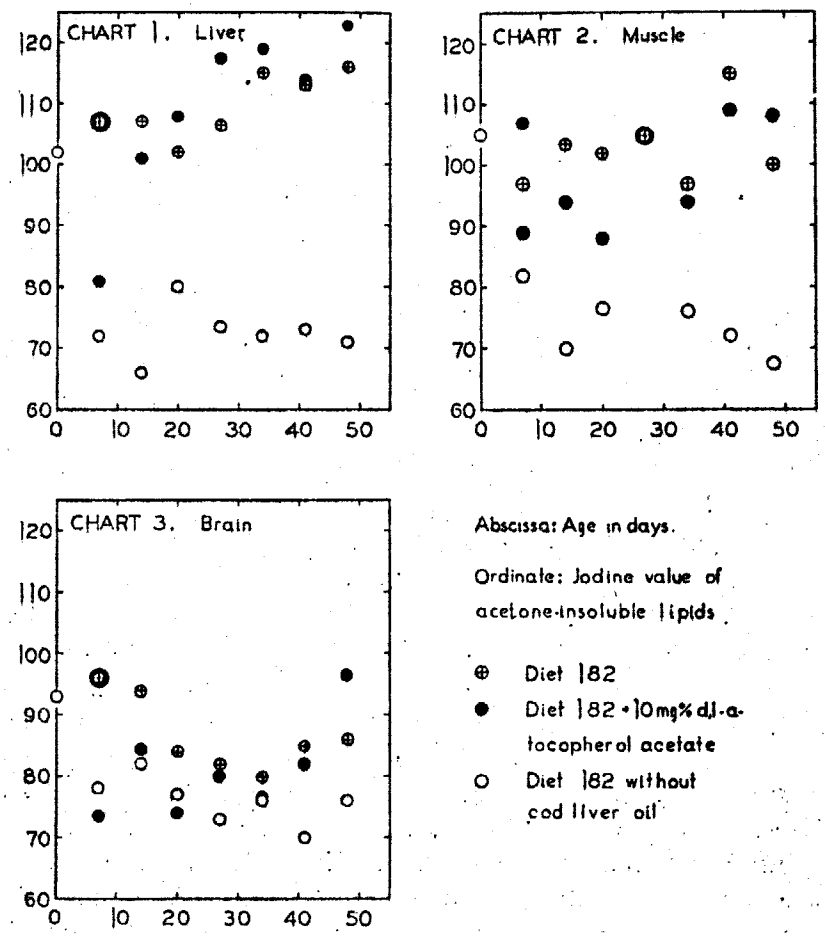
If fats merely destroy vitamin E in the body, it is difficult to explain why some fats favor exudates and others encephalomalacia, and why dietary fat is so essential for the development of symptoms in chicks but not so in rabbits. Certain fatty acids undoubtedly exert a damaging effect on the affected tissues, directly or indirectly but the nature of this mechanism remains to be investigated. Vitamin E deficiency seems not to incur a general inability to metabolize fat as manifested by ketonuria, lipemia or fatty liver. There is some evidence (Dam and Kelman, '42) that a deficiency of vitamin E decreases the ratio of phospholipids to other lipids in the blood plasma of chicks but the difference (20–40%) is not great enough to be of diagnostic value because of the considerable individual variation. The present studies have revealed no significant alteration in tissue lipids of deficient chicks, except for an increase in muscle cholesterol (170 mg. % with diet 182 against 104 mg. % when the diet contained 10 mg. % d, l-α-tocopherol acetate); a finding in harmony with observations on rats (Heinrich and Mattill, '43). Tests for rancidity in the fat tissue were negative.

Sinclair ('32) has shown that in rats the phospholipids take up highly unsaturated fatty acids from the diet. In order to find out whether vitamin E has any gross influence on this process I determined the iodine value[13] of the phospholipids of liver, muscle and brain from chicks reared on diet 182, diet 182 + 10 mg. % d, l-α-tocopherol acetate, and diet 182 without cod liver oil (vitamins A and D concentrate being given to all groups). Charts 1, 2 and 3 show that while there was a considerable difference in iodine value of liver and muscle phospholipids according to whether cod liver oil was given or not, the presence or absence of vitamin E did not cause any significant difference. It has further been shown that the iodine value of the subcutaneous depot fat is not significantly influenced by the presence or absence of vitamin E.

[13] The organs were ground with sodium sulfate immediately after killing by decapitation, the lipids extracted with chloroform at room temperature for 24 hours, evaporated in vacuo at body temperature and extracted six times with acetone. After dissolving the residue in chloroform the dry weight was determined in one aliquot and the iodine value in another, the method being that of Rosenmund and Kuhnhenn as described by Dam ('30).

Regarding the modifying influence of inositol, lipocaic, cholesterol, carbohydrates, and protein-carbohydrate ratios, it may be concluded that this influence is too complicated to be explained by general pro- or anti-oxidant effects.

Some observations relative to the response of rats to diets which produce exudates or encephalomalacia in chicks warrant brief mention here. Rats showed no such symptoms when fed a vitamin E deficient



diet from the time of weaning, even if the amount of cod liver oil in the diet is high. However, a peculiar buff discoloration of the subcutaneous and intraperitoneal fat tissue occurred without any signs of hemorrhage in (a) rats reared for 183 days on diet 182, to which was added 1% cholesterol, 1% NaCl and 60 mg. % of histamine dihydrochloride, and in (b) rats reared for 111 days on diet 182, modified by increasing the

cod liver oil from 5% to 20%. This change of the fat did not occur either in controls fed the same diets + 10 mg. % d, l-α-tocopherol acetate or in rats fed the unsupplemented diet 182 containing only 5% of cod liver oil. Should it be possible to demonstrate that this phenomenon is related to the buff coloration which develops in fat tissue in chicks receiving diet 182, after the reddishness of the fat tissue has disappeared, there would exist a bond of similarity in the response of these two species to vitamin E deprivation. In this connection it may be noted that a hemorrhagic tendency has been observed in the fetus of rats receiving a vitamin E low diet (Mason, '42, '43), which may have some relation to the exudative diathesis in chicks.

Rabbits will develop muscular dystrophy when placed on various vitamin E deficient diets, including the Pappenheimer and Goettsch diet 108, but nothing resembling exudates or encephalomalacia has been observed in them. Pappenheimer and Goettsch ('34) made the surprising observation that diet 108 also produces only muscular dystrophy in ducklings. Ten newly hatched ducklings reared for 33 days in the author's laboratory on the usual exudate-producing diet 182 did not show any definite signs of exudate or encephalomalacia. The reason for this different behavior of different species even when they are so closely related as ducks and chicks has not been explained, but it would not seem unlikely that differences in the fat-metabolizing enzyme system may play a rôle.

Symptoms corresponding to exudative diathesis and encephalomalacia have not been recognized in humans. The possibility that vitamin E might influence the capillary phenomena in certain forms of allergy was examined by Glaser and Dam ('44) with negative results.

SUMMARY

The symptoms of vitamin E deficiency in chicks — exudative diathesis and encephalomalacia — can be accelerated or suppressed by dietary changes which are unrelated to the vitamin E content of the diet.

Purified diets containing no added fat rarely produce exudates and never induce encephalomalacia. Both symptoms are accelerated by highly unsaturated fatty acids.

The fatty acids from cod liver oil, lard and linseed oil and commercial unsaturated C_{20} acids at a level of about 5% in the diet produce exudates as the main symptom, whereas fatty acids from hog liver favor the appearance of encephalomalacia. Oleic acid and thoroughly rancid cod liver oil are ineffective in producing either symptom.

A certain protein-carbohydrate ratio is optimal for the appearance of exudates, and the tendency to exudates is further enhanced by those inorganic salts which tend to accumulate in the extracellular fluid.

High lard (30%) favors encephalomalacia more than exudates when the carbohydrate-protein ratio is low. Inositol (1.5%) counteracts both symptoms. Lipoeaic (2%) counteracts only exudative diathesis. Cholesterol (1%) hastens exudates when the diet contains 5% cod liver oil and a low salt content, and counteracts encephalomalacia when the diet contains 30% lard. The iodine value of body phosphatides is influenced by dietary fat independently of the presence of vitamin E.

It is believed that the effect of the different fatty acids consists in a damage of tissue rather than in a general destruction of vitamin E, and that inositol, lipoeaic and cholesterol do not act simply through a general pro- or anti-oxidant effect.

The response of different species of animals to diets which produce exudates or encephalomalacia in chicks is discussed.

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Pharmacol. Rev. 9: 1-16, 1957

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

HENRIK DAM

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

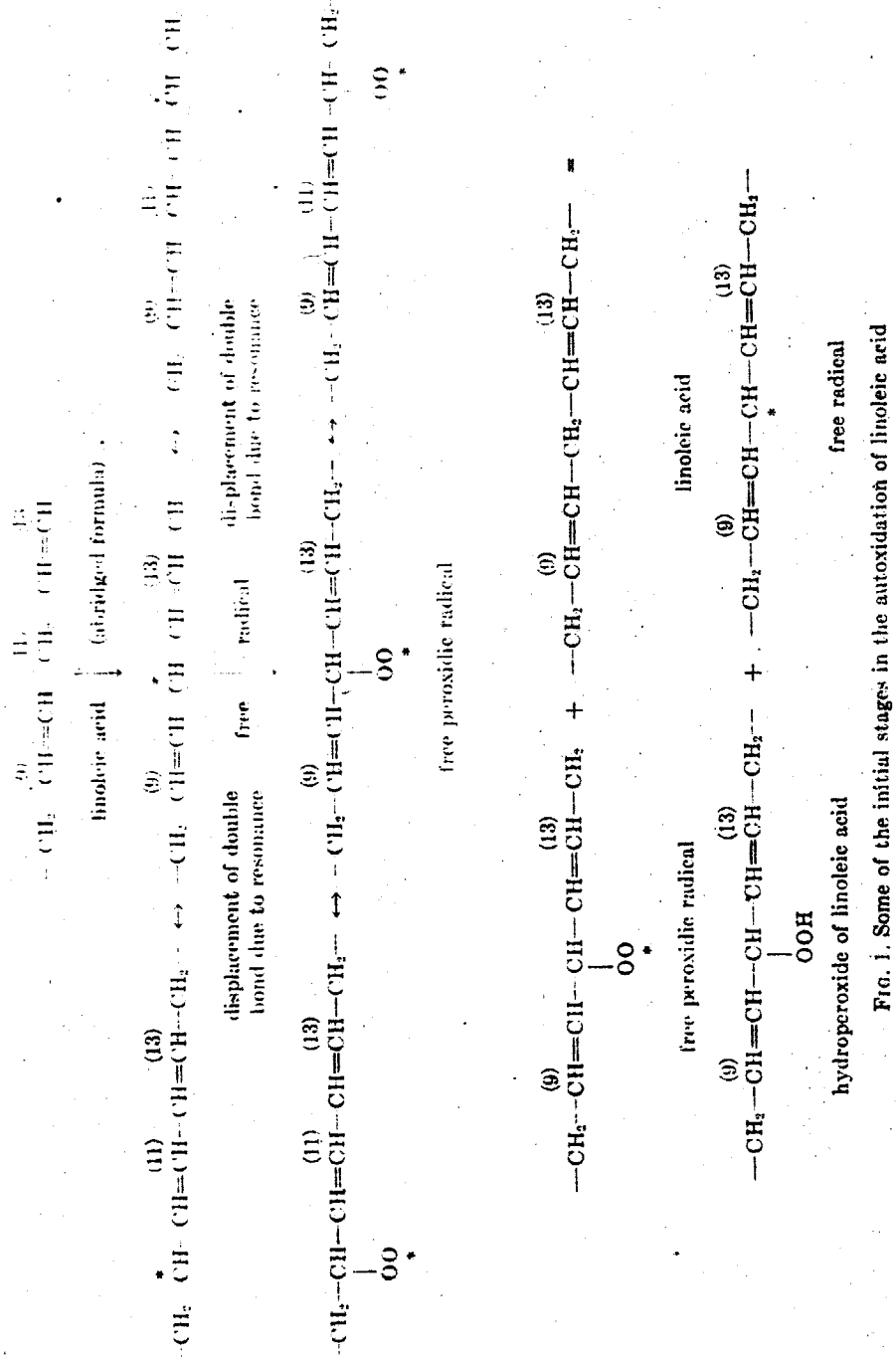

FIG. 1. Some of the initial stages in the autoxidation of linoleic acid

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 (11) 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 or when 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 K 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, 139 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 (86).)

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 carbohydrate and

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-Simmonds' salt mixture no. 185 supplemented with 13.5 mg KI, 139 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] Dicalcium 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 (13) 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" (34, 52, 53) 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 Dam (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 enzymatic 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 (39). 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 Coey (79) have found brown fat in pigs reared on a vitamin E-deficient diet containing cod liver oil. The rare disease scleredema 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* (49), 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).

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 Granados 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 first 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 enamel organ.

Hove and Harris (48) have shown that vitamin E increases the normal utilization of a suboptimal 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 (4000 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 11 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 accelerated 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, 44, 85). A more detailed review of this subject has been given by Himsworth (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

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 (59) 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 Søndergaard, 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 ameliorated the condition without resulting in higher concentrations of tocopherol

TABLE III

The signs of vitamin E deficiency discussed in the present article and their response to various substances.

Sign	Easily Autoxidizable Fat		Improvement Obtained by															
	Necessary for occurrence of sign	Aggravates or accelerates condition	Nordihydroguaiaretic acid		Disulfiram		Ascorbic acid		Cystine		Methylene blue		Thiodiphenylamine		Sulfonyl dyes*		Diphenylsulphenylenediamine	
			a	b	a	b	a	b	a	b	a	b	a	b	a	b	a	b
Exudative diathesis (chicks)	+			(+)		+		(+)		0		+		+				
Encephalomalacia (chicks)	+			(+)		0		+		0		+		+				+
Peroxidation and brown coloration of adipose tissue (chicks and rats)	+			+		+		(+)		0		+		+				
Brown uterus (rats)	?			0		0		0				+		+		+		
Depigmentation of incisors (rats)	+			+		+		0		0		+		+				
Low vitamin A in liver (rats, chicks)	+			(+)		+		0		0		+		+				+
Reproduction failure (female rats)	0										0†	0†						
Massive liver necrosis (rats)	0(?)	+						0		+		+						
												NB						
Lung hemorrhage (rats)		+																
Hemolysis with dialuric acid (rats)	0											(+) NB						
Muscular degeneration (chicks)	0	+	0		0		0		+		0							
Muscular degeneration (calves)		+						0				+						
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, Bindschedler's green, malachite green, methylviolet, rosaniline.
† formerly found positive with less thoroughly depleted rats and with diets containing traces of vitamin E.

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 Blaxter *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 dialuric 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 thiodiphenylamine (18), new methylene blue, Bindschedler's green (which does not contain sulfur), the triphenylmethene dyes—malachite green (apparently toxic to chicks), methyl violet and rosaniline—have effects in rats similar to those of methylene blue (72, 73, 74). Bindschedler'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-methoxy phenol (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 Søndergaard, 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 656 and 597 mV; at pH 7 this probably means between 236 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 incisor depigmentation and dialuric 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), roles 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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Effects of Diet on Fish Oil Toxicity in the Rat

BENJAMIN H. ERSHOFF
Western Biological Laboratories, Culver City, California

It is well established that the prolonged administration of fish oils will induce or accentuate symptoms of vitamin E deficiency in a number of animal species (Agduhr, '26a, b, '28; Madsen et al., '35; Dam, '43; Singsen et al., '55a). 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 or (2) an increased requirement for (or an impaired utilization of) tocopherol induced by (a) toxic substances in, or toxic metabolites of, fish oil and/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 muscular dystrophy in herbivora or encephalomalacia in chicks can readily be counteracted by the concurrent feeding of α-tocopherol (Dam et al., '38; Mackenzie and McCollum, '40). They can also be counteracted, however, by various non-tocopherol supplements. Thus, Ni ('36, '37, '38, '40) found that a water-soluble material derived from Chinese gelatin effectively prevented the occurrence of muscular dystrophy and encephalomalacia using diets 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 Dam et al. ('48, '51) observed that a number of apparently unrelated compounds including methylene blue, ascorbic acid and nordihydroguaiaretic acid had significant activity in preventing chick encephalomalacia using diets containing cod liver oil. Similar findings were obtained by Singsen et al. ('55b) with N, N′ - diphenyl - *p* - phenylenediamine (DPPD) and by Bunnell et al. ('55) with other antioxidants including 2, 6-ditertiary butyl-4-methoxyphenol (BHT), dibutyl and diamyl hydroquinones; di-sec-butyl- *p*-phenylenediamine; and 1,2-dihydro-2,2,4-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 coloration 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 regard (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

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that toxic effects may occur following fish oil administration due to factors other than the induction of a vitamin E-deficiency state.

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 μg; 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] Crystalets 500 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	86	119 ± 3.9
10% Crude sardine oil	29	56	84	120 ± 4.6
10% Menhaden oil	30	58	88	129 ± 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 48.0 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_3 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_3/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_3/gm, and the sardine oil approximately 500 U.S.P. units of vitamin A and 100 U.S.P. units of vitamin D_3/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:

$$\sqrt{\frac{\Sigma d^2}{n}}/\sqrt{n}$$

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)[1,2]

Dietary group	Average gain in body weight after 14 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
Basal 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	54
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, VioBin Corporation, Monticello, Ill.; corn oil, Corn Products Refining Co., Argo, Ill.; stripped lard, Distillation Products Industries, Rochester, (the tocopherol content of this material according to the manufacturers was less than 5 μg/gm); hydrogenated cottonseed oil, Best Foods, Inc., New York; and butter, Challenge Creamery Co., Los Angeles. The butter fat was prepared by melting the butter and removing the oil layer. The soybean oil, sesame oil, olive oil and cocoanut oil were obtained from the Pacific Vegetable Oil Corp., Los Angeles. The methyl linoleate was kindly provided by Dr. Roslyn B. Alfin-Slater, Department of Home Economics, University of California, Los Angeles. The purity of methyl linoleate as determined by iodine value assuming the contaminant to be methyl oleate 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 food 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 food 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 (Decker et al., '50; Cheng et al., '52), thyroid administration (Greenberg and Deuel, '50; Greenberg, '52), mineral oil administration (Bacon et al., '52; Ershoff and Greenberg, '54) and administration of succinylsulfathiazole, atabrine and other drugs (Bosshardt et al., '50; Bosshardt and Huff, '53). In each of the experiments indicated above a protective effect was obtained by increasing the fat content[4] 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

[4] The fats used in these experiments were either vegetable oils 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 tested. 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 feeding, findings were comparable to that obtained using the low-fat plus 10% 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, 6 times/week) were also without growth-promoting effect. DPPD and Santoquin, when fed at a 0.05% level in the diet and sesamol (the methylene ether of oxyhydroquinone, a constituent of sesame oil with antioxidant properties) when administered at a 0.05 or 0.017% level in the ration, counteracted completely the growth retardation caused by crude tuna oil when incorporated at a 10% level in the basal purified low-fat diet. Results are summarized in table 3.

TABLE 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 (6 animals per group)[1,2]

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 + 10% crude tuna oil	14
Basal low-fat diet + 10% crude tuna oil + following supplements:	
0.025% α-tocopherol	32
0.05% α-tocopherol	49
0.1% α-tocopherol	49
0.025% α-tocopheryl acetate	14
0.05% α-tocopheryl acetate	15
0.1% α-tocopheryl acetate	12
Tocopheryl phosphate (10 mg rat day 6 times weekly I.P.)	4
0.05% DPPD	65
0.05% Santoquin	70
0.017% Sesamol	72
0.05% Sesamol	74

[1] The average initial body weight of rats in the various groups ranged between 45.4 and 46.8 gm.

[2] Test supplements were obtained from the following sources: DL-α-tocopherol and DL-α-tocopherol phosphoric acid disodium salt from Nutritional Biochemicals Corp., Cleveland; DL-α-tocopheryl acetate (Dry Vitamin E Acetate Powder 25%), Hoffman-La Roche Inc., Nutley, N. J.; DPPD, B. F. Goodrich Chemical Co., Cleveland; and Santoquin, Monsanto Chemical Co., St. Louis, Mo. The sesamol used in the present experiment was a twice recrystallized product prepared by the Trubek Laboratories, Inc., East Rutherford, N. J., and supplied by the Western Utilization Research and Development Division Agricultural Research Service, U.S. Department of Agriculture, Albany, Calif.

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. Findings 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 desiccated 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 casein content of the diet by an additional 10% of the ration or supplementing the basal ration with defatted 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 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 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 (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 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; 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. 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, where as 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	28th
	gm	*gm*
Basal low-fat diet	64	132
Basal low-fat diet + 10% cottonseed oil	80	154
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[2]	12	41
Vitamins B, C and K[3]	16	40
5% Salt mixture[4]	24	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]	15	44
Aureomycin·HCl (1 gm/kg of diet)	22	54
10% Cellulose[8]	20	65
10% defatted fish flour[9]	50	116
10% Amino acid mixture[10]	44	101
0.6% DL-methionine	55	94
20% Alfalfa meal	83	158
10% Alfalfa meal	51	89
Desiccated 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 48.8 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 0.4% level in the diet: calcium flavonate glycoside, naringen (naringenin-5-rhamnosidoglucoside), hesperidin complex, hesperidin methyl chalcone and lemon bioflavonoid complex.

[8] Solka Floc BW 200, Brown Co., Boston.

[9] VioBin Corp., Monticello, Illinois.

[10] Schultze's amino acid mixture (Schultze, '57) consisting of the following: L-arginine monohydrochloride, 7.65; L-histidine monohydrochloride, 5.21; DL-isoleucine, 19.94; L-leucine, 18.97; L-lysine hydrochloride (95%), 11.06; DL-methionine, 5.40; DL-phenylalanine, 8.00; DL-threonine, 12.00; DL-tryptophan, 2.78; DL-valine, 21.60; DL-aspartic acid, 9.72; DL-alanine, 8.66; L-cystine, 5.01; L-glutamic acid, 36.74; L-tyrosine, 9.90; and glycine, 0.76.

[11] Extracted 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.

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 concentration in a number 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 graded amounts of vitamin E (in addition to that present in the basal ration) on the response 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 as the sole supplement, even at 1/6th the dosage of α-tocopherol used, in counteracting all symptoms 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 nonlipid 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 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 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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Vitamin E Deficiency in Cats[1]

S. N. GERSHOFF AND S. A. NORKIN[2]
Department of Nutrition, Harvard School of Public Health, Boston, Massachusetts

In recent years there have been several reports by veterinary groups of steatitis or "yellow fat" disease in cats (Cordy and Stillinger, '53; Coffin and Holzworth, '54; Munson et al., '58). In each instance the cats studied had for long periods of time been eating commercial fish diets, known to contain highly unsaturated fish oils. Similar conditions have been reported in a wide variety of vitamin E-deficient animals. The diets used generally contained highly unsaturated fish oils. In the present studies the effect of feeding cats purified diets with and without tuna oil and containing varying levels of vitamin E was investigated.

EXPERIMENTAL

Three- to six-month-old kittens of mixed breed and sex were used. Before being admitted to the laboratory they were dusted with an insecticide, dewormed and vaccinated against feline distemper. The cats were housed individually in metal mesh cages and food and water were maintained ad libitum. The diets fed contained the following, expressed in per cent: casein, 32; vitamin E-free lard,[3] 20; sucrose, 42.7; salts IV (Hegsted et al., '41), 4; cod liver oil, 1; and choline, 0.3. To each kilogram of diet the following were added, expressed in milligrams: thiamine hydrochloride, 4; riboflavin, 8; pyridoxine hydrochloride, 4; niacin, 40; calcium pantothenate, 20; folic acid, 1; biotin, 0.2; vitamin B_{12}, 0.05; and menadione, 1. Vitamin E was provided either as *dl*-α tocopherol (1 mg = 1.1 IU) or *d*-α-tocopheryl acetate (1 mg = 1.36 IU). Two types of tuna oil were used,[4] neither of which contained added antioxidants. One was a crude oil derived from the fish offal accumulated during the canning of tuna, the other a partially refined oil known as cooker oil derived from fats within the tuna muscle and under the skin. When used, tuna oil replaced 5% of the lard in the diets. Because of space limitations, the experiments requiring cooker oil were not carried out at the same time as the others. The cats were maintained with the experimental diets for periods up to 13.5 months. At various times during the experimental period, serum tocopherol determinations were obtained (Quaife et al., '49) and red blood cell (RBC) hemolysis studies were conducted (Gordon, '55).

At the end of the cooker oil experiments, mesenteric fat was taken from 5 vitamin E-deficient cats showing marked steatitis and from 5 normal appearing control cats fed diets containing cooker oil and analyzed for fatty acids by gas chromatography. Histologic examination of all cats in both experiments was made. Representative sections of all organs and fat from different locations were fixed in formalin and stained with hematoxylin and eosin. Fat sections from most cats were also stained with periodic acid Schiff, methenamine silver, Sudan IV, Sudan black B and carbol fuchsin.

RESULTS

Some of the results of these experiments are summarized in table 1. No significant differences were observed in the general appearance or growth of the cats maintained with diets with or without cooker oil regardless of the levels of vitamin E fed.

Four cats fed 5% of crude tuna oil and 17 IU of vitamin E/kg of diet failed to grow and were sacrificed in a moribund

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[1] Supported by the Fund for Research and Teaching, Department of Nutrition, Harvard School of Public Health, Boston, Massachusetts.
[2] Present address: Department of Pathology, Woman's Medical College of Pennsylvania, Philadelphia.
[3] Vitamin E free stripped lard was kindly provided by Distillation Products, Inc., Rochester, New York.
[4] The tuna oils were kindly provided by Star-Kist Foods, Inc., Terminal Island, California.

TABLE 1

Effect of feeding various levels of vitamin E to cats

No. cats	Months on experiment	Vitamin E	Tuna oil	Pigment[1] in fat	Myo-carditis	Myo-sitis
		IU/kg diet				
6	12½	0	0	0	+(4)	+(4)
4	2½(1),[2] 8(1), 13(2)	17	0	0	0	+(1)
3	9½(1), 13(2)	34	0	0	0	0
3	13½	68	0	0	0	0
4	2	17	5%[3]	0	0	0
2	10	425	5%[3]	0	0	0
6	8(2), 9½(4)	0	5%	+++(4), +(1)	0	0
4	10	27.5[4]	5%	+++(1), +(3)	0	0
5	10¼	34	5%	+(3)	0	0
4	10	110[4]	5%	±(1)	0	0
4	10¼	136	5%	0	0	0

[1] Globular pigment in the adipose tissue was visually graded 0 to 4 plus.
[2] Numbers in parentheses represent number of animals.
[3] Crude tuna oil, other cats received cooker tuna oil.
[4] Vitamin E provided by dl-α-tocopherol, other cats received d-α-tocopheryl acetate.

state after two months. These cats did not show any gross or microscopic changes except marked fatty vacuolization of liver cells throughout the lobules and fatty vacuolization of renal convoluted tubules. No hemorrhages, exudates or pigmentation were seen.

Two other cats receiving the same quantity of crude tuna oil in their diets but with 425 IU of vitamin E/kg of diet grew normally and showed no gross or histologic abnormalities when sacrificed after 10 months.

Steatitis, gross and microscopic fat pigmentation, was not observed in any of the cats fed diets to which tuna oil had not been added. Two cats fed cooker oil and no vitamin E, sacrificed after 8 months, showed no gross pigmentation. However, gross fat pigmentation was observed in cats that had been receiving cooker oil and zero or 27.5 IU of vitamin E/kg of diet for at least 9.5 months. Pigmentation involved the subcutaneous, abdominal, visceral and retroperitoneal fat. Compared with the cats not receiving vitamin E, the pigmentation in the 4 cats receiving 27.5 IU of vitamin E/kg of diet and killed after 10 months was less uniform and lighter and in two of these some lipid deposits showed only microscopic pigmentation. The subcutaneous tissue and peripancreatic adipose tissue in these animals appeared to be predominantly affected.

Microscopically depositions of yellow globular material were observed in all cats showing gross pigmentation and to a lesser extent in one of the cats not fed vitamin E but killed after 8 months and in three of the five cats receiving cooker oil and 34 IU of vitamin E/kg of diet. These focal accumulations of discrete yellow droplets (5 to 25 μ in diameter) were usually triangular or polygonal shaped because of their position in the interstices of normally appearing adipose tissue. The majority of these sites occurred in the immediate vicinity of blood vessels so that some of the larger foci formed cuffs around venules or arterioles. The smaller collections of pigment lay in crescents or ribbons of slightly basophilic granular cytoplasm at the periphery of fat cells, in association with increased numbers of round and oval nuclei. It cannot be definitely stated whether they were intracellular or extracellular, having orginated between groups of adjacent fat cells, but the first possibility is more probable. The larger accumulations appeared as large irregular patches between fat cells. Multiple nuclei were present in these foci without any recognizable cellular borders. The endothelium of the capillaries was swollen and the capillaries or venules were often surrounded by increased numbers of mononuclear cells, which occasionally extended along the vessel beyond the focus of pigment deposition. No areas of necrosis of fat were seen but occasionally, individual or clumped fat cells were ringed by mononuclear cells with different de-

grees of admixture of polymorphonuclear leukocytes. This inflammatory infiltrate was not always associated with the intracellular presence of globular pigment. Unlike a similar condition in swine, described by Davis and Gorham ('54), it is our impression that the process probably represents actual alteration of lipid within proliferating cells undergoing transition to lipocytes and probably to a lesser degree, to mature fat cells. A rare "signet ring" fat cell was seen, where a single vacuole was replaced by a large uniform yellow globule of pigment.

Numerous macrophages containing denser and darker globules of pigment could be seen in the adipose tissue and in the lymph nodes. In some of the cats that received no dietary vitamin E, the deposition of pigment in the medulla of the lymph node had almost a granulomatous character with formation of multinucleated giant cells. Histochemically, the globular pigment had staining characteristics similar to those described by other authors (Hass, '38; Dam and Granados, '45; Mason et al., '46) and is apparently related to the lipoid pigments of ceroid-lipofuscin type (Elftman et al., '49; Gedigk and Fischer, '59). The pigment is insoluble in the usual fat solvents, (acetone and xylol). It stained positive with Sudan IV and also with Sudan black B. It was usually acid-fast, staining red to pink. Methenamine silver and periodic acid Schiff stained a rare globule of pigment in the adipose tissue, but much more constantly, the pigment in the macrophages. In the fat, both stains usually stained a rim around the globule and the granular cytoplasm in the foci of accumulation of the lipoid pigment. We were unable to find in the organs of cats (heart, uterus, muscle) the fine granular pigment of the lipofuscin variety described in Vitamin E deficiency (Martin and Moore, '39; Mason and Emmel, '45; Elftman et al., '49; Gedigk and Fischer, '59). This pigment is apparently not related to dietary fats but to the duration of the vitamin E deficiency and the type and age of the animals used and may result from intracellular deposition and oxidation of unsaturated lipid-protein compounds in the cytoplasmic organelles of the cell. No myositis or myocarditis was seen in any of the cats fed cooker oil.

Cats not fed tuna oil showed no evidence of gross fat pigmentation. In three of six of these cats not receiving any dietary vitamin E there were changes in subcutaneous adipose tissue. These consisted in focal cuffing of capillaries and arterioles with mononuclear round cells. Focally, single or groups of fat cells were ringed by mononuclear round cells with some admixture of polymorphonuclear leukocytes. There was an apparent thickening of the usually fine rim of the fat cell surrounding the vacuole and the proliferation or presence of numerous nuclei in these areas. Focally, the slightly altered fat cells showed a fine yellowish granularity in the thickened rims of cytoplasm and similar granules were seen in some plump interstitial cells, probably macrophages or fibroblasts. The lymph nodes had numerous macrophages and swollen R.E. cells in the medulla which contained the fine granular pigment. These three animals, which did not receive vitamin E or tuna oil, also showed focal interstitial myocarditis with rare muscle fiber degeneration, focal myositis of the skeletal muscle, and prominent periportal infiltrates of mononuclear cells in the liver. In addition, in one of these animals focal mononuclear cell infiltrates were seen in the muscularis of the stomach and small intestine. Two of the other three cats in this group receiving no vitamin E or tuna oil also showed focal myocarditis and myositis. Pigment was not seen in any of the above lesions or in any of the other viscera.

In table 2 are shown the results of erythrocyte hemolysis studies done on the cats of all groups after they had been fed the experimental diets 5.5 months. Hemolysis data obtained from weanling rats fed two of the cat diets for 5 weeks are also presented. These data indicate that cat red blood cells are much more sensitive to hemolysis with hydrogen peroxide than those of rats fed the same diets and that relatively high levels of dietary vitamin E are required to increase the resistance of cat erythrocytes to peroxide hemolysis. The table also shows the relationship between dietary levels of vitamin E and serum tocopherol levels after 5.5 months on experiment. Detectable levels of serum tocopherol were observed in only one of the six cats not receiving any vitamin E.

In table 3 are shown the composition of mesenteric fat from animals fed cooker oil with gross steatitis and fat which appeared normal on gross and microscopic examination. These fat samples were all obtained from cats fed the experimental diets for 9 to 10 months. Statistically significant differences were found only in linoleic acid content, the fat of the vitamin E-deficient cats having considerably less than that of the controls.

DISCUSSION

The most prominent feature of these studies was the relative resistance of cats to gross signs of vitamin E deficiency. A wide variety of pathologic signs has been described in animals fed vitamin E-deficient diets for relatively short times, with muscle pathology being the most common observation. In this work mild myositis and myocarditis were observed in four of 6 cats maintained with vitamin E-deficient diets for 12.5 months, whereas the muscle of 6 cats fed vitamin E-deficient diets containing cooker oil appeared normal after periods up to 9.5 months. Growth depression was only observed in vitamin E-deficient cats fed crude tuna oil. Growth was normal in vitamin E-deficient cats not receiving tuna oil or those fed cooker oil. It is probable that deficiency signs would have become more pronounced if the dietary regimens had been maintained for longer periods. Vitamin E deficiency in animals is markedly influenced by the various constituents of the diet, particularly the quantity and composition of the fat. Encephalomalacia, exudative diathesis and gizzard necrosis, the chief symptoms of vitamin E deficiency in chicks were not observed when fat-free diets or diets lacking a major source of unsaturated fatty acids were fed (Dam, '44; Zacharias et al., '50). The addition

TABLE 2

Effect of feeding various levels of vitamin E on RBC hemolysis and serum tocopherol levels[1]

	Dietary vitamin E	Tuna oil	No. cats	Hemolysis	Serum tocopherol
	IU/kg diet			%	*mg/100 gm*
Cats	0	−	6	89	0.03
	0	+	6	100	
	17	−	4	82	0.15
	27.5[2]	+	4	100	
	34	−	3	84	0.50
	34	+	5	96	
	68	−	3	80	1.13
	110[2]	+	4	89	
	136	+	4	32	
Rats	17	+	6	40	
	68	+	6	5	

[1] Data obtained when cats had been fed diets 5½ months, rats 5 weeks.
[2] Vitamin E provided by *dl*-α-tocopherol, other animals received *d*-α-tocopheryl acetate.

TABLE 3

Composition of mesenteric fat of cats with and without steatitis

	Cats with steatitis[1]	Cats without steatitis[1]	
Percentage of fat as:			
Myristic acid	4.2 ± 0.4[2]	6.1 ± 1.1	
Myristoleic acid	0.3 ± 0.08	0.5 ± 0.15	
Palmitic acid	32.5 ± 1.9	29.4 ± 1.4	
Palmitoleic acid	2.8 ± 0.4	2.8 ± 0.2	
Stearic acid	9.1 ± 1.1	7.3 ± 0.6	
Oleic acid	40.9 ± 1.7	38.8 ± 2.9	
Linoleic acid	8.9 ± 0.7	13.0 ± 0.5	$P < 0.001$
C_{20} Acid	1.1 ± 0.5	1.1 ± 0.3	

[1] Five cats in each group.
[2] Values include standard errors of the mean.

of cod liver oil to these diets resulted in the production of these deficiency signs in chicks in one-to-three weeks.

However, a dietary fat source is not essential to the production of vitamin E deficiency. Mackenzie et al. ('39, '41) observed reproductive failure and paralysis in rats and muscular dystrophy in rabbits fed vitamin E-deficient fat-free diets for relatively short periods of time.

The appearance of steatitis or yellow fat disease has been associated with the feeding of vitamin E-deficient diets containing a source of unsaturated fatty acids in rats (Mason et al., '46), mink (Mason and Hartsough, '51; Gorham et al., '51; Lalor et al., '51), swine (Gorham et al., '51; Davis and Gorham, '54), and cats (Cordy, '54; Munson et al., '58). Current beliefs based primarily on the work of Hass ('38) and Dam and his associates (Dam, '49) are that a dietary source of long-chain fatty acids containing at least two unconjugated ethylenic linkages is necessary for the formation of the acid-fast pigments observed in steatitis. If fatty acids containing more than two unconjugated ethylenic linkages are fed, conditions become even more favorable for the appearance of steatitis. Peroxidation of the fatty acids probably precedes and may be a necessary stage in the formation of acid-fast pigments which probably represent further stages of oxidation and polymerization of the highly unsaturated fatty acids beyond the peroxide stage. Fat from cats with steatitis in these studies showed significantly less linoleic acid than fat from control animals receiving the same amounts and type of dietary fat. Levels of monounsaturated fatty acids such as oleic, palmitoleic and myristoleic acids were similar in the fat of both groups.

The problem of steatitis in domestic cats has been given practical significance by the many reports of veterinarians who have observed the disease in cats receiving diets deficient in vitamin E and containing a source of highly unsaturated fatty acids often provided by tuna oil. There is no evidence that steatitis as seen in cats is caused by some characteristic of fish oil unrelated to the increased need for dietary vitamin E that diets high in polyunsaturated fatty acids cause. Ershoff ('60) has reported fish oil toxicity manifested by growth inhibition and diarrhea in immature rats fed purified diets containing various fish oils as their sole fat constituent. The α-tocopherol, but not α-tocopheryl acetate provided partial protection against growth depression and full protection against diarrhea in his rats. Full and partial protection was also obtained with a variety of antioxidants and food supplements. In the present studies protection against steatitis and the relatively acute effects of crude tuna oil was provided by vitamin E supplementation. Cordy ('54) found no lesions in kittens fed a steatitis-producing commercial cat food when they were provided with daily supplements of 20 or 40 mg of *dl*-α-tocopherol.

Rose and György ('52) have reported that the hemolytic activity of hydrogen peroxide is related to the vitamin E content of erythrocytes. Cat erythrocytes appear to be much more sensitive to hemolysis by hydrogen peroxide than rat erythrocytes. Only at the highest levels of vitamin E supplementation, 136 IU/kg of diet was a protective effect on red blood cell hemolysis observed. It appears unlikely that the peroxide hemolysis test used here will be of much practical value in assessing the vitamin E nutriture of cats.

It is not possible at this time to quantitatively define the requirements of cats for vitamin E. The difficulty with which vitamin E deficiency symptoms were produced when tuna oil was not added to the experimental diets, although 20% of lard and 1% of cod liver oil were used, suggests that in any future studies of the vitamin E requirements of cats the composition and quantity of the fats fed will have to be emphasized.

SUMMARY

Purified diets containing varying levels of vitamin E, and with and without tuna oil as a source of highly unsaturated fatty acids, were fed to cats for periods up to 13.5 months. Steatitis was observed in cats receiving diets deficient in vitamin E and containing tuna oil. The linoleic acid content of adipose tissue from cats with steatitis was significantly lower than that

found in control cats. Vitamin E provided complete protection against steatitis in these experiments. Cats deficient in vitamin E, but not receiving tuna oil, showed relatively mild symptoms of deficiency, particularly muscle changes, after receiving the experimental diets for a year.

ACKNOWLEDGMENTS

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How polycyclic hydrocarbons cause cancer

Important as environmental contaminants, some polycyclic hydrocarbons are known to cause cancer in animals and they must therefore be a cancer hazard for man. Many years of research are now culminating in a reveal the mechanism by which these chemicals make a normal cell malignant

Dr Philip Grover
is a research worker at the Chester Beatty Research Institute, London

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.

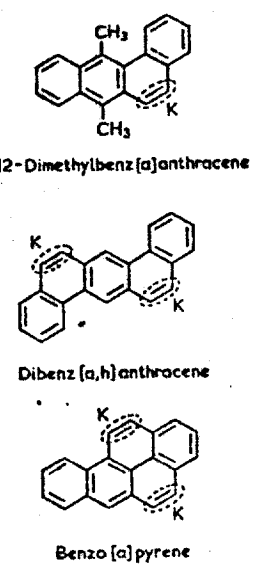

Figure 1 Chemical structures of some carcinogenic hydrocarbons

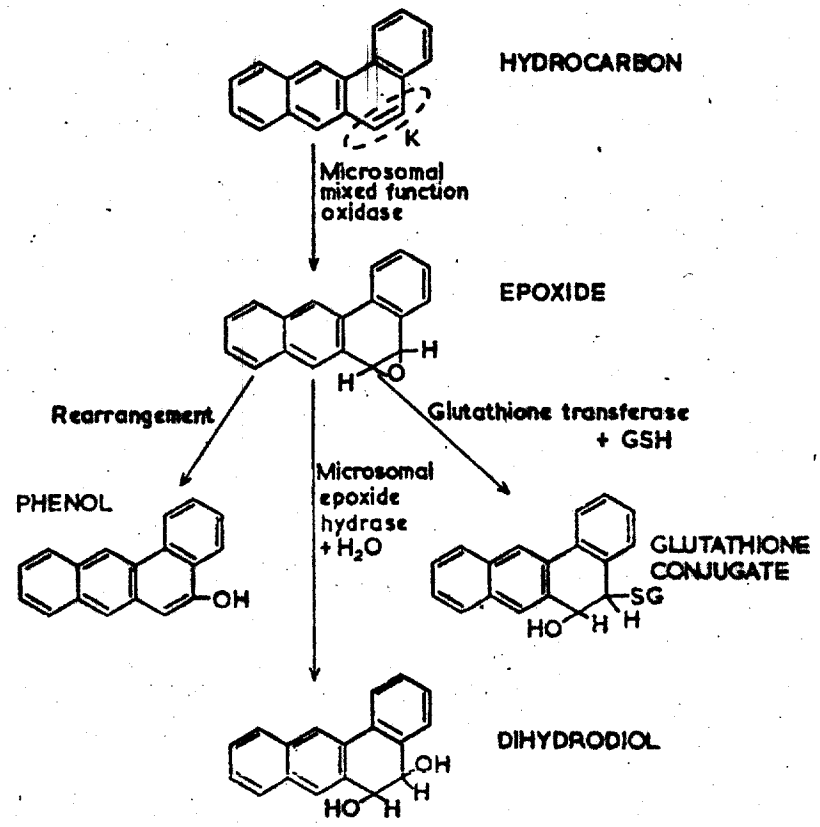

Figure 2 Metabolism at the K-region double bond of benz (a) anthracene

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.

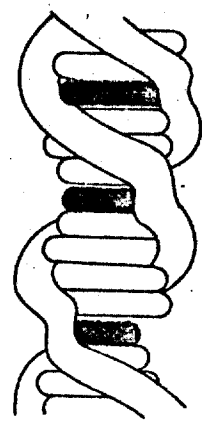

Figure 3 Intercalation of planar molecules (shown in black) into DNA

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.

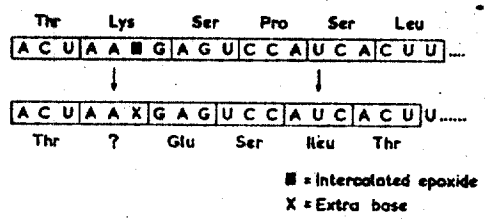

Figure 4 A mechanism involving DNA replication by which a hydrocarbon epoxide may induce a frameshift mutation

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CHAPTER 18

J. Hannewijk

Use of Fish Oils in Margarine and Shortening

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 let

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

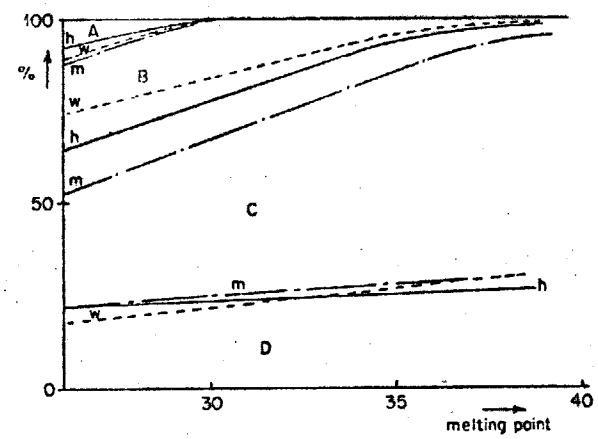

FIG. 42. COMPOSITION OF PARTIALLY HARDENED FATS WITH DIFFERENT MELTING POINTS

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, largely

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[2]

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:

[2] 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.

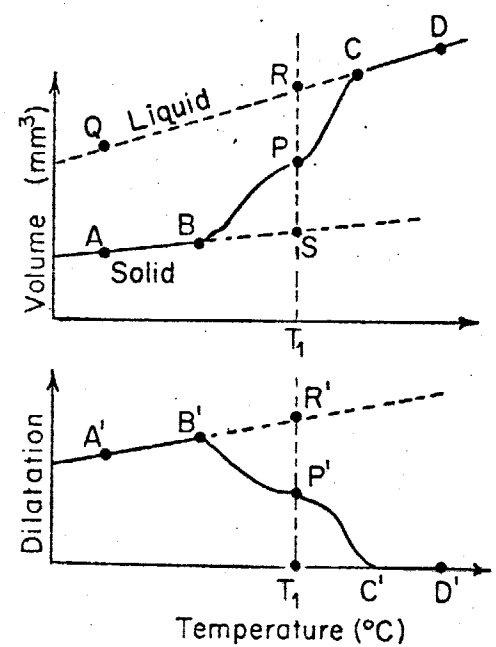

FIG. 43. VOLUME-TEMPERATURE AND DILATION-TEMPERATURE DIAGRAMS

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 to

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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) gave

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 variations

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 up

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

PHILIP L. HARRIS, PH.D. AND NORRIS D. EMBREE, PH.D.

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[5] 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

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TABLE I
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)	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. 02.[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	3.96	2.37	0.216	0.216	0.432
Lard	9.55	3.63	4.39	0.955	0.096	1.051
Margarine	9.42	2.45	5.37	0.848	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	7.69	0.699	0.699	1.398
Eggs	5.33	1.71	2.35	0.373	0.533	0.906
Meats, etc.	52.08	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	...	...	...	...	0.609
	(35%)†					
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 4, National Food Situation No. 96.[10] 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 percent 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

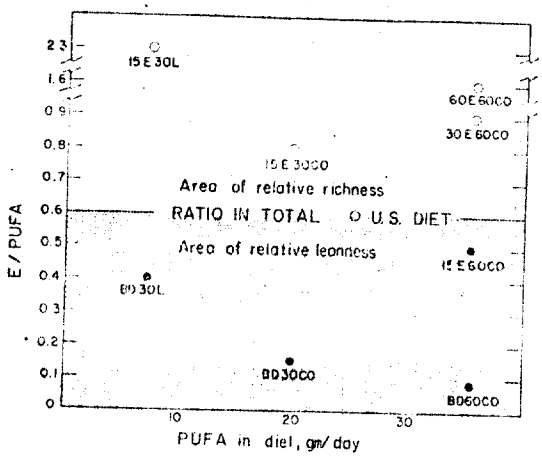

FIG. 1. Comparison of total U.S. diet, 1960, with Elgin experimental diets for ratio of E (milligram *d*-α-tocopherol) to PUFA (gram polyunsaturated fatty acids).

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.[6]

† 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 15E30L	3.0 + 13.6	4.0 + 3.1	2.3	Adequate
Horwitt 60E60CO	3.0 + 54.6	4.0 + 31.3	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	14.9	11.9 + 12.2	0.6	...
Horwitt 15E60CO	3.0 + 13.6	4.0 + 31.3	0.5	Inadequate
Horwitt BD30L	3.0	4.0 + 3.1	0.4	Inadequate
Horwitt BD30CO	3.0	4.0 + 15.6	0.15	Inadequate
Horwitt BD60CO	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[6]
(Corn oil 12%)	17.5	19.6	...	0.9		
Monkey	0.9	4.8	0.2	...	Muscle dystrophy and anemia	Fitch and Dinning[13]
(Lard 8% CLO 3 ml.)	5.9	4.8	...	1.3		
Bovine calf	38.8	30.3	1.3	...	Muscle wasting and paralysis	Blaxter et al.[16]
(Lard 28% CLO 2%)	39.0	22.5	...	1.7		
Bovine calf	10.1	16.3	0.6	...	Paralysis and death	Thomas and Okamoto[17]
(Lard 28%)	16.8	16.3	...	1.0		
Pig	9.5	25.1	0.4	...	Embryos died or young born with muscle dystrophy	Adamstone at al.[18]
(Wheat oil 1% CLO 1%)	43.0	25.1	...	1.7		
Rabbit	0.7	1.26	0.5	...	Creatinuria	Hove and Harris[3]
(Lard 6% CLO 2%)	1.4	1.26	...	1.1		
Rabbit	0.4	3.0	0.1	...	Elevated plasma aldolase and muscular weakness	Zuckerman and Marquardt[19]
(Lard 5% CLO 3%)	5.4	3.0	...	1.8		
Mouse	1.2	3.8	0.3	...	Resorption of fetus	Goettsch[20]
(Lard 20% CLO 2%)	1.7	3.8	...	0.4		
Rat	1.7	0.6	0.3	...	Insufficient lactation, uterine pigmentation	Evans and Emerson[21]
(Lard 22% CLO 2%)	5.0	0.6	...	0.8		
Rat	0.20	0.78	0.2	...	Testicular atrophy; muscular pathology	Filer et al.[1]
(Linseed oil 10%)	0.25	0.78	...	0.3		
Rat	0.4	8.8	0.05	...	Creatinuria	Century and Horwitt[22]
(Corn oil 15%)	0.7	8.8	...	0.1		
Rat	0.01	0.36	0.12	...	Resorption of fetus	Mason[23]
(Lard 18% CLO 2%)	0.09	0.36	...	0.25		
Rat	3.1	12.0	0.26	...	Testicular degeneration, tissue peroxides, anemia	Rasheed et al.[24]
(Menhaden oil 15%)	3.7	12.0	...	0.31		
Rat	0.6	3.6	0.2	...	Ceroid deposition and death	György et al.[25]
(Lard 18% CLO 2%)	1.9	3.6	...	0.5		
Rat	2.0	16.2	0.1	...	Hemorrhagic liver necrosis and death	Dam and Granados[26]
(CLO 5%)	8.7	16.2	...	0.5		
Rat, female	0.6	1.2	0.5	...	Erythrocyte hemolysis	Ward[27]
(Lard 10%)	0.7	1.2	...	0.6		
Rat, Male	1.0	1.2	0.8	...	Erythrocyte hemolysis	Ward[27]
(Lard 10%)	1.2	1.2	...	1.0		
Chicken	2.5	7.0 (LA)	0.4	...	Low hatchability caused by embryonic deaths	Olson et al.[28]
(Safflower oil 10%)	6.0	7.0 (LA)	...	0.9		
Chicken	5.0	32.0	0.2	...	Poor hatchability	Machlin et al.[29]
(Safflower oil 10%)	80.0	32.0	...	2.5		
Chicken	8.0	22.4	0.4	...	Encephalomalacia	Century et al.[30]
(Corn oil 4%)	16.0	22.4	...	0.7		
Chicken	3.0	11.0 (LA)	0.3	...	Encephalomalacia	Machlin and Gordon[31]
(Cottonseed oil 20%)	6.0	11.0 (LA)	...	0.6		
Chicken	0.5	4.0	0.1	...	Peroxidation of adipose tissue	Dam and Granados[32]
(CLO 5%)	2.2	4.0	...	0.6		
Turkey	1.0	2.1	0.5	...	Embryonic death, low hatchability	Jensen et al.[33]
(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 1.36 and 1.49, respectively, to get the equivalent quantities of d-α-tocopherol.[34]

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 (81 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.[16,35]

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:56 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[36] 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.[37] 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[38] 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.[39] 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 1960 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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THE EFFECT OF COD-LIVER OIL ON TUMOR GROWTH[1]

FRANCES L. HAVEN

(From the Department of Biochemistry and Pharmacology, School of Medicine and Dentistry, The University of Rochester, Rochester, New York)

INTRODUCTION

Sugiura and Benedict (1925) fed cod-liver oil and various vegetable oils and found them without influence on the percentage of takes obtained or on the rate of growth of the Flexner-Jobling rat carcinoma or the Bashford and Twort mouse carcinoma. Preliminary experiments in connection with the effect of dietary fats on growth and composition of tumors (Haven, 1935) had indicated that cod-liver oil inhibits the growth of a rat carcinosarcoma. This fact is further substantiated in the present paper.

EXPERIMENTAL

Tumor: The tumor used throughout the subsequent experiments was rat carcinosarcoma 256 obtained from the Institute of Cancer Research, Columbia University. Young male rats weighing from 70 to 100 gm. were inoculated subcutaneously in the groin by the trocar method. In each of the experiments all the rats were inoculated from the same tumor.

Diets: The diet used was Diet Number 262 (Sinclair, 1931) having the following percentage composition:

	Casein	Cane Sugar	Salt Mixture *	Fat	Yeast
Per 100 gm.	21.8	41.0	4.8	21.3	11.1
Per 100 cal.	18.0	34.0		40.0	8.0

* McCollum, E. V., and Simmonds, N.: J. Biol. Chem. 33: 63, 1918.

The fats used were cod-liver oil of iodine number 157 and, for comparison, coconut oil of iodine number 9. When coconut oil was fed, vitamins A and D were supplied by dissolving the unsaponifiable matter from 5 gm. of cod-liver oil in each 100 gm. of fat.

In each of the experiments half of the rats were placed on the cod-liver oil diet and half on the coconut oil diet on the day of inoculation and kept on this diet until the tumors were removed.

Measurement of Tumor Growth: The method of measurement of tumor growth involved weighing the tumor at the time of removal from the animal which, in the majority of cases, was from five to seven weeks after inoculation. To permit comparison of the growth of a tumor of one age with that of a

[1] The material presented in this paper is taken from a thesis submitted by the author to the faculty of the University of Rochester in partial fulfillment of the requirements for the degree of Doctor of Philosophy. (Received for publication, January 1936.)

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tumor of another age, the growth tendency of each tumor has been calculated by means of a formula of Bierich and Lang (1933) involving the weight of the tumor in grams upon its removal from the rat and its age in days from the date of inoculation, namely, $Wt. = a.t^3$; a (growth tendency of the tumor) $= \frac{\text{weight}}{\text{time}^3} \times 10{,}000$.

TABLE I: *Comparative Growth Tendency of Rat Carcinosarcoma 256 on Coconut Oil and Cod-Liver Oil Diets*

Experiment	Dietary Fat	No. Animals	Average Growth Tendency
1	Coconut	4	112
	Cod-liver	5	84
2	Coconut	9	145
	Cod-liver	3	117
3	Coconut	5	151
	Cod-liver	4	104
4	Coconut	3	172
	Cod-liver	5	128
5	Coconut	1	125
	Cod-liver	3	75
6	Coconut	3	130
	Cod-liver	7	115
7	Coconut	12	189
	Cod-liver	13	130
8	Coconut	7	203
	Cod-liver	10	169
av.	Coconut	44	164
	Cod-liver	50	124

TABLE II: *Average Residual Body Weights, Grouped According to Tumor Age*

Dietary Fat	Tumor Age (days)	No. Animals	Body Wt. (gm.)	Tumor Wt. (gm.)	Residual Body Wt. (gm.)
Coconut	30–38	15	199	22.5	177
	39–48	15	199	31.3	168
	49–57	14	204	38.8	165
Cod-liver	28–38	21	197	14.4	183
	39–48	21	188	26.3	162
	49–75	8	211	27.8	183

Results: Table I shows the average growth tendency of rat carcinosarcoma 256 on coconut and cod-liver oil diets as determined in seven different experiments carried out over the course of a year. The fluctuations in growth energy exhibited by a transplantable tumor account for the fact that the average growth tendency for coconut oil tumors in several of the experiments is below that for the cod-liver oil tumors in other experiments. It is significant, however, that in each experiment, the average growth tendency of the tumors in animals on the cod-liver oil diet is lower than that of the tumors in animals on the coconut oil diet. The average tumor growth tendency of the 50 animals on the cod-liver oil diet was 76 per cent of that of the 44 animals on the coconut oil diet.

In Table II the animals on each diet have been grouped according to tumor age. Comparison of the average tumor weight in each age group on the coconut oil diet with that in the corresponding group on the cod-liver oil diet reveals the poorer tumor growth on the cod-liver oil diet without the use of the formula for calculation of growth tendency. The residual body weight is obtained by subtracting the average tumor weight of each group from the average body weight.

Discussion

Hartwell (1927) and Harris and Moore (1928, 1929) reported that the incorporation of 12 to 15 per cent cod-liver oil in the diet inhibited the growth of rats. Such inhibition of growth of the host animal might account for the

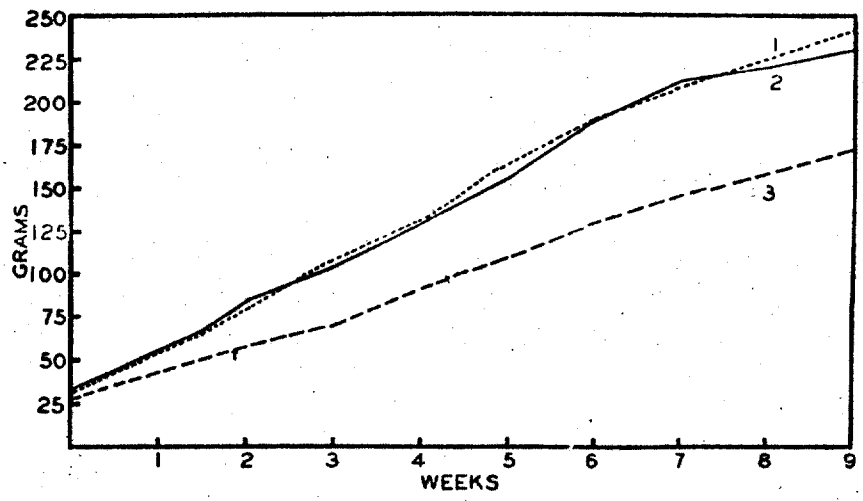

Fig. 1. Chart Showing the Increase in the Body Weight of Uninoculated Male Rats Placed on Diets at Weaning

Ordinate = Body weight in gms. Abscissa = Time in weeks from weaning. Curve 1, coconut oil diet; Curve 2, cod-liver oil diet; Curve 3, normal male rat growth curve (Donaldson: The Rat, Ed. 2, 1924, p. 175).

differences in tumor growth on the two diets. However, when the values for the body weights of the rats minus the weights of the tumors or residual body weights on the two diets are compared (Table II), they are found to be essentially the same for each tumor age group. Thus the cod-liver oil diet has retarded the growth of the tumor as such rather than exerting an effect on the tumor by a deleterious effect on the host animal.

Further confirmation of this point was obtained by growth studies of uninoculated rats on the two diets. In Fig. 1 are given the composite growth curves of rats on coconut oil and on cod-liver oil diets. Twelve young male rats were divided into two equal groups at weaning so that the rats in one group were litter mates of those in the other group. One group of rats was placed on the coconut oil diet, the other on the cod-liver oil diet. Comparison of the growth curve of the coconut oil group (Curve 1) with that of the cod-

liver oil group (Curve 2) reveals the fact that growth on the two diets was practically identical.

The possibility that the difference in tumor growth on the two diets might be due to a difference in vitamin content of the diets must be borne in mind. The coconut oil diet was adequate in respect to vitamins A and D due to addition of the unsaponifiable fraction of cod-liver oil. However, the cod-liver oil diet provided more of these vitamins than the supplemented coconut oil diet. Excess vitamin A was found by Ihara (1931) to promote the growth of a rat carcinoma. The results of our experiments would lead to the opposite conclusion. Vitamin D, on the other hand, has been reported by Sumi (cited by Shear, 1933) to exert no perceptible influence on the growth of a transplanted rat tumor. The inhibitory effect of cod-liver oil on tumor growth might be explained by the fact that vitamin D increases the "net absorption" of calcium, and calcium has been reported by many investigators to exert a slight retarding effect on tumor growth (Shear, 1933). In one experiment using the cod-liver oil diet alone, the addition of calcium gluconate to the diet of half of the tumor animals produced a slight acceleration rather than an inhibition of tumor growth.

Aside from vitamin content, coconut and cod-liver oil differ in their degree of unsaturation or iodine numbers. It is improbable that this difference would account for the effect on tumor growth, since linseed oil of iodine number 172 (cod-liver oil = 157) has been found to be devoid of any inhibitory effect (unpublished data). A third difference between coconut and cod-liver oil is in the length of chain of their constituent fatty acids, coconut oil being composed largely of short-chain acids and cod-liver oil of long-chain acids. In fact, cod-liver oil contains an abundance of C_{20} and C_{22} acids, which are absent from both coconut and linseed oils. It is possible that these long-chain acids might exert an inhibitory effect on tumor growth.

Summary

1. The rate of growth of rat carcinosarcoma 256 on a cod-liver oil diet is notably less than on a coconut oil diet.

2. The difference in tumor growth on the two diets is not due to differences in growth of the host animals since the growth curves of uninoculated rats on the two diets have been shown to be essentially the same and normal.

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

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

Fujikoshi Hospital, Toyama
Toyama Prefectural Central Hospital, Toyama

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

A LARGE amount of information was obtained on the relation between dietary fat and depot fat from an extensive study performed by HIRSCH and coworkers[1]. It was well documented that the depot fatty acid composition resembled that of dietary fat after an ingestion of sufficient amounts of oil over a period of years. In our study of long term ingestion of safflower oil, the linoleic acid content in depot fat reached 56 per cent in one case[2]. Therefore the pattern of depot fatty acid is regarded as a reflection of the dietary fat ingested during preceding years. The effect of dietary fat on the plasma lipid levels has been extensively studied in relation to atherosclerotic disease. The plasma lipid lowering effect of highly unsaturated fatty acids which are contained particularly in large amounts in fish oil has been described by several investigators[3][4][5], and the effect seems to be equal to linoleate or more than linoleate.

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

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

MATERIALS AND METHODS

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

Experimental procedures were as follows:

1) Tissues other than adipose tissue were carefully avoided.

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

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

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

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

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

RESULTS

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

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

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

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

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

tent between 34.9% and 39.5%, and 95% of the subjects had oleate content between 32.6% and 41.8% statistically. The second component was palmitic acid and had a mean of 21.6%. The coefficient of variation being 0.11, palmitate was also a constant component. The linoleic acid content was 14.8% on the average and this value was significantly higher than values seen in previous reports in the United States[1)11)12)13)]. The content of palmitoleic acid which was fourth component was quite labile, having a standard deviation of 2.5% to a mean of 10.3%. Similarly the stearic and myristoleic acid contents were labile, even though the amount of these acids was relatively small. In the contents of the above three acids, there existed close correlation as shown in Table II. Correlation coefficient being 0.78, palmitoleic and myristoleic acids increased or decreased closely in parallel and stearate showed clearly inverse correlation with other two acids.

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

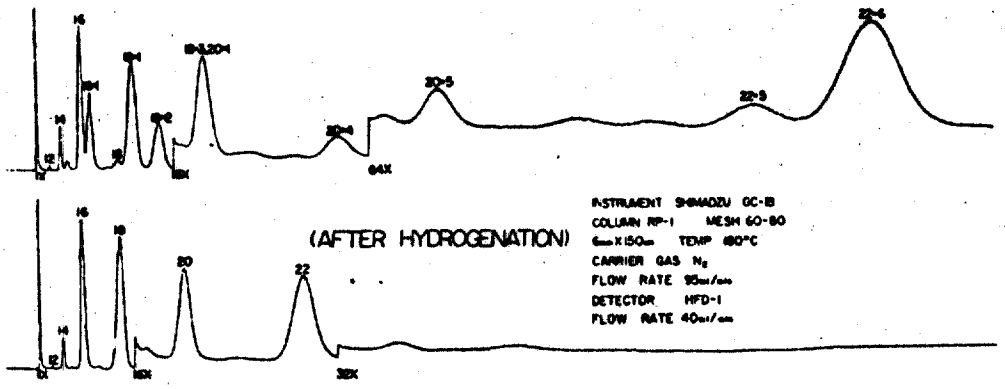
Table IV shows the fatty acid pattern divided by age groups. There was no significant difference in the content of major component fatty acids according to age. There seemed, however, to exist a tendency of an increase in the content of highly unsaturated acids as age increased.

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

TABLE II CORRELATIONS BETWEEN MYRISTOLEATE, PALMITOLEATE AND STEARATE CONTENTS IN DEPOT FAT.

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

Fig. 1 Gas-Liquid Chromatogram of Depot Fatty Acids



The case is a 65 y/o fisherman.

Discussion

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

The linoleate content, its mean being 14.8%, was considerably higher than that which appeared in previous studies performed in the United States[1)2)11)12)]. Shigeta and Hoshi[14)], Takahashi and Tanaka[15)], Goto and Nakamura[16)], Aizawa and Nakamura[17)] published similar results, so that the difference seems to be definite. Shigeta and Hoshi[14)] speculated that the difference was due to greater linoleate ingestion in Japan in proportion to the amount of total fat ingested, even though the absolute amount of linoleate consumption was clearly lower in Japan. We feel this is a very logical reason for the difference.

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

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

Other minor components are omitted.

Table IV Depot Fatty Acid Compositions Divided by Age Groups

Age	12	14	14=1	16	16=1	18	18=1	18=2	20	18=3, 20=1	18=4	20=2	20=3	20=4, 22=1	20=5	22=5	22=6	n
0–20	.4%	2.9%	.7%	21.3%	9.7%	3.4%	37.9%	14.8%	.4%	2.8%	.5%	.5%	.3%	1.3%	.3%	.4%	1.0%	22
21–30	.4	3.1	.8	21.7	9.9	3.1	36.7	15.6	.4	2.7	.4	.5	.3	1.0	.3	.4	1.1	65
31–40	.3	3.0	.8	21.9	10.7	2.8	36.7	14.9	.4	2.6	.4	.5	.3	1.0	.3	.5	1.0	47
41–50	.3	2.9	.8	21.4	10.6	2.9	37.5	14.7	.4	2.8	.4	.5	.3	1.1	.3	.5	1.1	40
51–60	.2	2.8	.8	21.2	10.9	2.7	38.0	13.7	.5	2.8	.5	.5	.4	1.3	.4	.6	1.2	24
61–	.3	2.9	.8	20.9	9.8	3.2	37.3	13.6	.6	3.0	.6	.5	.4	1.5	.4	.7	1.8	12

Expressed by means. Other minor components are omitted.

TABLE V DEPOT FATTY ACID COMPOSITION EXAMINED AFTER HYDROGENATIN

	12	14	15	16	17	18	19	20	21	22	23	24
Mean	.3%	3.3%	.3%	31.4%	.8%	56.7%	.1%	3.6%	.3%	2.8%	.1%	.2%
Standard deviation	.2%	.6%	.2%	2.8%	.3%	2.2%	.1%	1.1%	.3%	1.0%	.1%	.2%

25 randomly selected cases examined.

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

C14, C14 1, C16, C16 1, C18, C18 1 and C18 2 are the fatty acids which are discussed as common ones in depot fat in most occasions. Among of them, C18 1, C16 and C18 2 contents were fairly constant. C16 1, C14 1 and C18 contents were labile and there were considerable individual differences. However, there existed notable regularities in the relation of the content of these three acids. As shown in Table II, the palmitoleate and myristoleate contents were closely and directly correlated and the correlation coefficient reached 0.775. Contrarily the stearate content correlated inversely with that of palmitoleate and myristoleate. All of these correlations were highly significant statistically. On the other hand, greater contents of palmitoleate and myristoleate and smaller content of stearate are the most prominent characteristics of the peripheral adipose tissue as compared with the adipose tissue of the central part of the body, as documented in our previous study[18]. These facts indicate that there is an individual difference in depot fatty acid composition, similar to the difference between the body center and periphery, though not to such a great extent. There existed differences in the above three fatty acid contents in depot fat between male and female and the differences were highly significant statistically (p 0.01). Therefore we may say that the female has a somewhat peripheral type of fatty acid pattern of depot fat in comparison with the male.

SUMMARY

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

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

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

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

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

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

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

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

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

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

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

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

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

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

TABLE 1

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

	RAT NO.	FAT INTAKE MILK	(5 DAYS) FAT	FAT IN FECES (5 DAYS)	UTILIZATION
		gm.	*gm.*	*gm.*	%
Cottonseed oil	1	0.261	3.310	0.076	97.9
	2	0.194	3.128	0.113	96.6
	3	0.286	3.300	0.123	96.6
	4	0.267	3.152	0.052	98.5
Hydrogenated oil	1	0.260	3.011	0.165	95.0
	2	0.252	3.020	0.144	95.6
	3	0.308	3.470	0.134	96.5
	4	0.187	3.027	0.248	92.3

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

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

[1] Wesson oil was the product used.

[2] Crisco was the product used.

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

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

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

TABLE 2

Relative utilization by guinea pigs of oil before and after hydrogenation

Diet	*Lipids in dry feces, per cent*
Basal (extd.)	0.96
	0.97
	0.80
Basal plus cottonseed	1.69
	1.85
	1.85
Basal plus hydrogenated oil	5.43
	5.86
	5.46

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

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

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

[4] The hydrogenated oil used was Crisco.

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

TABLE 3

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

Fat supplement in diet	*Fat in feces, per cent*	*Utilization, per cent*
Castor oil	1.25	96.2
Soybean oil	1.54	94.5
Olive oil	1.60	94.5
Cocoanut oil	1.69	94.0
Salmon oil	2.03	94.0
Cod liver oil	1.65	93.8
Neatsfoot oil	1.64	93.5
Peanut oil	2.33	91.8
Butter	2.58	91.0
Cottonseed oil	3.36	87.4
Corn oil	3.35	86.5
Mutton tallow	5.22	79.8
Lard	6.70	75.2
Hydrogenated cottonseed oil	8.42	73.8
Beef tallow	7.05	72.0

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

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

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

TABLE 4

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

Oil supplement	*Tidwell-Holt method, per cent*	*Ether extract method, per cent*
Castor oil	2.31	1.25
Olive oil	2.34	1.60
Cottonseed oil	3.75	3.36
Hydrogenated oil	9.15	8.42

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

SUMMARY

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

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

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Relationship Between Molecular Structure and Flavor Perceptibility of Aliphatic Aldehydes

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

Abstract

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

Introduction

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

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

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

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

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

Experimental

Saturated aldehydes. Saturated unbranched aliphatic aldehydes (C_3–C_{12}) were purified by distilling commercially available products respeatedly; if necessary the purification took place via their sodium bisulphite addition product. The purity (according to the GLC analysis) was increased to 98% or more. The substances dosed gravimetrically were dissolved in paraffin oil with concn of 0.0001, 0.001 etc. up to 1000 ppm inclusive, and to guarantee a homogeneous

TABLE I

Flavor Potential of Saturated and Unsaturated Aldehydes Dissolved in Paraffin Oil

Substance	Threshold in ppm		Lea and Swoboda (5) (taste)
	Odor	Taste	
Propanal	3.6	1.6	1.0
Butanal	0.15	0.024	0.7
Pentanal	0.24	0.15	....
Hexanal	0.32	0.15	0.6
Heptanal	3.2	0.042	....
Octanal	0.32	0.068	0.6
Nonanal	13.5	0.32	....
Decanal	6.7	1.0	0.7
Undecanal	6.8	0.1	....
Dodecanal	3.0	0.046	0.4
2 *trans*-pentenal	2.3	0.32	....
2 *trans*-hexenal	10.0	2.5	....
2 *trans*-heptenal	14.0	0.63	....
2 *trans*-octenal	7.0	1.0	....
2 *trans*-nonenal	3.2	0.1	0.4
2 *trans*-decenal	33.8	5.5	....
2 *trans*-undecenal	150.0	4.2	....
2 *trans*-dodecenal	36.0	6.3	....
3 *cis*-hexenal	0.11	0.11	....
3 *trans*-hexenal	1.2	1.2	....
2 *trans*,4 *trans*-hexadienal	0.27	0.036	....
2 *trans*,4 *trans*-heptadienal	10.0	0.46	....
2 *trans*,4 *trans*-octadienal	1.0	0.15	....
2 *trans*,4 *trans*-nonadienal	2.5	0.46	....
2 *trans*,4 *trans*-decadienal	2.15	0.28	....
2 *trans*,4 *cis*-heptadienal	3.6	0.055	....
2 *trans*,5 *cis*-octadienal	15.0	0.21	....
2 *trans*,6 *cis*-nonadienal	0.01	0.002	....
2 *trans*,7 *cis*-decadienal	0.048	0.02	....
2 *trans*,6 *trans*-nonadienal	0.21	0.018	....
2 *trans*,7 *trans*-decadienal	1.0	0.32	....

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

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

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

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

The determined threshold values show in Table I.

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

Results and Discussion

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

TABLE II

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

Odor assessment	Composition of the mixture in paraffin oil	
	3 *cis*-hexenal (ppm)	2 *trans*, 4 *trans*-decadienal (ppm)
Decadienal	3.3	26.3
Decadienal	6.2	24.7
Decadienal	8.8	23.3
Decadienal	11.1	22.1
Decadienal + weakly green beans	13.2	21.0
Decadienal + weakly green beans	9.2	10.5
Only weakly green beans	1.4	1.6
Weakly green beans	0.1	.....
Green beans	1.0	.....

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

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

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

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

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

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

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

SUPPLEMENTATION OF HUMAN DIETS WITH VITAMIN E

A Statement of the

FOOD AND NUTRITION BOARD

DIVISION OF BIOLOGY AND AGRICULTURE

NATIONAL RESEARCH COUNCIL

(Prepared by the Committee on Nutritional Misinformation)

NATIONAL ACADEMY OF SCIENCES

June, 1973

NOTICE

The project that is the subject of this report was approved by the Governing Board of the National Research Council, acting in behalf of the National Academy of Sciences. Such approval reflects the Board's judgment that the project is of national importance and appropriate with respect to both the purposes and resources of the National Research Council.

The members of the committee selected to undertake this project and prepare this report were chosen for recognized scholarly competence and with due consideration for the balance of disciplines appropriate to the project. Responsibility for the detailed aspects of this report rests with that committee.

Each report issuing from a study committee of the National Research Council is reviewed by an independent group of qualified individuals according to procedures established and monitored by the Report Review Committee of the National Academy of Sciences. Distribution of the report is approved, by the President of the Academy, upon satisfactory completion of the review process.

Supplementation of Human Diets with Vitamin E

Among all the vitamins probably none has commanded more public attention or been the subject of more clinical controversy than vitamin E. The list of ailments claimed to be relieved by this vitamin includes most noninfectious diseases, e.g., heart disease, sterility, muscular weakness, cancer, ulcers, skin disorders, burns, and shortness of breath. As for apparently healthy subjects, vitamin E has been claimed to promote physical endurance, enhance sexual potency, prevent heart attacks, protect against the health-related effects of air pollution, and slow the aging process and alleviate its accompanying ailments.

How did these claims come about? To some extent they arose from a misinterpretation of the results of research on experimental animals. Vitamin E was shown to be among the factors required to prevent sterility in male rats and to permit normal pregnancy in female rats. This discovery naturally led to examination of the possible beneficial effect of the vitamin in connection with various problems of reproduction in men and women. The relevant tests on human subjects are difficult to evaluate because they cannot be conducted in a laboratory setting in which the effects of other factors can be eliminated. Some instances of beneficial effects were reported but, on the basis of results obtained over the past 35 years, leading scientific and medical opinion does not support the view that supplemental vitamin E has any value in preventing male impotency or sterility or in altering the outcome of pregnancy.

Advocates of vitamin E supplementation in human beings overlook the fact that an effect on reproduction in animals can be demonstrated only when the animals have been fed for long periods on diets free of vitamin E. The widespread presence of the vitamin in human diets has prevented a deficiency, such as seen in animals under experimental condition, from developing in man.

In most animal species that have been studied, severe vitamin E deficiency causes muscle wasting or dystrophy. Understandably, vitamin E has therefore been tested as a treatment for various muscle diseases of man, including hereditary muscular dystrophy. Again, results have been negative. The muscles of persons with dystrophy contain normal amounts of vitamin E, and there is no evidence that the condition in man is associated with a dietary deficiency of this vitamin.

Abnormalities in the heart muscle of vitamin E-deficient animals are less common and less severe than those seen in the skeletal muscle. In cattle and sheep, however, heart-muscle abnormalities can be severe, whereas severely deficient monkeys with skeletal dystrophy and bone-marrow failure have shown no cardiac involvement. Similarly, there is no evidence that cardiac disease is a consequence of vitamin E deficiency in man and, to date, extensive tests have failed to demonstrate therapeutic benefit from supplemental vitamin E.

Why has supplemental vitamin E been so ineffective in treatment of disease? Clearly, it is because the reproductive failure, heart disease, and muscular dystrophy observed in man are not attributable to dietary deficiency of this vitamin. Likewise, there is no satisfactory

scientific or clinical evidence that supplemental dietary vitamin E is beneficial in the treatment of such other conditions as burns, skin disorders, poor physical performance, and cancer.

Surveys of the United States population indicate that adequate amounts of vitamin E are supplied by the usual diet. The recommended daily dietary allowances of this vitamin range from 5 to 30 international units, depending upon age, sex, and physiologic state, and are deemed to exceed the actual needs of most individuals. Dietary vitamin E is supplied in substantial amounts by most vegetable oils as well as by margarine and shortening made from these oils, and significant inputs are made by many vegetables and by whole-grain cereals. Meats, fish, poultry, milk, eggs, legumes, fruits, and nuts also contribute to the dietary supply. Thus, supplementation of the diet with vitamin E is unlikely to be useful in alleviating any of the ailments mentioned above.

Are there any special cases in which supplementary vitamin E is beneficial? Some physicians prescribe vitamin E for premature infants, who frequently have low blood levels of the vitamin because of limited transfer from the mother's blood before birth. Patients afflicted with conditions that interfere with normal digestion or absorption of fats and fat-soluble vitamins require supplements of these vitamins, one of which is vitamin E. Such individuals should be under the care of a physician; for others vitamin E supplements are unnecessary. Self medication with vitamin E in the hope that a more or less serious condition will be alleviated may indeed be hazardous, especially when appropriate diagnosis and treatment may thereby be delayed or avoided.

Summary

Misleading claims that vitamin E supplementation of the ordinary diet will cure or prevent such human ailments as sterility, lack of virility, abnormal termination of pregnancy, heart disease, muscular weakness, cancer, ulcers, skin disorders, and burns, are not backed by sound experimentation or clinical observations. Some of these claims are based upon deficiency symptoms observed in other species. Careful studies over a period of many years attempting to relate these symptoms to vitamin E deficiency in human beings have been unproductive. The wide distribution of vitamin E in vegetable oils, cereal grains, and animal fats makes a deficiency in humans very unlikely. Premature infants or individuals with impaired absorption of fats may require supplemental vitamin E, but they should, in any event, be under the care of a physician.

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Effects of dietary fish oil on the composition and stability of turkey depot fat

By T. S. NEUDOERFFER and C. H. LEA

Food Research Institute, Low Temperature Research Station, Cambridge

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1. Beef fat (2·5%) and anchovy oil (2·5%; 2·5% plus 0·02% ethoxyquin; 5%) were incorporated into isocaloric cereal-based diets A, B, C and D and given to turkeys from 2 to 10 weeks of age. Diets A, B, and C contained 13 and diet D 26 i.u. α-tocopheryl acetate/kg. The lipids of diets B and D, which contained fish oil without ethoxyquin, autoxidized in the feeding troughs, but not seriously in the brief period of exposure permitted. 2. The birds all remained healthy and grew well, the only nutritional effects of the fish oil being a depressed storage of vitamin A in the liver at both levels of feeding (prevented by ethoxyquin) and a slightly adverse effect, at the higher level only, on food conversion ratio. 3. The skin fats of the birds given fish oil contained seven major and two minor fatty acids, derived from the fish oil, which were not present in the skin of the control group given beef fat, as well as one major fish oil acid present in much greater concentration in the birds given fish oil than in the controls. All these acids were present in the skin fats at about half or two-thirds of their concentrations in the dietary lipid, except for acid 22:5, which reached two or three times this level. 4. The stabilities towards autoxidation of the skin fats decreased in the order A > C > B > D, as their content of polyunsaturated fatty acids increased, but the greater stability of group C as compared with B was probably due, in the main, to the higher tocopherol content of the skin fat of the birds on the ethoxyquin-containing diet. Fishy flavours which developed on cooking showed a similar relationship to diet.

Though adequate supplies of polyunsaturated fatty acids (PUFA) are necessary for the animal body to produce lipids of proper biological function, adverse effects can ensue if such acids are present in the diet in concentrations that are high in relation to those of vitamin E and other substances of related protective activity. In chicks, when the diet is deficient in vitamin E, polyunsaturated acids of the linoleic–arachidonic series cause encephalomalacia, and Dam & Sondergaard (1964) have shown that approximately 0·8 mg D-α-tocopherol is required to balance each g of linoleic acid present in the diet. Neither selenium nor sulphur-containing amino acids protect against this sign of vitamin E deficiency, though certain synthetic antioxidants do so, e.g. ethoxyquin (EMQ; 1,2-dihydro-6-ethoxy-2,2,4-trimethylquinoline). On diets low in selenium and vitamin E (or other biologically active antioxidant) the giving of PUFA causes or greatly increases the severity of exudative diathesis. On diets low in sulphur amino acids and vitamin E, muscular dystrophy develops, though this sign is less dependent on the fat content of the diet and some evidence has recently been produced disputing any direct relation between muscular dystrophy in the chick and increased susceptibility of the tissue lipids to oxidation (Desai, Calvert & Scott, 1964). Nevertheless, Calvert, Desai & Scott (1964) found that the amount of vitamin E required to prevent muscular dystrophy in chicks increased with the amount of linoleic acid ingested up to 0·5% (though thereafter no further increase in vitamin E was required up to 2·5% linoleate), and Miller, Leong, Knobl &

Gruger (1965) have demonstrated that sufficiently high levels of highly unsaturated fish oil esters or glycerides will cause both these vitamin E deficiency syndromes in the presence of normally effective levels of protectants. Miller *et al.* (1965) assess the relative protective potencies of selenium, DL-α-tocopheryl acetate and EMQ for this purpose as approximately 1:500:3750, on a weight basis.

It may be that PUFA increases the requirement for vitamin E and related protective substances partly because of increased destruction of the vitamin by free radicals produced during autoxidation of the unsaturated fatty acids, either before or during absorption. Probably more important, however, is the resulting increased deposition of these readily peroxidizable fatty acids in important lipid structures, such as the membranes of mitochondria and lysosomes, where their biological function could be damaged by peroxidation under the influence of catalysts now believed to consist of both haem proteins and inorganic iron (Wills, 1966).

Another important consequence of giving fats containing high levels of PUFA to non-ruminant meat animals is an increased tendency for the development of 'fishy' odours and flavours on cooking: at lower dietary levels of PUFA 'off' flavours may result which are not recognizable as 'fishy'. Taints of this kind probably arise from peroxidation of the increased quantities of oxidatively unstable fatty acids present in the tissues, with subsequent fission to volatile, malodorous fragments.

In a previous experiment (Lea, Parr & Carpenter, 1960) we failed to detect any fishy taint in the eggs or flesh of hens on diets containing 8% of fresh or stored herring meal, equivalent to a dietary content of nearly 1·4% of chloroform–methanol extractable fish lipid or approximately 1·0% of ether or light petroleum extract. More recently (Lea, Parr, L'Estrange & Carpenter, 1966) we have found that the presence of fish oil (in fresh fish meal), at a level of 2·2% of the diet, caused serious tainting of the flesh of roast turkeys; the same amount of oil oxidatively polymerized by storage of the meal before feeding caused only negligible tainting. Stabilization of the oil in fish meal by the use of antioxidants is therefore likely to have an adverse effect on the flavour of the meat produced, though the protein of stabilized meal may be better nutritionally because the oxidized oil can cause damage to the protein (Lea *et al.* 1960; Carpenter, Morgan, Lea & Parr, 1962). At levels likely to be met in practice the oxidized oil of stored fish meal is not itself directly toxic to chicks (Carpenter, Lea & Parr, 1963).

In a recent review of the rather contradictory literature on the subject of tainting by fish oil Fry, van Walleghem, Waldroup & Harms (1965) have concluded that 'off' flavours most commonly appear when the fish oil content of the diet reaches 1·5–2·0%, indicating a necessity to keep it below 1·0–1·5%. There have been some reports that the addition of extra tocopherol or of synthetic antioxidants to diets containing fish oil or meal reduces tainting of the flesh of pigs or poultry to a limited degree, but in other instances no apparent improvement has resulted.

Since the physiological and organoleptic consequences of feeding fish oil to poultry presumably stem alike from the transference of some of the characteristic PUFA (with or without modification) from the fish oil to the body tissues, it was of interest to apply the improved techniques now available for lipid analysis to trace this trans-

ference into the storage depot fat and into the lipids of the muscular tissue. The present paper, therefore, compares diets containing anchovy oil at the 2·5% (with and without antioxidant) and 5% levels with a control containing 2·5% beef fat, for their effects on the growth of turkeys, and on the chemical composition, oxidative stability and palatability of the skin fat (mainly triglyceride). In a subsequent paper the detailed composition of the fractionated lipids of the breast and leg muscle will be presented, to establish the extent to which individual PUFA of the fish oil enter individual lipids of the muscle.

EXPERIMENTAL

Thirty-six 1-day-old female turkey poults, of a strain (10/20 Broad Breasted White) of fast-maturing birds specially suited for slaughter at 5–6 lb weight, were obtained from Norfolk Manor Farms Ltd, Great Witchingham Hall, Norwich. For the first 2 weeks the birds were fed on a 'young bird diet' which, with the exception of a slightly higher protein content (26·3% *v.* 22·1%) was the same as the diet given subsequently to control group A. The birds were then weighed and eight randomized groups of four birds each selected to minimize differences between group weights. Four sets of two groups each were then given diets A, B, C and D respectively for a further 8 weeks. After the 2nd week on the experimental diets, when the birds were seen to be tolerating the diets well, one from each cage was discarded, leaving six birds on each diet. Feeding was *ad lib.* but residual food was removed from the troughs every 24 h and discarded, to minimize autoxidative changes in the lipids.

Diets

Each of the four diets contained 92 parts of a common basal mixture supplying the necessary growth factors in an economically feasible form. To this was added beef fat or fish oil and maize starch or cellulose powder, as indicated below, to adjust the four diets to the same calorific value.

Diet A: (beef fat control) 92 parts basal diet, 2·5 parts beef fat and 5·5 parts maize starch.

Diet B: 92 parts basal diet, 2·5 parts anchovy oil and 5·5 parts maize starch.

Diet C: as diet B, but with the addition of 0·02% EMQ.

Diet D: 92 parts basal diet, 5·0 parts anchovy oil and 3·0 parts cellulose powder (Whatman Standard Grade). The vitamin E content of this diet was raised by doubling the amount of Rovimix E (Roche Products Ltd) added.

Immediately after mixing, all four diets were packed under nitrogen in A 2½ cans, the end seams of which were coated with a low-melting alloy (Wood's metal) to ensure complete gas-tightness. Storage was at −10° until a few hours before opening for use.

Basal mixture. The 92 parts basal diet contributed: ground wheat 28, finely ground barley 28, extracted soya-bean meal 17, dried unextracted yeast 5, casein 8, sterilized steamed bone flour 2 and mineral, vitamin, antibiotic premix (in bone flour) 4. The premix, which was similar to that used in a previous experiment (Lea *et al.* 1966) with the addition of vitamin B_{12} (0·02 ppm of the final diet), contributed to the diet

7700 i.u. vitamin A/kg and 13 i.u. vitamin E/kg as α-tocopheryl acetate (26 i.u./kg for diet D).

Fats used. Beef fat was rendered from fresh beef adipose tissue. The anchovy oil, of Peruvian origin, in solution in light petroleum, was passed through a column of activated silicic acid and alumina to remove autoxidation products and other impurities, followed by distillation and deodorization with steam under reduced pressure to remove the solvent. After removal of a sample for fatty acid analysis by gas chromatography both fats were packed in sealed cans under nitrogen and stored at −80° until required.

Oxidation in the feeding troughs. Spare troughs half filled with diet were held under conditions similar to those in which the birds were feeding, and representative samples were removed daily to test for oxidation. The lipid from each sample was extracted by shaking with chloroform for 0·5 h, after which the peroxide value (pv) was determined on the extracted fat by the method of Lea (1952).

Performance of the birds

Weight increase and food consumption were measured throughout the experiment, and after the birds were killed their livers were weighed and analysed for vitamin A content (Lea, Parr, L'Estrange & Carpenter, 1964).

Analysis of the lipids

Extraction of the fat. Since practically no abdominal fatty tissue had been laid down, the skins were examined as the main fat storage depot. For this purpose the skins of each group were combined, cut up with scissors and mixed, and a representative sample was freeze-dried. The fat was then extracted from the dry tissue with peroxide-free ether, in darkness under nitrogen in an apparatus of the hot extraction type, and the solvent removed at low temperature under reduced pressure.

Conversion to methyl esters and analysis by gas chromatography. The fat (1 g) was refluxed in 400 ml dry 5% (w/v) sulphuric acid–methanol, with the addition of 25 ml benzene to ensure solution, for 4 h under nitrogen. The methyl esters were washed and dried by the method of Bowyer, Leat, Howard & Gresham (1963). After removal of a sample for determination of the iodine value a 10% (w/v) solution of the methyl esters in pure isooctane containing 5 mg % BHT (2,6-di-tert.butyl-4-methyl-phenol) was prepared for gas chromatography.

Hydrogenation. To assist identification of the very complex mixture of fatty acids present in the anchovy oil a sample (0·2 g) of the methyl esters from this oil was hydrogenated in methanol solution for 16 h at 37° with Adam's platinum oxide catalyst (50 mg), and the recovered saturated esters were subjected again to gas chromatographic analysis.

Thin layer chromatography. As a further aid to the identification of individual fatty acids the anchovy oil methyl esters were fractionated according to degree of unsaturation on a 20 × 20 cm 20% silver nitrate–silica gel H plate, using a modification of the method of Kenney, Komanowsky & Wrigley (1965). The sample of methyl esters (80–100 μl of the 10% solution in isooctane) was applied as a narrow band 2 cm from the bottom of the plate, and developed in a constant temperature room at 1°

with hexane-benzene (20:80, v/v) containing, per 100 ml, 10 mg BHQ (butylhydroquinone, Neudoerffer & Lea, 1966), which was found to be the most suitable solvent. We preferred to make the separated ester bands visible by spraying with rhodamine-fluorescein (rhodamine 100 mg and dichlorofluorescein 32 mg dissolved in ether 150 ml, ethanol 70 ml and water 17 ml), rather than with water as used by Kenney *et al.* (1965). After elution of the four separated fractions from the support with 3 × 5 ml light petroleum the solvent was distilled off and the residue redissolved in 0·2 ml isooctane containing 5 mg BHQ 100 ml for gas chromatographic analysis.

Gas chromatographic analysis. Analysis of the fatty acid methyl esters was carried out on a Pye Series 104, model 24 (dual column) flame ionization gas chromatograph, but only one of the columns was used. The 5 ft glass column (purchased from W. G. Pye and Co. Ltd, Cambridge) was packed with 100–120 mesh Celite, carrying 10% of polyethylene glycol adipate (PEGA) as liquid phase, and the temperature was programmed at 4°/min from injection at 150° to 200°, after which the analysis was completed isothermally at 200°. The detector temperature was held at 220° throughout, in a specially constructed compartment, to maintain uniformity of response. Argon at a flow rate of 40 ml/min was used as carrier gas and a 1:1 mixture of hydrogen and nitrogen supplied the flame. The voltage applied to the jet of the flame detector was increased from 45 to 90 V, to extend its linear dynamic range. With a sample injection of 1·5 μl of a 10% solution of the esters in isooctane the attenuation of the amplifier sensitivity was varied between 2000 and 50000 times (i.e. full-scale deflection varied from 2×10^{-9} to 5×10^{-8} A) and the chart speed was usually 30 in/h.

For calibration, mixtures of methyl myristate, palmitate, stearate, oleate, linoleate, linolenate, arachidonate, eicosapentaenoate and docosahexaenoate (obtained from the Hormel Foundation, Austin, Minn. and the National Institutes of Health, Bethesda 14, Md) were prepared and assayed repeatedly. The last three of these esters were less pure (about 95%) than the others (> 99%) and were themselves assayed by gas–liquid chromatography (GLC), so that appropriate corrections could be made to the compositions of the mixtures containing them.

Tocopherol determination. Tocopherol (presumed to be mainly α- because of less efficient absorption and deposition of the other homologues) was determined on portions of the ether extracts of the skin by a slightly modified version of the method of Erickson & Dunkley (1964) using 4,7-diphenyl-1,10-phenanthroline. Replacement of the 0·1 M-orthophosphate specified by a 0·2 M solution was found to give more complete protection against photoreduction of residual ferric iron, and elution with 20 ml solvent, in place of the 10 ml suggested, was found to be necessary to achieve complete recovery of added tocopherol. A standard reference curve was constructed for hexane:benzene (2:3, v/v) solutions of pure DL-α-tocopherol.

Stability of the depot fat. For comparison of the stabilities of the skin fats towards autoxidation 0·2 ml portions were pipetted into a series of small glass cups and held at 60°, with determination of the peroxide value (pv) at suitable intervals, as previously described (Lea, 1960), but omitting the peroxide 'starter'.

RESULTS

Oxidation in the feeding troughs. The rates of peroxidation of the lipids of the four diets when stored in the feeding troughs under the conditions of the 1st week of the experiment (27° and bright fluorescent lighting) are shown in Fig. 1. Thereafter the temperature at the feeding troughs (which were remote from the infrared heating lamps) was reduced to 20°, from which it fell gradually to 15° at the conclusion of the experiment. The illumination was also much less bright than during the 1st week.

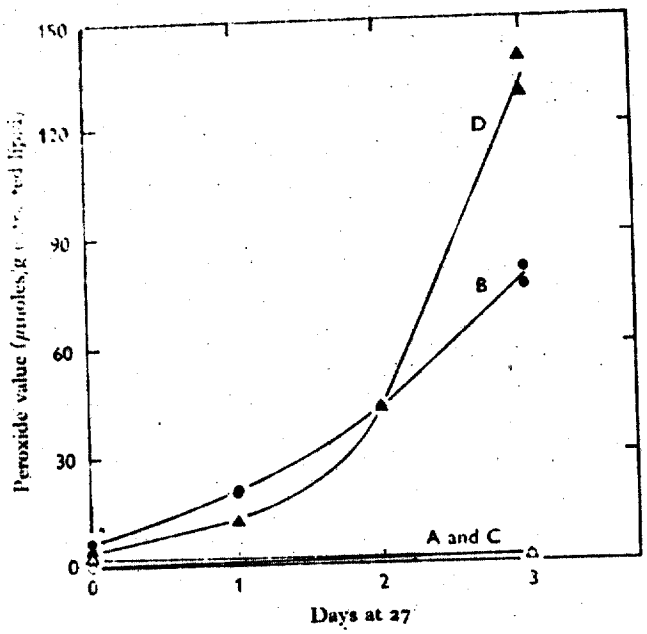

Fig. 1. Peroxidation of dietary lipids in the feeding troughs of turkeys. Diet A, 2·5% beef fat; diet B, 2·5% anchovy oil; diet C, 2·5% anchovy oil + EMQ; diet D, 5·0% anchovy oil.

Table 1. *Response of the turkeys to the experimental diets containing different fats*

Diet	Live-wt gain 2–10 weeks (g/poult)	Food conversion ratio (food consumed/wt gained)	Liver wt (as % body-wt)	Vitamin A reserves (i.u./liver)
A (2·5% beef fat, control)	2516	2·38	1·49	7060
B (2·5% anchovy oil)	2574	2·43	1·50	5680
C (as B, +0·02% EMQ)	2564	2·41	1·50	7280
D (5·0% anchovy oil)	2552	2·58	1·59	5550
Standard error of treatment means*	±38	±0·041	±0·055	±248

* In the analysis of variance the food conversion and the vitamin A content of the livers were significantly affected by treatment. In the former, diet D was just significantly higher than the other three ($P < 0·05$); in the latter, diets A and C were higher than diets B and D ($P < 0·05$).

On both these counts, therefore, peroxidation of the lipids of diets B and D during the exposure of less than 24 h in the trough would have been considerably slower during most of the experimental period than is shown in Fig. 1.

Table 2. *Composition of the skin lipids of the turkeys on diets containing different fats*

Diet	Lipid content of the skin (%)	Tocopherol content of the lipid (μg/g, as α-)
A (2·5% beef fat, control)	18·4	22
B (2·5% anchovy oil)	10·2	20
C (as B, +0·02% EMQ)	9·8	38
D (5·0% anchovy oil)	16·8	19

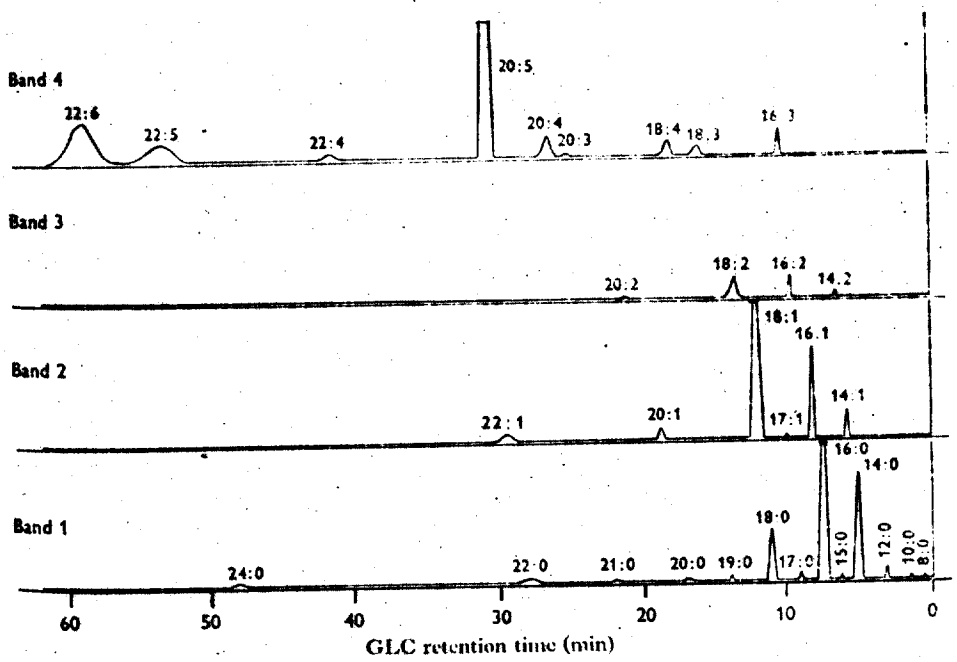

Fig. 2. Fractionation of anchovy oil methyl esters according to unsaturation on a silver nitrate–silica gel thin layer plate at 1°.

Performance of the birds. Growth and apparent health were normal on all four diets (Table 1). There did, however, appear to be a slightly inferior food conversion ratio ($P < 0·05$) and perhaps a slightly higher liver weight ($P > 0·05$) when 5% anchovy oil was given, as compared with values on the other three diets. Replacement of beef fat by anchovy oil slightly depressed storage of vitamin A in the liver, as previously observed (Lea *et al.* 1966), but diet D (5% anchovy oil), which also contained extra vitamin E, was not appreciably worse in this respect than diet B (2·5% anchovy oil). Vitamin E is known to conserve or protect vitamin A. Addition of EMQ with 2·5%

anchovy oil (diet C) restored the liver vitamin A to the level on the control (2·5% beef fat) diet A.

Analysis of the lipids. The fat contents of the skins of all the birds were rather low (Table 2), but negligible values for lipid phosphorus showed that the extracted lipid was virtually all neutral fat. Whereas fish oil may have marginally reduced the tocopherol content of the depot fat (diets B and D), EMQ (diet C) apparently raised it considerably. With the method of tocopherol estimation used, a recovery of 96% of added α-tocopherol was obtained and no interference by carotenoid with the determination was experienced.

Table 3. *Results of analysis of the anchovy oil methyl esters after hydrogenation*

	Hydrogenated esters	
Ester of acid	Found	Calculated from determined composition of the unhydrogenated esters (Table 4)
12:0	0·1	Trace
14:0	8·0	8·0
15:0	0·5	0·4
16:0	28·0	27·6
17:0	1·1	1·2
18:0	21·2	21·2
19:0	0·5	Trace
20:0	27·8	27·8
21:0	0·5	Trace
22:0	12·1	12·2
24:0	0·2	Trace
	Total 100·0	Total 98·4

Thin layer chromatography of the methyl esters of anchovy oil separated them cleanly into four fractions (Fig. 2), the fastest moving band (no. 1) containing the saturated components, followed by bands 2 and 3 of monoenes and dienes respectively, leaving polyenes in a fourth band near to the origin. To obtain sharp separation of dienes from polyenes it was essential to avoid overloading the plate.

Hydrogenation of the methyl esters of anchovy oil reduced their iodine value from 196·2 to 1·2 and greatly simplified the gas chromatographic pattern. Comparison of the analysis of the hydrogenated esters with that expected from the direct analysis of the unhydrogenated esters (using in the latter, identification facilitated by the separation achieved on the thin layer silver nitrate plate) gave satisfactory agreement (Table 3). Further confirmation of the validity of the identifications used was afforded by good agreement between the iodine value calculated for the fatty acid composition found (194·8) and that of the original ester mixture analysed (196·2).

The fatty acid compositions of the basal diet, of the fats added to it, and of the skin fats of the four groups of turkeys are summarized in Table 4. For ease of reference, the acids have been arranged in three groups according to whether they were major (> 1%), minor (1·0–0·1%) or very minor (< 0·1%) constituents.

Stability and flavour of the skin fats. The fats extracted from the skins of the birds

on diets B and D, despite precautions against autoxidation during handling and extraction, already showed small peroxide values (3·2 and 2·2 μmoles/g respectively), whereas those from the birds on diets A and C were zero. The colour of the extracted fats also increased progressively from A (white to cream) through C (definitely yellow) and B (deeper yellow) to D (very yellow). On incubation at 60° the fats developed peroxide in the same order (Fig. 3), the beef fat control diet (A) giving the most and the 5% anchovy oil diet (D) the least stable fat.

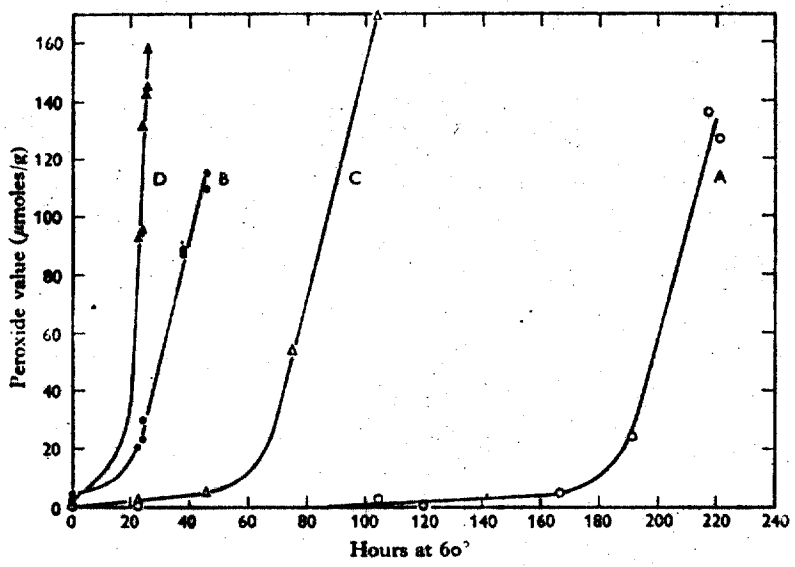

Fig. 3. Relative rates of autoxidation at 60° of the skin fats of turkeys on diets with different fats. Diet A, 2·5% beef fat; diet B, 2·5% anchovy oil; diet C, 2·5% anchovy oil + EMQ; diet D 5·0% anchovy oil.

Taste panel results on the roasted breast and leg meats from this experiment will be given in Part 2, together with the composition of the muscle lipids to which they directly relate. Full taste panel tests could not be done on the skin tissue alone, through lack of material, but it was obvious that fishy taints were present in all the groups given fish oil, with D certainly the most tainted and C probably less tainted than B.

DISCUSSION

Oxidation of the dietary lipids. Peroxidation of unstable lipids such as fish oils during the storage and feeding of experimental diets containing them can invalidate the results obtained unless a stabilizing antioxidant is also present, and this is not always practicable in view of possible nutritional effects. In the experiments of Rasheed, Oldfield, Kaufmes & Sinnhuber (1963), in which 15% fresh clay-bleached menhaden oil was given to rats in a semi-synthetic basal diet, toxic signs including anorexia, steatitis and death were attributed to rapid and extensive oxidation of the oil during 24 h exposure of the mixed diet before consumption. The same authors also

quote an experiment in which menhaden oil mixed in unspecified proportion with a synthetic diet increased in thiobarbituric acid (TBA) value from 32 to 1220 in 24 h at room temperature. On the other hand, we have previously found (Lea *et al.* 1966) that 1·5% anchovy oil incorporated into a normal type of cereal-based diet for feeding to turkeys did not oxidize appreciably in 24 h at 32°. The experiment now reported, in which 2·5% or 5·0% anchovy oil was included in a similar diet, represents an intermediate example, in that peroxidation of the oil during 24 h exposure at 27° was readily detectable (Fig. 1), but was too small to be nutritionally serious. Probable reasons for the diversity of these results are (*a*) the smaller proportions of oil used in our experiments, and (*b*) the protective action of natural antioxidants present in the cereal, soya and yeast constituents of the normal diet. Some of the nutritional effects of fish oils reported in the earlier literature may have been due rather to peroxidized fish oils.

Performance of the turkeys. The turkeys tolerated 5% of anchovy oil surprisingly well (Table 1), a result, however, in general agreement with the recent observation of Leong, Knobl, Snyder & Gruger (1964) that at 5% of the diet menhaden oil was equal to maize oil in feeding value for broiler chickens. Our result was, no doubt, influenced by the presence in the diet of 26 i.u./kg of added stabilized α-tocopheryl acetate and Leong *et al.*'s by the presence of 0·0166% EMQ.

Gas chromatographic analysis of the lipids. GLC provides a rapid means of separating and tentatively identifying the components of mixtures of fatty acid esters, but with so complex a mixture as a fish oil some supplementary aid is advisable to assist differentiation between close-running pairs such as the esters of 17:0 and 16:2, 18:4 and 20:1, and 20:5 and 22:1, where the opposing effects of increasing chain length and unsaturation can make identification difficult. For this purpose a preliminary fractionation of the ester mixture on a thin layer silica–silver nitrate plate, and an additional GLC analysis of the fully hydrogenated ester mixture were both found useful.

Quantitative operation can present more difficulty. Though a majority of users of the GLC technique have observed (or, more usually, assumed) proportionality between peak area and weight percentage (Horning, Ahrens, Lipsky, Mattson, Mead, Turner & Goldwater, 1964), some authors have found better agreement with mole percentage (Gruger, Nelson & Stansby, 1964), and the difference between these two methods of calculation can be large for a fish oil containing major components so far apart in molecular weight as C14 and C24. Still others (Kilgore & Luker, 1964; Pons & Frampton, 1965) have found it necessary to use a different conversion factor for each individual fatty ester. Because of these conflicting reports it has become fairly common practice to test the performance of the instrument and the conditions used on test mixtures of known composition, but these have often consisted of saturated esters only, or of saturated plus unsaturated esters up to linolenate (18:3). Only very rarely have esters of the 20:4, 20:5 and 22:6 acids been included, though these late-running components are the most likely to suffer loss under the conditions of high temperature or long time required to elute them. Gerson, McIntosh & Shorland (1964) reported losses of 51% and 86% during separation of the methyl esters of the C_{20} and C_{22} acids of shark-liver oil. In repeated runs with known mixtures con-

taining these highly unsaturated esters we found no evidence of any systematic loss or of deviation from proportionality between chart area and weight percentage, and no correction factors have been applied to any of the results reported in this paper.

Effect of dietary lipids on the composition of the skin fats. Replacement of 2·5% beef fat in the diet by the same amount of anchovy oil raised the iodine value of the skin

Table 4. *Fatty acid composition of the dietary fats, and of the skin fats of the four groups of turkeys receiving diets A–D with different fats*

Carbon number	Probable identity	Basal diet* IV 105	Beef fat† IV 47	Anchovy oil‡ IV 196	Skin fats A IV 68	B IV 99	C IV 94	D IV 133
			Major constituents (> 1%)					
14·00	14:0	1·1	3·5	7·8	1·7	4·5	4·3	5·9
16·00	16:0	22·4	24·0	14·0	25·0	26·3	27·0	22·4
16·49	16:1	1·8	4·7	9·2	7·0	6·7	6·6	7·9
17·20	16:2	—	—	2·1	—	0·7	0·7	1·1
17·70	16:3	—	—	2·0	—	0·7	0·6	1·1
16·95	17:0	Tr	1·4	1·2	0·7	1·0	1·0	1·0
18·00	18:0	6·7	18·9	2·2	10·3	8·7	8·0	6·1
18·40	18:1	19·3	41·0	17·3	37·4	20·8	21·4	19·7
18·95	18:2	43·2	2·9	1·2	13·9	15·8	15·5	13·9
19·80	18:3	4·7	0·4	0·4	1·3	1·5	1·7	1·6
20·20	18:4	—	—	3·0	—	0·8	0·7	1·3
20·35	20:1	Tr	Tr	2·1	0·5	1·2	1·2	1·5
21·75	20:4	—	—	2·2	—	0·5	0·5	0·7
22·48	20:5	—	—	23·4	—	5·9	4·9	10·0
24·55	22:5	—	—	3·0	—	2·2	2·2	2·6
24·85	22:6	—	—	7·3	—	1·9	1·7	3·0
			Minor constituents (1·0–0·1%)					
14·55	14:1	—	1·0	0·2	0·5	0·4	0·3	0·4
15·00	15:0	—	0·7	0·4	0·3	0·5	0·4	0·5
17·35	17:1, 18 br	—	0·8	—	0·4	—	—	—
20·00	20:0	Tr	Tr	Tr	0·5	0·4	0·6	0·6
22·20	22:1	—	—	0·1	—	0·1	0·1	0·1
23·55	22:4	—	—	1·0	—	Tr	Tr	Tr
			Very minor constituents (< 0·1%)					
10·00	10:0	—	Tr	Tr	Tr	Tr	Tr	Tr
12·00	12:0	—	Tr	Tr	Tr	Tr	Tr	Tr
14·75	15 br	—	Tr	NS	NS	NS	NS	NS
15·60	14:2, 16 br	—	0·4	Tr	0·1	Tr	Tr	Tr
16·80	17 br	—	0·8	NS	Tr	NS	NS	NS
19·02	19:0	—	Tr	Tr	Tr	Tr	Tr	Tr
19·50	20 br	—	Tr	Tr	Tr	Tr	Tr	Tr
19·65	19:1	—	—	Tr	Tr	Tr	Tr	Tr
20·90	20:2	—	—	Tr	Tr	Tr	Tr	Tr
20·95	21:0	—	—	Tr	—	Tr	Tr	Tr
21·30	20:3	—	—	Tr	Tr	Tr	Tr	Tr
22·00	22:0	—	—	Tr	—	Tr	Tr	Tr
24·00	24:0	—	—	Tr	—	Tr	Tr	Tr

Iodine values (IV) are the means of observed and calculated, rounded to nearest unit. Tr = < 0·1%; NS (no significant amount) = ≤ 0·1%; — = absent.

* Contributes 2·3% lipid to all diets.

† Contributes 2·5% to diet A. Also contains traces of 14 br and 19 br acids.

‡ Contributes 2·5% to diets B and C, 5·0% to diet D.

fat by about 30 units, and doubling the amount of anchovy oil raised it by another 30 units (Table 4).

The lipid of the basal diet, 2·3% in all four diets, was very much richer in linoleate than either the beef fat or the anchovy oil, and was presumably the main source of this acid in the skin fats; its concentration, therefore, did not vary very much. The beef fat contained more branched chain saturated acids, all in small amount, than did the other dietary fats; some of these branched chain acids were detectable also in the skin fat of the birds from group A. The anchovy oil contained seven major and two minor acids which were not present in either of the other dietary fats, and all of these were also present in the skin fats of groups B, C and D, which had received fish oil, but were absent from the skin fats of group A, which had not. One major acid (20:1), present at 2·1%, in the anchovy oil and at the trace level only in the other dietary fats, was also present at much higher levels in the skin fats of the groups given fish oil.

Table 5. *Transference of individual fatty acids from dietary fish oil to the skin lipid of turkeys receiving diets B, C and D*

Acid	Concentration in anchovy oil (%)	Concentration in skin fat: No. of times level in diet B	C	D	As % of level in dietary lipid* B	C	D
16:2	2·1	13·3	13·3	10·5	64	64	76
16:3	2·0	14·0	12·0	11·0	67	58	80
18:4	3·0	10·7	9·3	8·7	51	45	63
20:1†	2·1	13·3	13·3	9·5	64	64	69
20:4	2·2	9·1	9·1	6·4	44	44	46
20:5	23·4	10·1	8·4	8·5	48	40	62
22:5	3·0	19·4	29·4	17·3	141	141	126
22:6	7·3	10·4	9·3	8·2	50	45	60
Mean (excluding 20:1)		11·6	10·7	9·0	55	51	65

* Total dietary lipid = 4·8% for diets B and C, 7·3% for diet D.
† Present also in skin fat of group A and calculations therefore based on increases.

The efficiencies with which the eight major fatty acids of fish oil were transferred from the dietary to the skin lipid followed a generally similar pattern, except for acid 22:5. When the fish oil level was 2·5% (diets B and C) the other seven acids were deposited in the skin fats at a level about eleven times greater than their concentrations in the whole diet, or approximately half their concentrations in the dietary lipid. When the fish oil content of the diet was raised to 5·0% (diet D) the corresponding figures were approximately nine times and two-thirds (Table 5). Acid 22:5, however, accumulated between two and three times as efficiently with diets B and C and nearly twice as efficiently with diet D. This could be the result of a more efficient deposition or less efficient mobilization of this particular acid, but it could, alternatively, be due to chain lengthening of a small proportion of the 20:5 acid, of which the anchovy oil contained as much as 23·4%, before deposition.

Marion & Woodroof (1963) gave 1% of menhaden oil to chickens and found approximately 1% of the 20:5 acid in the skin fat, but no acids of longer chain length were

detected, though they were present in the lipids of the muscle. Reed (1964) found that deposition of PUFA in the depot fat of rats did not follow the proportions in which they were present in the cod-liver oil given; though the 20:5 and 22:6 acids were present in the cod-liver oil in a ratio of nearly 2:1 the depot fat of rats receiving the oil showed elevated contents of hexaene but not of pentaene acids. The method (alkali isomerization) used for analysis of the depot fats gave fatty acid composition according to degree of unsaturation, but not according to chain length. In our experiment with turkeys there was no difference between the behaviour of the 20:5 and 22:6 acids.

Effect of diet on fat stability and flavour. The reason for the greatly reduced stability of the skin fats of the birds given fish oil as compared with the control group given beef fat (Fig. 3) can be seen from their compositions. The fats of groups A, B and D all contained approximately the same amount of tocopherol (Table 2) and the large differences in their stability therefore derived, presumably, from the higher levels of unsaturation in the groups given fish oil. The total concentrations of fatty acids with three or more double bonds in groups B, C and D were respectively 13·5, 12·3, and 20·3%, mainly pentaene and hexaene, whereas in group A the polyene level was only 1·3, all linolenate (18:3). The considerably greater stability of skin fat C as compared with B is not likely to have been due to its only slightly lower PUFA content, but was more probably due to a sparing of tocopherol by the antioxidant (EMQ) in this diet, which resulted in a tocopherol level nearly twice as high as in the other skin fats. Whether traces of EMQ itself might have been deposited in the skin fat of group C could not be determined with the methods of estimation and limited amounts of material available. The extra tocopherol in diet D did not result in increased deposition in the depot fat, presumably owing to a destructive effect of the high level of fish oil present in this diet.

Even dietary linolenic acid in sufficient quantity can cause 'off' flavours described as 'fishy' in roast turkey, as shown by Klose, Mecchi, Hanson & Lineweaver (1951) who gave 5% of linseed oil, but fishy flavours resulting from dietary additions of marine animal oils probably arise mainly from oxidative degradation of fatty acids still more highly unsaturated. The skin, because of its high fat content and maximum exposure to high temperature and oxygen during cooking, is particularly susceptible to the development of 'off' flavours of this kind.

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Hydrogenated Marine Fat, Effect on Growth and Liver Weight in Young Rats given a High Fat, Low Protein Ration

L. R. NJAA, O. R. BRÆKKAN, G. LAMBERTSEN and F. UTNE

Government Vitamin Laboratory, Norwegian Fisheries Research Institute, Bergen

Abstract. Hydrogenated marine fat and some other commercial fats were fed to young rats in a low protein diet at a level of about 50% of the calories. The diet was marginal in choline, α-tocopherol and linoleic acid. The experimental procedure involved littermate control and equalized feeding within litters.

Rats given hydrogenated marine fat, as well as those given hydrogenated soya bean oil and hydrogenated rapeseed oil showed less weight gain than rats given lard and herring oil. When the conditions were not complicated by the occurrence of fatty livers, the rats given the hydrogenated fats showed a higher liver weight: final body weight ratio than the rats given the natural fats.

Supplementing the hydrogenated marine fat with 2.5% sunflower seed oil improved its caloric efficiency.

Additions of α-tocopherol were without any effect.

There was a high degree of correlation between the growth efficiency of calories above basal needs and the liver weight: final body weight ratio.

Key Words
Young rat
Hydrogenated marine fat
High fat diet
Growth
Liver weight

Hydrogenated marine fat (HMF) is used in the production of margarine. Few studies report on the nutritive value of HMF. BRÆKKAN *et al.* [1968] fed a moderate level of HMF (m.p. 32°C) to young rats on a semisynthetic diet. The rats given HMF grew slightly slower than the control rats given a mixture of lard and olive oil (9:1) of the same melting point (m.p.). UTNE *et al.* [1966] gave graded amounts of 2 types of HMF to growing mink. The melting points were 44 and 33°C, and lard (m.p. 38°C) was used as a control. Growth was best on the lower melting HMF, followed by lard and the higher melting HMF. The content of fat and fatty acids in the faeces was highest in the higher melting HMF, followed by the lower melting HMF and lard. LAKSESVELA [1966] found in experiments with chicks that hydrogenation of herring oil to give HMF's with melting points ranging from 31 to 50°C

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reduced the digestibility of the fat progressively. The degree of hydrogenation had, however, little effect on the growth of the chicks. HERSTAD [1970] used 2 types of HMF (melting points 43 and 33°C) as supplements to broiler feeds. He found that the feed utilization was best with the lower melting type and that soya bean oil was slightly better utilized than HMF. AAES-JØRGENSEN and HØLMER [1969] gave partly-hydrogenated herring oil and similarly hydrogenated arachis oil and soya bean oil as the sole dietary fat to young rats and studied the development of essential fatty acid deficiency and the changes in the fatty acid composition of lipids from the testes. Essential fatty acid deficiency and lipid changes in the testes developed on all the hydrogenated fats, but the effects were most profound with partly-hydrogenated herring oil.

We now report growth studies with young rats given HMF and some other fats in a high fat, low protein diet which was also low in choline. This type of diet, which was also used by PLATT [1939], was chosen because it is reported to be suitable for detection of differences between different types of fat. LUCAS and RIDOUT [1967] make the following statement on this point: 'BENTON *et al.* point out that the difference in the effect of different fats can be shown only when the diet is deficient in either protein or choline and that under normal conditions (adequate diet) the type of fat has no effect on the deposition of liver lipids. We agree with this and also with DAMODARAN and SIVA RAMAN that the response to fats fed with an unbalanced diet gives no indication of the nutritive value of the fat when fed in a normal ration.'

Seven experiments were performed over 2 years using different batches of commercial available HMF. Each experiment lasted 4 weeks and the 4-week weight gain was used as a criterion for fat utilization, further, littermate control and equalized feeding within litters were applied. The ratio between liver weight and final body weight was also recorded because results with oxidized fats [JOHNSON *et al.*, 1957], laboratory heated fats [POLING et al., 1960, POTTEAU *et al.*, 1970] and polymerized marine oils (own unpublished results) have shown that this ratio may be an indication of some unknown changes in fat metabolism.

Materials and Methods

Fats. The fats used were commercially available, HMF, hydrogenated soya bean oil (HSO), and 85:15 mixture of hydrogenated rape seed oil and tallow (R+T), a 1:1 mixture of coconut oil and tallow (C+T), a herring oil (HO) and lard. In the tables the fats are

Table I. Some analytical data on the fats used

	Lard	HO	Hydrogenated fats		
			HMF	HSO	R+T
Melting point	34–40	–	36–40	40.5	36
Percent trans-bonds (in sum of fatty acids)	0	0	45	50	38
Percent 18:2-acids (incl. isomers in hydrogenated fats)	7.5–8.5	1.1	1.5–2.0	0.8	–
Percent C_{20}-acids (saturated and monoenes)	1.5	15.8	13–18	0	8.0
Percent C_{22}-acids (saturated and monoenes)	0.1	22.4	15–20	0	38.4

identified by the codes given in parentheses. Some information on these fats are given in table I.

Lard was used as a standard of reference and the melting points of the other fats, except that of herring oil, were adjusted near to that of lard. All fats except lard contained little linoleic acid, and the hydrogenated fats contained 38–50% of trans double bonds, calculated on the basis of the total fatty acids. As the experiments were done over 2 years, several samples of HMF and lard were used, and these were not analyzed individually for their fatty acid composition. The type of HMF used has been analyzed for its fatty acid composition [Lambertsen *et al.*, 1966] and for its isomer content [Lambertsen, 1971]. HMF had fatty acids of chain lengths from 14 to 24 carbon atoms and about 35% of saturated and monoene C_{20} and C_{22} acids. 75% of the monoenoic acids had trans configuration at their double bonds, and the positions of the double bonds showed a statistically normal distribution around a maximum at the middle of the chain. This isomer composition was interpreted to indicate that the fatty acids had been isomerized to an equilibrium state in the hydrogenation process.

The mixture of coconut oil and tallow, which was not analyzed for its fatty acid composition, was included as a fat having a wide range of fatty acid chain lengths (C_{10}–C_{18}) compared to lard with 65% C_{18}-acids and to hydrogenated soya bean oil with 90% C_{18}-acids. Herring oil, a raw material for HMF, does not contain 'unnatural' isomers and has less saturated acids and more highly-unsaturated acids than HMF. The fatty acids of herring oil have been analyzed by Lambertsen and Brækkan [1965]. The batches of lard used were of Danish origin supplied in 250 g packages.

Samples of HMF from different oil refineries are identified by a, b, and c. The numbers 1, 2.5, 4, 5 and 10 given together with the fat codes indicate percentage additions of sunflower seed oil (containing 60% linoleic acid) to the experimental fats. 5 r designates addition of 5% sunflower seed oil stripped of α-tocopherol by molecular destillation. The additions of α-tocopherol are indicated by α-toc_1, α-toc_2 and α-toc_3 which refer respectively to 0.07 and 0.032% α-tocopherylacetate, and to 0.031% saponified α-tocopherylhydrogen succinate in the diet.

Diets. The fats were tested at the 28-percent level (corresponding to about 50% of the calories) in a diet which further consisted of 44% partly dextrinized potato starch, 9% sucrose, 10% spray dried egg albumine, 4% salt mixture [SURE, 1941] and 5% oat hulls. The vitamin supplements given in 1% starch, were per 10 g diet: Thiamine, riboflavine and pyridoxine 50 μg, vitamin B_{12} 0.1 μg, d-biotin 1 μg, folic acid 10 μg, d-calcium pantothenate 150 μg, niacin 250 μg, *p*-aminobenzoic acid 500 μg, i-inositol 2.5 mg, cholin chloride 5 mg, menadion 25 μg. Vitamin A and D were given by dropper 2 times weekly or mixed into the diet in amounts corresponding to 200 IU vitamin A and 20 IU vitamin D per rat per week.

Experimental procedure. Each experiment comprised 4 treatment groups of 6 rats except experiment G which comprised 5 groups of 5 rats. The rats were 4–5 weeks old at the start of the experiment. 6 (or 5) litters of 4 (or 5) rats of the same sex and of similar body weight within litters were allotted to the treatment groups so that each litter was represented in all treatment groups. The rats were kept in separate cages with wire bottoms, suspended over trays covered with filter paper. The urine was absorbed by the paper, thus the separation of spilled food and faeces was facilitated. The diets were mixed weekly and stored at 2–5° C. In all experiments except in experiment F and G the rats were put directly on to the experimental diets, in experiments F and G all rats were given the lard diet for 1 week before the experiment started. The appetite factor was eliminated by keeping the food intake constant within each litter throughout the experiment, including the preliminary periods in experiments F and G. The rat with the lowest food intake determined the amount offered to the other rats within the litter. Spillings were returned to the feeding cup several times daily and by careful supervision the littermates were induced to eat the same amount of food during the 4-week feeding period. The rats were weighed weekly. In 2 experiments (A and B) faeces were collected from each rat during the last 2 weeks, air dried and weighed. After 4 weeks on the experimental diets the rats were weighed and killed, the livers were removed and weighed.

Analytical methods. Fat in the livers was determined by ethyl ether extraction in a Soxhlet apparatus of individual livers (experiment A) or of samples of pooled livers (experiment B) after grinding with anhydrous sodium sulphate. Fat in faeces was determined by ethyl ether extraction of samples of dry faeces without and with previous treatment of the samples with 18 N sulphuric acid. The extraction procedure was described by SQUIBB *et al.* [1958]. The amount of fat extracted without previous acid treatment was termed free fat, the amount extracted after acid treatment was termed free plus bound fat. The fat fractions used to determine the fatty acid chain length composition of faeces fat were obtained by first extracting extensively with ethyl ether, then the faeces residue was acidified with hydrochloric acid and the bound fat was extracted by continued use of ethyl ether. From these fractions methyl esters were prepared, these were completely hydrogenated and analyzed by GLC.

The fatty acid composition of some of the fats used have been analyzed at intervals, partly in connection with other experiments. Details of the analytical procedures including GLC were described by LAMBERTSEN *et al.* [1966]. The percentage of trans double bonds was determined by IR-spectrophotometry of the total fatty acid methyl esters [LAMBERTSEN, 1971].

The trans-values given, therefore, refer to the sum of the saturated, monoenoic and polyenoic fatty acids in the fat (table I).

Protein (N × 6.25) was determined by a micro Kjeldahl technique.

Dry matter was determined by drying in an oven at 105°C for 2 h.

Ash was determined by heating in an oven at 800°C for 4 h.

Calcium was determined in the ash by oxalate precipitation and titration with potassium permanganate.

Details of the experiments

Experiment A. HMF was compared with 2 natural fats, lard and a mixture of coconut oil and tallow (1:1) and further with hydrogenated soya bean oil.

Vitamins A and D were given by dropper 2 times weekly in 50 mg sunflower seed oil which supplied about 5 μg α-tocopherol and 9 mg linoleic acid per rat per day. Individual livers were analysed for fat. Faeces collected from each rat during the last 2 weeks of the experiment were weighed air dry and analysed for free and free plus bound fat, and for nitrogen.

Experiment B. 3 samples of HMF from different oil refineries were compared with lard. One package of lard was used each week, the melting point varied from package to package between 34 and 40°C. Vitamin A and D were supplied as in experiment A. Fat, protein and ash were determined in the pooled livers from each group. Faeces collected from each rat as in experiment A were analysed for free and free plus bound fat, ash and calcium.

Experiment C. HMF was compared with the hydrogenated soya bean oil as in experiment A and with hydrogenated rapeseed oil to which had been added 15% tallow to raise the melting point to 36°C. Sunflower seed oil containing fat soluble vitamins was either given by dropper as in experiments A and B or for comparison mixed with HMF.

Experiment D. HMF containing 1% sunflower seed oil was compared with HMF containing 4% sunflower seed oil and extra α-tocopherol, with lard and with herring oil.

Experiment E. HMF without added sunflower seed oil was compared with HMF with added α-tocopherol and with HMF with 5% added sunflower seed oil which had been stripped of α-tocopherol by molecular distillation. Lard was included as a standard of reference. Vitamins A and D were given by dropper as in experiments A–C, except that arachis oil was used as vehicle instead of sunflower seed oil to reduce the intake of α-tocopherol and linoleic acid.

Experiment F. HMF was compared with HMF containing 5% added sunflower seed oil, with HMF containing 5% sunflower seed oil stripped of α-tocopherol and with HMF to which was added α-tocopherol corresponding to the concentration in the original sunflower seed oil. Vitamins A and D were mixed into the experimental fats.

Experiment G. HMF containing graded amounts of sunflower seed oil (0, 2.5, 5 and 10%) was compared with lard. Vitamins A and D were given as in experiment F.

Statistical treatment of the results. 4-week weight gains, liver weight: final body weight ratio and values of liver and faeces components determined on individual rats were treated in analyses of variance and the significance of the treatment effect was ascertained. When significant treatment effects were found, the treatment sum of squares was subdivided into comparisons with one degree of freedom as described by Cochran and Cox [1950]. Of

these comparisons only the significant ones are given in the tables, the significance levels being indicated by 3–1 asterisks corresponding to the levels 0.001, 0.01 and 0.05, respectively. For instance: In experiment A (table II) the following comparisons were made between weight gains: HMF (2) against the mean of the 3 other groups ($\frac{1+3+4}{3}$), hydrogenated soya bean oil (3) against the mean of the 2 remaining groups ($\frac{1+4}{2}$), and finally lard (1) against the mixture of coconut oil and tallow (4). Only the 2 first comparisons were significant and are indicated in the table, 2 vs. $\frac{1+3+4}{3}$ is given on the same line as HMF with 3 asterisks, indicating that this comparison is very highly significant.

An attempt was made to correlate the liver weight: final body weight ratio with the efficiency of caloric utilization for growth. As the starting weight and the food intakes were not the same in all experiments this was taken into account by calculating the efficiencies of the calories consumed above the basal requirement. In the variable thus calculated: $(C—C_0)/\triangle W$, C is the calorie intake obtained by multiplying the food intake by the calorie content of the diet (4.8 kcal/g). C_0 is the amount of calories required for basal metabolism obtained from the formula $C_0 = 70$ kcal $W^{0.73}$. 28, where W is the mean body weight in kg and 28 is the length of the experimental period in days [MILLER and PAYNE, 1964]. $\triangle W$ is the 4-week weight gain in g.

Results

The 4-week weight gains and the liver weight: final body weight ratios are summarized in table II. The results in experiment A showed less weight gain in the group given HMF than in the other groups, further the group on hydrogenated soya bean oil grew less than the rats given the 2 natural fats. The liver weight: final body weight ratio increased with decreasing weight gain. These findings were substantiated when 3 different samples of HMF were compared with lard in experiment B and when HMF was compared with hydrogenated soya bean oil and hydrogenated rapeseed oil in experiment C. There was no significant difference between the 3 samples of HMF (experiment B). Hydrogenated soya bean oil and hydrogenated rapeseed oil gave similar weight gains, but the liver weight: final body weight ratio was significantly lower for the group given hydrogenated soya bean oil than for the 3 other groups (experiment C). There was no significant effect of giving the sunflower seed oil containing vitamins A and D in the diet instead of by dropper.

In experiment D was tested whether the effects found for HMF were inherent in unhydrogenated herring oil, and further whether these effects were due to low intakes of linoleic acid and α-tocopherol. There was a tendency for the group given 4% sunflower seed oil and α-tocopherol in HMF to grow better than the group given 1% sunflower seed oil. The 2 HMF groups grew

Table II. Weight gain and liver weight: final body weight ratios in rats given HMF and some other commercial fats

Experiment	Group	Fat	Starting weight g	Food eaten g	Weight gain g	Significance of comparison	Liver weight/ final body weight ratio g/g	
A	1	Lard	65.1	175	38.4		0.031	
	2	HMF	65.4	176	22.2	vs. $\frac{1+3+4}{3}$ ***	0.040	vs. $\frac{1+3+4}{3}$ **
	3	HSO	65.5	176	31.7	vs. $\frac{1+4}{2}$ **	0.034	vs. $\frac{1+4}{2}$ **
	4	C+T	65.5	175	35.8		0.031	
B	1	Lard	53.7	175	40.2	vs. $\frac{2+3+4}{3}$ ***	0.030	vs. $\frac{2+3+4}{3}$ ***
	2	HMF_a	53.7	175	26.6		0.047	
	3	HMF_b	54.0	176	23.3		0.049	
	4	HMF_c	52.7	176	24.2		0.047	
C	1	HMF	51.1	164	24.0	vs. 3+4***	0.042	
	2	HMF 1	51.1	164	25.9		0.041	
	3	HSO	50.9	164	30.7		0.035	vs. $\frac{1+2+4}{3}$ ***
	4	R+T	50.1	164	29.7		0.040	
D	1	Lard 1	59.3	184	46.5		0.038	
	2	HMF 1	59.5	184	38.7	vs. 1+4**	0.036	
	3	HMF 4 α-toc_1	59.0	184	41.1		0.038	
	4	HO 1	58.6	184	44.6		0.046	vs. $\frac{1+2+3}{3}$ **
E	1	Lard	68.5	208	46.4	vs. $\frac{2+3+4}{3}$ **	0.034	
	2	HMF	69.3	208	34.3		0.042	vs. 1+4***
	3	HMF α-toc_2	68.1	208	32.9		0.043	
	4	HMF 5 r	69.5	208	36.5		0.036	
F	1	HMF	60.2	198	31.8		0.045	
	2	HMF 5	60.3	198	41.0	vs. 1+4***	0.036	vs. 1+4***
	3	HMF 5 r	59.6	198	42.4		0.036	
	4	HMF α-toc_2	60.9	198	29.5		0.048	
G	1	Lard	95.0	288	68.9	vs. $\frac{2+3+4+5}{4}$ ***	0.046	vs. $\frac{2+3+4+5}{4}$ **
	2	HMF	95.8	288	56.4	vs. $\frac{3+4+5}{3}$ *	0.041	vs. $\frac{3+4+5}{3}$ *
	3	HMF 2.5	95.5	288	61.9		0.037	
	4	HMF 5	95.5	288	60.1		0.035	
	5	HMF 10	96.4	288	60.9		0.036	

less than the 2 groups given the natural fats, lard and herring oil. Fatty livers were observed in some of the rats in the herring oil group and to a smaller extent in the lard group, accounting for the higher liver weight: final body weight ratio observed in these groups. In experiment E the tendency observed in experiment D for the supplemented group was investigated further by separate additions of α-tocopherol and sunflower seed oil stripped of α-tocopherol. The HMF groups showed no significant growth differences but all grew slower than the lard group. The liver weight: final body weight ratio was of the same magnitude in the HMF group with added stripped oil and in the lard group, the mean of these 2 groups were significantly lower than in the 2 other HMF groups. In experiment F the rats given HMF containing 5% sunflower seed oil and 5% of the stripped oil grew significantly better and had lower liver weight: final body weight ratios than the rats given HMF without supplement or supplemented with α-tocopherol alone. Experiments E and F showed that when HMF was supplemented with α-tocopherol in amounts corresponding to the content in 5% sunflower seed oil neither weight gain nor liver weight: final body weight ratio changed significantly as compared with the values found for the unsupplemented groups. In fact, weight gains were slightly less and the liver weight ratios slightly higher in the groups supplemented with α-tocopherol than in the unsupplemented groups. The effect of graded amounts of sunflower seed oil was studied in experiment G. The growth was significantly improved when HMF was supplemented with sunflower seed oil, the 2.5% level showing maximum effect as no further improvement was found with the 5- and 10-percent levels. The weight gains in the supplemented groups did not reach the level attained in the lard group. The supplemented groups showed lower liver weight: final body weight ratios than the unsupplemented group. The high liver weight: final body weight ratio in the lard group was due to the occurrence of fatty livers.

The analyses of the livers in experiments A and B (table III) showed lower fat contents in the groups given HMF or hydrogenated soya bean oil than in the groups given lard or the mixture of coconut oil and tallow. In experiment A, where individual livers were analyzed, the difference was very highly significant. The fat levels found were, however, below the range usually found in fatty livers [LUCAS and RIDOUT, 1967]. The dry matter, protein and ash contents of the pooled livers in experiment B showed only small differences. The faeces collected during the last 2 weeks of experiments A and B weighed more from rats given HMF than from the other rats. This was due to more fat being excreted in the HMF groups. This difference in fat excretion was

Table III. Chemical analyses of livers and faeces of the rats in experiments A and B

	Experiment A				Experiment B			
	1 Lard	2 HMF	3 HSO	4 C+T	1 Lard	2 HMF_a	3 HMF_b	4 HMF_c
Liver								
Dry matter, %					29.9	29.2	30.5	33.6
Fat, %	5.2	3.0	3.2	4.3	7.9	4.2	3.6	3.8
Protein, %					23.6	24.2	24.6	24.7
Ash, %					1.47	1.48	1.58	1.54
Fat comparisons	1+4 vs. 2+3***							
Faeces								
Weight, g	10.5	12.4	10.6	10.4	10.1	11.8	12.0	11.4
Weight of fat free, g	8.1	8.0	7.7	7.8	7.6	8.0	7.7	7.5
Free fat, % of eaten	0.64	1.80	1.49	0.80	0.69	1.63	1.71	1.37
Free plus bound fat, % of eaten	8.9	16.7	11.1	9.9	9.6	14.5	16.7	15.1
Nitrogen, % of eaten	16.6	19.4	14.8	14.2				
Calcium, % of fat free					2.98	2.80	3.00	2.91
Ash, % of fat free					13.0	11.6	11.9	11.7
Free fat comparisons	2+3 vs. 1+4***, 2 vs. 3*				1 vs. $\frac{2+3+4}{3}$***			
Free plus bound fat comparisons	2+3 vs. 1+4***, 2 vs. 3***				1 vs. $\frac{2+3+4}{3}$***			
Nitrogen comparisons	2 vs. $\frac{1+3+4}{3}$*** 1 vs. $\frac{3+4}{2}$*							

For code, see text.

seen both for free and for free plus bound fat. The data given for fat free faeces are the differences between faeces weight and the weight of free plus bound faeces fat. The HMF rats lost up to about 8.5% of the calories as fat in the faeces whereas the other rats lost about 4–5%. In experiment A the HMF group also lost slightly more nitrogen in the faeces than the other groups. In experiment B the fat free faeces contained more ash in the lard group than in the other groups, but there was no corresponding differences in the calcium contents.

The chain length analyses of the experimental fats used in experiment A and of the corresponding fat fractions extracted from the faeces (table IV) showed a general agreement between food fat and faeces fat. There was,

Table IV. Experiment A: Fatty acid proportions by chain length of diet fats, and free (f) and bound (b) faeces fat fractions

Fatty acid chain length	Group 1			Group 2			Group 3			Group 4		
	Lard	f	b	HMF	f	b	HSO	f	b	T+C	f	b
12	–	–	–	–	–	–	–	–	–	31.1	11.9	5.7
14	1.2	1.6	0.6	10.4	4.7	1.8	–	0.5	0.1	11.9	12.5	9.5
16	29.4	22.7	23.3	24.9	14.7	12.4	9.4	12.6	19.1	19.4	26.4	34.2
18	67.9	64.2	73.6	20.2	20.8	18.0	90.6	82.5	79.2	37.6	45.9	49.5
20	1.5	7.6	2.0	20.6	24.0	27.8	–	2.9	1.1	–	2.6	0.8
22	–	3.9	0.5	23.8	35.8	40.0	–	1.5	0.5	–	0.7	0.3

however, a tendency for the longer chain lengths to be more concentrated in the faeces fraction than in the food fats and a correspondingly lower concentration of the short chain fatty acids.

Discussion

At the start of these experiments we assumed that the long chain saturated and trans monoene fatty acids present in HMF would be more difficult to metabolize than those in lard, particularly at the high fat, low protein and low choline levels used. However, the first 3 experiments showed that this was not the case. All fats tested were well digested. The different digestibility of HMF and lard (table III) could not explain the growth effect: The calorie efficiencies for lard and HMF (A1 and A2 in fig. 1) were 13.5 and 24.5, respectively. When the calorie intakes were corrected by the calorie content in the faeces fat, these values changed only to 12.4 and 21.3. The slower weight gain of the groups given HMF compared to the lard groups point in fact to a slightly increased catabolism of the HMF fatty acids. The low choline level used did not result in fat accumulation in the livers of the HMF groups. Table III shows that the fat content in the livers were higher in the lard groups than in the HMF groups, and the few cases of fatty livers observed were confined to 2 lard groups and the herring oil group. Experiments D–F showed that α-tocopherol did not influence the growth differences. The α-tocopherol content of HMF varies between 25 and 100 μg/g

[LAMBERTSEN *et al.*, 1964] and the amounts present seem to be adequate under our experimental conditions. Experiments E–G showed that growth improved when sunflower seed oil was added to HMF, 2.5% addition giving optimal effect.

The results obtained from 29 experimental groups in 7 experiments were compared in the graph given in figure 1. This graph shows that calories above the basal level required for 1 g of weight gain and the liver weight: final body weight ratio were highly correlated (r = +0.92). The observations on HMF with up to 1% added sunflower seed oil show a high degree of spreading along the regression line, whereas those with 2.5% or more added sunflower seed oil cluster together near the lower part of the regression line. The 1-percent level thus seems to be marginal. In the lower set of values we also find one observation on HMF with 1% added sunflower seed oil (D2), the observations on hydrogenated soya bean oil (A3 and C3) and one obser-

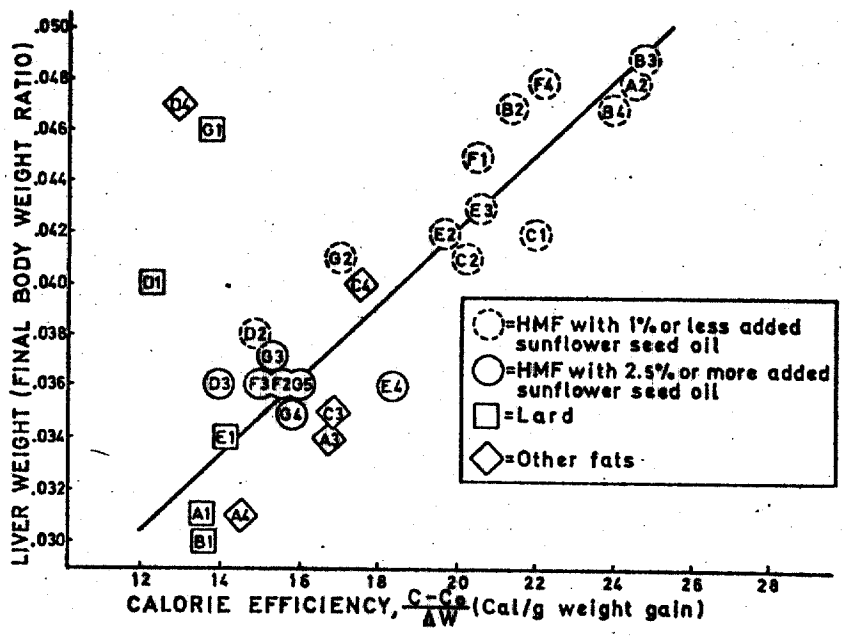

Fig. 1. Linear regression of liver weight: final body weight ratio (Y) on efficiency for growth of calories consumed above basal needs (×). Regression equation Y = 0.0015* × +0.012, correlation coefficient r = +0.92. Calculation based on 26 points excluding the groups with fatty livers (D1, D4 and G1). C = calories consumed, C_0 = basal calories, ΔW = 4-week weight gain. Letters refer to experiments, numbers to groups.

vation on lard (E1). Two observations on lard (A1 and B1) and the observation on the mixture of coconut oil and tallow (A4) are found at the lower end of the line. The observation on hydrogenated rapeseed oil (C4) is found near the middle of the line. The graph illustrates the effects of adding sunflower seed oil to HMF on its calorie efficiency as well as on the liver weight: final body weight ratio. We have pointed out [BRÆKKAN *et al.*, 1968] that the content of about 2% 18:2 fatty acids in HMF is mainly in the form of other isomers than true linoleic acid, and the effects of adding sunflower seed oil to HMF are probably due to its content of linoleic acid.

The requirement of linoleic acid for the rat is not well established. HOLMAN [1960] estimated the requirement for the maintenance of a normal trienoic: tetraenoic ratio in tissue lipids to be 1% linolate calories in the diet. The 2.5-percent level of sunflower seed oil in HMF corresponds to about 0.5% linolate calories in the diet. This may be regarded as an estimate of linoleic acid requirement for the proper utilization of HMF for growth on a diet which in contrast to that used by HOLMAN [1960] is low in protein and choline and which is high in a fat of a type very different from those used by him. No symptoms of fatty acid deficiency were seen in any groups in this study. The hydrogenated soya bean oil showed calorie efficiencies and liver weight: final body weight ratios near those found for HMF with 2.5% or more added sunflower seed oil, in spite of its low residual linoleic acid content. Probably the lack of long chain fatty acids accounts for this. In comparison the one group given hydrogenated rapeseed oil gave a decidedly higher liver weight: final body weight ratio.

CHENITI *et al.* [1967] found that sunflower seed oil improved the utilization for growth of partly-hydrogenated rapeseed oil. The rapeseed oil was fed at about 40% of total calories and the linoleate levels corresponding to the 3 levels of sunflower seed oil tested were about 1, 2 and 3% of total calories. Although the experimental conditions were very different from ours, the requirement for added linolate for maximum improvement of hydrogenated rapeseed oil was not very far from that found by us to be required for improved utilization of HMF. In both cases the levels found to give maximum effect did not differ much from HOLMAN's [1960] estimate of total linoleate requirement.

We mentioned in the introduction that high liver weight: final body weight ratios have been reported in rats fed laboratory heated fats or polymerized fats. Generally the high ratios were found in rats showing growth depression or impaired food utilization. KAUNITZ *et al.* [1955] reported that the growth depression and liver enlargement observed in rats given heated and aerated

cottonseed oil could be counteracted by mixing untreated oil into the diet, or to a lesser extent by giving untreated oil by dropper. POLING *et al.* [1960] showed that unsaturated fats which had been damaged in the laboratory by long heating at high temperatures under oxidative conditions reduced food utilization and caused increases in liver weight. ALEXANDER [1966] pointed out that POLING *et al.* [1960] make no mention of how often the diets were mixed and of storage conditions. This also apply to KAUNITZ *et al.* [1955]. ALEXANDER [1966] found that the untoward effects of heated fats could be counteracted by daily mixing of the diet and by keeping it stored under refrigeration. The conditions in these experiments may well have been such that the linoleic acid supplied was oxidized in the stored diets. POTTEAU *et al.* [1970] found growth depression and enlarged livers in rats given soya bean oil heated in an inert atmosphere. Analyses showed that about 75% of the linoleic acid present in the original oil were found in the heat-treated oil. Therefore, their results can not be ascribed to the destruction of linoleic acid.

Altogether this indicates that liver enlargement is observed when the liver is subjected to a metabolic stress. Such stress conditions may due to a deficiency of an essential substance (fatty acids, vitamins) or to the presence in the diet of substances which are inadequately metabolized. The liver enlargements mentioned above all pertain to the fat metabolism, but similar enlargements have been observed in vitamin deficiencies [GUGGENHEIM and DIAMANT, 1959; CHATTERJEE *et al.*, 1970].

In conclusion, HMF in the type of diet used gave a slower growth and an increased liver weight: final body weight ratio compared to 'natural' fats (lard and a mixture of coconut oil and tallow). The 2 criteria measured were positively correlated as shown in figure 1. Three dietary factors were marginal in the diet. Of these the low choline content resulted in fatty livers in 2 of the lard groups, and in the herring oil group, i.e., in 3 groups given 'natural' fats. The increased liver weight: final body weight ratios seen in the HMF-fed groups were not caused by an increased fat content in the livers, and were, therefore, probably not related to a choline deficiency.

A supplementation with α-tocopherol did not improve the HMF diets as judged by our criteria.

HMF as well as the 2 hydrogenated vegetable fats were all very low in linoleic acid. Supplementing HMF with sunflower seed oil improved calorie efficiency and lowered the liver weight final: body weight ratio, 2.5% giving maximum effect. However, supplementation with sunflower seed oil did not result in calorie efficiencies equal to those found for 'natural' fats. This residual difference may be caused by a combined effect of long chain fatty

acids (20 and 22 carbon atoms) of low unsaturation and a substantial content of 'unnatural' isomers (trans and positional) in HMF. Hydrogenated soya bean oil with a similar isomer content but without long chain fatty acids showed better growth efficiency than unsupplemented HMF and hydrogenated rapeseed oil, the latter containing 'unnatural'isomers as well as long chain fatty acids. Herring oil containing natural long chain fatty acids gave good growth whereas the liver weight: final body weight ratio was obscured by the occurrence of fatty livers.

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Essential Fatty Acid Properties of Tuna, Herring and Menhaden Oils[1]

O. S. PRIVETT, F. J. PUSCH, R. T. HOLMAN AND W. O. LUNDBERG
The Hormel Institute, University of Minnesota, Austin

The limited evidence that is presently available indicates that the structures of the major polyunsaturated fatty acids of marine oils are not of the linoleic acid type (Klenk, '57; Farquhar et al., '59; Stoffel and Ahrens, '58; Whitcutt, '57; Silk and Hahn, '54; Montag et al., '57; and Whitcutt and Sutton, '56). Nutritional studies (Privett et al., '59) demonstrated that the dermal symptoms of essential fatty acid (EFA) deficiency in rats were not relieved by the polyunsaturated fatty acids of tuna oil given at a rate of 100 mg per animal per day. Nevertheless marine oils do not appear to be entirely devoid of essential fatty acid activity. Menhaden, herring and whale oils were estimated to contain 4.4, 7.9 and 6.4% of essential fatty acids, respectively, by a rat bioassay method based on growth under conditions of restricted water intake (Thomasson, '53a, b).

In the present study, observations were made of the effects of tuna, menhaden and herring oils on the dermal syndrome of EFA deficiency in the rat as a prelude to the isolation and characterization of the fatty acids responsible for the EFA activity of these oils.

EXPERIMENTAL

Two nutritional experiments were performed. In the first, 50 male weanling rats of the Sprague-Dawley strain were maintained ad libitum on a fat-free diet (Privett et al., '59) for 16 weeks. At the end of this period the growth rate of the animals had reached a plateau and all animals exhibited distinct dermal symptoms of EFA deficiency. The rats were then divided into 5 equal groups. Group 1 was continued on the basal (fat-free) diet and served as a control group. Groups 2, 3, 4 and 5 received the basal diet in which 10% by weight of menhaden, tuna, herring and corn oils, respectively, were substituted for sucrose. The corn oil group served as the positive control for the experiment. The rats were weighed and scored for dermal symptoms of EFA deficiency each week for an additional 12 weeks, and the experiment was then terminated.

The second experiment was conducted in two parts. In the first part 50 male weanling rats were divided into 5 equal groups, and fed ad libitum the same fat-free diet used previously. Groups 1, 2, 3 and 4 were administered orally 200 mg per day of menhaden, tuna and herring oil ethyl esters and ethyl linoleate, respectively. Group 5 received no supplement and served as the negative control for this experiment. The animals were scored for dermal symptoms of EFA deficiency and weighed each week of the experiment for 14 weeks. At this point the administration of 200-mg quantities of esters was discontinued.

In the second part of the experiment, which followed immediately, the same groups of rats were fed the basal diet in which had been substituted for sucrose, 10% by weight of the oil of the same esters that had been administered orally; the ethyl linoleate group was given corn oil. The feeding was continued for an additional 14 weeks during which all of the animals were scored for dermal symptoms and weighed each week as before. The purpose of the second phase of this experiment was to confirm that the dermal symptoms of EFA deficiency could be cured, and the growth affected by feeding a larger amount of oil in the diet.

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RESULTS AND DISCUSSION

The results of the first experiment are summarized in table 1. It is evident from the dermal scores at the end of the 16th and 28th weeks that all of the fish oils cured the dermal symptoms of EFA deficiency and stimulated growth equally as well as corn oil. Figure 1 shows the results on the growth rate of the EFA-deficient animals of feeding 10% of fish oil.

In the first part of the second experiment in which 200 mg of the fish-oil esters were administered daily, the dermal symptoms of fatty acid deficiency developed equally as fast and as severely in animals receiving the fish-oil esters as in the animals fed the fat-free diet (table 2).

In contrast, the growth rates of rats fed the fish-oil esters compared favorably

TABLE 1

Average growth and gross symptoms of animals in experiment 1

Group no.	Supplement	Average weight		Average score[1]	
		Initial 16th week	Final 28th week	Initial 16th week	Final 28th week
		gm	*gm*		
1	Fat-free	232 ± 4.8[2]	252 ± 8.2[2]	5.2 ± 0.3[2]	5.6 ± 0.1[2]
2	10% Menhaden oil	237 ± 7.6	354 ± 15.4	6.0 ± 0.2	1.0 ± 0.4
3	10% Tuna oil	219 ± 15.2	329 ± 16.8	5.6 ± 0.2	0.7 ± 0.2
4	10% Herring oil	235 ± 11.9	354 ± 15.1	5.2 ± 0.2	1.4 ± 0.2
5	10% Corn oil	223 ± 4.0	340 ± 8.1	5.6 ± 0.3	0.1 ± 0.1

[1] Animals scored on basis of dermal symptoms of tail and legs, 0 to 7.
[2] Standard error of mean.

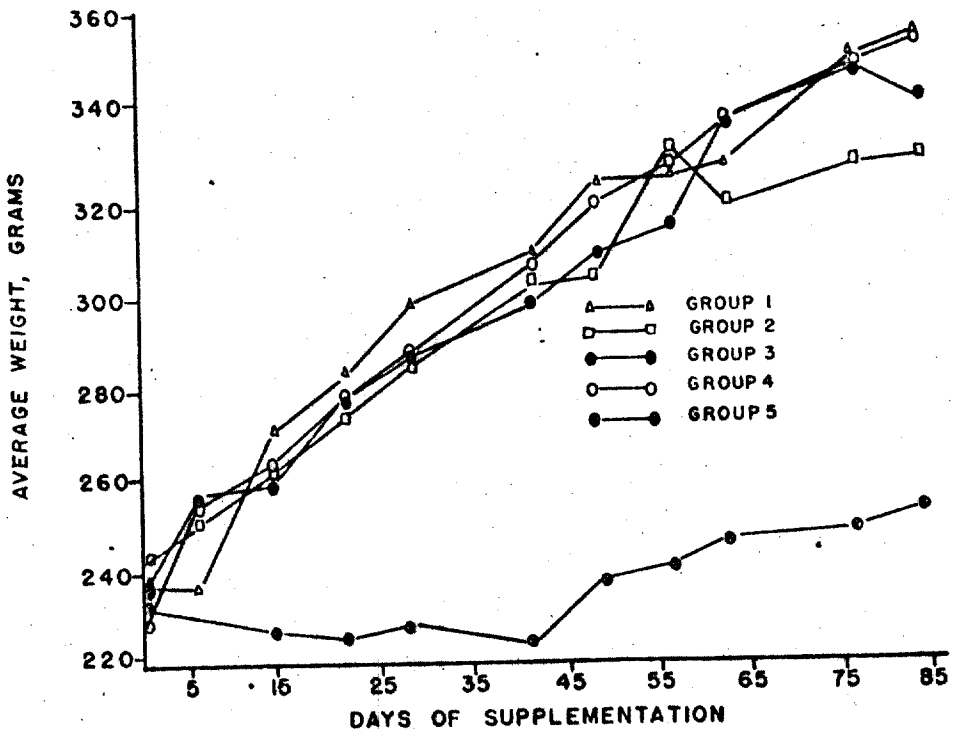

Fig. 1 Average growth rate of rats in experiment 1. Group 1 received the fat-free basal diet; groups 2, 3, 4 and 5 received the fat-free basal diet plus 10% of menhaden, tuna, herring and corn oil, respectively.

with that of the group receiving the ethyl linoleate, and were significantly greater than for the animals fed the fat-free diet as shown in table 2.

When the amounts of these oils were increased to 10% in the diet, in the second phase of this experiment, the dermal symptoms of EFA deficiency were cured. The cure occurred earlier in the animals receiving the corn oil, followed in order of decreasing effectiveness by tuna, menhaden and herring oils (fig. 2).

TABLE 2

Average weights and dermal scores[1] of animals in experiment 2

Group no.	Part 1			Part 2		
	Supplement 200 mg/animal	14th week		Supplement 10%	28th week	
		Av. weight	Av. score[2]		Av. weight	Av. score[2]
		gm			gm	
1	Menhaden oil ethyl esters	290 ± 10.4[1]	5.0 ± 0.4[1]	menhaden oil	351 ± 11.8[1]	0.8 ± 0.2[1]
2	Tuna oil ethyl esters	308 ± 9.8	4.8 ± 0.3	tuna oil	357 ± 15.0	0.6 ± 0.1
3	Herring oil ethyl esters	290 ± 10.4	4.8 ± 0.3	herring oil	352 ± 13.1	1.6 ± 0.2
4	Ethyl linoleate	314 ± 9.0	0.2	corn oil	361 ± 12.5	0
5	Fat-free	245 ± 3.0	5.2 ± 0.2	fat-free	258 ± 5.7	5.5 ± 0.2

[1] Standard error of mean.
[2] Animals scored on basis of dermal symptoms of tail and legs, 0 to 7.

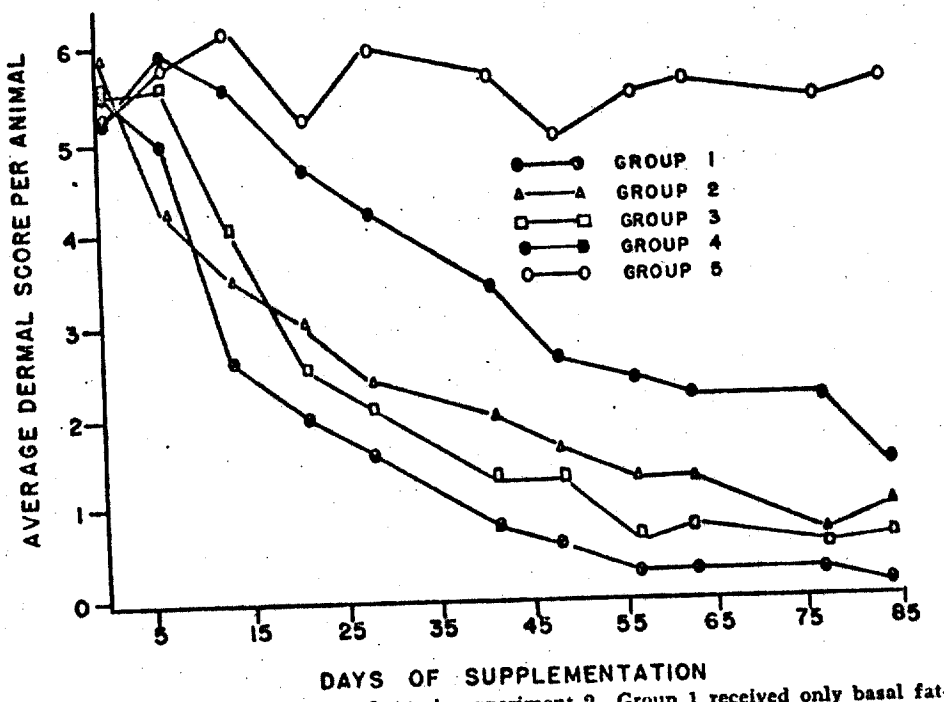

Fig. 2 Average dermal scores of rats in experiment 2. Group 1 received only basal fat-free diet; groups 2, 3, 4 and 5 received basal diet plus 10% of menhaden, tuna, herring and corn oils, respectively.

Since the oral administration of 200 mg per rat per day of tuna, herring or menhaden oils did not prevent the development of the dermal symptoms of EFA deficiency, if the prophylactic dose of linoleic acid is assumed to be about 20 mg per day (Holman, '56), and if "sparking" of polyunsaturated acids by small amounts of linoleate is assumed to be inoperative, these oils may be judged to contain less than 10% of EFA of the linoleate family. If the "sparking" effect on the cure of dermal symptoms (Privett et al., '55) operates with all polyunsaturated acids and one half of the oils are assumed to consist of polyunsaturated acids, the content of linoleate-type acids in these oils may be estimated to be less than 2%.

Inclusion of 10% of fish oils in the diet of rats having pronounced dermal symptoms completely relieved the symptoms. Thus the approximately one gram of oil consumed per rat per day may be estimated to contain at least 20 mg of linoleate-type acids equal to 2% of the oil.

The results presented here indicate that tuna, herring and menhaden oils contain small amounts of linoleate-type acids. In the case of menhaden oil, the estimate of about 2% of acids of the linoleate family agrees in magnitude with an analysis by gas chromatography of this oil by Farquhar et al. ('59). Thomasson's values are somewhat higher than ours which were estimated from dermal symptoms. His data were obtained by a method of assay which uses water restriction, with growth as a criterion, and which therefore may be sensitive to the activity of the acids of the linolenate family. These fish oils contain large amounts of acids of the linolenate family which undoubtedly bestow the growth promoting activity to the oil; and these acids probably account for the ability of the oils to lower serum cholesterol levels in humans (Ahrens et al., '59) and in rats.[3]

SUMMARY

Tuna, menhaden and herring oils cured the dermal symptoms of EFA deficiency and stimulated growth of EFA-deficient rats when fed at a level of 10% in the diet.

It was shown that although severe dermal symptoms of EFA deficiency developed with feeding 200 mg of the above oils in the form of their ethyl esters, the growth rate was not affected. The content of acids of the linoleate family is estimated to be approximately 2% of these oils, using dermal symptoms of EFA deficiency as the criterion.

[3] Peifer, J. J., and W. O. Lundberg 1959 Effect of unsaturated acids and fish oils on plasma and tissue lipides from hypercholesteremic rats. Federation Proc., *18:* 300 (abstract).

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Nutritive Value of Marine Oils

I. MENHADEN OIL AT VARYING OXIDATION LEVELS, WITH AND WITHOUT ANTIOXIDANTS IN RAT DIETS[1,2]

A. A. RASHEED, J. E. OLDFIELD, J. KAUFMES AND R. O. SINNHUBER
Departments of Animal Science and Food Science and Technology, Oregon State University, Corvallis, Oregon

Certain marine or vegetable oils when fed to experimental animals have caused various types and degrees of abnormalities. Restricted growth (Anglemier and Oldfield, '57); reduced efficiency of feed utilization (Oldfield and Anglemeir, '57); deposition of soft fat susceptible to oxidation (Barnes et al., '43; Lea, '53); and incidence of steatitis or "yellow fat disease" in mink (Lalor et al., '51), pigs (Davis and Gorham, '54), cats (Coffin and Holzworth, '54) and rats (Mason et al., '46) are among symptoms reported.

Although marine oils have been used as dietary sources of some essential nutrients (especially vitamins A and D) for humans, farm animals and fowl, they have seldom been used as the sole or major lipid component of diets. However, certain animals, notably mink, consume high fish diets and thus may derive considerable of their energy supply from fish oils. Menhaden and perhaps other marine oils, are commercially available in quantities which would allow such use and constitute a significant potential source of energy. Due to their high degree of unsaturation, fish oils pose problems of instability which affect both palatability of the diet and quality of the animal product (Brown, '31). There is evidence that many of these problems are oxidative in nature (Barnes et al., '43; Johnson, '56; Matsuo, '61) and that antioxidants may be useful means of protection.

The studies reported herein were concerned with the preparation of marine oils for feeding and the consequent effects on laboratory rats. Three separate experiments were conducted. The first utilized menhaden triglyceride fraction (MTG)[3] separated from the oil by molecular distillation. The triglyceride was used at three widely different levels of oxidation. The second experiment tested the effectiveness of antioxidants added to previously oxidized clay-bleached[4] menhaden oil, and the third followed a similar pattern using a fresh oil. Evaluation of the quality of both diet and animal body lipids was made by the thiobarbituric acid (TBA) procedure of Sinnhuber and Yu ('58). This procedure determines malonaldehyde, which is formed in lipid peroxidation (Zalkin and Tappel, '60).

To obtain a clear definition of the problems involved, oxidative levels considerably higher than those generally encountered in edible marine oils were used.

EXPERIMENTAL

Experiment 1. Four groups of 10 male albino rats each were used. The rats were caged individually and fed for a 21-day period on diets composed as shown in table 1. The various B-vitamins were weighed out in quantities listed and made up to one gram with starch. The MTG was oxidized in closed glass containers under a slight positive oxygen pressure in the light at room temperature and in this way, successive aliquots having TBA numbers[5] of 9, 519, and 1082 and with peroxide

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[1] Technical Paper no. 1588 Oregon Agricultural Experiment Station, Corvallis.

[2] Carried out under Contract no. 14-17-0001-352, U.S.D.I. Bureau of Commercial Fisheries. Appreciation is tendered M. E. Stansby and staff, Fisheries Technological Laboratory, Seattle, for counsel and assistance in conducting this project.

[3] Prepared at the Fisheries Technological Laboratory, U.S.D.I., Seattle, Washington. The product may be referred to as being free from nonsaponifiable materials and polymers.

[4] Clay bleaching is a process utilized for removal of odors, free fatty acids and pigments from the oil. Charcoal is also used with the clay in this process.

[5] TBA number = mg malonaldehyde/1000-gm sample.

TABLE 1
Composition of basal diet

	gm
Cornstarch	644
Fibrin, beef, washed	180
Menhaden triglyceride (MTG)	100
Glucose	6
Salt mix[1]	40
B-vitamin mix	10
Cellulose, wood, purified	20
Total	1000
	B-vitamin mix
	mg
Thiamine	0.6
Riboflavin	1.2
Pyridoxine	0.4
Niacin	5.0
Ca pantothenate	4.0
Inositol	100.0
Choline Cl	200.0
p-Aminobenzoic acid	2.5
	μg
Biotin	1.0
Folic acid	1.0
Cyanocobalamin	1.0

[1] Jones and Foster ('42).

values (POV) of 2, 125 and 310 were obtained. These were used in diets for groups 1–2, 1–3 and 1–4 respectively. Diet 1–1 contained 10% of non-antioxidant treated lard replacing the MTG.

The prepared oils were measured out in appropriate daily aliquots and stored in vials at − 10°C to prevent changes in their oxidation levels. Each day the MTG or lard was mixed with other dietary ingredients and immediately placed before the animals. Refused feed was recorded daily and the feed dishes were thoroughly cleaned prior to refeeding. Animal weights and feed consumption were determined weekly. After 21 days on experiment the animals were killed with ether and samples of blood, fat, liver and kidney tissues were removed for further study.

Experiment 2. Six groups of 4 each male and female rats were established, and the animals individually fed rations formulated as shown in table 1 but having clay-bleached menhaden oil substituted at a 15% level. This oil replaced the MTG used in experiment 1, plus 5% of the cornstarch in the basal ration. The clay-bleached menhaden oil was oxidized in a manner analogous to that used with the MTG. Samples having the following characteristics were prepared: (a) low oxidation level; POV, 2.6, TBA, 35.3; (b) medium oxidation level; POV, 15.5, TBA, 48.8; (c) high oxidation level; POV, 61.0, TBA, 162.0. A lard-supplemented diet was fed as a control to lot 2–1. Lots 2–2, 2–3 and 2–4 received diets containing the low, medium and high oxidation levels of oils, respectively. Lots 2–5 and 2–6 received the highly oxidized oil plus the *in vivo* antioxidants, *dl*-α-tocopheryl acetate or 1,2-dihydro-6-ethoxy, 2,2,4-trimethylquinoline (ethoxyquin),[6] respectively. The α-tocopheryl acetate was added at the level of 0.0044%, and the ethoxyquin was added at the level of 0.0125% in the diet. Storage and incorporation of the oil in the diet and general feeding practices were similar to those in experiment 1. After 100 days on experiment the rats were killed with ether and samples of blood, brain, fat, heart, kidney and liver tissues were removed for further study.

Experiment 3. Four groups of 10 male rats each were used. The animals were individually fed ration formulas similar to those described in experiment 2, but using clay-bleached menhaden oil (POV, 3.0; TBA, 86.0) from the stock previously used in experiment 2, and substituting casein for beef blood fibrin. Lots 3–1 served as the control group and received 15% lard instead of the oil. Lot 3–2 received 15% of unprotected menhaden oil while lots 3–3 and 3–4 received the same amount of oil with either α-tocopherol or ethoxyquin added. Further experimental oxidation of the oil was not carried out in this experiment. The antioxidants were premixed with the daily aliquots of oil prior to freezing and storage at levels of 0.005% and 0.0125% of the total diet, respectively. Care of the animals and assembly of data were carried out as previously described. The experiment lasted 6 weeks.

RESULTS

Experiment 1. Body weights and feed consumption data are presented in table 2. Growth performance was similar in groups 1–1 and 1–2 which were fed lard and fresh MTG, respectively. Groups 1–3 and 1–4, fed medium and highly oxidized MTG

[6] Ethoxyquin (Santoquin) is a product of the Monsanto Chemical Co., St. Louis, Missouri.

TABLE 2

Performance of rats fed menhaden triglyceride (MTG) ration

Group diet description	Body weights					Feed consumption			
	Start	Week 1	Week 2	Week 3	Mean Gain	Week 1	Week 2	Week 3	Mean Intake
	gm	*gm*	*gm*	*gm*	*gm*	*gm*	*gm*	*gm*	*gm*
1-1 Lard	73	91	108	115	42	64	69	68	201
1-2 Fresh MTG	70	87	108	121	50	60	65	69	194
1-3 Medium oxidation MTG	73	80	99	108	35	54*	64	64	183
1-4 High oxidation MTG	73	71**	80**	91*	21*	46**	48**	53*	146**

* Indicates significant difference from control (1–1) group ($P < 0.05$).
** Indicates significant difference from control (1–1) group ($P < 0.01$).

TABLE 3

Effect of menhaden triglyceride on liver and kidney weights, hemoblogin and hematocrit levels

Group	Average Liver wt		Average Kidney wt		Average Hemoglobin	Average Hematocrit % RBC
	gm	%[1]	*gm*	%[1]	*gm/100 ml*	
1–1	4.11	3.57	1.04	0.90	15.4	32.6
1–2	5.35**	4.44	1.13	0.94	13.0**	33.2
1–3	4.88*	4.51	1.09	1.01	13.3**	33.6
1–4	4.40	4.40	0.97	1.03	13.6**	34.3

[1] % of total body weight.
* Indicates significant difference from control (1–1) group ($P < 0.05$).
** Indicates significant difference from control (1–1) group ($P < 0.01$).

showed correspondingly lower gains as the level of oxidation was increased. Statistical significance was reached in comparison with the control (lard) diet only in the case of the highly oxidized MTG ration (group 1–4); however, gains with the fresh MTG-supplemented diet (group 1–2) were consistently greater ($P < 0.01$) than those on the highly oxidized MTG and overall mean gains with the same diet (1–2) were significantly ($P < 0.05$) greater than those fed the medium oxidized MTG mix. Feed consumption was generally directly related to gains except that the lard-fed group ate slightly more than the group fed fresh MTG. Again, there was no significant difference in feed consumption between the lard-fed and fresh MTG-fed groups. Both of these groups (1–1 and 1–2) ate significantly more than group 1–4 which received the highly oxidized MTG.

The weights of certain organs are recorded, along with blood characteristics, in table 3. Livers tended to be larger in all groups fed MTG than in the lard-fed group, regardless of state of oxidation of the triglyceride. This is further emphasized when percentages that liver weights make up of total body weights are calculated. Similar observations apply to kidney weight:body weight percentages. Blood from rats fed MTG was significantly lower in hemoglobin than that from lard-fed rats. Differences among the 4 groups with respect to hematocrit were not significant.

Lipid peroxidation using the TBA procedure to measure malonaldehyde, determined on liver and kidney tissues taken from all animals at necropsy as well as on 24-hour collections of urine and feces from single randomly chosen animals from each group, are presented in table 4. The TBA numbers of liver and kidney tissues reflect the oxidation level of the dietary fat. Values were lowest in rats fed fresh

TABLE 4

Thiobarbituric acid (TBA) number[1] of certain tissues and excreta of rats fed menhaden triglyceride (MTG)

Group	Tissues Liver	Tissues Kidney	Excreta Urine	Excreta Feces
1–1	1.97	3.42	0.0	0.0
1–2	1.73	5.61	14.0	61.6
1–3	4.40	8.24	115.0	232.0
1–4	6.83	11.60	201.0	589.7

[1] TBA number = mg malonaldehyde/1000-gm sample.

MTG and lard and increased as triglycerides of increasing rancidity were fed.

The TBA numbers for 24-hour collections of feces and urine also reflect the condition of the oils fed. Based on the amounts of urine and feces voided, the following total excretions of malonaldehyde have been calculated: 0.0 for group 1–1; 0.1063 mg for group 1–2; 0.5214 mg for group 1–3; and 0.4010 mg for group 1–4. The higher excretion from group 1–3 than from group 1–4 (highly rancid MTG) probably reflects the considerably higher food intake by the former (table 2).

Experiment 2. Average body weights of male and female rats at 4-week intervals are presented in table 5. Values recorded are average weights of not less than three rats per group. Groups were dropped from experiment when only two rats survived. Mortality was higher among the males than the females.

From the first interval (table 5) body weights of all groups fed fish oil, except group 2–6, tended to be lower than those in the lard-fed control group (2–1). Protection was afforded by ethoxyquin, but not by *dl*-α-tocopheryl acetate, under the conditions of the experiment. Differences were statistically significant ($P < 0.05$) between groups fed the most highly oxidized oil (group 2–4) and the control or ethoxyquin-treated groups. The *dl*-α-tocopheryl acetate added to the already highly oxidized oil had little protective effect among the females at the level used. Group 2–5 females were significantly lighter than the controls.

The growth-depressant effects of the less highly oxidized oils became more apparent during the 5- to 8-week period when weights of all rats receiving unprotected fish oils were significantly lower than those of the controls. Mortalities commenced during the 5- to 8-week period also and were apparently influenced by the oxidation level of the oil; occurring first in the highly oxidized oil lot (2–4), next on the medium oxidation level (group 2–3) and so on (table 6). A sex difference in mortality rates is evident, the males being more frequently affected. The growth and mortality patterns established during the first two intervals were extended through the third. Feed consumption data are presented in table 7.

TABLE 6

Mortalities and abnormal fat coloration[1] of rats fed menhaden oil at various oxidation levels

Groups	Experimental interval			Total mortality
	1–4 weeks	5–8 weeks	9 weeks – end of trial	
2–1	—	—	—	0
2–2	w	—	y y y	4
2–3	—	y y y	y y y y	7
2–4	y y	—	y y y y y	7
2–5	—	w	y	2
2–6	—	—	w	1

[1] Each letter represents one mortality; w = white fat, y = yellow fat.

TABLE 5

Average body weights at 4-week intervals for rats fed menhaden oil at various oxidation levels

Groups		Experimental interval					
		1–4 weeks		5–8 weeks		9–12 weeks	
		Males	Females	Males	Females	Males	Females
		gm	*gm*	*gm*	*gm*	*gm*	*gm*
2–1	Lard	186	149	257	195	283	215
2–2	Low oxidation oil	171	133	170*	143**	—	135**
2–3	Medium oxidation oil	159	136	163*	152*	—	139**
2–4	High oxidation oil	122*	108*	—	134**	—	—
2–5	High oxidation oil + vitamin E	164	97*	227	150**	231*	154**
2–6	High oxidation oil + ethoxyquin	192	143	253	180	248	202

* Indicates significant difference from control (2–1) group ($P < 0.05$).
** Indicates significant difference from control (2–1) group ($P < 0.01$).

TABLE 7

Average weekly feed intake for male and female rats fed menhaden oil at various oxidation levels

Groups	1–4 weeks		5–8 weeks		9–12 weeks	
	Males	Females	Males	Females	Males	Females
	gm	*gm*	*gm*	*gm*	*gm*	*gm*
2–1	276	265	316	300	332	271
2–2	260	237	233*	204**	190*	193*
2–3	247	238	227*	216**	—	204*
2–4	200*	187*	—	204**	—	168**
2–5	231	190*	310	248	285	223*
2–6	304	258	326	281	324	265

* Indicates significant difference from control group 2–1 ($P < 0.05$).
** Indicates significant difference from control group 2–1 ($P < 0.01$).

TABLE 8

Effects of menhaden oil and antioxidants on rat weight gains

Group		Initial wt	Wt gain at weekly intervals					
			1	2	3	4	5	6
		gm	*gm*	*gm*	*gm*	*gm*	*gm*	*gm*
3–1	Lard	58.4	86.7	115.1	138.7	175.6	219.6	289.4
3–2	Fresh oil	58.8	80.4**	89.8**	102.5**	125.3**	140.4**	169.0**
3–3	Oil + vitamin E	59.8	89.1	110.1	133.1	171.5	211.0	250.6
3–4	Oil + ethoxyquin	58.4	87.8	107.3	131.7	167.2	203.3*	236.2*

* Average weights significantly different from those of lard group ($P < 0.05$).
** Average weights significantly different from those of lard group ($P < 0.01$).

Feed consumption by the control (2–1) and the ethoxyquin-treated oil (2–6) groups was equal but higher than that of the other groups. The three groups fed the unprotected oils showed progressive and significantly lower feed intakes, the depression occurring first in the case of the most highly oxidized oil (group 2–4). The *dl*-α-tocopheryl acetate did not improve feed intake, particularly among the female rats, at the level used.

Before experiment 2 terminated, 21 out of the 48 rats involved had died. Observations at necropsy showed that yellowish coloration of depot fat appeared about two weeks after feeding of the most highly oxidized oil commenced and slightly later with the oils of lower oxidation levels. Mortality rate was higher among groups fed medium and highly oxidized oils. Addition of the antioxidants to diets of groups 2–5 and 2–6 retarded pigmentation of the depot fat and protected the animals from early death. Data in table 6 represent number of mortalities from each treatment group as well as depot fat color noted at necropsy.

Experiment 3. Data concerning body weights of the experimental animals at weekly intervals are listed in table 8.

Among the oil-fed groups, improved gains were afforded by the antioxidants, more so by the *dl*-α-tocopherol than by ethoxyquin. Better performance was obtained with α-tocopherol in this study over that in experiment 2 (where the acetate form was used) possibly because of higher levels and because the chemical form in this latter case permitted some *in vitro* antioxidant activity.

Feed intake and efficiencies of feed conversion were calculated for the entire trial, and are included in table 9. Differences noted were consistent throughout the trial, and did not change from week to week. Net gains are listed for comparison. The average absolute weights of livers, left kidneys, spleens and testes are listed in table 10 together with the percentages that these organs make up of total body weight. Even though total body weights were less, the liver weights of rats fed menhaden oil protected by antioxidants were significantly greater ($P < 0.01$) than those of the con-

TABLE 9

Relationship of weight gains and feed consumption by rats fed menhaden oil and antioxidants

Group	Net gain	Total feed intake	Feed consumed/ gm gain
	gm	*gm*	*gm*
3–1	201.8	606.0	3.02
3–2	108.7**	425.3**	3.95**
3–3	190.8	574.6	3.03
3–4	177.8**	577.7	3.26*

* Indicates significant difference from lard group 3–1 at ($P < 0.05$).
** Indicates significant difference from lard group 3–1 at ($P < 0.01$).

trol group. In group 3–2 which received fresh oil, although the absolute liver weight was low, the percentage that liver represented of total body weight was again elevated. Kidney weights were significantly affected only by the fresh oil diet (group 3–2) in which case they made up a larger percentage of body weight. Spleen weights tended to follow the same pattern as livers, being heavier in all three oil-fed groups than those of controls. Testes weights indicated retardation of development in the fresh oil-fed group (3–2). Lack of normal pigmentation of the upper incisors was also noted in this group.

Blood hemoglobin and hematocrit were determined and results are listed in table 11. Values for the rats fed the fresh-oil diet were significantly lower than those for the control or ethoxyquin-protected oil diet groups ($P < 0.01$). The *dl*-α-tocopherol was effective against lowering of hemoglobin level, but not against depression of hematocrit.

Liver characteristics, in terms of dry matter and ether extract contents and TBA values, are also included in table 11. There were no statistically significant differences among any of these items. Although considerable variation occurred in TBA values, all these were extremely low as compared with oxidized oils in the diets and were considered indicative of a low level of oxidation. There was no evidence of steatitis in any animals on this experiment.

DISCUSSION

Growth. Evidence has been presented to indicate a difference in the growth response of rats to type, level and condition of lipid fed. Fresh menhaden triglyceride supported growth similar to that of lard when both were given at a 10% level in the diet for three weeks (table 2). Clay-

TABLE 10

Effects of menhaden oil and antioxidants on organ weights

Group	Body wt	Liver		Left kidney		Spleen		Testes	
	gm	*gm*	%	*gm*	%	*gm*	%	*gm*	%
3–1	260.4	10.12	3.88	1.010	0.389	0.60	0.23	2.66	1.02
3–2	169.0	9.66	5.76**	0.740**	0.439*	0.56	0.39**	1.82**	1.05
3–3	250.6	12.95**	5.19**	0.979	0.391	0.75*	0.30*	2.73	1.09
3–4	236.2	12.16**	5.17**	0.969	0.411	0.72	0.31**	2.61	1.11

* Indicates significant difference from lard group 3–1 ($P < 0.05$).
** Indicates significant difference from lard group 3–1 ($P < 0.01$).

TABLE 11

Effects of menhaden oil and antioxidants on blood and liver

Group		Blood characteristics		Liver characteristics		
		Hemoglobin	Hematocrit	D.M.[1]	E.E.[2]	TBA no.[3]
		%	%	%	%	
3–1	Lard	13.88	38.9	28.51	11.73	4.6
3–2	Fresh oil	10.78**	29.8**	27.10	8.25	9.2
3–3	Oil + vitamin E	12.62	32.2**	27.96	9.28	4.04
3–4	Oil + ethoxyquin	13.33	35.3	27.48	10.42	5.1

[1] D.M. indicates dry matter in liver homogenates.
[2] E.E. indicates ether extract of dry liver samples.
[3] TBA number = mg malonaldehyde/1000-gm sample.
** Indicates significant difference from control group or those fed ethoxyquin-protected oil diet ($P < 0.01$).

bleached oil (POV, 2.6; TBA, 35.3) at a 15% dietary level also supported growth similar to lard but only for 4 weeks following which growth depression resulted (table 5). Slightly more oxidized clay-bleached oil (POV, 3.0; TBA, 86.0) failed to support growth equivalent to lard from the second week (table 8). These results are in agreement with previous observations, at this station, of growth depression in pigs following feeding of 8.25% sardine oil in the diet (Anglemier and Oldfield, '57). However, normal growth was obtained with 5% menhaden oil (Oldfield and Anglemier, '57). This difference of effect shown in experiments 1, 2 and 3 may be due to the composition of the lipid used (pure triglyceride vs. clay-bleached oil) as well as to the degree of oxdiation and the level of the latter lipid. Clay-bleaching of the oil may have rendered it free from all natural tocopherols and could have contributed pro-oxidants (i.e. metals), therefore increasing its susceptibility to rapid oxidation when exposed to room temperature for the 24-hour feeding periods. It has been noted elsewhere (Buxton, '42) that fish liver oils treated with activated carbon had a shortened induction period and an increased rate of peroxide formation.

As the degree of MTG oxidation increased, a corresponding decrease in growth rate became evident (table 2). Similarly, growth depression was obtained as the level of oxidation of the clay-bleached oil was elevated (table 5). There was also an increase in mortality rate, more so among the males than the females, which corresponded to the increased level of oxidation (table 6). Sex difference in rate of mortality may be due to the lower growth stress in females or to the higher content of vitamin E in the female rat as shown by Edwin et al., ('61).

Ethoxyquin was effective in preventing growth depression caused by highly oxidized oil (experiment 2; table 5). It was less effective, however, when added to the oil in experiment 3 where a beneficial response occurred during the first 4 weeks only (table 8). The level used was the same in both trials. This situation suggests that the rapidly oxidizing oil used in experiment 3 may have created a deficiency of an essential metabolite, such as vitamin E, whose role was not filled by the synthetic antioxidant. Alternatively, it may mean that a higher antioxidant level was necessary to protect oils in earlier stages of oxidation than in later ones.

The α-tocopheryl acetate used in experiment 2 at a dietary level of 0.0044% was ineffective against growth depression caused by highly oxidized clay-bleached oil (table 5). This was partly due to its unavailability as an antioxidant *in vitro* prior to ingestion with the diet. According to Ames[7] tocopherol esters have no antioxidant activity until hydrolyzed to free the tocopherol, thus it could be assumed that oxidation levels above those established initially (POV, 61.0; TBA, 162) may have been reached during the period when the diet was before the rats. On the other hand, the *dl*-α-tocopherol used in experiment 3 acted immediately as an antioxidant when mixed with the oil thus giving both *in vitro* and *in vivo* antioxidant action. The higher level used (0.005%) may have been an added advantage.

Feed intake. No difference in the feed intake due to the feeding of fresh MTG or lard was noted in the first short-term (3-week) experiment (table 2). When fresh clay-bleached oil was fed, however, feed intake was reduced suggesting the susceptibility of this oil to oxidative rancidity during the period of exposure in the feeding pans. Sinnhuber[8] has demonstrated the extent of oxidative changes in menhaden oil mixed with a synthetic diet which changed from an initial TBA value of 32.5 to a value of 1220 over a 24-hour period at room temperature. Reductions in feed intake by pigs fed oxidized sardine oil have been reported (Anglemier and Oldfield, '57).

Addition of antioxidants to the oil (experiment 3; table 9) improved feed intake to a level only slightly below that of the lard-fed group of rats, and as shown in table 9, total feed intake in both protected groups was not significantly lowered from the control group. The α-tocopherol was more effective in improving efficiency of

[7] Ames, S. R. 1957 Vitamin E (alpha tocopherol) in Poultry Nutrition: Recent Developments. Tech. Presentation no. 57-1. Distillation Products Industries, Rochester, New York, March.

[8] Unpublished material, Department of Food Science and Technology, Oregon State University, 1961.

feed utilization than ethoxyquin at the levels used. As the level of oxidation was increased in both MTG and clay- bleached oil there was a corresponding decrease in feed intake (tables 2 and 7).

Organ size. Evidence is presented in both experiments 1 and 3 indicating that feeding menhaden oil, either as a pure triglyceride or as a clay-bleached oil, led to liver hypertrophy (tables 3 and 10). The extent of this condition is evident when the percentages that liver weights make up of total body weights are compared (table 10). The degree of oxidation of the oil, and the type and amount of antioxidant used appear to influence the degree of hypertrophy. As the level of oxidation of MTG increased, there was a corresponding decrease in absolute liver weights (table 3).

Hypertrophy of the liver in rats fed menhaden oil was not due to alterations in the ratio of tissue components dry matter content as well as ether extractable materials in liver homogenates showed only slight differences among the various groups (table 11). Kaunitz et al.[9] however, have indicated that an increase in liver lipid content occurred as a result of feeding oxidized fat.

Hypertrophy of the kidneys was demonstrated when rats were fed clay-bleached oil but not MTG, and when the percentages that kidney weights make up of total body weights were compared. When comparisons were on an absolute weight basis kidneys from rats fed clay-bleached oil were smaller compared to those from lard-fed or protected-oil groups (table 10). In this study, the effect of antioxidants in overcoming the hypertrophic effect of menhaden oil was demonstrated. It was reported by Kaunitz ('60) that larger livers and kidneys were obtained from rats fed polymers of oxidized lard or cottonseed oil. An increase in kidney weights was also shown in swine fed polymerized menhaden oil (Oldfield and Anglemier, '57).

Results of experiment 3 also demonstrated the hypertrophic effect of fresh oil on the spleen (table 10). The absolute weight of this organ was higher in both protected oil groups than in the control group, whereas this was not evident in the unprotected group because of the relatively smaller size of animals. The hypertrophic effect was further demonstrated in all three oil-fed groups when the percentages that spleen weights make up of total body weight were compared with the unprotected group showing the highest values. This study also demonstrates some slight reduction by both antioxidants of the hypertrophic effect of the oil.

Development of the testes (normally late developing organs) was evidently retarded by feeding unprotected fish oil (table 10). This specific growth retardation may have its cause in an induced vitamin E deficiency. Retarded growth of testes in mice was demonstrated by Horikawa ('58) as a result of vitamin E deficiency. Damage to rat testes as a result of feeding rancid fat was also reported (Kaunitz, '60).

Blood characteristics. Feeding menhaden oil or its triglyceride to rats led to a small but significant reduction in blood hemoglobin irrespective of the oxidation level of the oil (tables 3 and 11). Both antioxidants used in experiment 3 were effective, ethoxyquin more so than alpha tocopherol in preventing reduction of hemoglobin.

Rats fed MTG showed no significant changes in their blood hematocrit. Those fed clay-bleached oil, however, showed a significant reduction in hematocrit values, a condition which was effectively overcome by the use of ethoxyquin but not by α-tocopherol. Hematocrit values closely followed those of hemoglobin with respect to the effects of both the oil and the antioxidants (table 11).

It has been shown that low blood hemoglobin may result from decreased formation of the pigment due to protein malnutrition or iron deficiency (Follis, '58), vitamin E deficiency (Indovina, '51) or alteration in the rate of transfer of ferric to ferrous iron (Anonymous, '58). Rats receiving the unprotected menhaden oil mixed with a vitamin E-free synthetic diet may have been exposed to some if not all of the above circumstances.

Incisor depigmentation. Depigmentation of the upper incisors was observed in rats

[9] Kaunitz, H., C. A. Slanetz, R. E. Johnson, B. Puetzer, G. B. Levy and E. Windholz 1955 Influence of heated and aerated cottonseed oil on fat content of liver and kidney. Federation Proc., 14: 408 (abstract).

fed the unprotected menhaden oil in experiment 3. A similar symptom has been observed by other workers who have related it to iron deficiency (Follis, '58), low dietary protein (Irving, '58; Anonymous, '59), and to deficiencies of vitamins A and E (Irving and Richards, '39; Irving, '42; Garton et al., '52; Moore and Mitchell, '55; Golberg and Smith, '60). In this work, the simultaneous lowering of hemoglobin and hematocrit levels is suggestive of lowered availability of iron induced by a deficiency of vitamin E. Lewis et al. ('51), among others, demonstrated that the latter may be occasioned by the presence of highly unsaturated fish oil in the diet.

Steatitis and malonaldehyde level. Malonaldehyde, which is measured by the TBA reaction, is indicative of the extent of lipid peroxidation. Malonaldehyde is only one carbonyl that results when polyunsaturated fats undergo autoxidation. This very reactive substance probably does not exist in the free state, but results from the action of TBA on lipid hydroperoxides or malonaldehyde derivatives.

Results obtained from these experiments indicate the effect of the oxidized lipid on the condition of liver lipids, depot fats and TBA values of excreta. Malonaldehyde concentrations of certain tissues and excreta of the animals in experiment 1 were directly related to the levels in the diet lipids (table 4). Although the animals were apparently able to excrete most of the malonaldehyde, measurable levels accumulated in the liver and kidneys. These levels were not, however, indicative of any extensive oxidation. Subsequent experience has shown that this compound also accumulates in the blood, in which connection it may be a useful diagnostic criterion for oxidized oil effects. It seems probable that a causal relationship exists between malonaldehyde retained in the animal body and toxicity symptoms which developed. In experiment 3, TBA values for livers were similar to those obtained for rats fed medium and highly oxidized MTG in experiment 1 (tables 4 and 11).

An important observation in both experiments 2 and 3 was the effectiveness of the antioxidants at the levels used in preventing steatitis or "yellow fat" disease. In experiment 2, both *dl*-α-tocopheryl acetate and ethoxyquin performed well in this respect. Since the oil was considerably oxidized initially in experiment 2 (POV, 60) and since the acetate form is inactive *in vitro*, it is obvious that the effect took place after ingestion by the animal. The consistent observation of yellow to brown fat deposits in the animals which died on experiment 2 (table 6) indicates the importance of steatitis in the syndrome of toxic effects due to ingesting oxidized oils. Steatitis has been shown to occur in many species of animals fed different types of oils, and has been effectively prevented by α-tocopherol and its esters, (Davis and Gorham, '54; Gorham, et al., '51; Lalor et al., '51; Robinson and Coey, '51).

SUMMARY

1. Pure triglyceride separated from fresh menhaden oil proved the equivalent of lard in supporting growth of laboratory rats when added to a semisynthetic basal diet at a 10% level. When experimentally oxidized in glass containers to high peroxide levels (POV = 125 to 310) the triglyceride caused steatitis, enlarged livers and caused high levels of malonaldehyde in the blood and excreta of the animals.

2. Clay-bleached menhaden oil was shown to undergo extensive oxidation changes over a brief period (24 hours). Such "fresh" oil caused toxic symptoms in rats when fed at a 15% dietary level. Symptoms included anorexia, steatitis, lowered hemoglobin levels and death, and they became more extensive or severe as the level of oxidation of the dietary fat increased.

3. Antioxidants, ethoxyquin or α-tocopherol were effective in causing remission of most of the symptoms attributed to presence of the oxidized fats in the diet. Considerable protection was obtained *in vivo* even after the oil had been allowed to oxidize (POV = 60;) however, most effective protection was achieved when the antioxidants were added to the fresh oil. Differences were shown in antioxidant activity relating to the form of tocopherol and level used.

4. The nature of the metabolic changes resulting from feeding oxidized menhaden oil was discussed, and possible nutrient relationships involved are postulated.

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Damage to Proteins, Enzymes, and Amino Acids by Peroxidizing Lipids

W. T. ROUBAL[1] AND A. L. TAPPEL

Department of Food Science and Technology, University of California, and Bureau of Commercial Fisheries, Davis, California

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Transient free-radicals are produced in peroxidizing lipid-protein reaction systems. The pattern of damage to proteins, induced by these radicals, is similar to that observed in the case of radiation damage; proteins and enzymes lose solubility and constituent amino acids are destroyed. Lipid peroxidation damage appears to be about one-tenth as effective as radiation damage. Amino acid destruction was measured in lipid-peroxidation damaged γ-globulin, catalase, serum albumin, hemoglobin, and and ovalbumin. Among the most labile amino acids are methionine, histidine, cystine, and lysine. Major products of lipid peroxidation-cysteine interaction are hydrogen sulfide and cystine.

Transient free-radicals, generated in peroxidizing unsaturated lipid-protein mixtures, participate in the chain of reactions leading to considerable damage. Chemical evidence for damage to cytochrome c has been reported (1). A recent paper describes the mechanism for lipid peroxidation induced free-radical polymerization of proteins and enzymes (2); it was also shown that soluble polymeric materials are produced in an irradiated cytochrome c solution.

This paper reports evidence for the radiomimetric effects of lipid peroxidation intermediates to proteins and amino acids.

MATERIALS AND METHODS

Experiments conditions of the reaction systems have been given (2); ethyl arachidonate was used unless otherwise indicated. Proteins and enzymes used in experiments on yield of insoluble protein were: trypsin (2X-crystallized, Mann), pepsin (2X-crystallized, Mann), α-chymotrypsin (3X-crystallized, Mann), ovalbumin (crystalline, Nutritional Biochemicals Corp.), and hemoglobin (2X-crystallized, Sigma). In order to initiate and maintain peroxidation in reactions containing nonmetalloproteins, the buffer employed was 0.05 *M*, pH 7.0 phosphate containing 10^{-4} *M* ascorbate and 10^{-5} *M* copper sulfate. Control reactions were run in lipid-free systems at 37°.

In studies of lipid peroxidation damage to constituent amino acids, the proteins and enzymes were: γ-globulin (Bovine, Calbiochem), catalase (Crude, Sigma), bovine serum albumin (Crystalline, Pentex, Inc.), hemoglobin and ovalbumin of the purity and source given above. Lipid peroxidation was allowed to proceed until sufficient protein was insolubilized; insoluble proteins were extracted and stored as previously described (2). Amino acid analyses of the acid hydrolyzed insoluble protein were made either with a Beckman or Technicon automatic amino acid analyzer.

In studies of peroxidation damage to cysteine, a reaction system composed of 1 gm of L-cysteine, 1 gm of ethyl arachidonate, and 8 ml of buffer was employed. Hydrogen sulfide was measured by the method of Marback and Doty (3). Nonvolatile cysteine degradation products were characterized by thin-layer chromatography using silica gel G plates and a migrating solvent composed of phenol–acetic acid–water (70 wt.:10 vol.:20 vol.). Spots were detected with the polychromatic copper-ninhydrin spray devised by Moffat and Lytle (4).

RESULTS

Expression of protein insolubilized per mole of peroxy radical in Table I is appro-

[1] Present address: U. S. Bureau of Commercial Fisheries, Technological Laboratory, 2725 Montlake Blvd., Seattle, Washington 98102.

priate because it allows a comparison with protein damage by ionizing radiation where product formation per free radical is given as ionic yield. Peroxy radicals are approximately equal to oxygen reacted in this well known lipid peroxidation mechanism involving free-radical intermediates.

Table II gives the percentage loss for each amino acid in reacted protein or enzyme. Extent of oxidation was 2.5, 1.6, 1.9, 1.9, and 1.6 moles of oxygen per mole of lipid for γ-globulin, catalase, bovine serum albumin, hemoglobin, and ovalbumin reaction mixtures, respectively. Lysine, histidine, tyrosine, methionine, and cystine, in descending order, are the most labile amino acids in γ-globulin; in catalase, lysine, serine, valine, methionine, and histidine are the most labile amino acids. Glycine, cystine, histidine, alanine, and valine are labile in bovine serum albumin while tyrosine, methionine, lysine, and histidine are labile in hemoglobin. Finally, methionine, histidine, threonine, proline, and glycine are the most labile amino acids in ovalbumin.

TABLE I

PROTEIN INSOLUBILIZED BY PEROXIDIZING LIPID

Protein	Moles O_2/mole lipid	Yield (moles protein insolubilized/mole peroxy radical)
Trypsin	0.28	0.012
Pepsin	0.15	0.017
Ovalbumin	0.29	0.0087
α-Chymotrypsin	0.22	0.0025
Cytochrome c	0.25[a]	0.018
Hemoglobin	1.03[a]	0.0021

[a] Lipid consisted of a mixed ethyl ester containing 73% docosahexaenoate and 17% eicosapentaenoate.

Peroxidizing arachidonate-cysteine interaction leads to sulfhydryl cleavage with the production of hydrogen sulfide. The data of Fig. 1 show that H_2S production increased in a linear fashion with extent of lipid peroxidation. Of the nonvolatile products of lipid-cysteine interaction, only cystine and a trace of alanine could be detected on thin-layer chromatograms (Fig. 2). Both cystine and alanine would be expected to react further to yield both ninhydrin-positive and ninhydrin-negative products and could account for the low yield of alanine at the termination of the experiment. However, no intermediate oxidation products of cysteine or cystine, based on spot color, could be identified. No in-

TABLE II

LIPID PEROXIDATION DAMAGE TO AMINO ACIDS OF PROTEINS

Amino acid	% Amino acid loss				
	γ-Globulin	Catalase	Bovine serum albumin	Ovalbumin	Hemoglobin
Lysine	58.8	42.4	40.8	21.6	58.6
Histidine	51.8	18.2	54.1	38.3	57.6
Arginine	26.5	8.2	11.5	Slight damage	24.1
Aspartic acid	11.3	0	39.8	0	7.2
Threonine	14.8	10.9	46.2	28.0	37.7
Serine	21.4	22.4	43.2	25.0	43.0
Glutamic acid	21.1		41.0	25.0	39.0
Proline	16.0		Slight damage	27.9	38.8
Glycine	21.2	12.0	82.5	27.6	53.8
Alanine	18.8	10.3	50.0	24.5	33.7
½-Cystine	32.8	—[a]	64.0	—[a]	—[a]
Valine	21.0	21.4	47.7	25.8	31.3
Methionine	38.3	20.3	47.5	80.3	58.5
Isoleucine	20.0	12.3	42.8	21.4	—[a, b]
Leucine	22.2	2.6	34.4	5.4	32.8
Tyrosine	50.7	10.5	45.2	8.5	91.0
Phenylalanine	32.0	6.8	44.1	20.9	36.4

[a] Amino acid analyses were not suitable for an accurate measurement of loss.

[b] These amino acids are only present in small amounts in the protein.

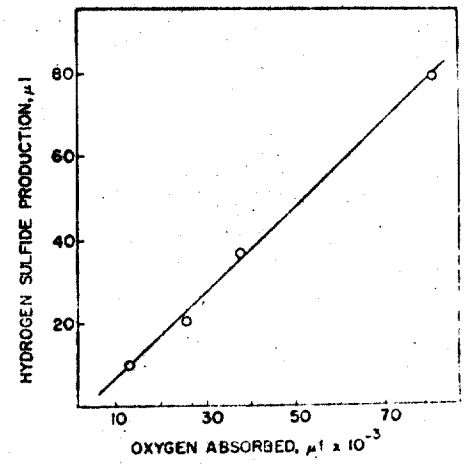

FIG. 1. Formation of hydrogen sulfide as a function of oxygen absorption in a cystein-arachidonate system.

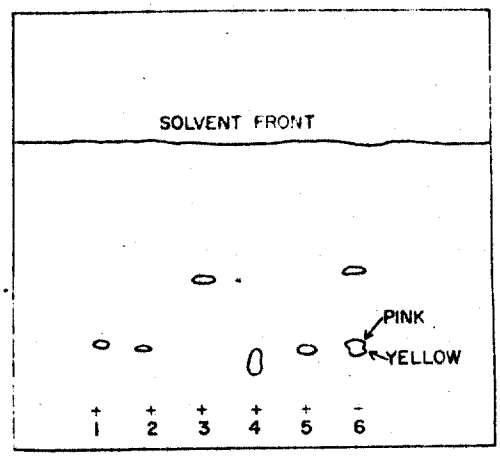

FIG. 2. Thin-layer chromatography of cysteine-arachidonate reaction products.

		R_f	Color
1	Cysteine standard	0.28	Pink
2	Cystine standard	0.27	Yellow
3	Alanine standard	0.50	Pink
4	L-Cysteic acid standard	0.22	Blue
5	L-Cysteine sulfinic acid standard	0.27	Blue
6	Aqueous cysteine-arachidonate reaction products	0.29 (Pink and yellow), 0.57 (pink)	

solubilized material was produced even under prolonged exposure of reaction mixture to high oxygen tension; tests for sulfate and free sulfur were negative.

DISCUSSION

Comparison of lipid peroxidation damage with that of ionizing radiation is appropriate because both reactions involve free-radical intermediates and because analogous information is available from studies of radiation damage to proteins and cysteine. Results of lipid peroxidation damage in Table I can be compared to radiation damage to proteins. Yield values of protein damaged per ion pair are 0.05, 0.03, 0.10, and 0.48 for invertase, catalase, cytochrome c, and ribonuclease, respectively (5). Another yield value is 0.20 for trypsin (6). Lipid peroxidation damage is less than radiation damage, an inequality caused by the biphasic lipid reaction system; many of the peroxy free-radicals are prevented from reacting with protein. Since both radiation and lipid peroxidation give rise to free-radical intermediates, it does not seem surprising that both types of damage should show similarities; Haissinsky has indicated that both ionizing radiation and lipid peroxidation may have a similar mechanism of damage at the molecular level (7).

The relationship between radiation and peroxidation damage is also able to explain the observation that the over-all pattern of amino acid damage in proteins is similar in both processes. In irradiated ovalbumin, histidine, cystine, methionine, phenylalanine, and threonine suffer greatest damage (8). Irradiation studies with hemoglobin show the most radiolabile amino acids to be methionine, histidine, threonine, tyrosine, and phenylalanine (8). Likewise, cystine, methionine, phenylalanine, histidine, and tyrosine are the most radiolabile amino acids of catalase (9).

Irradiated proteins often show an increased alanine content with increase in dose; however, the content of this amino acid drops at still higher dosage and remaining amino acids suffer increased destruction. Studies of lipid peroxidation damage to proteins have shown that there are no increases in alanine. Furthermore, α-amino-n-butyric acid, produced by cleavage of the terminal CH_3S group from methionine, has not been detected. Evidently, the magnitude of peroxidation damage was sufficient to destroy these primary damage products.

Hydrogen abstraction reactions, initiated by a variety of free-radical intermediates, would account for disulfide production:

$$\left.\begin{matrix}\mathrm{ROO}\cdot\\ \mathrm{R}\cdot\\ \mathrm{RO}\cdot\\ \mathrm{HO}\cdot\end{matrix}\right\} + \mathrm{R'SH} \rightarrow \mathrm{R'S}\cdot + \left\{\begin{matrix}\mathrm{ROOH}\\ \mathrm{RH}\\ \mathrm{ROH}\\ \mathrm{H_2O}\end{matrix}\right.$$

followed by $\mathrm{R'S}\cdot + \mathrm{R'S}\cdot \rightarrow \mathrm{R'SSR'}$. Free-radical cleavage of the R—SH bond would lead ultimately to H_2S production. The radiolability of sulfur amino acids is well documented (10, 11).

The lability of histidine is of interest. Data of Table II show this amino acid to be labile in most of the examples given. Histidine acts prooxidatively to initiate a rapid uptake of oxygen in arachidonate emulsions with the formation of soluble polymeric products which are Folin reagent-positive (2). Recently, Saunders and Hampson have shown that the prooxidative effect of histidine in methyl linolenate emulsions appears to be associated with both the imidazole and amino groups (12). In the presence of activators, the histidine complement of β-casein is photolabile (13).

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PROBLEMS DISCOURAGING USE OF FISH OIL IN AMERICAN MANUFACTURED SHORTENING AND MARGARINE

Maurice E. Stansby

Pioneer Research Unit - Northwest Fisheries Center[1]

Seattle, Washington 98112

[1] National Marine Fisheries Service, NOAA, U. S. Department of Commerce

SUGGESTED KEY WORDS

Fish oil

Shortening

Margarine

Hydrogenation

Monoglycerides

Diglycerides

Flavor, reversion of

ABSTRACT

Prior to 1950 California sardine oil was a component of many margarines and shortenings of American manufacture with about 140 million pounds of fish oil used per annum at peak years. With the disappearance of commercial quantities of California sardines after 1950, fish oil dropped out of use for edible purposes in the United States. In intervening years various circumstances such as adoption of margarine standards of identity without provision for the use of fish oils has discouraged use of other American fish oils (e.g., menhaden oil) in domestic production of edible fats. Of the various problems - uniformity of quality, legal requirements, public image, and price considerations - the latter problem will be the most difficult to overcome before fish oils can be in demand for such uses. Satisfaction of legal and sanitation requirements can be met only by costly improvements in manufacturing facilities for preparation of the fish oil, yet the main inducement for use of fish oils is their availability at competitively low cost.

INTRODUCTION

Twenty-five years ago more than 100 million pounds of fish oil, primarily pilchard (i.e., California sardine) oil, were used in manufacture of American produced margarine and shortening; today no American fish oil is so used. Prices of fish oils often fluctuate widely. When this has occurred, American fish oil manufacturers have felt that had they had access to margarine and shortening uses as an outlet for their product, the price fluctuations would have been less severe. In all other countries the principal use of fish oils is in the edible oil field. Yet there are a variety of problems which would have to be overcome before such markets could be available to the American fish oil industry. In this article, the historical developments which have brought about the situation here are traced and the problems which would have to be overcome to alleviate the situation are considered.

PRE-1920 PERIOD

The chemistry of preparing hydrogenated fats from triglycerides containing unsaturated fatty acids was worked out and reported between 1897 and the first few years of the twentieth century. By 1910, with the chemical basis of the process well documented, industrial research was under way toward perfecting methods for efficient manufacture of hydrogenated fat for use in edible products. Fish oils were among the first oils considered in this early work. E. Bedford and C. E. Williams (3) were among the first in this field, filing on December 11, 1911 for a patent on methods for hydrogenating fish oils. The first U. S. patent on methods for hydrogenating fish oil was granted to Vilbushevitch in 1912 (14).

During the decade 1910 to 1920, applied research brought the hydrogenation industry in European countries from a tiny endeavor to almost a full-grown operation. The nucleus for the later gigantic Unilever Company began with research operations and a very small output at a small concern started in 1911 at Joseph Cross Field near Warrington, England. By 1916 this concern, renamed Unilever, was, according to Lassen (8), already using fish oils.

Although by 1920 the use of hydrogenated fish oils in Europe was a fair-sized industry, operations had yet to get under way in this country. Even the use of vegetable oils for hydrogenation lagged far behind that of most European countries. Failure to use fish oil in margarine as early as was done in Europe was due, at least in part, to the fact that margarine itself was not as important a food commodity here as in Europe. Repressive legislative made sale of margarine illegal in some states, or in others subject to such restrictive practices that the margarine could only be sold as a white, colorless product.

Although hydrogenated fish oil was not manufactured in the United States during the decade ending in 1920, during World War I fish oil for edible use was included in demonstrations of fish cookery conducted by the Bureau of Fisheries.

1920 TO 1951 PERIOD

Shortage of vegetable oils in the United States during World War I had focused attention upon the possibility of using fish oil in margarine or shortening. Considerable research and experimentation was carried out during the first part of the decade starting in 1920. Research by

Richardson, Knuth, and Milligan (10) investigated the types of fatty acids produced during the hydrogenation of American species of fish oils, especially menhaden. Meantime, applied research on California sardine oil was under way at two hydrogenation plants in the Los Angeles area. This oil was the type which looked the most promising of any American fish oils to use for hydrogenation. California sardines had become a major source of fish during World War I. Markets for sardine oil had not been firmly established and much of it was going into the low-priced outlet for use as soap. Furthermore, California law prohibited the taking of sardines for other than food use so that only the waste from sardine canneries could be processed into oil if it was to be used for industrial purposes. If, however, a market for use of sardine oil could be developed where the oil was used in an edible product, it was argued (1) that whole sardines could be legally rendered. These possibilities spurred research to find wider markets for sardine oil, especially in the field of an edible oil. By 1925, 120,000 pounds of California sardine oil were hydrogenated and used in margarine. By 1928, the annual use of sardine oil in margarine had risen to 15 million pounds, and in 1936, a peak use in this way of 40 million pounds was reached.

An indirect use of fish oil in shortening accounted for a much larger annual consumption than showed up in published statistics for use of fish oil in edible products. This use involved the preparation of hydrogenated mono- and diglycerides from sardine oil by partial glycerolysis and hydrogenation. Mixtures of mono- and diglycerides have been added to shortening for many years (2) (12) in the preparation of superglycerinated shortening. This product gets its name from the process of preparing the

mono- and diglycerides by heating the oil with glycerine. The resulting mixture of mono- and diglycerides is added at a level of 2.5 to 3 percent to make the superglycerinated shortening. Such shortening is much in demand in the baking industry, especially for the preparation of cakes. Its use permits a higher ratio of sugar to flour to be used in cake without resulting in a deterimental texture. Superglycerinated shortenings are widely used in bakeries and for general household use. Their application to deep-fat frying is not successful owing to lowering of smoke temperature.

A very large volume of mono- and diglycerides was made from California sardine oil at a hydrogenation plant at Tacoma, Washington, during the 1930's and 1940's. This product was used not only in this country but also was shipped abroad, and the volume of sardine oil used reached at one time a peak of about 100 million pounds per year.

The last year that California sardine oil was available was 1951. After that year, the fish disappeared almost completely. During the late 1940's when a shortage of sardine oil first occurred, the large glycerolysis operation supplemented its oil supply with herring oil from Alaska and British Columbia. The supply of this and other oils on the Pacific Coast was not sufficient to sustain this production after complete disappearance of the California sardine. Furthermore, the increasing demand for hydrogen in the Tacoma area for the manufacture of ammonia left too little for other uses. As a result of these factors, this large-scale glycerolysis-hydrogenation operation ceased in 1951, as did also the use of fish oil as a triglyceride source for margarine and shortening manufacture in the United States.

PERIOD SINCE 1951

In the years immediately following 1951, no fish oil whatever was made into shortening or margarine in this country. Principal reasons for this situation stem from changes in the U.S. Food and Drug regulations. The standards of identity promulgated for margarine did not permit the use of fish oil. While there was no such restriction on use of fish oil in shortening, new sanitary requirements for raw materials going into food products precluded the use of the ordinary commercial fish oil for this purpose. All existing plants for manufacture of fish oil were far from meeting the new sanitation requirements for manufacture of ingredients of food products. The only case of which we know where fish oil was included in an American manufactured shortening since 1951 occurred during 1967. A large manufacturer of shortening used fish oil for a short time in their product marketed here. The oil used was herring oil imported from Iceland and manufactured in a plant having adequate sanitation for processing of food for human consumption. The inclusion of fish oil was discontinued because of the apprehension that inclusion of the required labeling statement which indicated presence of a marine oil would reduce the volume of sale of the product.

Since 1951 fish oils have continued to be used in increasing volume by most countries other than the United States. Most European countries are using considerable quantities in this way, especially northern European countries such as Great Britain and the Scandinavian countries. Japan also uses much hydrogenated fish oil, and this practice is beginning in South America, especially in Peru and Chile. Canada, prior to 1950, had legal restrictions which prevented hydrogenated fish oil from being

used in this way. Since that time, however, these restriction have been removed. Canada uses much domestic herring oil and some imported menhaden oil in their margarine and to a lesser extent in shortening.

In the period since 1951, the margarine and shortening industry has made the transition from the situation where cottonseed and coconut oils were the principal sources for their production to one where soybean oil is now the principal source. This situation should be of interest to the fishing industry. Soybean oil resembles fish oil in that it contains considerable quantities of ω-3 fatty acids. These fatty acids cause a serious reversion problem. During storage, there is a tendency for the original, somewhat unpleasant flavor which had been removed by hydrogenation and other refining to return. Nevertheless research has largely overcome this problem.

PROBLEMS IN USE OF FISH OIL IN SHORTENING IN THE UNITED STATES

There is a variety of reasons given by different individuals in the American shortening industry for the failure of fish oil to be looked upon as a highly desirable raw material source. In this section these reasons will be examined and evaluated.

A. Reversion

Early (prior to 1952) users of fish oil in this country mentioned flavor reversion as a major problem which was at that time a greater one than when vegetable oils were employed. Before considering reversion problems, a discussion will be given on the nature of reversion.

Two kinds of flavor reversion occur with hydrogenated oils. One of these results in the return of the flavor of the original oil itself.

Soybean oil has a "green" or "grassy" flavor. It is sometimes described as "beany." This flavor is generally disliked. Immediately after hydrogeneration and other refining steps are completed, this type of flavor is absent. It may return, however, after prolonged storage, especially when the product is shortening. Shortening is stored at room temperature for a year or longer, whereas margarine is held under refrigeration for, at most, only a few months. Furthermore, margarine contains flavor components, e.g., diacetyl, which may mask small degrees of flavor reversion.

Similar reversion can occur with fish oil except that instead of a "grassy" or "beany" flavor, the typical "fishy" flavor returns. A slight "fishy" flavor in a shortening would probably be considered to be a worse defect than a slight "grassy" or "beany" flavor.

There is an entirely different kind of flavor reversion that can occur with any hydrogenated oil. The process of hydrogenation results in a flavor peculiar to the hydrogenation process itself. This flavor is variously described as a "hardening" flavor, a "tallowy" flavor, a "candle-like" flavor, or a "cardboard" flavor. This flavor disappears as a result of refining steps which follow hydrogenation. It can, however, revert upon storage. These flavors and their reversion properties occur alike with hydrogenated soybean oil, hydrogenated fish oil, or in fact with any hydrogenated oil.

Hannewijk (4) ascribes the return of this "hardening" flavor to a very slight increase in bound aldehyde content which occurs upon storage. Bound aldehydes form as a result of oxidation so that this form of flavor reversion is also one involving oxidation. Hannewijk (4) also describes a very odd situation with regard to this type of flavor

reversion. The reverted flavor is sometimes not noticed when the product is tasted indoors, but when the taster goes outdoors and inhales fresh air (even as long as a half-hour after tasting), he experiences "an offensive taste in the throat." The hardening type of flavor in hardened soybean oils, linseed oil, and marine oils according to Hannewijk (4) is caused by the presence of 6-trans-nonenal, an aldehyde oxidation product which has a taste threshold value of only 0.0003 parts per million.

Since both types of flavor reversion result from oxidation, the degree of unsaturation of the fish oil (and changes that occur during hydrogenation) is important in determining the stability against flavor reversion. This makes hydrogenated sardine oil more vulnerable to reversion than hydrogenated menhaden oil. Very likely the severe difficulties experienced with flavor reversion in shortening made with California sardine oil could be much less acute in shortening made from hydrogenated menhaden oil.

Flavor reversion has been an equally difficult problem with soybean oil. The very extensive research carried out the past 20 years on soybean oil reversion has overcome most of these problems. Because both soybean oil and fish oil have similar types of fatty acids which are both highly polyunsaturated and of the ω-3 type, there is good reason to believe that experience of American shortening manufacturers with soybean oil can be adapted to overcoming similar problems with fish oils. This has certainly been the case with handlers of fish oil in margarine and shortening abroad. There is actually a feeling abroad that from the standpoint of reversion, fish oils are perhaps easier to handle than soybean oil.

B. Miscellaneous Technical Problems

There are a number of miscellaneous problems which potential users of fish oil mention as giving difficulties. One of these is the statement that the quality of fish oils is less uniform than is that of most other oils. By quality is generally meant such factors as free fatty acid content and color. The variation also extends sometimes to composition, e.g., iodine value may vary from batch to batch.

From the standpoint of hydrogenation costs, the content of sulfur compounds in fish oil is sometimes excessive. High sulfur content poisons the hydrogenation catalyst, requiring more to be used. This problem is one of considerable current concern to hydrogenators, and at the Eleventh World Congress, ISF, several papers discussed it. Mørk (9) described extensive research on the mechanism of the action of sulfur in fish oils as it affected catalyst poisoning and Urdahl et al. (13) showed that fish oils prepared from very freshly caught fish contained considerably less sulfur than did those made from spoiled fish. Recent legislation in Denmark requires all fish, including those to be used for fish oil production, to be iced with at least 20% of their weight of ice, and research is under way to develop mechanical icing equipment for use aboard vessels which would make it economically feasible to ice fish destined for fish meal and oil production (5).

Most problems of uniformity can be overcome by more careful quality control or by blending of different batches of oil.

C. Public Image of Fish Oil

The image of fish oil in the mind of the public varies from one region of the world to another. In many countries a statement on the

label of margarine or shortening that the product contains oil of marine origin causes little or no difficulty. There is a strong belief by many that such a statement on an American product would cause strong buyer resistance. Perhaps this situation stems partly from the buildup of the idea that vegetable oils in a shortening are far superior to animal fat. This situation, of course, developed years ago when the then newly introduced hydrogenated vegetable oil shortenings provided superior resistance against oxidation and resulting rancidity than was possessed by lard. Even today many shortenings stress the wording "100% vegetable oils."

When sardine oil was added to shortening in the United States prior to 1952, it was common to use it only in a second-grade product under a different label of a given manufacturer. There is considerable belief in the American shortening industry that such a practice would be necessary today should fish oil be used. Use of fish oil in only second-grade shortenings would have an adverse effect on the price that could be paid for the fish oil.

There is one possible way to avoid this problem. In past years when a mono- and diglyceride product was added to a shortening, the additive was classified as a chemical, and there was no requirement to indicate the source of components of added chemicals. If this is still the case, it should be possible to add hydrogenated mono- and diglycerides prepared from fish. While only 2.5 to 3 percent of a superglycerinated shortening can be of this mono- and diglyceride mixture, the volume of shortening of this kind is so great that a very large proportion, if not all, of the American menhaden oil production would be required to saturate the market.

D. Price

The principal, perhaps the only reason that the American shortening industry might be interested in using hydrogenated fish oil in its product is the factor of price. Yet the reason the fish oil industry wants to develop markets for their product in shortening is to stabilize price -- hopefully to increase it substantially.

The steps necessary to improve American produced fish oil to a point where it can be used in shortening -- improving the manufacturing plants with respect to sanitation and making the general quality of the oil more uniform -- will raise the cost of production. Probably this will increase the selling price of the fish oil. Is there still room between the price the shortening industry is paying for its raw material and this increased manufacturing cost of fish oil, to add a further price increment to improve the profit margin of the fish oil industry and still leave a price attractive to the shortening industry? The answer to this question may be one of the most critical factors in determining whether fish oil will again be used by the American shortening industry.

E. Legal Restrictions

When American fish oil started to be used during the 1920's as a component of shortening and margarine, there were no legal restrictions concerning its use, and this situation continued for many years. Today, and since use of fish oils in the United States for edible purposes has all but disappeared, new requirements established by the Food and Drug Administration have been adopted relating to the sanitation conditions of the plant in which the food component is prepared. Unfortunately few, if any, fish oil plants in this country meet such requirements for

preparation of a component of food for human consumption. If fish oil is to be used in food in this country, it is quite universally agreed that most plants will have to be modified to meet required standards.

Since the early days when American margarine manufacturers used fish oil in their product, a standard of identity has been set up for margarine by the Food and Drug Administration. Because fish oil was not included in these standards, it cannot now be used in margarine. While it is possible to change standards of identity, such changes require extensive hearings with new evidence submitted, and at best are very time-consuming and lengthy.

There is some fear on the part of the fishing industry that the Food and Drug Administration might take some administrative action toward barring the use of fish oil in shortening on some other grounds. These might relate to the question of whether hydrogenated fish oils are a safe or desirable component of a shortening, or whether, from esthetic grounds, a product made from whole fish is desirable. Fish oils were used in this country extensively for many years up to 1952 in both shortening and margarine. Today hydrogenated fish oils are used more than ever before in northern European countries, in Canada, in Japan, and in some South American countries. In no place has either of the two above-mentioned reasons ever been used to bar use of hydrogenated fish oils in shortening.

Another problem in using marine oils, particularly menhaden oil, for edible products has recently arisen as a result of Codex Alimentarius standards (recommended but not yet adopted) for international trading purposes, where the amounts of iron and arsenic are to be limited

to 0.1 parts per million. In menhaden oil each of these trace elements is often present at levels above those limits. Research on the trace element, sulfur, as previously mentioned has shown that use of the freshest possible fish will diminish the amount of this element. The same may be true for iron and especially for arsenic, and restriction of production of menhaden oil to use of only fresh raw material might overcome this problem. This might mean that the fish would need to be iced at sea as is being done in Denmark for herring and certain other species, which would increase production costs considerably.

Since recent reports of the adverse dietary effects of certain components of peanut oil such as erucic acid, e.g., Kritchevsky et al. (7), upon fatty infiltration of the myocardium with subsequent fibrotic changes in experimental animals, the question as to whether hydrogenated herring or other fish oil might pose similar problems has concerned manufacturers of edible products containing such fish oil. Currently, a large cooperative investigation on this matter between governmental laboratories in Canada and Norway (6) is under way. Until results of this or of similar investigations have been concluded, we cannot yet assess whether such factors may be a further deterrent to wider use of fish oils in edible food products. Other nutritional aspects of fish oils have been reviewed by Stansby (11).

In contacts with individuals outside the fishing industry who might be concerned about the use of hydrogenated fish oils in shortening, of the five main potential sources of problems discussed above--reversion, miscellaneous technical problems, public image, price, and legal

restrictions--the last mentioned of these was the one which seemed to be of least concern, on the assumption that the fish oil would, in any case, have to be made in plants possessing adequate sanitation. The matter of price of the fish oil would appear to be the most critical in determining the possibility for use of hydrogenated fish oils in edible fats of American manufacture.

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Food Science Pioneer Research Laboratory, Bureau of Commercial Fisheries,
Seattle, Wash.

Nutritional Properties of Fish Oils

M. E. Stansby

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I. Introduction

Fish oils are much more complex in chemical structure than are other fats or oils. They contain many classes of lipids as well as a very diverse group of fatty acids ranging from saturated to highly polyunsaturated. Because of this varied composition, these oils are of considerable interest from the standpoint of biochemistry, metabolism, nutritional properties and in some cases pharmaceutical action.

Research on fish oils has lagged behind that on most other lipid materials. The very complexity of the fish lipids from the standpoints of difficulty of separation of components and the great instability of the polyunsaturates discouraged many of the earlier workers in the lipid field from carrying out any more than desultory investigations upon them. In the past 15 years or so, however, with introduction of many new techniques a considerable volume of research papers dealing with fish oils have appeared.

The purpose of this review paper is to discuss our knowledge of fish oils primarily from the viewpoint of nutritional properties. However, a considerable amount of background material will be presented with respect both to the chemistry of fish oils and to sources of fish oils. The chemistry will be discussed chiefly with regard to the many classes of lipids found in fish oils and with considerable emphasis on the diverse fatty acid make-up of the chief class, triglycerides. The topic of oxidation and stability will also be considered briefly. The source of fish oils will be discussed primarily with respect to the way

in which the diverse chemical entities found in fish originated and found their way into the fish as well as a consideration of the way these substances occur in different types of tissue or organs of the fish. Some brief treatment will be included of the chief sources of commercial fish oils and of their geographical distribution.

Nutritional properties will then be discussed first with respect to the various actions of the fatty acids, then with regard to the nutritional properties of other important classes of lipids.

II. Chemistry of Fish Oils

A. Introduction

The chemical composition of the *average* fish oil differs from that of most other naturally occurring oils and fats in having a greater variety of lipid compounds, in possessing larger quantities of fatty acids with chain length exceeding 18 carbons, in having a much greater proportion of highly polyunsaturated (more than 4 double bonds/molecule) fatty acids and in having polyunsaturates primarily of the $\omega 3$[1] rather than the more usual $\omega 6$. Most all fish oils possess these properties to a greater or lesser extent in common. There are, however, vast differences in composition of oils from different species of fish or even from oils of the same species of fish taken from different geographical areas. Such variations will be discussed in this section. There is another type of wide variation in fish oil composition which depends upon the part of the fish from which the oil is made. Not only is the liver oil, for example, quite different in composition from that of the oil of the flesh of the same fish, but also oil from different parts of the flesh (e.g. dark *versus* light muscle or depot fat *versus* cell constituents) may vary widely in chemical composition. Most of the discussion of oil within different parts of the fish will be given in the next section (Source of fish oil).

B. Classes of Lipids

All fish oils contain triglycerides and in many cases these are the only main components. With some species of fish and especially with fish

[1] The designation of the position of the first double bond related to the methyl end of the molecule by the ω numbering systems is used in this article.

liver oils, other classes of lipids such as hydrocarbons or ether linked compounds, e.g. alkoxyglycerides may be a major component.

1. Triglycerides and fatty acids

Fish body and liver oils have the polyenoic fatty acids preferentially located in the β position, yet in marine mammals the triglycerides of the blubber have the polyunsaturates primarily in the α position [BROCKERHOF and HOYLE, 1963]. The ethylenic bonds in fish oils occurring in the *cis* configuration are present as a methylene interrupted type (divinylmethane structure) [PRIVETT, 1956; KLENK, 1958].

The carbon chain length in fish oil fatty acids commonly exceeds C18, and a considerable portion of the fatty acids contain 20, 22 and to a limited extent, 24 carbon atoms. The total proportion of these long chain fatty acids usually amounts to $^1/_4$–$^1/_3$ of the total and sometimes approaches ½. This high incidence of long chain fatty acids contrasts sharply with the situation usual with most oils and fats of vegetable or animal origin where the proportion of fatty acids with more than 18 carbon atoms often is of only trace proportions, seldom exceeds 5% and is frequently less than 1% (especially in most vegetable oils).

The highly polyunsaturated fatty acid content of fish oils is far higher than that of any other common naturally occurring oils or fats. Most frequently these 5 and 6 double bonded fatty acids range in proportion of total fatty acids from 15–30%. They very seldom are less than 10% and occasionally range up to 50%. Five and six double bonded fatty acids in oils and fats of vegetable or animal origin seldom equal even 1%.

In addition to the highly polyunsaturated fatty acids fish oils contain moderate amounts of other polyunsaturates (2, 3 and 4 double bonds) and considerable quantities of both saturates and monoenes.

Another unusual feature of fish oils is the presence of relatively large amounts of odd carbon chain (mostly C15, 17 and 19) fatty acids. While these ordinarily total about 1–3%, with at least one species, mullet, they total 10% or more (GRUGER *et al.*, 1960). Odd chain fatty acids have been reported hitherto [HARTMAN, 1957] as occurring in triglycerides of the fat of ox, sheep and butter. The extent to which they occur in fats from these sources is considerably less, however, than that in the oils of some fish.

Nearly all of the polyunsaturates of fish oils ranging in chain length from C18–C22 are of the ω-3 type, i.e. they are of the linolenic acid family rather than of the linoleic acid family (ω-6 type) as occurs pre-

Table I. Usual range of fatty acid contents of most common species of marine, freshwater fish and shellfish

Fatty acid No. C atoms	No. double bonds/molecule	Saltwater fish %	Freshwater fish %	Shellfish %
14	0	2 –8	2 –6	2 –6
15	0	0.3–7	0.3–1	0.5–3
16	0	10 –30	10 –20	15 –25
16	1	2 –11	7 –11	2 –11
16	2	0.5–2	0.5–1.5	0.5–2
17	0	0.7–2.5	0.5–1.5	0.5–2
18	0	2 –6	3 –4	4 –9
18	1	12 –28	18 –28	5 –18
18	2	1 –3	4 –6	0.5–2
18	3	0.5–1.2	3 –5	0.5–2
18	4	0.7–4	1 –2	0.5–5
19	0	0.5–2	—	—
20	1	1 –10	1 –3	2 –9
20	4	0.5–4	2 –4	1.5–5
20	5	6 –14	5 –7	9 –22
22	1	1.5–9	0.5–3	0.2–12
22	5	0.6–3	2.5–4	1 –8
22	6	8 –20	8 –20	11 –27
24	1	0.5–2	1 –4.4	0.5–1

dominantly in other oils and fats. The proportion of ω-6 fatty acids in fish oils is very small, under 10%, often below 5%. The oils of freshwater fish usually have somewhat higher proportions (around 10%) of ω-6 fatty acids than the marine oils.

The usual ranges of fatty acids occurring in the body oils of most species of fish is shown in table I. It is apparent that there is a considerable variation in the amount of any given fatty acid. For example the C20 monoene varies in saltwater species over a range of ten times and most fatty acids have a range spread of three times or more. Freshwater fish oils would appear to have somewhat greater proportions of 18:2 (linoleic acid) though still far less than vegetable oils and a lesser amount of the C20 pentaene than do the corresponding saltwater species. The oils of shellfish on the other hand appear to be unusually high in the C20 pentaene and of the C22 hexaene.

In table II are shown the fatty acid contents reported for oils made from different species of fish. Because as will be shown later, there is

a relatively wide variation in fatty acid content of different batches of oil made from the same species of fish and because the samples of fish from which the results are reported for in table II were not in any case large, these values are only very approximate. They can not be considered to be entirely representative of the fatty acid content of all oils made from the same species. This fact can indeed be seen by comparing values reported by two investigators for the fatty acid content of two batches of oil from Atlantic cod. The considerable difference in composition is most likely largely due to actual differences in composition of the different cod body oils employed. Nevertheless there is sufficient difference in fatty acid composition of oils made from different species to show certain trends. For example, pink salmon, which is well known to contain oil which is the most unstable of any of the species of salmon indeed as unstable as the oil of any fish has a very high content of 22:6 and 22:5 fatty acid (over 32%). Sablefish, on the other hand, widely known to contain oil which is highly stable, has only about 7% of these same highly polyunsaturated acids.

The fatty acids of the oil of any given species of fish are not of constant proportion from fish to fish, but vary depending upon the feed which the fish has been consuming as well as with other factors. Even large batches of many tons of commercial fish oil (such as menhaden) prepared at different times or places will vary considerably with respect to fatty acid composition. Table III gives fatty acid composition of several very large sized commercial batches of menhaden oil made at different plants. Menhaden oil made from fish in southern areas (e.g. Gulf of Mexico) has on the average lower iodine number than oil from fish caught farther north. The content of saturates is lower and of polyunsaturates higher in the menhaden oil made from fish caught in northern waters.

The fatty acid content of fish oils has been reviewed comprehensively by GRUGER [1967] and for further details the reader is referred to this reference.

2. Phospholipids

Our most complete knowledge of the nature of phospholipids which occur in fish concern those found in tuna. KATADA, ZAMA and IGARASHI [1960] and more recently SHUSTER, FROINES and OLCOTT [1964] have carried out extensive investigations with this species, the former using *Thunnus orientalis*, the latter albacore tuna, *Thunnus alalunga*. The predominant phospholipids occurring in these species and probably

Table II. Fatty acid content of body[1] oil

Name of species		Fatty acid[2] content in percent						
Common name	Scientific name	14:0	15:0	16:0	16:1	17:0	18:0	18:1
Clam, Littleneck	Protothaca stiminea	3.2	0.8	23.8	9.6	1.3	5.4	10.8
Cod, Atlantic	Gadus morhua	1.8	0.5	33.4	2.4	0.9	4.0	11.8
Cod, Atlantic	Gadus morhua	0.4	0.1	24.1	1.1	0.1	3.4	7.8
Cod, Pacific	Gadus macrocephalus	1.0	+	18.2	3.6	NK	4.7	14.5
Cod Liver Oil	Gadus morhua	2.8	0.4	10.7	6.9	1.2	3.7	23.9
Crab, Blue	Callinectes sapidus	2.2	0.9	15.2	11.2	1.9	7.2	17.6
Haddock	Melanogrammus aeglefinus	1.1	0.5	22.7	4.7	1.5	5.3	15.1
Haddock, Liver Oil	Gadus aeglefinus	2	+	16	6	1	3	30
Halibut, Pacific	Hippoglossus stenolepsis	2.8	0.3	15.1	8.9	0.7	3.4	25.7
Herring, Pacific	Clupea harengus pallasi	7.6	0.4	18.3	8.3	0.5	2.2	16.9
Mackerel, Common	Scomber scrombrus	4.9	0.5	28.2	5.3	1.0	3.9	19.3
Menhaden	Brevoortia tyrannus	8.0	0.5	28.9	7.9	1.0	4.0	13.4
Mullet, Striped	Mugil cephalus	4.6	6.3	17.3	11.0	0.8	5.0	8.4
Oyster, Pacific	Crassostrea gigas	2.7	0.9	21.4	4.6	1.4	4.0	8.5
Perch, Ocean	Sebastes marinus	4.6	0.6	12.6	8.0	1.0	3.6	22.0
Pilchard	Sardinops sagax; Sardinops caerulea	7.6	0.6	16.2	9.2	0.7	3.5	11.4
Salmon, Chinook	Oncorhynchus tshawytscha	3.7	0.4	16.6	9.2	1.1	5.8	29.1
Salmon, Chum	Oncorhynchus keta	2.2	0.6	17.0	4.1	1.1	3.2	21.4
Salmon, Coho	Oncorhynchus kisutch	3.7	0.5	10.2	6.7	0.9	4.7	18.6
Salmon, Pink	Oncorhynchus gorbuscha	3.4	1.0	10.2	5.0	1.6	4.4	17.6
Scallop, Sea	Placopecten magellanicus	1.9	0.7	23.0	2.0	0.8	5.3	5.2
Sole, Lemon	Microstomus kitt	4.3	0.7	16.5	14.4	1.4	2.4	12.2
Trout, Rainbow	Salmo gairdneri	2.1	0.8	11.9	8.2	1.5	4.1	19.8
Tuna, Albacore	Thunnus germo	3.7	1.0	29.3	6.3	1.2	6.1	16.6
Tuna, Bluefin	Thunnus thynnus	4.5	0.6	22.1	2.8	0.8	6.1	21.7
Tuna, Skipjack	Katsuwonus pelamis	7.0	1.0	24.0	6.3	1.1	3.0	16.2
Tuna, Yellowfin	Neothunnus macropterus	2.6	0.6	27.1	4.4	2.1	7.5	17.8
Whitefish, Lake	Coregonus clupeaformis	2.2	0.3	12.1	10.5	1.1	4.0	27.2

[1] Two liver oils are included for comparison.
[2] The figure before the colon is chain length; that after is the number of double bonds.

Table III. Variation in fatty acid content of large batch of menhaden oil made from fish caught in Gulf of Mexico compared to that from a large batch of fish caught in the North Atlantic

Fatty acid	% of fatty acid	
	Gulf of Mexico	North Atlantic
14:0	13.5	10.6
15:0 + 17:0	3.1	3.4
16:0	22.4	23.6
18:0	2.6	3.0
Total saturates	41.6	40.6
16:1	17.3	14.8
18:1	14.1	12.6
20:1	1.6	2.7
22:1	0.9	1.0
Total monoenes	33.9	31.1
18:2	0.9	1.2
18:3	0.4	1.0
18:4	1.1	0.9
20:4	0.8	0.8
20:5	13.5	13.1
22:5	1.4	1.4
22:6	3.3	6.5
Total polyunsaturates	21.4	24.9
Total hexaenes	3.3	6.5

in fish in general are lecithin (about half of the phospholipids) and cephalin (about ¼).

The range of fatty acids present in the lecithin and cephalin of albacore tuna as reported by SHUSTER *et al.* [1964] are shown in table IV. The ranges are for those occurring in four different fish which were analyzed. As will be noted a very high variation occurred from fish to fish. Palmitic and oleic were the chief saturated fatty acids occurring with the former being predominant in lecithin, the latter in cephalin. As with the triglycerides of fish, the C20:5 and 22:6 fatty acids were the principal polyunsaturates present in both lecithin and cephalin.

3. Hydrocarbons

Hydrocarbons occur in high quantity in the liver oil of several shark; about 90% of the liver oil of *Centrophorus uyato* [HELLER *et al.*, 1957]

Table IV. Principal fatty acids in albacore tuna lecithin and cephalin[1]

Fatty acid[2]	Content in cephalin %	Content in lecithin %
12:0	trace to 1.3	trace to 0.3
14:0	trace to 3.6	0–9.4
16:0	4.6–13.8	22.2–34.0
16:1	trace to 3.2	0–4.8
17:0	trace to 5.3	0–0.5
18:0	14.8–28.5	3.7–5.4
18:1	9.2–17.5	9.8–29.9
18:2	trace to 1.5	trace to 1.8
18:3	trace to 1.9	0–1.0
20:4	2.9–4.1	2.5–5.6
20:5	trace to 6.7	2.3–11.6
22:6	6.1–47.4	14.0–50.4
Total unsaturated	26.5–68.1	39.2–72.9

[1] Data from SHUSTER, FROINES and OLCOTT [1964].
[2] Figure before colon is carbon chain length; that after the colon is number of double bonds.

is squalene. Smaller quantities of pristane and zamene are also present in such oils [CHRISTENSEN and SØRENSEN, 1951].

Body oils of fish may also contain hydrocarbons. Thus LAMBERTSEN and HOLMAN [1963] have shown that herring contains 0.05% hydrocarbons which were separated into 8 branched-chain and 21 straight-chain compounds. Among the branched chain hydrocarbons, pristane (70%) and squalene (13%) occurred as the principal components.

4. Ether-linked compounds

The presence in shark of alkoxydiglycerides yielding upon hydrolysis such glyceryl ethers as selachyl, chimyl and batyl alcohols has been known for a long time [TSUJIMOTO and TOYAMA, 1922; HEILBRON and OWENS, 1928]. Later KARNOVSKY, RAPSON and SCHWARTZ [1948] showed that these substances occurred also in many other shark body oils. The fatty acid content in both the ester- and ether-linked portion of dogfish flesh and liver oil has been determined by MALINS, WEKELL, and HOULE [1965]. A rather complete review of both the chemistry and nutritional properties of such ether-linked compounds has recently been made by SNYDER [in press].

(5) Wax esters

Wax esters in which a fatty acid ordinarily of a low degree of unsaturation is esterified to a fatty alcohol usually of 16 or 18 carbon chain length occur sometimes in very high proportions of both the body and liver oils of certain species of fish. Thus AGAWA, HIRAO and KOMORI [1953] found such waxes in the liver oil of a cod, and NEVENZEL, RODEGKER and MEAD [1965] found them in both the liver and flesh oils of the castor oil fish, *Rivettus pretiosus*. In the latter case, the flesh oil was over 90% wax esters, the liver oil about 4%. The fatty acids were principally monoenes with 18:1, 20:1 and 22:1 accounting for 91% of the total.

C. Miscellaneous Components

Of the lipid components contained in fish not already mentioned, sterols are perhaps the most important. KRITCHEVSKY, TEPPER, DI TULLO and HOLMES [1967] have shown the presence of the following sterols in clams, scallops, and oysters: a C26 sterol, 22 dehydrocholesterol, cholesterol, brassicasterol, 24 methylene cholesterol and two different C29 sterols (one not present in oysters). Of these, cholesterol occurred in the greatest quantity and is also present in other fish and shellfish. The quantity occurring in several species is shown in table V. The amount present in fish is lower (under 100 mg/100 g wet fish) than in shellfish and crustaceans (usually 150–200 mg/100 g). The other sterols reported to be present in certain shellfish occur in considerably smaller amounts except for 24 methylene cholesterol (about 20 mg/100 g) and brassicasterol (about 15 mg/100 g).

Other components of fish lipids include vitamins and pigments. A discussion of pigments is beyond the scope of this review and vitamins will be treated only briefly. Vitamins A and D occur in widely varying amounts in the liver and body oils of fish [STANSBY and HALL, 1967] ranging from high values of up to 700,000 units vitamin A/g in halibut liver oil and up to 250,000 units vitamin D/g of certain tuna liver oils, down to values usually under 100 units/g in many body and some liver oils. For the average medium oil content fish however the vitamin A content per gram of wet flesh ranges from 10–100 units with an average of about 25 [STANSBY and OLCOTT, 1963]. Vitamin A and D, formerly obtained in the United States primarily from fish livers is now, because of the availability of the cheaper synthetic product, no longer manufactured here using fish as a source. In other

Table V. Quantity of cholesterol in certain species of fish and shellfish[1]

Species	Cholesterol content mg/100 g
Fish:	
Haddock	90
Pollock	75
Salmon	95
Shellfish and crustaceans:	
Clam	190
Crab	140
Lobster	170
Oyster	150
Scallop	175
Shrimp	200

[1] Data of KRITCHEVSKY, TEPPER, DITULLO and HOLMES [1967].

countries, e.g. Norway, Great Britain and Japan, fish livers are still an important source of Vitamin A [STANSBY, 1967a].

D. Oxidation

In this section of this review only a brief discussion of chemical oxidation of fish oils is presented. The reader is referred to Section III, Stability and Deterioration, in the book 'Fish Oils' [STANSBY, 1967a] for greater detail.

Fish oils, although differing chemically to a considerable extent from animal and vegetable oils, oxidize by the same general mechanism as for most lipids the theoretical basis for which was developed during the 1940's by FARMER [1946], BOLLAND [1946] and others. Fish oil fatty acids, however, have up to five methylene interrupted groups, where oxidation can take place. Compared to linoleic acid with only one or linolenic acid with two, many of the fish oil fatty acids are far more vulnerable to oxidation, and hence their rate of oxidation is far in excess of that of the fatty acids in most other oils.

With most polyunsaturates of fish oils of the ω-3 type the kind of oxidation products resulting may vary from those obtained from the more usual ω-6 variety.

Fish oils generally oxidize in a manner which results in a less distinct break in the induction curve and antioxidants are less effective

in prolonging the induction period than is the case with the same antioxidants applied to animal or vegetable fats and oils [LISTON, STANSBY and OLCOTT, 1963]. Oxidation in the oil of fish is very markedly affected by the presence of hematin compounds acting as prooxidants. The hematin compounds occur in fish primarily in the dark muscle and oxidation in this dark or red muscle may occur at a rate up to 100 times as rapid as takes place in the light muscle [BROWN, VENOLIA, TAPPEL, OLCOTT and STANSBY, 1957].

Fish oils with their high content of polyunsaturates can oxidize to a very great extent. Such extensive oxidation takes place, for example, when paint or other protective coatings containing fish oil oxidize. The extent of oxidation, however, which takes place in fish used for human food, and stored under ordinary handling conditions (e.g. in ice or frozen) is very small. The extent of oxidation in oil in iced fish is in fact almost imperceptible and there appears to be some relationship to the presence of growing bacteria in fish and reduction of oxidation rate [LISTON, STANSBY and OLCOTT, 1963]. When frozen fish is stored at usual temperatures, e.g. −20 °C, oxidation proceeds more rapidly, but the degree of oxidation at which obnoxious oxidative rancidity flavors develop—peroxide values of 5 or less—is at a very early stage of oxidation often still in the induction period as compared to the extreme oxidation occurring when fish oils thoroughly oxidize [STANSBY, 1967c].

The types of odors developing when a highly purified menhaden oil oxidizes have been described by STANSBY and JELLINEK [1965]. These follow through a pattern of first a loss of the initial normal, fresh, species characteristic flavor or odor to a particular 'fishy' type flavor or odor and later to the usual rancidity found in most oxidized oils. A more thorough discussion of odors and flavors in oxidizing fish oils has been given by STANSBY [1967b].

III. Sources of Fish Oil

A. Introduction

So far we have been considering primarily the chemistry of fish oils. In this section will be discussed just how the fish oils and their component fatty acids arise and where they occur within the flesh of the commercial fish. Some brief treatment will be also given to the subject of commercial fish oils.

B. Oil in Fish

1. Food chain in the sea

The many unique lipids found in fish come unaltered from the plankton and other feed of the fish, by metabolism in the fish of components of the fish's diet or as a combination of both processes. There is good evidence that the latter combination process prevails in most instances. In this section the transfer of lipid substances from the fish's feed to the fish will be examined briefly.

Early work by LOVERN [1935, 1951] and others compared the fatty acid composition of the oil in the fish with that in its feed. It was shown, for example, that when the fatty acid composition of the feed showed a seasonal variation, the fatty acids in the fish changed in the same way. In more recent work, KLENK and EBERHAGEN [1962a, b] have isolated, from plankton, polyunsaturated fatty acids identical with those found in fish. Fish have also been fed definite fatty acids and the effect on deposited fat observed. Thus KELLY, REISER and HOOD [1958a, b] showed that feeding of linseed oil, cottonseed oil, or herring oil would influence the fat of a freshwater fish to resemble that of the fat ingested and that the long chain polyunsaturated fatty acids originally present in the fish all but disappeared if they did not occur in the oil fed. KAYAMA, TSUCHIAYA, and MEAD [1963a] showed that by feeding a phytoplankton, *Chetoceros simplex* to a crustacean, *Antemia salina* as the only feed, the fatty acids of the fat of the crustaceans resembled those of the phytoplankton. When, however, these crustaceans were fed as the sole food of guppies *Lebistes reticulatus*, although the fatty acid pattern resembled that of the fat of the original phytoplankton to a small degree, the resemblance was far less than in the former case.

2. Metabolism in fish

Our knowledge of metabolism in fish has lagged behind that of such work with other species. It was not until BROWN and TAPPEL [1959] carried out work with carp liver mitochondria that it was confirmed that the fatty acid co-enzyme A derivatives of fish are oxidized making use of the same mechanism of β oxidation as elsewhere and making use of the same cofactors-Cytochrome C, ATP, Mg^{++} and a tricarboxylic cycle member. These workers also showed that this mechanism readily oxidized the long chain polyunsaturates such as docosahexaenoic acid.

The inability of fish in common with other organisms to synthesize the essential fatty acids was demonstrated by KLENK and KREMER [1960] in experiments in which polyunsaturates were formed during incubation of fish liver slices with acetate-1-^{14}C. In other work by NICOLAIDES and WOODALL [1962] it was demonstrated that chinook salmon maintained from the time of hatching on a completely linoleic acid-free diet developed deficiency symptoms and showed a lowered gain in weight. Fish, however, can readily change linoleic and linolenic acid, supplied in the diet to other members of the ω-6 and ω-3 polyunsaturate families of fatty acids respectively as was shown in such work as that of MEAD, KAYAMA and REISER [1960] and of KAYAMA *et al.* [1963b]. These and similar experiments resulted in a proposal for conversion pathways of polyunsaturates in fish by KAYAMA and TSUCHIYA [1962] such as is shown in figure 1. In this figure chain elongation is shown as long arrows which cut across the horizontal lines. The short arrows represent desaturation. The solid arrows show reactions already demonstrated to occur in fish and the dashed lines steps which are assumed to occur.

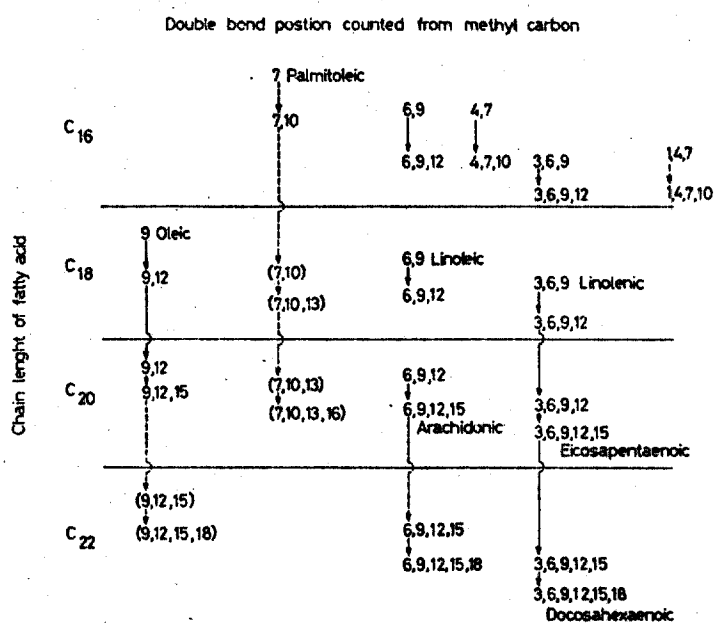

Fig. 1. Possible conversion pathways of polyunsturated acids in fish.

Much research remains to be done to bring our knowledge of metabolism in fish abreast of that for other organisms. In addition to gaps in knowledge in pathway conversions shown by the dashed lines in figure 1, almost nothing is known about the mechanism for the formation of esters such as glycerides, phospholipids or waxes. Our knowledge of the function of lipids in fish is also very incomplete. In such cases as the presence of up to 90% in the flesh of *Cristivomer namaycush siscowet* (Siscowet trout occurring in Lake Superior) [THURSTON, 1962] and of large wax ester deposits in certain species of fish [NEVENZEL, RODEGKER and MEAD, 1965] the lipids must act in some way other than as a mere energy source. MEAD and KAYAMA [1967] have suggested that in these instances the lipids may represent a means of controlling buoyancy in lieu of employment by the fish of an air bladder.

3. Environmental effects

The effect of feed on fatty acids deposited in fish has already been mentioned. Certain other environmental effects occur. It has long been known that the geographical area or the season wherein the fish is caught effects fatty acids deposited in the tissue. These effects in addition to being influenced by different types of feed are often largely a matter of effects of water temperature [LOVERN, 1942; SWAIN, 1953]. It has been long observed in commercial fisheries that a species like menhaden taken from both warm water and from cold waters produces oil of a higher iodine value, the colder the water from which the fish is taken. These observations receive confirmation from a number of experiments. FARKAS and HERODEK [1964] showed that as the environmental temperature decreased, the content of C20 and C22 fatty acids in freshwater crustacean plankton increased. KAYAMA, TSUCHIYA and MEAD [1963a] found that guppies raised in 17°C water contained a higher proportion of C22 fatty acids than did those raised in 24°C water. KNIPPRATH and MEAD [1965] likewise found an increase in 22:6 and other polyunsaturated fatty acids in rainbow trout after they were transferred from water at about 12°C to water at about 5°C. The fatty acid change occurred in oil from both the flesh and from composite organs (liver, heart, and spleen). JOHNSTON and ROOTS [1964] found a similar effect for polyunsaturated fatty acids from the brain of goldfish held in water at different temperatures. On the other hand, REISER *et al.* [1963] were unable to find any significant change in the pattern of fatty acids in mullet or goldfish raised in waters at

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13 °C and 23 °C. In this experiment, however, the fish were first depleted of long chain fatty acids and then fed with linoleic and linolenic acids.

4. Cellular *versus* depot fat

The lipids of fish occur primarily as phospholipids associated with cells as non-depot fats to an extent of about 0.7%. This type of lipid scarcely ever exceeds 1% [LOVERN, 1962]. The fish may or may not contain, in addition, depot fats which ordinarily consist of triglycerides and where phospholipids are absent or at most occur in very small quantity. Fish like cod and haddock with 1% or less total oil have little or no triglyceride with substantially all the lipid occurring as non-depot fat. Species of fish like herring or salmon with often 10% or even considerably more total oil have a large proportion of their lipid occurring as triglycerides.

5. Light *versus* red muscle

The oil is divided in fish between light and dark (or red) muscle. Ordinarily, but not invariably, the oil content of the dark flesh is higher (sometimes ten times or more) than in the light flesh. The dark muscle (usually of a dark red color) occurs predominantly as a 'v' shaped strip beneath the lateral line, but also in other areas, to a lesser extent, such as along the dorsal region.

BARETS [1961] has given a comprehensive discussion of the anatomy and function of the red and light muscle in fish. The red muscle is described as 'slow' muscle because it is believed to function as a means of propelling the fish through the water during slow, steady swimming. The light muscle sometimes referred to as 'rapid' muscle is believed to function primarily where bursts of speed of short duration are needed. GEORGE [1962] points out that the red muscle, provided with a relatively large supply of mitochondria, hematin compounds and enzyme systems such as lipase and succinase dehydrogenese, acts primarily to aerobically convert fat into energy. The white muscle, on the other hand, with limited mitochondria and associated lipid converting enzyme systems operates largely as a converter of carbohydrate (glycogen) into energy. These ideas correlate well with observations on migrating fish such as salmon which are provided with an extensive dark muscle system and which live exclusively off from stored depot fats during extended spawning migrations. More dormant species such as bottomfish like cod, on the other hand, do not store much depot fat, do not go on long spawning migrations

where stored fat is needed as an energy source, and do not have very extensive dark muscle systems.

The red muscle was once thought of as being exclusively an organ-like liver for carrying out some metabolic functions but having no muscular activity purpose [BRAEKKAN, 1956]. As shown by BUTTKUS [1963] red muscle contracts during rigor actually to an extent three times as great as does white muscle. There would seem to be no doubt that the red muscle does possess important function with respect to muscular activity in the fish as well as carrying out important metabolic activity particularly as the most efficient site for conversion of lipid to energy.

6. Oil in flesh *versus* organs

In addition to its occurrence in differing amounts in the flesh of fish, oil also occurs in certain organs particularly in the liver in highly variable proportions. In fish like cod, where almost all of the flesh oil occurs in form of phospholipids associated with the cells, the liver is large (about 10% of body weight), is very oily (up to 80%) and consists largely of triglycerides. In species of fish like salmon or herring which contain a preponderance of their flesh lipids as triglycerides occurring in depot fats, the livers are small (1–3% of body weight) and relatively non-oily (under 5%).

Table VI. Types of fish based on oil and protein content of the flesh

Category	Type of fish	Prototype Species	Moisture %	Protein %	Oil %	Ash %
A	Low oil-high protein (under 5%) (15–20%)	Pacific cod *Gadus macrocephalus*	81.5	17.9	0.6	1.6
B	Medium oil-high protein (5–15%) (15–20%)	Mackerel *Scomber scrombrus*	67.5	18	13	1.5
C	High oil-low protein (over 15%) (under 15%)	Siscowet lake trout *Cristivomer namacush siscowet*	52.5	11.3	36.0	0.53
D	Low oil-very high protein (under 5%) (over 20%)	Skipjack tuna[1] *Katsuwonus pelamis*	72.4	26.2	0.7	1.5
E	Low oil-low protein (under 5%) (under 15%)	Butter clams *Saxidomus nuttall*	83.0	13.3	1.3	1.9

[1] Analysis of light meat only. This part is the only one that is ordinarily canned.

7. Oil content of different species of fish

The oil content of the flesh of fish, as has been previously noted, varies over very wide limits from minimum values of about 0.6% up to maximum values of about 90%. Not only does this wide variability occur from species to species, but also the oil content of different fish of a single species may vary over a range of ten times or more. It is not, therefore, possible to present any data on average oil content of the different species of fish which would have much significance.

STANSBY [1962] has proposed a system for classifying composition of the flesh which is based upon a purely arbitrary consideration of oil and protein content. The 5 categories in this classification system are shown in table VI.

Oil content ranges in categories A, B and C are (A) under 5%, (B) 5–15%, and (C) over 15%. Most fish come under categories A or B. Category A includes both those species like bottomfish (e.g. cod) which possess little or no depot fat, and a number of species which have only modest quantities of depot fats. Category B includes most species which contain fairly high quantities of depot fats. Since many of these species vary widely in oil content from season to season, they may at one season or another have so little oil as to drop down temporarily to category A or so much as to occasionally lap over into category C. Most crustaceans and shellfish (except shrimp) with very low protein content and also low oil content fall into category E and a few special species with very high protein as well as low oil contents like tuna and halibut fall into category D. Some of the most important species of fish are classified by this system in table VII.

The range of oil contents in the livers of some species of fish are listed in table VIII. Note by comparison with table VII how fish with low oil content in the flesh have high oil content in the liver and vice versa and that the relative size of the livers is also generally related inversely to the oil content of the flesh.

C. Commercial Fish Oils

1. Introduction

Fish oils are manufactured in widely scattered areas of the world from in some instances whole fish, in others, from trimmings from cannery or fillet operations. The oil may be used as a component of food, as an animal feed, or for a variety of industrial purposes. In this section is

Table VII. Types of composition of the flesh of some important species of fish

Species	Category	Remarks
Anchovies	B	Some caught at certain seasons or in certain localities may fall in category C
Carp	A	Some caught at certain seasons or in certain localities may fall in category B
Cod	A	
Flounder	A	
Haddock	A	
Hake	A	
Halibut	D	
Herring	B	Some caught at certain seasons or in certain localities may fall in category C
Mackerel	B	Some caught at certain seasons or in certain localities may fall in category C
Mullet	A	Borders on category B
Ocean perch	A	
Pollack	A	
Rockfishes	A	
Salmon	B	Some species of salmon, chum (Oncorhynchus keta), Atlantic (Salmo salar), and pink (Oncorhynchus gorbuscha) may border on category A
Sardines	B	Some caught at certain seasons and in certain localities may be category C
Tuna	D	Albacore tuna (Germo alalunga) when caught in certain areas is in category B. Bluefin tuna (Thunnus thynnus) borders on category B
Whiting	A	
Clams	E	
Crabs	A	
Oysters	E	
Scallops	A	
Shrimp	A	

Category A = oil < 5%, protein 15–20%; category B = oil 5–15%, protein 15–20%; category C = oil > 15%, protein < 15%; category D = oil < 5%, protein > 20%; and category E = oil < 5%, protein < 15%.

presented a brief description of the methods used in manufacturing such commercial fish oil followed by a short description of some of the different varieties of fish oil and their use and method of preparation in different geographical areas.

2. Manufacture of commercial fish oils

Most fish oil is manufactured in an operation which produces both fish oil and a dried protein product, fish meal, which is used primarily

Table VIII. Oil content and liver size of the livers of certain species of fish

Species	Liver Ocean from which caught	Proportion of round weight %	Oil content %
Cod	Atlantic	7	20–60
Herring	Pacific	2	5–25
Menhaden	Atlantic	2	5–20
Rockfish	Pacific	1.5	5–25
Sablefish	Pacific	2	10–25
Salmon, chum	Pacific	2	2–6
Salmon, king	Pacific	2	4–8
Salmon, pink	Pacific	2	4–6
Salmon, silver	Pacific	2	4–6
Salmon, sockeye	Pacific	2	5–8
Shark, dogfish	Pacific	10	50–72
Shark, sleeper	Pacific	12	40–55
Shark, soupfin	Pacific	10	55–72
Swordfish	Atlantic	2	8–35
Tuna, albacore	Pacific	2	7–20

as a poultry feed supplement. By far the most common manufacturing procedure, known as the wet rendering process, involves cooking the wet fish to coagulate protein and to release moisture and oil followed by pressing away the oil and water, separating the oil from the water by centrifuging and drying of the solid phase to produce the meal. Usually the aqueous phase containing dissolved protein, amino acids, and vitamins is concentrated to produce condensed fish solubles (also used in poultry feed). The crude oil is generally given a 'polishing' in a high speed centrifuge to remove small amounts of water or proteinacious material.

Small quantities of oil and (meal) are produced by the dry rendering method in which the fish is cooked and dried in one operation, followed by pressing of the oils from the meal. Very small quantities of fish oil are currently produced by a solvent process. Such a method may in the future form the basis of producing large quantities of fish protein concentrate (FPC). If this develops into a large scale operation larger quantities of oil may be produced in the future by such a method. Oil produced by either the dry rendering or by the solvent extraction processes are generally of darker color than when produced by the wet

rendering method, and they then require additional and special refining procedures. Oil made as a part of an FPC operation unlike that by most other processes meets US Governmental standards for use as a food product for human consumption. For a more complete description of these processes see PIGOTT [1967].

Fish liver oils are manufactured by cooking the livers immediately after catching of the fish with water and centrifuging the oil from the water. For highest quality of medicinal oil the livers may be cooked aboard the fishing vessel. Low oil content livers which often contain very high vitamin A content usually give an insufficient yield by a simple cooking process and they are, therefore, frequently cooked with dilute alkali to partially solublize the protein, followed by centrifuging away the oil. AURE [1967] gives much greater detail concerning methods of producing fish liver oils.

Fish oils are usually refined by some additional processing steps which are used to remove free fatty acid, stearine, and odor, and to improve color of the oil [CHANG, 1967]. Stearine which consists of some of the most saturated fatty acid that may be solid at relatively high temperatures and might separate out when the fish oil is chilled, are removed by 'winterizing' i.e. by cooling the oil to about 0–1.5 °C and, while cold, pressing away the solid stearine. Free fatty acids are neutralized with either a sodium hydroxide or sodium carbonate solution. Color is bleached using special clays or activated carbon. Bleaching also reduces odor and flavor. Hydrogenation removes all odor and flavor, but, of course, converts the fish oil to a more nearly fully saturated, solid form. This process is used when the fish oil is to be incorporated into edible margarine or shortening and it has been described by HANNEWIJK [1967].

3. Anchovy

The anchovy fishery of Peru extending also into Chile, produces the raw material for the world's largest production of fish oil (and fish meal). This large industry is of relatively recent origin, the production having increased by a factor of over 15 since 1958. Anchovy oil is one of the most highly unsaturated of important commercial fish oils and it has an especially high content of eicosopentaenoic acid. The oil is manufactured in relatively new plants established during the past decade. The usual wet-rendering process is used throughout this industry and alkali refining and bleaching are carried out at several of the manufacturing plants. Most of the anchovy oil is exported to

Europe and other areas and it is largely used (after hydrogenation) in margarine and shortening. In recent years an increasingly larger amount is being hydrogenated in Peru for domestic consumption for human use. The anchovy fish oil industry has been described in detail by SANCHEZ TORRES [1967].

4. Menhaden

The menhaden industry is the basis for by far the largest fish oil production in the United States. In some recent years, the catch of menhaden has approximated the catch of all other species combined. The fishery is carried out off the Atlantic Coast from New York southward to Florida and around into the Gulf of Mexico. Important production areas include coast areas in New Jersey, Delaware, Virginia, North Carolina, Florida, Mississippi, Louisiana and Texas. Within recent years there has been a relatively larger proportion of the production taking place in the southern segment of the industry in the Gulf of Mexico with fish in the northern areas becoming relatively scarce and with a downward trend in total production of the entire industry.

Menhaden oil is produced entirely by the conventional wet rendering process. Details concerning production methods and of the menhaden industry in general have been described by LEE [1963] and by JUNE [1963] respectively. More than half the oil is exported to Europe for hydrogenation and use in margarine and shortening (use of commercial fish oil as ordinarily currently produced in the United States for human food applications even after hydrogenation is not permitted by Federal authorities in this country). The remainder finds application in protective coatings and for a wide variety of other industrial uses [FINEBERG and JOHANSON, 1967].

5. Herring

Herring oil is produced in widely separated areas of the world. Relatively large production takes place in Norway and Iceland with smaller production in Sweden, Denmark, West Germany, Canada (largely British Columbia) and to a small extent in the United States (Alaska and Maine, the latter only by use of trimmings from sardine (herring canneries).

Herring oil is of somewhat lower iodine number (unsaturation) than is menhaden or anchovy and contains a relatively large proportion of monoenes.

6. Other oils

Fish liver oils are no longer produced to any significant extent in North America and South Africa because of competition from synthetic vitamin A. Cod liver oil is still an important commodity in such European countries as Norway, Iceland and Great Britain [SAND, 1967]. Liver oil is produced in Japan from cod, pollack and shark liver to a considerable extent [SONE, 1967].

Pilchard oil was once a very important commodity produced on the West Coast of North America (California, Washington and British Columbia) its production at one time exceeding that of menhaden. Because of disappearance of pilchard in this area this industry has ceased. Pilchard oil production is of an important magnitude in South Africa as is also that of Maasbanker oil [DREOSTI, 1967].

In Japan, in addition to fish liver oil, there is a sizable production of saury oil with lesser quantities of squid oil, mackerel oil, and sardine oil [SONE, 1967].

7. Uses of commercial fish oils

The various uses of fish oils have been described in considerable detail by FINEBERG and JOHANSON [1967], DE SESA [1967], HANNEWIJK [1967], and DYER [1967]. In this brief description, the uses of menhaden oil which are reasonably typical of world-wide uses of fish oils will be chiefly emphasized.

A preponderant part of all fish oils is used for hydrogenation whereby the unique chemical characteristics of fish oils are destroyed in the process of changing them over into a more saturated form. Hydrogenated fish oil is used in substantial quantity in shortening and margarine in northern European countries and to lesser extents in Canada, South America, Japan, in fact in most other countries except the United States. Nevertheless about half of the menhaden oil (all of which is produced in the United States) is exported for hydrogenation and used abroad in edible shortenings and margarine. About 15% of the menhaden oil is used directly without hydrogenation in domestic poultry and animal feeds.

A most important use of menhaden (and other) oils, second only to use in food and feeds is as a drying oil for use in protective coatings. Originally fish oil was added directly to paint or varnish. During drying, strong fishy odors developed which restricted use of these coatings for uses such as for painting the outside of buildings. Today much of the menhaden oil destined for the domestic protective coating

field is first converted into another chemical form which eliminates many of these odor problems. For example alkyd resins are prepared by reaction of the menhaden oil with phthalic anhydride and the alkyd then used in the paint. In other cases a maleated oil is produced by reaction of the menhaden oil with maleic anhydride or a co-polymer is produced by reaction with cyclopentadiene. These various derivatives improve the characteristics of fish oil not only from the odor stand-point but also aid in preventing undesirable yellowing which sometimes limits use of fish oils in varnish or other coatings.

Usually a little over 10% of menhaden oil is used in preparation of lubricants. Soaps of menhaden oil such as those of lithium, barium, sodium, aluminum, or lead are prepared and added to other lubricating oils to form a grease. Another 10% or somewhat less of the menhaden oil production goes into the making of fatty acids. The long carbon chain (C20 and C22) of many fish oil fatty acids impart special qualities not found in other fatty acids. The fatty acids may be used as such or employed as a starting material for preparation of a wide variety of other chemical derivatives.

Derivatives of fish oils are used in a very wide range of miscellaneous industrial applications including in leather tanning, ore flotation, printing inks, glazing and caulking compounds, water repellents, core oils in foundries, and as corrosion inhibitors.

IV. Nutritional Properties

A. Introduction

Under this heading will be considered various effects of fish oils and their component constituents when ingested or otherwise applied to animals including humans. Metabolism of fish oils, already treated briefly with respect to metabolism in fish will be included in a more general way along with nutritional properties. This latter topic will also include pharmaceutical and related effects. Most of the discussion will relate to fatty acids, the principal chemical component of fish oils. Briefer mention will be made of other classes of components such as ether linked compounds and phospholipids.

B. Fatty Acids

1. Essential fatty acids (EFA) in fish oils

Before we consider the situation with respect to the EFA role of

certain fish oil fatty acids we should clarify the meaning of EFA for the purpose of this discussion. As has been pointed out by STANSBY [1967c] the historical development of our concepts of EFA was based upon research with animal or vegetable oil, and certain aspects of the role of fish oils with respect to EFA concepts do not lend themselves to proper interpretation unless we clearly understand this situation. When BURR and BURR [1929] first enunciated their ideas on EFA, the important function of these acids was restricted to their effects in supporting growth and in curing of dermal symptoms. In the years immediately following this discovery the curing of the dermal symptoms became the basis for the assay procedure used to measure EFA content. We know now, of course, that this assay procedure measures primarily fatty acids of the ω-6 series (linoleic acid family), but that fatty acids of the ω-3 series (linolenic acid family) although unable to cure the dermal symptoms completely not only do support growth but also possess certain other important functions such as transportation of cholesterol. For the purpose of discussion in this article, we will, therefore, consider that fatty acids of the linoleic acid family to be 'classical EFA' but recognizing that fatty acids of the linolenic acid family display certain EFA properties and may be considered to be A's perhaps in a more limited sense.

PRIVETT, PUSCH, HOLMAN and LUNDBERG [1960] showed that tuna, herring, and menhaden oil cured dermal symptoms in rats (and that these oils therefore contained classical EFA) when included in the diet at moderately high concentrations (10%) but that they were relatively ineffective at lower concentrations [PRIVETT, AAES-JØRGENSEN, HOLMAN and LUNDBERG, 1959]. The amount of linoleic acid family of fatty acids was estimated from these experiments to be about 2% in these fish oils. Present day knowledge of the fatty acid composition of the oils of fish [GRUGER, 1967] indicates that this figure of 2% is probably not far from the correct value for the content of linoleic acid family of fatty acids of these commercial fish oils. Body oils of marine fish may average a little higher perhaps about 3% and with some species as high as 6%; the content of linoleic family of fatty acids in the oil of freshwater fish is somewhat higher, perhaps in the range of 5–9%.

2. Growth effects

In the experiments of PRIVETT *et al.* [1960] the rats fed for 16 weeks on a fat-free diet followed by supplementation for 12 weeks with 10%

Table IX. Supplementing effect on growth of rats of addition of 10% menhaden, tuna, herring, or corn oil to a fat-free diet [DATA of PRIVETT, PUSCH, HOLMAN and LUNDBERG, 1960]

Supplement	Initial weight after 16 weeks on fat-free diet	Final weight after 12 further weeks on supplemented diet	Weight gain grams
None (fat free)	232±8	252±8.2	20
10% menhaden oil	237±7.6	354±15.4	117
10% tuna oil	219±15.2	329±16.8	110
10 herring oil	235±11.9	354±15.1	119
10% corn oil	223±4	340±8.1	117

menhaden, tuna, or herring oil showed growth response at least equal to that of the positive control where corn oil was the supplementing oil (see table IX). The final weights of animals on all of these additives after 28 weeks averaged 344 g as compared to only 252 g for the animals on the negative control with no fat in the diet.

An effect of feeding of linoleic and linolenic acid simultaneously upon metabolism rates of these two families of fatty acids was indicated by work of KLENK and OETTE [1960], MARCO, MACHLIN, EMERY and GORDON [1961] and MACHLIN [1962]. A series of experiments by MOHRHAUER and HOLMAN [1963a, b] and summarized by HOLMAN [1964] showed that when the ratio of linolenic to linoleic acid increased, the conversion of linoleic to arachidonic acid was progressively inhibited. HOLMAN [1964] also points out that this is a more general situation such that fatty acids of any one family repress the metabolism of another. Thus oleate fed with linoleate will also repress the conversion of the latter to arachidonate. Even saturated fatty acids repress the metabolism of unsaturated ones. These experiments dealing only with linolenic acid so far as ω-3 fatty acids are concerned is not directly applicable to fish oils which are very low in linolenic acid itself although very high in fatty acids of the linolenic acid family.

More recent research, however, indicates that this repression of metabolism of linoleic to arachidonic acid occurs not only when linolenic acid itself is the inhibitor, but also when other members of the linolenic acid family including the typical ω-3 fatty acids common in fish oils are present in considerable quantity. Thus BRENNER and

PELUFFO [1966] using *in vitro* experiments involving incubation of labeled fatty acids with adenosine triphosphate, reduced nicotinamide adenine dinucleotide, reduced nicotinamide adenine dinucleotide phosphate, magnesium chloride, coenzyme A, glutathione, nicotinamide, sodium fluoride, and sodium cyanide in oxygen at pH 7 and 35 °C showed that docosahexaenoic acid decreased the percentage of desaturation of linoleic acid. Likewise MOHRHAUER, CHRISTIANSEN, GAN, DEUBIG and HOLMAN [1967] showed by similar means that chain elongation is inhibited by both eicosapentaenoic acid (ω-3) and docosahexaenoic acid (ω-3).

3. Effects of feeding fish oils

A large part of our evidence concerning nutritional properties of fish oils is based on more practical feeding tests in which farm animals or poultry were employed as test animals. A vast literature reviewed by KARRICK [1967] documents such experiments. In much of this type of work it soon became apparent that when fish oil was fed in a proportion of the total feed beyond some small limit, problems of imparting of a fishy taste to the meat or eggs of the product from the animal resulted. This and other considerations have limited the use of fish oils in animal feeds to a considerable extent.

a) Digestibility, adsorption, and metabolizable energy

The coefficient of digestibility for fish oil when consumed by many animals [ARTMAN, 1964; DEUEL, 1954; LEOSCHKE, 1959; REDER, 1942] has been reported to be high. STEENBOCK, IRWIN and WEBER [1936] reported that halibut liver oil and cod liver oil were absorbed more rapidly than were corn oil, lard, or partially hydrogenated fats. These results were based upon experiments employing rats, Metabolizable energy of fish oil is generally reported to be relatively high, e.g. 3700 cal/lb for menhaden oil for chicks as compared to 2900 cal/lb for tallow [RENNER and HILL, 1958].

b) Fish oil in poultry feed

Insofar as growth only is concerned, fish oils have been found to give excellent growth in quantities up to 12% [ARTMAN, 1964] and mixtures in the ratio of 1:1 of tallow and fish oil increased the utilization of the former. Both growth and feed utilization efficiency have been reported to be improved by use of fish oil in the diet of poultry by many workers, e.g. EDWARDS *et al.* [1961, 1962, 1963].

Chicken flesh very readily takes on an objectionable fishy flavor when the birds are fed fish oils [CARRICK and HAUGE, 1926; DANSKY, 1962; HARDIN, MILLIGAN and SIDWELL, 1964]. Safe levels of fish oil in the diet low enough to prevent this problem are probably no higher than 1% of the diet. Turkeys are even more susceptible than are chickens and fishy flavor has been noted in turkey flesh in cases where as little as 0.4% fish oil occurred in the birds' diet [KLOSE *et al.*, 1952]. These flavor problems can usually be alleviated to a considerable extent by removing the birds from a diet containing fish oil some time before slaughter [KARRICK, 1967]. Stability of frozen poultry flesh toward oxidative deterioration in cold storage can also be decreased by including excessively large amounts (e.g. 2%) of fish oil in the feed [KLOSE *et al.*, 1953; SCHREIBER *et al.*, 1947].

When fish oils are included in the diet of hens, egg production efficiency improves [EDSON, 1932]. Fishy flavor is not ordinarily imparted to the eggs as it would be to the flesh of the fowl. Thus VENDELL and PUTNAM [1945] found no fishy flavor whatever in eggs from hens which had been consuming for 30 days a strong-smelling, low grade sardine oil at levels up to 14 times the recommended amount.

c) Fish oil in swine feed

Both texture and flavor or pork can be adversely altered if the swine consume a diet with excessively high proportions of fish oil. The depot fat readily takes on the chemical characteristics of the fish oil [ELLIS and ISBELL, 1926] and a yellow discoloration may also result [BROWN, 1931]. Restrictions of fish oil content in the ration to 0.5% has been reported to eliminate all these problems [KARRICK, 1967]. Elimination of fish oil from the pigs' rations 30 days before slaughter is also reported to be an effective remedy [FRASER, STOTHART and GUTTERIDGE, 1934]. OLDFIELD and ANGLEMIER [1957] also reported improvement in pork color, flavor and odor from pigs fed polymerized fish oil but at the expense of growth efficiency.

d) Fish oil in cattle fodder

Fish oil when fed to cattle can result in the same effects as when included in swine rations, but the effects are usually considerably less [BEARD, SCHULTZ and CULBERTSON, 1935; DAVIS and MAYNARD, 1938; NICHOLSON *et al.*, 1962]. Muscular dystrophy can result if too high a quantity of fish oil is fed to calves, and this effect can be controlled by inclusion of supplementary tocopherol [BLAXTER *et al.*, 1953a, b, c].

Fish oil fed to cows lowers the fat content of the milk as well as increasing the degree of unsaturation of the milk fat [BROWN and SUTTON, 1931]. These changes result from alteration in the pattern of fatty acids in the rumen which is brought about by incorporation of fish oil in the feed.

Acetic acid and butyric acid in the rumen decrease and propionic acid increases [SHAW and ENSON, 1959].

e) Fish oil in the diet of other animals

Herring oil fed to goats alters the butter fat content in a manner similar to that which occurs when it is fed to cows [GRAHAM and CUPPS, 1938]. Mink, although not fed fish oils directly, often are fed fresh fish which contain fish oil. As with feeding of other unsaturated oil to mink, oil in fish can cause steatitis unless an adequate supplement of tocopherol is included [LALOR, LEOSCHKE and ELVEHJEM, 1951]. Cats, if fed a diet consisting almost exclusively of fish can also develop steatitis. Here again the solution is to provide an adequate quantitiy of tocopherol [GERSHOFF and NORKIN, 1962].

4. Polyunsaturates effects in animal feeds and human food

We have seen, in a previous section, that fish oils with their relatively high content of polyunsaturated fatty acids are highly unstable, subject to rapid oxidation. There has also been, in other discussions included in the foregoing material, suggestion that in some instances the presence of the polyunsaturates or their oxidation metabolites may raise nutritional problems. The purpose of this section of the review is to consider further what these effects are and under what circumstances they may be of serious consequence.

Of the two types of effects of polyunsaturates, those involving oxidation and those relating to the nature of the unoxidized condition, the former will be discussed first. We must first have a clear distinction between the varying extents of oxidation which fish oils can reach under different methods of handling of either the oil itself or the oil in fish flesh. The oil in fresh or frozen fish may oxidize during storage resulting in flavors described as 'fishy' or rancid [STANSBY, 1967b]. When such fish reach a point of marginal acceptability so far as human food is concerned with a somewhat pronounced unpleasant flavor, the extent of oxidation is exceedingly minute. Only a small fraction of 1% of the oil has actually been oxidized, no loss whatever in fatty acid content can be measured, nor is there any detectable drop in

iodine value. This situation occurs because the carbonyl compounds resulting from oxidation of oil and which cause the unpleasant rancid flavor are so exceedingly flavorful that a mere 0.01% or so will cause exceedingly unpleasant flavors in fish. Peroxide values of fish which correspond to definite rancidity may be in the range of 0–2 or when the flavor is pronounced and exceedingly objectional usually no higher than 5–10, and such levels of oxidation can never cause any adverse nutritional problems [STANSBY, 1967c].

Protection of oils in fresh or frozen fish destined for human consumption is well provided both by the fact that the fish is generally shielded from direct contact with oxygen from the air by being provided with a barrier such as packaging material and by storing at a low temperature where the chemical oxidation reaction is retarded. Of even greater significance is the built-in factor of flavor such that any sample of fish in which oxidation had taken place to an extent capable of impairing nutritive value would have resulted in a flavor so disagreeable that at least in Western nations, it would have been rejected on the basis of its exceedingly unpalatable flavor.

With animal feed, especially fish meal, conditions for oxidation are far more favorable than with fish stored for human consumption. The fish meal is stored in burlap or paper bags with relatively free access to air. The meal is held without refrigeration and during storage oxidation occurs at such a rate that the temperature of the meal rises from the heat produced by the oxidation to levels which sometimes even creates a fire hazard. Under these storage conditions, a much later stage of oxidation is reached. Iodine values may fall from 150 to 80 or less. A considerable portion of the fatty acids of the oil is destroyed. Even if there were no toxicity caused by the end products of oxidation, the energy value and the growth stimulating value of the fatty acids are lost when these are destroyed.

When oils oxidize, two types of products with possible adverse nutritional effects which form are peroxides and carbonyls. High levels of peroxides cause intestinal irritation, diarrhea, and weight loss [ANDREWS *et al.*, 1960]. In their experiments, soyabean oil with high peroxide values (over 1000) when fed to rats caused them to lose weight. When the peroxide value of the oil was lower the effect diminished until, at peroxide values of 100, no difference in weight gain was apparent from that of the unoxidized oil, and no other toxicity symptoms occurred.

Fish oil or fish oil in fish meal seldom has peroxide values as high

as 100. The peroxides of fatty acids of fish oils are so unstable that, instead of accumulating, they usually break down almost as fast as they form resulting usually in relatively low peroxide values.

KANEDA, SAKAI, ISHII and ARAI [1955] showed that when fish oils oxidize it is the peroxides, not the carbonyls, which cause toxic effects. GROOT and KLEIN OBBINK [1953] showed that oxidized cod liver oil when fed to rats resulted in impaired growth rate only when peroxide values exceeded 50. RASHEED, OLDFIELD, KAUFMES and SINNHUBER [1963] showed that oxidized menhaden oil fed to rats gave toxic symptoms when peroxide values exceeded 125. CARPENTER, LEA and PARR [1963] found no retarding of growth of chicks when herring oil with a peroxide value of 142 was fed at a 6% level in the diet.

A potentially more serious condition may, under certain circumstances, occur as a result not of the end products of the oxidation process itself but rather from the effect of the oxidation upon other components of the diet during storage before consumption as a result of free radical action. These types of effects will now be considered. ROUBAL [1966] has shown that such free radical damage can occur to proteins, amino acids, or enzymes. The level of peroxide in the oxidizing lipids in this work was relatively high (peroxide value of 220). At these levels of oxidation, protein damage was quite severe. Evidence that such type of damage occurs in commercial fish meal is also shown indirectly by experiments of CARPENTER, LEA and PARR [1963] where fish meal was used in which the oil was badly oxidized and which gave poor growth and yet when the extracted oil was fed no retardment in growth occurred. It was shown that oxidizing of the lipid had made some of the amino acids of the protein unavailable.

These effects, where free radicals formed during oxidation of lipids and damaged the proteins, would never be apparent at the very low levels of oxidation which go on in fish being handled fresh or frozen for human consumption. As was seen in an earlier section, oxidation in such a case results in peroxide values of about 10, usually much less. At such low levels of oxidation free radical damage to protein would be insignificant.

The second main type of potentially adverse effect of polyunsaturates involves *in vivo* effects which may occur when the unoxidized polyunsaturate is fed to animals. Adverse effects of this type have been observed with animals fed diets which consisted either almost exclusively of fish or where highly polyunsaturated concentrates from fish oil were fed in considerable quantity. Thus encephalomacia has been

observed in chicks fed either large quantities of unsaturated vegetable or fish oils without adequate tocopherol supplementation [DAM, 1944] or fed concentrates of fish oil polyunsaturates [MILLER *et al.*, 1964]. The incidence in mink of yellow-fat disease [LALOR *et al.*, 1951] and of steatitis in cats [GERSHOFF and NORKIN, 1962], both mentioned previously, are other examples of this effect. In such cases these conditions can be remedied by adding an adequate tocopherol supplement to the diet. Effects like these have never been noted in the case of fish consumed by humans. Even in areas of the world where fish is eaten in relatively large quantity by most of the population, there is no evidence of such conditions. In Japan, for example, IMAICHI *et al.* [1965] show that as a result of eating very large quantities of fish, some members of the population lay down in their depot fat significant amounts of C22 fatty acids. Such individuals, however, fail to develop conditions mentioned above. In the case of the animals which live on diets fed to them, it is very easy for them to get so much fish that tocopherol content is inadequate, a condition not met with in human diets where much other food always is included.

5. Cholesterol depressant effect

a) Introduction

The idea that cholesterol is somehow connected with heart disease is not a new one. In 1843 VOGEL, noting that cholesterol was present in relatively large amounts in the arteries of atherosclerotic victims, proposed this cholesterol accumulation as a cause for the disease and as early as 1838 LECANU had shown that cholesterol occurred in human blood. Work continued during the 19th Century, VIRCHOW [1856] suggesting that atherosclerosis occurred when cholesterol precipitated out as a solid layer in the arteries.

As early as 1908 IGNATOWSKI was able to induce atherosclerosis in rabbits by feeding them a diet high in meat and dairy products. At this stage, it was believed that the cholesterol arose entirely from its content in ingested food, and it was not until the work of RITTENBERG and SCHÖNHEIMER [1937] and BLOCH and RITTENBERG [1942] that it was realized that much of the cholesterol was formed by metabolism within the body.

Although a connection between heart disease, cholesterol, and high fat in the diet was suggested much earlier [WIDAL *et al.*, 1913] experience during World War II in which enforced dietary changes caused noticeable changes in the incidence of atherosclerosis [MALM-

ROS, 1950] gave impetus to research on diet and heart disease. Initially much work was done connecting dietary habits with atherosclerosis incidence, and the relationship between high dietary fat content and high blood cholesterol levels with accompanying low incidence of heart disease levels received much attention [KEYS, 1952]. KINSELL *et al.* [1952, 1953, 1954] were the first to relate the type of fat (i.e. saturated *versus* polyunsaturated) to serum cholesterol levels.

b) Effect of fish oil on serum cholesterol levels

Soon after it was noted that the type of fat or oil in the diet was related to the serum cholesterol levels, the effect of fish oils was studied by several workers on both man [BRONTE-STEWART *et al.*, 1956; MALMROS and WIGAND, 1957; AHRENS *et al.*, 1959] and animals [PEIFER *et al.*, 1960; HOWE and BOSSHARDT, 1962a, b; KAHN *et al.*, 1963a, b]. Results of some of this work on dietary fish oils compared to dietary oil of vegetable origin is shown in table X.

It is apparent that for each species, man, rat, chick and mouse, fish oils lowered serum cholesterol to a greater extent than did corn oil or linoleate (corn oil is predominately linoleate). The extent of lowering of the serum cholesterol level in these experiments was about twice as great for the fish oil as for the corn oil or linoleate for man and for the rat and ranged up to 12 times as great in the chick.

Figure 2 from the work of PEIFER *et al.* [1960] depicts the changes in serum cholesterol levels in rats fed diets in which one-third of the fat (the other two-thirds was tallow) consisted of palmitate, oleate, linoleate, linolenate, tuna oil or menhaden oil. There was no decrease of serum cholesterol levels when palmitate or oleate were fed for 5 weeks. The other fat additives lowered serum cholesterol levels, with the lowering caused by linoleate being considerably less than that for the fish oils or linolenate. Of particular interest is the fact that the two fish oils showed a lowering effect immediately after they were included in the diet. After 2 weeks serum cholesterol had dropped significantly in rats on the fish oil diets but was either unchanged or actually increased for rats on the other diets. This more *rapid* lowering of serum cholesterol levels seems to be specific to fish oil and has been consistently noted.

The quantity of fish oil needed to effect a given decrease in serum cholesterol level is considerably less than that of corn or other vegetable oils. HOWE and BOSSHARDT [1962a, b] found a cod liver oil concentrate to be ten times as effective, weight for weight, as corn oil

Table X. Comparative effects of fish oil with corn oil or linoleate on serum cholesterol depressio man and animals. Data of PEIFER [1967]

Species	Reference	Test lipid	Proportion of test lipid to total dietary lipid %	Test period weeks	Lowering of serum choleste %
Man	AHRENS *et al.*, 1959	corn oil	100	6	—29
		menhaden oil	100	8	—57
Rat	PEIFER *et al.*, 1960	linoleate	33.3	4	—31
		menhaden oil	33.3	4	—51
		tuna oil	33.3	4	—59
Chicken	KAHN *et al.*, 1963	corn oil	50.0	1	— 5
		cod-liver oil	50.0	1	—63
Mouse	HOWE and BOSSHARDT, 1962	linoleate	100	1.7	— 4
		corn oil	100	1.7	— 4
		cod-liver oil	100	1.7	—26

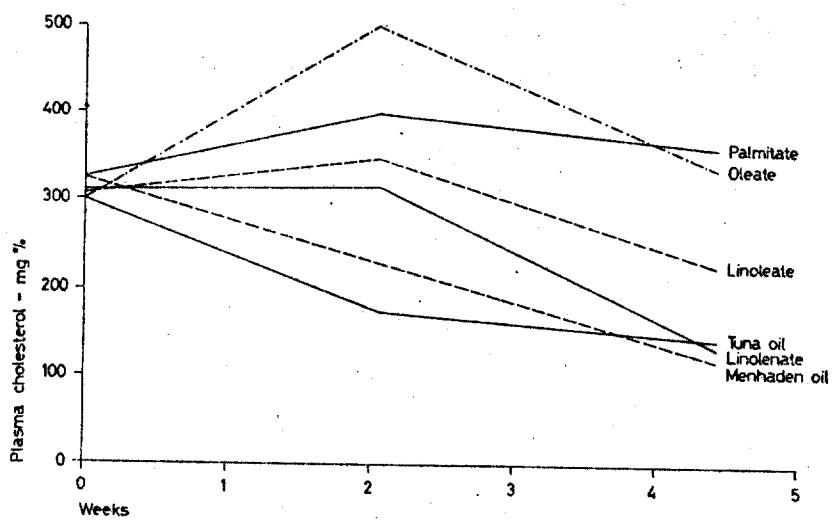

Fig. 2. The effects of feeding specific fatty acids and fish oils on the plasma cholesterol levels of hypercholesteremic rats [PEIFER, 1960].

in lowering serum cholesterol in mice. KINGSBURY *et al.* [1961] found that less than ¼ as much cod liver oil was as effective in lowering serum cholesterol in man as the 40% level of vegetable oils ordinarily employed [KINSELL *et al.*, 1954]. PEIFER *et al.*, 1965; PEIFER, 1966, have obtained significant lowering of serum cholesterol in the rat when less than 4% of the fat calories were supplemented as fish oil.

Cholesterol depressant effects have been noted for oils prepared from a wide variety of species of fish as shown in table XI. The effect is found in oils from the whole fish, from fish liver oils, and from fish body oils.

Not only is serum cholesterol brought to a lower level by use of fish oil than by vegetable oils, but also the same holds true for the lowering of plasma triglycerides which, with some individuals may be abnormally high when their serum cholesterol level is normal [ALBRINK and MANN, 1959]. In one case, AHRENS *et al.*, [1959] found that with an initial plasma triglyceride level of 810 mg/100 ml, corn oil intake resulted in a minimum value of 486. When this treatment was followed by menhaden oil intake, the value fell to 230, but when ingestion of corn oil was resumed, the value returned to 435.

The results so far discussed have been obtained with fish oil being consumed by man or animals. Presumably similar results would be expected if the oil were fed in the form of fish flesh which contains oil. In order to confirm this point, PEIFER *et al.* [1962] fed rats freeze-dried flesh of menhaden, coho (silver) salmon, mullet and ocean perch. In another part of the experiment oil extracted from these species and to another group of animals, the oil-free fish tissue was fed. Results of these experiments are given in table XII. Three of the species used were moderately oily. The fourth species, ocean perch, contains only a few percent oil, and it was not, therefore, possible to get as much oil into the diet as with the other species. In spite of the fact that when the ocean perch flesh which supplied only 2.5% of fish oil to the diet (as compared to about twice this amount for the other species) quite as great a cholesterol depressant effect occurred. All the species showed very nearly the same cholesterol depressant activity whether the flesh containing the oil or the oil in the extracted form was fed to the rats. Feeding of the fat-free flesh, however, resulted in either no change or a small increase in serum cholesterol levels.

Somewhat analogous but less comprehensive experiments involving consumption of fish by man have been carried out by HARLOW and MORTON [1962] and by IMAICHI *et al.* [1963]. Although data from these

Table XI. Species of fish for which cholesterol depressant effect has been demonstrated

Species	Source of oil	Reference
cod	liver	HAUGE-NICOLAYSEN, 1959; HOWE and BOSSHARDT, 1962a, b; KAHN *et al.*, 1963a; KINGSBURY *et al.*, 1961
cuttlefish	liver	KANEDA and ALFIN-SLATER, 1963
dogfish	liver	PEIFER *et al.*, 1965; WOOD *et al.*, 1961
halibut	liver	WOOD *et al.*, 1961
menhaden	whole fish	AHRENS *et al.*, 1959; PEIFER *et al.*, 1960, 1962, 1965
mullet	body	PEIFER *et al.*, 1962
ocean perch	body	PEIFER *et al.*, 1962
pilchard	body	BRONTE-STEWART *et al.*, 1956
salmon (coho)	body	PEIFER *et al.*, 1962
sardine	body	MILLER *et al.*, 1962
seal	body	BRONTE-STEWART *et al.*, 1956
tuna	whole fish	PEIFER *et al.*, 1960
whale	body	MALMROS and WIGAND, 1957

Table XII. Cholesterol depressant effect of feeding to rats whole fish, oil-free whole fish, and the extracted oil. Data of PEIFER *et al.* [1962]

Fishery product in diet	Fish oil in diet %	Fish oil polyunsaturates in diet %	Change in serum lipids %
None (tallow control)	0	0	—
menhaden flesh	4.9	13	—51
silver salmon flesh	5.3	12	—62
mullet flesh	3.8	7	—49
ocean perch flesh	2.5	5	—61
menhaden extracted oil	4.9	13	—56
silver salmon extracted oil	5.3	12	—68
mullet extracted oil	3.8	7	—48
ocean perch extracted oil	2.5	5	—63
menhaden oil-free flesh	0.0	0	0
silver salmon oil-free flesh	0.0	0	+20
mullet oil-free flesh	0.0	0	+12
ocean perch oil-free flesh	0.0	0	+ 9

tests are very limited, the trend seems to be the same as in the animal experiments of PEIFER *et al.* [1962] just described.

c) Mechanism for cholesterol depression action

There has, in the past, been much confusion as to just what types of polyunsaturates possess the cholesterol depressant activity and how this relates to presence of saturated fatty acids. The picture is still far from clear. In much of the literature we see use of the terminology introduced by KEYS *et al.* [1957] in which the ratio of polyunsaturated fatty acids (P) to that of the saturated fatty acids (S), expressed as P/S is used as a measure of the effectiveness of an oil. Although this ratio is still used today it is not always applicable especially in the case of fish oils. Fish oils contain a high proportion of saturated fatty acids, the percentage sometimes exceeding 40 and frequently the proportion of saturated acids exceeds that of the polyunsaturates. This would give a P/S ratio of less than 1 which is very low as compared to vegetable oils where often more than half of all the fatty acids are linoleic resulting in P/S far above unity. If the P/S ratio were a valid measure of cholesterol depressant activity of oils, we would have to predict that fish oils were far inferior to all vegetable oils. Yet we have just seen that on the contrary, fish oils are superior to vegetable oils in this respect. The discrepancy, of course, lies in the fact that when we add up all the polyunsaturates we make no distinction between dienes with only two double bonds like linoleic acid common in vegetable oils and 4, 5 and 6 double-bonded fatty acids such as are so prevalent in fish oils.

It was postulated earlier that it is the classical EFA which possess the cholesterol depressant activity. That this can not be the case is obvious from the fact that fish oils possessing much less content of classical EFA than other polyunsaturated oils, nevertheless have the greatest cholesterol depressant activity. Moreover HAUGE and NICOLAYSEN [1959] showed that when young rats were placed on a special diet which both depleted their supply of EFA and raised their serum cholesterol level, either cod-liver oil or linolenate resulted in greater depression of serum cholesterol levels than did linoleate.

The degree of total unsaturation such as might be measured as iodine value has sometimes been considered to be an indication of cholesterol depressant activity of an oil. This measure is also quite unreliable. PEIFER *et al.* [1965] fed to rats a series of fractionated menhaden oil ethyl esters as well as corn oil, margarine, butter, and tallow.

Table XIII. Fatty acid compositions of tallow-test lipid mixtures. From PEIFER, LUNDBERG, ISHI and WARMANEN [1965]

Fatty acid	Tallow	Butter	Marga-rine	Corn oil	MO-1	MO-2	MO-3	MO-4	MO-5
8:0–15.0		5.4			4.1	5.2		1.9	
16:0	25.5	24.9	21.2	21.3	36.7	22.8	18.8	17.4	17.0
18:0	24.9	20.6	18.0	17.7	18.7	17.3	19.0	17.3	16.6
16:1	1.7	1.8	1.1	1.1	2.8	8.5	2.2	2.3	1.1
18:1	41.1	35.9	46.0	36.9	29.0	37.7	44.2	28.4	27.4
16:2–4					+			1.9	1.7
18:2	1.2	1.4	3.5	17.4	0.8	1.1	1.3	1.4	2.5
18:3	0.3	0.6	0.5	0.5	0.2	0.3	0.2	0.8	0.2
20:1							4.6		
20:3								1.1	
18:4+20:2								0.4	2.7
20:4								0.4	0.3
20:unsaturated								2.6	+
20:5					0.5	+	1.9	9.3	15.7
22:5								2.4	1.1
22:6					+	?	+	3.7	6.9
Total PUFA									
Dienes	1.0	1.8	3.4	15.5	0.8	—	0.8	2.1	0.7
Trienes	0.2	0.7	0.1	0.4	0.2	—	0.4	0.5	0.1
Tetraenes					0.9	—	1.1	4.5	6.2
Pentaenes					1.2	—	2.6	9.5	16.2
Hexaenes					0.4	—	1.8	5.6	9.7
% S+O[1]	98.5	97.8	95.6	81.1	98	98.3	95	73.1	66.7
% PUFA	1.5	2.2	4.4	18.9	2	1.7	5	26.9	33.3
Total FA[2]	94.7	90.6	90.3	94.9	92.8	92.9	92.2	91.3	93.2
Iodine number	41	44	80	124	49	93	127	267	353

[1] Percent 'S+O' is the g of saturated (S) and monounsaturated (O) acids present in 100 g of dietary lipids. Similarly, % PUFA is the amount of total polyunsaturated acids found in the same amount of the tallow-test lipid mixtures. These values are based on the results obtained by GLC.

[2] Total fatty acids found in the different diets. All calculations were based on a 95% fatty acid composition for triglycerides and 92% fatty acid composition for the ethyl esters.

Fatty acid content of the menhaden oil methyl esters and other test lipids is shown in table XIII. The most saturated fraction of the menhaden oil esters, MO-1, with an iodine value of only 49 gave equivalent cholesterol depressant effect as the corn oil with an iodine value of 124. The menhaden oil ester fraction MO-4 with iodine value of 266 gave a somewhat greater cholesterol depressant effect than did fraction MO-5 with an iodine value of 353. These results (table XIV) show

clearly that total unsaturation as measured by iodine value is not a measure of cholesterol depressant effect of an oil.

It might appear that several different polyunsaturated fatty acids were involved or even some monoenes or saturates with perhaps some of these acting as synergists to increase the activity of certain polyunsaturates [PEIFER, 1967].

Another possible explanation for the superior cholesterol depressant effect of fish oil fatty acids may be in the preponderance of ω-3 fatty acids present in these oils. If such fatty acids are somehow more effective in cholesterol transport than the more usual ω-6 types, such a fact could account for the greater effectiveness of fish oils without resorting to any reference to degree of unsaturation. Well over 90% of the polyunsaturates of fish oil are ordinarily of the ω-3 variety whereas in most edible vegetable oils the percentage is usually well under 5%. We have no specific data, however, which lends itself to testing of such a hypothesis.

Certainly much work employing purified fatty acids found in fish oil is needed before this point can be clarified. A bottleneck to carrying out such research is the fact that so many different fatty acids, several of them unavailable commercially, are present in fish oils. Until, however, such experiments are carried out, our picture of the mechanism of the cholesterol depressant activity of fish oils will remain obscure.

d) Fatty acid pattern change in tissues

When hypercholesterolemic animals are fed diets containing different types of fat, not only are there changes in serum cholesterol levels but also changes take place in the fatty acid patterns in the different tissues. Table XV shows the tissue fatty acid pattern in the heart tissue of hypercholesterolemic rats after 1 month on diets containing different fats [PEIFER *et al.*, 1965]. Serum cholesterol levels fell from the initial value of about 300 mg/100 ml to values in the range of 140–175 when fish oil or linolenate were fed and to 238 when linoleate was used. At the same time the hexaene and pentaene contents rose in all heart tissues. Initial hexaene content of heart tissue in rats having normal serum cholesterol level is, according to PEIFER *et al.* [1965], not higher than 0.3 mg/g of heart tissue. Even the hypercholesterolemic rats on tallow or linoleate had more than double this amount (0.7). Rats on the fish oil or linoleate diets had seven to seventeen times this much hexaenoic acid in the heart tissue. A similar pattern change took place for

the pentaenoic acids. Build-up of hexaenoic acid in the heart tissue increased from diet to diet as terminal serum cholesterol level decreased.

Since the hexaenoic acid content of the heart tissue showed a marked increase for rats on diets (tallow, linoleate) which contained very little of this polyunsaturate in the oil being fed (actually only trace

Table XIV. Relative effects of menhaden oil fractions[1] and of other oils and fats on plasma cholesterol levels of the rat

Treatments	I.V. of supplements	Total PUFA in dietary[2] fat mix %	Plasma lipids, mg/100 ml		
			TC	Pl	TC/Pl
Tallow	41	1.5	524	145	3.6
Butter	44	2.2	499	151	3.3
Margarine	80	4.4	401	142	2.8
Corn oil	124	18.9	297	123	2.4
Men. oil Fr.					
MO-1	49	2.0	255	126	2.0
MO-2	93	1.7	206	113	1.8
MO-3	127	5.0	142	125	1.2
MO-4	266	26.9	112	77	1.5
MO-5	353	33.3	160	89	1.8

[1] PEIFER *et al.* [1965].
[2] The diets contained 10% fat, 1/3 of which was the indicated test lipids and 2/3 was tallow. The test lipids had widely different iodine values (I.V.) and the test lipid-tallow mixtures contained polyunsaturated fatty acids (PUFA) ranging from 1.5–33.3% of the total fat calories.
I.V. = Iodine value TC = Total cholesterol Pl = Phospholipids

Table XV. Change in heart tissue fatty acids resulting from feeding hypercholesterolemic[1] rats different kinds of fat for 1 month. Data of PEIFER *et al.* [1965]

Oil fed	Fatty acid-content in mg/g of tissue					Final serum cholesterol level mg/100 ml
	Hexaenoic acids	Pentaenoic acids	Tetraenes	Trienes	Dienes	
Tuna oil	4.5	1.0	2.5	0	0.7	140
Menhaden oil	5.1	1.8	3.5	0	0.9	169
Linolenate	2.1	1.8	2.2	0.6	0.6	175
Linoleate	0.7	0.8	5.0	0	3.3	238
Tallow	0.7	0.3	2.1	0.7	1.3	344

[1] Initial serum cholesterol value about 300 mg/100 ml.

amounts too small for ordinary GLC analysis to detect, see table XIII), it would appear that there is a need for such polyunsaturates in hypercholesterolemic animals and that the body mobilizes them from wherever they are available. With diets containing fatty acids of the linolenic acid family (as with fish oils) the highly polyunsaturated fatty acids (C22:6, C20:5) are already present or can be readily metabolized from linolenic acid itself.

These results might suggest that serum cholesterol depression, while easily possible with dietary fats containing predominately dienes and tetraenes of the ω-6 family is more efficient with a wider combination of fatty acids including ω-3 types such as occurs in fish oils. Much more research is needed to further elucidate what takes place in such cases.

6. ω-3 fatty acids

A paper has recently appeared [Bernsohn and Stephanides, 1967] linking ω-3 fatty acids and more specifically docosahexaenoic acid with multiple sclerosis. These authors show that in areas of the world where ω-3 fatty acids occur in some quantity in the diet such as where much fish is eaten or, where certain seed products containing linolenic acid are eaten, multiple sclerosis is almost unknown. The disease is most common in areas where very little fatty acids of the ω-3 type are found in the diet. Furthermore Kishimoto *et al.* [1967] have found that the human brain which normally contains considerable 22:6 ω-3 fatty acids, for subjects who have had multiple sclerosis contains less than half the normal amount of this fatty acid. In their experiment, they found that the 22:6 ω-3 fatty acid content in the average normal appearing white matter of the brains of several multiple sclerosis victims averaged 1.5% of total lipids as compared to an average value from control (non-multiple sclerosis patients) of 3.5%.

Bernsohn and Stephanides [1967] have developed the theory that lack of docosahexaenoic acid or its precursor, linolenic acid in the diet is one factor in development of multiple sclerosis. They recognize, of course, that even in areas of high incidence of multiple sclerosis and of low intake of ω-3 fatty acids, most individuals do not contract multiple sclerosis. They hypothesize, therefore, that in those individuals in such geographic areas who do develop the disease, in addition to the lack of a safe quantitiy of ω-3 fatty acids in the diet, some other factors such as faulty enzyme system for conversion of linolenic acid to docosahexaenoic acid are present. In the presence of

adequate dietary ω-3 fatty acids, these other factors would not be sufficient to allow the disease to develop. This theory is receiving attention in laboratories of BERNSOHN and STEPHANIDES as well as elsewhere. Still to be determined is whether ω-3 fatty acid supplementation is effective only at a very early age while the brain is developing or to what extent it is important with older indivuals. Also evidence is being sought as to whether supplementation of diets of multiple sclerosis patients with ω-3 fatty acids can reverse the trend of the disease.

Needless to say, should these findings be positive, the importance of fish in the diet as a source of ω-3 fatty acids and indeed of its high content of the effective component, docosahexaenoic acid, would be much greater than presently recognized. Fish oil fatty acids are usually about 30–40% of the ω-3 type whereas vegetable oils contain usually under 2%. Furthermore the ω-3 fatty acids of animal and vegetable fats and oils are only precursors for the apparently desired docosahexaenoic fatty acid and none of this acid is present in edible oils or fats other than fish oils where the content approximates 10% of the total fish oils. Thus consumption of only a very small quantity of fish could provide docosahexaenoic acid in substantial quantity without need for its perhaps less efficient metabolism from other ω-3 precursors. Thus the old adage 'fish is a brain food' may be not a mere superstition but rather a recognition of a relationship between docosahexaenoic acid and its requirement in the brain.

7. Discussion and summary

Fish oil fatty acids, as we have seen, possess nutritional properties in common with fatty acids from other sources both in providing metabolizable energy and as growth stimulants. Although fish oil polyunsaturated fatty acids in common with any of the ω-3 type lack certain EFA properties as exemplified by inability for complete curing of dermal symptoms, there is some indication that these same ω-3 fatty acids may, of themselves, possess other functions which are lacking in the ω-6 series.

The antagonistic effect of one fatty acid family upon another has been mentioned. The effect of the ω-3 and ω-6 families upon each other may be of concern when fish oils, high in ω-3 fatty acids are used in animal feed or for human food together with other food which contains predominantly members of the ω-6 fatty acid family. This effect of depressing the conversion of fatty acids of one family is

dependent upon the presence of large excesses of members of the other family. In almost every case of use of fish in either animal feed or for human consumption there will be a large excess of ω-6 fatty acids from other components of the diet. Insofar as animal feed is concerned, the amount of fish used is nearly always greatly limited by the need to prevent imparting of a fishy flavor to the meat or eggs so that oil or fat from non-fish source with ω-6 type of fatty acids predominates. Likewise in any human diet, at least in Western countries, ω-6 type fat or oils always greatly exceeds in quantity that from fish sources. This means that under normal conditions there will be no critical suppression of conversion of linoleic to arachidonic acid by inclusion in the diet of fish containing ω-3 type fatty acids. What will happen is that the excess of ω-6 fatty type acids in the diet will decrease the conversion of what little linolenic acid is present to docosahexaenoic acid and other related ω-3 fatty acids so that there will be less of these formed than would have been if fish oils or linolenic acid were the sole type of oil or fat present. It is here, of course, that the beneficial effect of fish oils show up. If indeed the higher members of the ω-3 fatty acid family such as docosahexaneoic acid are needed for some important function, they already occur preformed in fish oils and it is unnecessary for them to be metabolized from linolenic acid. Thus inclusion of fish or fish oils in a diet insures the presence of the higher members of the ω-3 family for whatever benefit they may impart.

Some preliminary evidence indicating nutritional effects of fish oils beyond those found in other oils or fats has been presented. These deal with properties concerned with superior cholesterol depressant action and beneficial effects for maintaining normal levels of docosahexaenoic acid in the brain and other tissues with a possible relationship to multiple sclerosis or other conditions. These effects need a great deal more documentation and elucidation before they can be accepted as being firmly established.

Certain potentially harmful effects of fish oils based upon their high content of polyunsaturates have been considered. It appears that under normal conditions there is no possibility that accumulation of oxidation products of harmful effect can be a cause for concern in either human or animal food. The *in vivo* effect of the presence of unoxidized polyunsaturates in a diet while of no concern at the level fish is included in our diet, may be potentially hazardous for captive animals if fish oils predominate in the diet. This effect, however, is readily avoided by adequate tocopherol supplementation.

Fish thus has a place in the diet of animals and man. Future research may further substantiate the role of fish (at present only partially documented) as a source of ω-3 fatty acids which may well play a more important nutritional role than previously suspected.

C. Miscellaneous Classes of Lipids

1. Introduction

Nearly all of the well documented evidence concerning nutritive value and related pharmaceutical activity of fish oils (aside from vitamin properties only being incidentally mentioned in this review) deal with fatty acid components. Nevertheless a considerable body of literature exists in which claims are made as to beneficial effects of fish oils or their components and which are believed by their proponents to be due either to specific classes of components of fish oils other than fatty acids or to unidentified components. In this section, this type of material will be covered. Since in most instances there is uncertainty as to precisely which class of compound may be responsible for a given action, to facilitate discussion the treatment will be made according to observed effects rather than by classes of compounds. Two principal types of activity have been extensively reported. One of these, dealing with many healing effects ranging from curing of wounds and burns to tuberculosis alleviating action has a very extensive literature. A second quite different and more recent field covers work on reticuloendothelial stimulation with application to phagocytosis.

2. Healing effects

a) Wound and burns

For many years fish oils have been reputed to effect rapid healing of burns and wounds. It is, of course, unquestionable that the pain from a fresh burn can be immediately and effectively alleviated by keeping air away and that this can be readily achieved by applying a solid fat such as petrolatum or butter or a liquid oil such as mineral oil or a fish oil. The question which, however, has perhaps never been resolved is whether use of any of these products actually promotes better healing.

The early literature contains many papers indicating a healing property of vitamin A for wounds [LOLTAN, 1935; SANDOR, 1936; PROTO, 1936] when applied topically to the wound or when taken orally [LAUBER, 1936a, b]. Oddly enough those investigators who reported

effective healing by topical application usually found no effect for dietary supplementation and vice versa. Other investigators in these early periods could find no effect of vitamin A on wound healing or they even found an inhibiting effect [LAMBERT and ROCHOLL, 1935]. Along with investigations on vitamin A promotion of wound healing are, of course, several papers in which fish liver oils was the source of the vitamin A [DRIGALSKI, 1934; LESSER, 1950]. Other workers who believed fish liver oils promoted wound healing believed that this result was achieved because of a bacteriostatic action of other components of the oil such as oxidation products like peroxides and aldehydes [SPANHOFF and JACOBSEN, 1950] or even certain fatty acids [SOLOMIDES, 1949].

The occurrence in many fish liver and body oils of alkoxydiglycerides which yield upon hydrolysis such glyceryl ethers as chimyl, selachyl, and batyl alcohols has more recently been suggested as the component of fish oils primarily responsible for wound healing [BODMAN and MAISIN, 1958]. These workers found that such compounds were greatly effective in promoting the healing of deep-seated lesions caused by irradiation therapy. They developed a theory based upon the fact that the glycerol ethers and plasmalogens are found in bovine and human tissues in foetus or in tissues the first few days after birth [HARTREE, 1964] in much higher concentration than at later stages. They predicate that in prenatal cell development, glyceryl ethers play a role in cell development before triglycerides are present and that gradually the role of the glycerol ethers is taken over by triglycerides. From these considerations they explain their findings that deep-seated wounds are more rapidly healed by topical application of the glyceryl ethers which would facilitate regeneration of new cells. BODMAN and MAISIN [1958] found no healing effect of glyceryl ethers on burns; in fact, if anything, topical application resulted in a slight impeding of curing of burns.

Fish oils are currently added to several commercial ointments for the purpose of increasing healing of wounds, burns and skin irritation. Because of the conflicting evidence on the role of fish oils used topically to promote healing, the US Bureau of Commercial Fisheries and the Dermatology Department of Mayo Clinic carried out a series of experiments in which various fish oil preparations were applied to hairless mice which had been given wounds or burns under controlled conditions [STANSBY, ZOLLMAN and WINKELMANN, 1967]. Treatments used consisted of topical application of (1) a high vitamin A fish liver

oil, (2) a typical fish body oil with negligible vitamin A content, (3) a fish body oil to which glyceryl ethers had been added, (4) a commercially available ointment containing cod-liver oil, (5) a laboratory prepared cod-liver oil ointment and (6) a mineral oil control. The length of time required to effect curing of the wound or burn was measured for each treatment. No significant difference was observed in the healing time for any treatment of either the wounds or burns except that in most cases the use of any oil including mineral oil gave small improvement in healing time over use of no treatment at all. It was observed that during the healing process, the fish oil treated samples in many cases seemed to result in a cleaner lesion which gave the impression of a more rapid healing. The elapsed time of healing (point where scabs fell off of wound or burn) was not, however, any shorter than that for the control animals.

b) Irradiation damage

Workers at the Radiumhemmet, Karolinska Institute, Stockholm, have carried out extensive work on the use of glyceryl ethers taken orally in connection with irradiation treatment. This work is described in considerable depth by BROHULT [1963]. In early research the workers in this group found that certain bone marrow fractions (which contain alkoxydiglycerides) when given orally to children suffering from leukemia resulted in an increase in white cell and thrombocyte count. The unsaponifiable fat portion of the bone marrow preparation gave the best results, leading to the belief that glyceryl ethers might be the principal active components. In subsequent work alkoxydiglyceride concentrates, prepared from fish liver oils (dogfish and Greenland shark) were employed. When these were fed to patients suffering from irradiation leucopenia, the same protection against drastic drop in white cell and thrombocyte count occurred as those with leukemia patients treated similarly. This type of effects has not only been observed over years of research by this group, but also has been confirmed by workers at other laboratories [LINMAN, 1958; SUKI and GROLLMAN, 1960]. Tests have been run using both glyceryl ether concentrates prepared from fish liver oils and with synthetic batyl alcohol.

Contrary to these results are those of CARLSON [1966] in whose work little or no significant hematopoietic effect could be demonstrated when chimyl, selachyl, or batyl alcohols were fed to dogs or rats at dose levels of 6 mg/kg of body weight and a slight, marginally

significant such effect was obtained when doses were used of up to 2400 mg/kg of body weight. Similar negative results with rats have been reported by SNYDER [in press].

3. Effects with miscellaneous diseases

A disease of cattle, bracken poisoning, is said to be alleviated by feeding batyl alcohol [EVANS, EVANS, EDWARDS and THOMAS, 1957]. Since this condition caused by the cattle having consumed a type of fern results in damage to bone marrow similar to that encountered with irradiation, there would appear to be a relationship between the action of glyceryl ethers in these two conditions. On the other hand DALTON [1964] was unable to reproduce such results for bracken poisoning treatment with glyceryl ethers.

Fish oils, especially cod liver oil, have been reported to have favorable properties in connection with curing of tuberculosis [GETZ, 1955]. He has indicated [GETZ, 1960] that faulty vitamin A metabolism in tubercular patients could be offset by natural vitamin A concentrates which, he believes, also contain fragments of the vitamin A molecule of an unidentified nature more readily utilized by such patients. Along quite another vein, EMMERIE, ENGEL and KLIP [1952] report a fraction isolated from cod liver oil (containing batyl alcohol) which inhibits the growth of tubercle bacilli.

CALCAGNA [1935] reports on the use by natives of South America of certain fish oils in the treatment of leprosy.

CHALMERS, WOOD and SHAW [1966] describe a means of treatment of inflammatory diseases such as rheumatoid arthritis or gouty arthritis with fish liver oil concentrates of glyceryl ethers. Their supporting evidence is based upon rat feeding tests with the anti-inflammatory action measured by the granuloma pouch test of ROBERTS and NEZAMIS [1957].

4. Cosmetics

Fish oils and derivatives obtained from them are sometimes used in cosmetics. The most commonly used substance of this kind is squalane. It is the hydrogenated derivative of squalene, the unsaturated hydrocarbon which occurs not only in abundance in the liver oils of several shark but is also an important constituent of surface lipids of the skin [ROSENTHALL, 1964]. Originally the squalene as it occurs in the shark liver oil was used directly in the cosmetic cream but it was found that due to instability against oxidation, the unsaturated squalene soon

developed undesirable oxidative products. This lead to the present practice of use of the hydrogenated product, squalane.

Fish oils or fish oil fatty acids have sometimes been used directly in cosmetics with the belief that some of the unsaturated fatty acids or in other instances the content of vitamin A were of value [GOLDRICK, 1966]. In recent years there has been a vogue for incorporation of turtle oil in cosmetic creams.

5. Reticuloendothelial stimulation

Effects of a different kind which have been reported to be influenced by fish oil components relate to the enhancement of the reticuloendothelial action which results in an increase in the phagocytic effect. HELLER, PASTERNAK, RANSOM and HELLER [1963] reported the isolation of a lipid fraction from the liver oil of certain sharks which acts as a stimulating agent for reticuloendothelial systems. The lipid fraction, which has not been identified, is extracted with ethanol, ether, and hexane and is separated and concentrated by various solvent and chromatographic procedures. The activity of this lipid concentrate was evaluated by measurement of stimulation of phagocytosis by the carbon clearance technique. An increase of 50% survival of mice treated with this fraction over controls was obtained when mice were injected with *Salmonella typhimurium*. RINGLE, HERNDON and BULLIS [1966] have also tested this same lipid fraction from shark for its resistance to shock but with negative results.

D. Discussion and Summary

The role of fish lipids other than fatty acids is even less clear than is the case with polyunsaturated fatty acids. Such effects as that of a reticuloendothelial stimulant by some unknown component of shark liver is still very obscure and will require a great deal more evidence both in replicating the effect itself and in learning what classes of compounds are involved and how they operate.

The case for desirable effects in using alkoxydiglycerides or their hydrolysis products, glyceryl ethers, is somewhat better established although still not universally accepted. On the one hand, the fact that several quite distinct groups of workers sometimes working independently and without knowledge of what other workers had found came up with the same type of action of glyceryl ethers certainly must carry some weight.

On the other hand much of the earlier work was based upon inadequate number of animals or patients used in the research or use of inadequate controls. As more thorough clinical or animal work involving more adequate numbers of tests and controls are used these effects are being indicated to be less important and in some cases are being completely disproved. A great deal more research will have to be carried out before we can be certain which of the various effects which have been described have a genuine basis in fact and warrant a practical application of fish oils for various purposes.

V. General Summary and Conclusions

In this review, the chemical and nutritional properties of fish oils have been considered in some detail with some background information included on sources of fish oils.

The highly diverse fish oil fatty acids varying from fully saturated ones making up about one-third of the total to polyunsaturates ranging in amount upward from one-quarter to nearly one-half have been discussed. The unique characteristics of the fish oil fatty acids including presence sometimes of substantial quantities having odd carbon chain length, the presence of both very long carbon chain—up to C24—and of very highly polyunsaturated acids with up to six double bonds/molecule as well as the predominance of ω-3 rather than the usual ω-6 family of fatty acids have been mentioned.

Brief discussion has been given of such topics as the origin of fatty acids in fish either via food chain in the sea or during metabolism in the fish; occurrence of fish oils in the flesh *versus* in certain organs, in cells *versus* in depot fats and in light *versus* dark muscle; differences in amounts and type of oil in different species of fish; commercial production and uses of principal industrial fish oils especially from the South American anchovy, American menhaden and various herring industries.

The nutritional properties of fish oil fatty acids have been examined in some detail. Special attention has been paid to the unique predominance of the ω-3 family of fatty acids in fish oils. Relationships between the different families of fatty acids in metabolism have been considered. It has been indicated that incorporation of moderate amounts of fish oils in diets does not upset the important conversion of linoleic to arachidonic acid yet insures by reason of the presence of preformed

docosahexaenoic and other ω-3 family fatty acids that these will be available when and if needed.

The use of fish oils in feeds for different animals has been described as well as for special pharmaceutical uses such as for cholesterol depressant action. The superior activity of fish oil for this purpose as compared to the use of various vegetable oil polyunsaturates has been pointed out.

Finally a number of effects of non-fatty acid components of fish oils which have been claimed to be important have been discussed. These include use of such components as glyceryl ethers and hydrocarbons for a variety of purposes. It has been pointed out that these purported effects—wound healing, radiation damage alleviation, reticuloendothelial stimulation, etc.—need a great deal more research before they can be firmly established and generally accepted.

VI. Summary

Fish oil fatty acids differ from those in other fats and oils especially with regard to being more polyunsaturated, being largely members of the ω-3 rather than the more usual ω-6 family. These properties result in some nutritional effects which differ at least in extent from the effects of fatty acids from other sources. Evidence is beginning to accumulate to indicate that docosahexaenoic acid, the end metabolite of the ω-3 family has desirable nutritional effects not previously recognized.

Glyceryl ethers and some other non-fatty acid components of fish oils are reported to have special nutritional properties, but much more evidence is needed before these claims can be fully substantiated.

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Author's address: Mr. M.E. STANSBY, Food Science Pioneer Research Laboratory, Bureau of Commercial Fisheries, *Seattle*, Wash. 98 102 (USA).

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DDT RESIDUE LEVELS IN SOME U.S. FISHERY PRODUCTS AND THE EFFECTIVENESS OF SOME TREATMENTS IN REDUCING THEM

by

V.F. STOUT, F.L. BEEZHOLD and C.R. HOULE
U.S. Department of Commerce
National Oceanic and Atmospheric Administration
National Marine Fisheries Service
Technological Laboratory
Seattle, Washington 98102, U.S.A.

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1 INTRODUCTION

Because of the extensive use of DDT since World War II, the ease with which it disperses, and the stability of the chemical, it has become a widespread pollutant of our environment. For this reason, the National Marine Fisheries Service[1/], which is concerned with the wholesomeness of fishery products, has been determining the concentration of total DDT (that is, of DDT and its metabolites, TDE and DDE) in food fish and shellfish, in fish protein concentrate, in the products from industrial fish, and in edible oils. Methods for reducing the DDT content have been examined.

2 FOOD FISH AND SHELLFISH

The aim of our work on food fish and shellfish was to determine the residue levels in the edible portion, and in those species with high residue levels to attempt to reduce the levels to meet the U.S. Food and Drug Administration interim tolerance for edible flesh, 5 ppm on a wet-weight basis. Both marine and freshwater species were studied.

2.1 Marine species

2.1.1 Monitoring

Several species (Table I) of marine fish and shellfish collected from the open waters of the Pacific Ocean and from the coastal waters of the Northeast Pacific Ocean were examined. Although all species shown are edible, several are used primarily for industrial or bait purposes or are not fished commercially. The findings are conveniently considered in relation to the geographic areas of Washington and Oregon (Stout, 1968, for some of the data) and of southern California.

None of the samples from the Pacific Ocean or Washington and Oregon contained more than 0.5 ppm total DDT. These residues of less than 10% of the current FDA limit for fish constitute some of the lowest levels found for fish in the United States. DDT usage depends on the intensity of farming, the weather, and the density of the population. Washington and Oregon have a short growing season, a winter cold enough to kill many pests, and a relatively low population density. Thus, relatively little DDT has been used in these areas, so the marine life contains little DDT.

In southern California, farming is intensive and the growing season is long. There are as many as three crops a year. Also, the density of population is high, and the winter mild. It is not surprising, therefore, that some of the fish from this area contain excessive residues of total DDT. In our study of species from this area we gave particular attention to jack mackerel, inasmuch as it has been involved in FDA action.

During the fall of 1969, the FDA seized jack mackerel from southern California because some samples contained more than 5 ppm total DDT. Since that time, high levels of residues have been found in the livers and edible portion of other species of fish taken from Santa Monica Bay, an area not fished by commercial fishermen. The livers from one sample, for example, contained 1,026 ppm total DDT; fillets of the same fish contained 57 ppm. Although the relation between residues in the livers and flesh was not constant, the concentration of residues in the liver was always greater than that in the flesh. In fish with low levels of DDT, such as those from the coastal waters of Washington, the ratio of DDT in the liver to that in the flesh was greater than 100. When the concentration of DDT was high, the ratio decreased in an irregular, unpredictable way to as low as 5. Generally, it was in the range of 20 to 50.

1/ Formerly the Bureau of Commercial Fisheries

Table I. Species of marine fish and shellfish collected both on the high seas and off the coast of the Northeast Pacific and examined for the presence of DDT and its metabolites

Common name	Scientific name	Location[c]
Dungeness crab	*Cancer magister*	NW
Pink shrimp	*Pandalus jordani*	NW
Pacific herring	*Clupea harengus pallasi*	NW[d]
Northern anchovy	*Engraulis mordax*	NW[d]
Coho salmon	*Oncorhynchus kisutch*	NW[ab]
Sockeye salmon	*Oncorhynchus nerka*	High seas[ab]
Chinook salmon	*Oncorhynchus tshawytscha*	NW[ab]
Pacific cod	*Gadus macrocephalus*	NW
Pacific hake	*Merluccius productus*	NW[d]
Jack mackerel	*Trachurus symmetricus*	Cal
White croaker	*Genyonemus lineatus*	Cal[b]
Albacore	*Thunnus alalunga*	NW[ab]
California scorpionfish	*Scorpaena guttata*	Cal[bd]
Pacific ocean perch	*Sebastodes alutus*	NW
Starry rockfish	*Sebastodes constellatus*	Cal[b]
Vermillion rockfish	*Sebastodes miniatus*	Cal[b]
Blue rockfish	*Sebastodes mystinus*	Cal[b]
Bocaccio	*Sebastodes paucispinis*	Cal[b]
Rosy rockfish	*Sebastodes rosaceus*	Cal[b]
Treefish	*Sebastodes serriceps*	Cal[bd]
Yellowtail rockfish	*Sebastodes flavidus*	NW
Sablefish	*Anoplopoma fimbria*	NW[ab],Cal[b]
Rex sole	*Glyptocephalus zachirus*	NW
Dover sole	*Microstomus pacificus*	NW,Cal[b]
English sole	*Parophrys vetulus*	NW,Cal[b]
Starry flounder	*Platichthys stellatus*	NW
Sand sole	*Psettichthys melanostictus*	NW

[a] Only the livers of these fishes were analyzed.
[b] Office of the Secretary, U.S. Department of Interior, Laboratory, Sabine Island, Gulf Breeze, Florida analyzed the livers. For the relation between residues in the livers and the edible flesh, see the text.
[c] NW is Pacific northwest area of the United States--i.e., Washington, Oregon, and occasionally British Columbia, Canada; Cal is California, High seas is lat. 45-55° N, long. 155-177° W, that is, south of the Aleutian Is.
[d] Industrial or bait species or not fished commercially.

2.1.2 Attempts to lower residue levels

Several experiments were done with jack mackerel to determine if simple precanning process alterations would reduce significantly the level of DDT in the flesh. Relevant to this work were the findings of Ritchey (1969, and references therein) who studied the effects of cooking methods on DDT residues in chickens fed diets containing DDT. He and his coworkers found that the reduction in the residue levels paralleled somewhat the loss of fat by rendering. Frying and pressure cooking were most successful; baking effected very little change even though the fat content was reduced. The greatest reduction of total residues was 38% on a dry-weight basis or 17% on a wet-weight basis, which is the basis used by the FDA. In most cases of high residues, a greater reduction would be needed to provide a practical solution. For example, flesh found to contain 10 ppm (wet) might after cooking contain only 8 ppm (wet), but it

would still be unacceptable according to the current tolerance. Fish presumably would act in a similar way to chicken. Our prospects for success, in reducing DDT level in fish, therefore, were few.

Nonetheless, we studied the effects of cooking time and temperature on residues of DDT in the flesh of jack mackerel. After being placed in the can these fish are commonly cooked for about 15 min at about 96° C. Following this, salt is added, the cans are sealed and given a sterilizing cook. We altered precook time from 15 to 60 min and temperature of precook from 85° to 100° C. No reduction in residues in the drained fish was observed. In fact, as the cooking time was increased, the level of DDT on a wet-weight basis increased, because of the reduction in the moisture content of the fish. Similarly, soaking the jack mackerel in water overnight at 3° C and precooking them in upright cans or in inverted cans to permit drainage during heating did not reduce the residues, but instead increased them slightly even after we corrected for the loss of water. Heat may possibly free DDT that is bound to protein in the raw fish (the control) and that is not extractable in the analytical procedure.

Three experiments were carried out. In each experiment fish were divided into lots of equal numbers of fish. These lots were 19 to 52 fish taken at random from large landings. A later study in which 25 fish from a large landing were analyzed individually showed the tremendous variation in DDT, oil, moisture, and size among individuals from a supposedly homogeneous lot. The concentrations of total DDT in the flesh ranged from 2.1 to 17.4 ppm; oil content ranged from 1.1 to 6.3%; and moisture content from 71.3 to 76.7%. The weight ranged from 123.8 to 386.7 g.

Because of this wide variation, ground, well mixed jack mackerel were used in subsequent work. No reduction of DDT residues resulted from precooking the ground flesh in water or 1.5% NaCl brine.

These experiments show that no simple means exist for decreasing the residues of DDT in jack mackerel and still producing the normal canned product. As has been found with chicken, DDT is too closely associated with muscle components to be removed by conventional processing procedures.

The level of DDT in jack mackerel dropped early in 1970, so fishing was started again, but the threat of excessively high levels still continues. A cyclic rise and fall related to the season of the year or to the age of the fish may be suggested. In the fall, fish could be accumulating residues applied throughout the agricultural growing season. We found that those fall-caught fish having high residues were also large and therefore older; a relation between age and residue content would not be surprising. In support of this, we found little residue in a sample of small fish caught in December. Large fish caught one month later contained residues in excess of 5.0 ppm. Jack mackerel caught in late winter and early spring contained low levels of DDT. In June, the fleet was still catching small fish containing low levels of DDT.

Interpreting these findings on a seasonal basis alone is difficult. During the winter season, for example, the agricultural application of DDT is low but heavy winter rain would cause major runoffs into marine waters.

The data for the 25 fish analyzed individually showed some correlation between size and residue levels. That only the largest fish in an admittedly small sample had high residue levels suggests that older or faster growing fish accumulate more DDT. Additional work is needed to relate season and age of fish to DDT residue content.

2.2 Freshwater species

In the Spring of 1969 the occurrence of excessive residues of DDT in freshwater fish was brought to the attention of the general public by the seizure of Lake Michigan coho salmon containing up to 20 ppm DDT and up to 0.32 ppm dieldrin: the maximum permissible content of dieldrin in the edible portion is 0.30 ppm.

At about the same time excessive levels of DDT residues were being reported in chub (Coregonus hoyi).

The seriousness of the DDT residue problem in freshwater fish was shown by the data of the National Pesticide Monitoring Program of the U.S. Bureau of Sport Fisheries and Wildlife (Henderson, 1969). In 12 of 44 lakes and rivers sampled, some or all of the fish contained more than 5 ppm DDT, and in 8 of these locations, the mean for all fish examined exceeded the tolerance level.

Work has been in progress at the National Marine Fisheries Service Laboratory at Ann Arbor, Michigan, to study methods of reducing the residues in local fish. A partial solution has been found for salmon (Reinert, 1969). Analyses of various portions showed (Table II) that DDT is concentrated in areas of high fat content. Removal of the skin and the fatty deposits in the dorsal median, lateral line, and belly flap areas of fish with high residues would therefore reduce the contamination substantially.

Table II. Distribution of DDT in Lake Michigan coho salmon[a]

Sample	Total DDE + DDT[b] (ppm)
Whole fish	12.2
Whole eviscerated steak	14.9
Flesh (muscle)	5.7
Dorsal median fat	62.8
Laterial line fat	34.3
Abdominal adipose	92.3
Belly fat	69.7

[a] Reinert, 1969
[b] TDE is not found in significant amounts in Great Lakes fish.

The situation with other fish is less readily improved. Trimming is practical only in a product of high value such as salmon. Furthermore, in many fatty fish such as chub, residues are so evenly distributed that trimming would be ineffective. Cooking chub, for 15 min at 96° C in open cans filled with boiling water or 1.5% NaCl brine or with no added liquid caused little reduction in the level of residues. The drained liquid portion of the product contained little oil. Only if substantial rendering of fat occurred during processing would any removal of pesticides be anticipated. Extended high temperature cooking might well be successful in reducing the residues, but the product would probably be unpalatable.

3 FISH PROTEIN CONCENTRATE

Studies (Stout, MS) on the development of methods for production of fish protein concentrate (FPC) required information about the fate of the pesticides during processing. The lipophilicity of organochlorine pesticides suggested that DDT would be concentrated into the byproduct oil. Table III indicates the validity of this inference by showing that FPC made from Pacific herring both by the isopropyl alcohol extraction method (Bureau of Commercial Fisheries Technological Laboratory, College Park, Maryland, 1966) and by the aqueous fractionation method (Spinelli, 1968) contained a barely detectable amount of DDT. The largest concentration of pesticide occurred in the oil portion. Phospholipids are potent solubilizing agents for organic molecules, which may explain the concentration of the pesticides in Miscella 1 of the isopropyl alcohol extraction method. Fortunately, because of the fractionation of the DDT into Miscella 1, the edible-oil fraction contains a very low level of DDT, especially after being washed with water, which tends to remove any of the suspended phospholipids and associated compounds.

Table III. Fate of pesticide residues during FPC production

Sample	Total DDT (ppm) Isopropyl alcohol extraction	Total DDT (ppm) Aqueous fractionation
Whole raw Pacific herring	0.097	0.097
Separated raw herring[a]	0.084	0.084
FPC	<0.002	0.006
Oil		1.12
Miscella 1[b]	1.75	
Miscellas 2 + 3[c]	0.33	
Miscellas 2 + 3, washed with H_2O	0.28	

[a] Skin and bones removed.
[b] Mainly phospholipids, not usually considered edible.
[c] Triglycerides, potentially for food use.

4 INDUSTRIAL FISHERY PRODUCTS

Fish meal and it accompanying oil are used in poultry feeds. The residues in meal contribute to the pesticide burden imposed on poultry and contribute to the load in foods derived from the animals.

Residue levels in menhaden (Brevoortia tyrannus and B. patronus) meal and oil are shown in Table IV. These data were obtained in 1969. As we found with FPC, the meal contains less than one-tenth as much DDT as does the oil. Because meal contains up to about 15% oil, processes that reduce the oil content would also reduce the pesticide content. The Food and Drug Administration tolerance of 5 ppm for DDT residues in the edible portion of fish flesh does not apply to fish meal or oil.

Table IV. Range of pesticide residue levels in menhaden oil and meal in 1969

States	No. of samples	Total DDT (ppm) Oil	Total DDT (ppm) Meal
Middle Atlantic	6	4.4-9.4	0.29-0.61
Chesapeake	2	2.8-4.0	0.21-0.25
South Atlantic	12	2.1-7.2	0.10-0.32
Gulf of Mexico	2	2.2-2.3	0.10-0.11

The concentration of pesticides in oil suggests that removal of DDT from fish oil may become necessary. Deodorization of oil in the refining process of edible vegetable oil (Saha, 1970; Smith, 1968) removes almost all of the chlorinated hydrocarbon pesticides originally present. Unfortunately deodorization is too expensive for animal feed products so we tried to find simple methods to reduce the residue content of fish oil. Washing with cold or hot water, steam distillation at atmospheric pressure, or partitioning with 55 and 90% (v/v) aqueous isopropyl alcohol, however, effected no change in the residue level.

5 PESTICIDES IN EDIBLE OILS

Most of the work on the elimination of chlorinated pesticides has been done with vegetable oils. Fish oils, which are of primary interest in this paper, differ markedly in chemical makeup from vegetable oils, so the problems encountered with fish oil are different. Accordingly, in this section, we first consider the pertinent work that has been done with vegetable oils and then the work we have done with fish oils.

5.1 Elimination from vegetable oils

Recent studies by Smith et al. (1968) on soybean and cottonseed oils and by Saha, Nielsen, and Sumner (1970) on rapeseed oil have demonstrated that vacuum steam deodorization at 6 mm Hg pressure and at temperatures in the range of 230-260° C will remove as much as 95% of the chlorinated pesticide content. Other commercial processing techniques such as solvent extraction, steam refining, alkali refining, bleaching, and hydrogenation were reported to have little effect in eliminating chlorinated pesticides in these vegetable oils. The mechanism of the removal of chlorinated pesticides was indicated to be a forced volatilization of the pesticides in the presence of steam at the high temperatures used in the deodorization process.

5.1.1 High temperature requirements

The minimum temperature (i.e., 230° C) deemed essential for the removal of chlorinated pesticides from vegetable oils by vacuum steam deodorization coincides with the maximum temperature used in the United States for deodorization of fish oils. In the United States, fish oils are deodorized at 190-230° C, whereas in Europe they are deodorized at 170-190° C (Chang, 1967). Fish oil is not presently used in food products in the United States, but it is used in margarine and other edible fat products in Europe. The lower deodorization temperature limits in Europe are probably much more desirable for critical applications, as in foods. Owing to the more unsaturated nature of fish oils in comparison with vegetable oils, high temperature processing of the former leads to a greater degree of thermal degradation and polymerization.

5.1.2 Steam distillation

Vacuum steam distillation is used in the processing of fats and oils for two basically different purposes. One purpose is to remove off flavors and odors after an oil has been hydrogenated. The second--and less common purpose--is to remove free fatty acids. In this application, it is called "steam refining" and is used on degummed oils. In the present work, we have used both applications in our attempts to gain insight into the best ways of removing chlorinated pesticide residues from fish oils.

5.2 Removal from fish oils

5.2.1 Vacuum steam refining

Table V presents data on the effectiveness of vacuum steam refining of two crude bleached Pacific herring oils containing closely similar concentrations of chlorinated pesticides. The two oils were subjected to the same conditions during the steam refining except for the temperature of distillation. From the data, the effectiveness of the removal of the chlorinated pesticides does not appear to depend on the temperature in the range studied. In both cases, as shown in Table V, p,p'-DDT was eliminated to below the limit of detection.

Although the rate of removal of pesticide residues is more rapid at the higher temperature, the quality of the fish oil is undoubtedly better retained at the lower temperature.

5.2.2 Hydrogenation and deodorization

In a preliminary experiment, to obtain artificially high levels of both p,p'-DDT and p,p'-DDE, we added these pesticides to a bleached menhaden oil. By hydrogenation, we then eliminated 90% of the p,p'-DDT present. The p,p'-DDE was not found to decrease to such a degree. The mechanism of this decrease in the concentration of p,p'-DDT during hydrogenation was not investigated by us. The phenomenon had previously been observed by Gooding (1966).

Table V. Partial removal of pesticide residues from two bleached[a] Pacific herring oils by steam refining

Distillation temperature[b]	Pesticide	Pesticides (ppm) Original oil	Refined oil	Condensate fraction
190 to 200° C	p,p'-DDE	0.73	0.47	40.3
	p,p'-TDE	0.59	0.53	21.3
	p,p'-DDT	0.37	BDL[c]	BDL
	Total	1.69	1.00	61.6
230 to 240° C	p,p'-DDE	0.82	0.49	34.1
	p,p'-TDE	0.70	0.62	24.5
	p,p'-DDT	0.44	BDL	BDL
	Total	1.96	1.11	58.6

[a] Bleached with activated clay (4% weight of oil) for 30 min at 8 mm Hg and 80° C.
[b] Deodorized for 3 hr at 8 mm Hg with 9 wt % steam based on the wt of the oil.
[c] BDL: below detectable limit.

The pesticide p,p'-DDE, a product of the natural breakdown of p,p'-DDT, is often the chlorinated pesticide found in highest concentrations in marine animals. Table VI presents data on the effectiveness of removal of p,p'-DDE from menhaden oil containing added amounts of this substance. The table shows that the combination of hydrogenation and deodorization is effective in reducing the level of this pesticide. The deodorization process, did not, under the conditions used, reduce the pesticide level in menhaden oil at as high a rate nor to as great a degree as Smith et al. (1963) and Saha, Nielsen, and Sumner (1970) found with vegetable oils.

Table VI. Partial removal of artificially high residues of p,p'-DDE from bleached menhaden oils by hydrogenation and vacuum steam deodorization

Sample	Residues of p,p'-DDE (ppm) Trial 1	Trial 2	Trial 3
Original	63.1	23.4	12.0
Hydrogenated oil[a]	51.8	21.9	10.5
Deodorized oil[b]	28.7	16.8	8.2

[a] Melting point: 42° C.
[b] Deodorized for 3 hours at 12 mm Hg and 190-200° C with 10 wt % steam based on the wt of the oil.

The major difference in the application of deodorization to the removal of pesticides from fish oils, compared with work of these authors on vegetable oils, is the lower temperature that is nearly mandatory in work on fish oils to prevent thermal degradation. Because of this requirement for a lower temperature, the duration of the deodorization must be extended if a substantial reduction in the pesticide level is to be accomplished.

6 SUMMARY

The residue levels of DDT and its metabolites were examined in fish from waters off the Pacific Coast of the United States. The effects of simple alterations in the pre-

canning process for jack mackerel were studied. The fate of pesticides during production of fish protein concentrate was followed. Pesticide levels in menhaden meal and oil from both Atlantic and Gulf of Mexico fisheries were studied. Methods for lowering the DDT content of fish oil were examined.

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Nutritional aspects of processing and chemical additives on fats and oils

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J. P. K. VAN DER STEUR
Unilever N. V., Rotterdam, The Netherlands

OILS AND FATS are important human foods. They not only provide a concentrated form of the energy that people need to do their daily job, but they also include certain factors which are essential to life and which the human body does not synthesise. We have to ensure, therefore, that man's diet includes adequate amounts of fats, containing enough of the essential constituents.

Some oils and fats can be consumed in the form in which they are recovered from their natural sources. These include olive oil which is obtained by crushing ripe olives at the place of cultivation, and milk fat which is separated from fresh milk by centrifuging and is converted into its ultimate form as butter or ghee by churning and kneading and gentle heating. These products can be made in an appetising form, however, only if they are obtained fresh from the raw materials. The are available only in limited quantities. The same applies to beef tallow and lard which if freshly rendered, can be used without refining. But only a limited proportion of the two latter fats can be obtained in this way.

In most cases the raw materials for edible oils and fats are obtained in countries far away and the oils and fats cannot always be produced locally from the seeds or fruits owing to economic conditions. In cases where this is done—for instance with plantation palm oil—the taste of the fat prevents it from being used for direct consumption by the pampered Westerner. Moreover the quality of the product would deteriorate during transport over long distances. Coconut fat that could be obtained from fresh nuts in tropical countries has an attractive flavour but is not to everyone's taste.

In the vast majority of cases the oils and fats obtained from oil seeds and fruits contain substances that make them unsuitable for human consumption in that form, mainly because of their flavour or odour or appearance. Before becoming commercial products for human consumption, these oils and fats first have to be purified in order to remove the off-flavours, together with the colouring matter and free fatty acids.

On the other hand many of them have also to be processed to impart the required consistency to them. In some countries oil is used in the kitchen for the preparation of food. Other countries are more accustomed to using fats with a solid consistency, such as butter or margarine, or hard fats such as lard and beef tallow. Endeavours must therefore be made to process the raw materials so that they also will comply with the consumers' requirements regarding consistency.

It is the task of the oils and fats industry to process the raw materials made available to it by the world economy so as to make edible products which will guarantee a good diet and will suit living habits in different countries.

The main constituents of oils and fats are the triglycerides, esters of glycerol with a great variety of fatty acids. Many fatty acids can be synthesised by the human body; some, however, such as the essential fatty acids (cis-cis linoleic acid, arachidonic acid, etc.) cannot. Oils and fats also contain much smaller quantities of other constituents which are saponifiable and contain fatty acids as part of their molecules, such as phosphatides and waxes. In the natural oils and fats there are, moreover, unsaponifiable constituents such as hydrocarbons, e.g. squalene, sterols, e.g. phytosterols and cholesterol, and—in some of them—fat-soluble vitamins. Many oils and fats, especially the vegetable ones, contain vitamin E (tocopherol) which is practically all conserved during processing. Some contain vitamin A or carotene.

RAW MATERIALS

On the whole, oils and fats are obtained from animal fat tissue, from the flesh of oil fruits or from seeds. These cannot always be processed while fresh, and storage may lead to deterioration. This may be caused by bacteria and insects or by the effects of moisture, high temperature and atmospheric oxygen. These factors may in turn accelerate the effect upon the fat in fatty tissues of the fat-splitting and oxidising enzymes, the lipases and lipoxydases, which such tissue always contains. The result is that free fatty acids are formed on the one hand and mono- and diglycerides on the other, and further on some oxidation may take place. The better the raw materials are handled, the better the

quality of the crude oil will be and consequently of the refined oil.

Let us now discuss the main operations applied by the oils and fats industry one by one and see how far they influence the nutritive values of oils and fats.

PROCESSING

In order to separate the fat and to convert it into an edible form, we find that some of the following chemical and physical operations are necessary: *a*) separation from tissues, fruit or seed; *b*) degumming; *c*) neutralisation; *d*) bleaching; *e*) deodorising. Besides this the oils and fats industry applies other operations to make the fats more suitable for the purposes for which they are used. These are: *f*) hardening; *g*) esterification; *h*) mixing; cooling, kneading and—for special products such as margarine—emulsifying.

Separation of oils and fats. Before oils and fats are separated the tissue and the seeds must be finely divided in order that the fat released from the cells can run off properly. This is followed by heating which, in the case of animal tissue, already releases a substantial part of the fat. This is the process applied, for instance, in melting out beef tallow, lard and whale oil. Some fat remains which is absorbed in the cell walls and can later be separated by pressing or centrifuging. The same applies to the flesh of fruit, for instance palm fruit.

In the case of seeds, the fat is often more difficult to remove. After crushing, the material is heated and most of the fat is removed by pressing at high-pressure and temperatures of about 100°C.

Besides this process based on heating and pressing, extraction is often used to obtain a higher yield of oil. The extractant used nowadays is almost always a low-boiling petroleum fraction: usually technically pure hexane with a boiling point of about 66°C. Solvents used in the past, such as trichlorethylene, which has the advantage of being noninflammable, have been completely abandoned. The reason for this is that it brings into solution many more substances (nonfats) from the seeds which cause trouble during refining. Moreover trichlorethylene when brought into contact with water very easily forms hydrochloric acid, which may corrode the steel of the apparatus.

For economic reasons a combination of expelling and extraction is often applied. A fairly high oil content remains in the pressed seed which is then crushed again and solvent-extracted. After extraction a meal is left with a low oil content (0.5–1.0%) and a solution of oil in solvent, the miscella. From this miscella the solvent is distilled off and finally volatilised by blowing through steam.

It has sometimes been thought that the solvent might contain higher aromates having a toxic effect. Extraction solvent is so well fractionated, however, that there will certainly be no higher fractions in it. After refining the edible oil is completely free from solvent. This has been proved, inter alia, by using extracting agents containing radioactive tracers. After the solvent had been distilled off and after neutralisation, washing and drying under vacuum at 90°C, all the radioactivity had vanished.

Degumming. When the oils and fats are removed from their natural sources (tissues, seeds) some constituents other than triglycerides are also partly dissolved in the oil. In many cases it is useful to remove such substances, e.g. phospholipids, before further refining is commenced. This applies particularly to soybean oil, for instance, in which quantities of phospholipids amounting to several per cent are often found after extraction from the beans. These can easily be removed by swelling the phospholipids with small quantities of water, after which they can be separated by centrifuging. This product is the well-known soya-lecithin. For degumming, chemical agents are also often used, for instance sodium chloride solutions, phosphoric acid, citric acid, acetic acid, acetic acid anhydride, hydrochloric acid, etc. It has been found that many of these substances are separated more easily under the influence of these agents. This degumming usually takes place at temperatures below 100°C. The agents do not appear in the degummed oil and the nutritional value is not affected by this operation.

The phosphatides that are removed may be important food constituents. Their presence makes an adequate refining into tasteless fats impossible, but the lecithin may be purified separately and subsequently re-added to margarine to improve the frying properties. It is also used to improve the viscosity of chocolate in manufacture.

Neutralisation. As I have already mentioned, some splitting has occurred in the oils and fats through storing the seed or the fatty tissue under the influence of moisture and lipases, as a result of which free fatty acids and mono- and diglycerides have been formed from the triglycerides originally present. Although in many cases, i.e. with long-chain acids (C_{16} and higher) these fatty acids do not adversely affect the taste of the oil, it is advisable to remove them, as they affect refining of the oil and the use of the final product (low smoke point).

The fatty acids combined with caustic soda in solution to form soaps are removed as an aqueous phase that settles at the bottom of the neutraliser vessel and can be drawn off or removed by centrifuging. This alkaline aqueous phase also contains part of the colouring matter and other unwanted substances. This is an additional advantage of the neutralisation process, as the latter might adversely influence the taste of the oils and fats in further processing. Remains of the soap phase are removed as well as possible by washing with water. This entire operation which is carried out at temperatures below 100°C definitely does not remove any integral constituent of the diet.

Bleaching. After the fatty acids have been neutralised, we have to remove as much as possible of the colouring material that is still present. Strongly coloured oils are difficult to make tasteless. At the same time the bleaching

process eliminates the soap residues left in the oil after neutralisation and washing with water.

This bleaching is done by treating the oils and fats under vacuum with bleaching earth at a temperature of 90°–100°C. Some fats, such as coconut- and palm-kernel oil, are treated with small quantities of activated carbon in addition to bleaching earth. The decolouring is for the greater part a purely adsorptive action at the surface of bleaching earth and activated carbon.

In addition, however, slight changes may occur in unsaturated oils and fats under the influence of the bleaching earth. The extent to which this takes place depends partly upon the past history of the crude materials.

Mitchell and Kraybill (8) first observed that under the influence of bleaching earth characteristic changes occur in the ultraviolet spectra of edible oils. Absorption bands develop at 268 mμ when the oil contains linoleic acid and 268, 300 and 316 mμ when linolenic acid is also present. These absorption bands indicate the presence of conjugated trienes or tetraenes, which are formed only when the oil has been previously oxidised. The trienes are formed from primary hydroxy fatty acids or hydroperoxides by the splitting off of water under the influence of bleaching earth. The absolute concentration of these trienes and tetraenes is however very small, viz. about 0.1%.

Polymerisation may also occur under the influence of bleaching earth, especially in oils with a high unsaturated fatty acid content. The normal test on polymers in oil (Hügel test) is negative, but more sensitive methods for detecting dimers have taught us that while the content in crude oil is 0–0.01% it goes up to 0.01–0.08% in neutralised and bleached soybean oil, to 0.02% in sunflowerseed oil, to 0.03% in cottonseed oil and to 0.02% in rapeseed oil.

Nevertheless these reactions occur to such a small extent and the compounds are of such a kind that no influence on the food value is to be expected from them.

Deodorising. After gums, fatty acids and colouring material have been removed, it is necessary to eliminate the off-odours and flavours from the crude oils and fats. The usual method that has been applied for many years is steaming under vacuum. The oil is pumped into vacuum containers, heated to 160°–230°C (normally 180°C) under a constant stream of superheated steam which entrains the odoriferous substances. This method can be effected batchwise, semicontinuously or continuously and its big advantage is that treatment under high vacuum prevents oxidation, which might lead again again to deterioration of the refined oil. The steaming time and the quantity of steam passed through are determined by the taste, the operation being continued until the fat is tasteless.

During the deodorisation process some chemical reactions can take place under the influence of steam and high temperature. Some saponification will occur through which free fatty acids will be formed that are distilled off. In addition to this, heating may also cause polymerisation. The same sensitive methods as mentioned before have shown that steaming has only very little effect on the formation of polymers.

Deodorisation of sunflowerseed oil at 240°C, a temperature which is not applied normally (180°C) increased the dimer content from 0.02% to 0.07% and 0.08%. With soybean oil the dimer content when deodorised at 170°C increased from 0.05% to 0.08% and at 235°C from 0.05% to 0.10%. In groundnut oil and hardened groundnut oil the dimer content was increased by bleaching and deodorisation from 0.01% to 0.06%.

Experiments so far have not indicated even the slightest effect upon the growth of rats attributable to these extremely small quantities of dimers. Only if polymerisation is increased up to contents of 10% and 20% in the treated oil is growth affected, because the highly polymerised portions are no longer digested and pass out of the body again without having been used.

Considering all the processes we have discussed so far, i.e. degumming, neutralising, bleaching and deodorising, we can say that the fat that is obtained has in all likelihood the same nutritional value as the starting product, but has become more palatable. As to the latter, our views will hardly differ. To elucidate what I have said regarding nutritional value, let me mention comparative experiments we made with rats.

These animals had been fed diets with a high content (50–70 cal.%) of an unrefined fat; as controls served groups which were fed comparable amounts of the same, but refined fats. According to Thomasson these diets with an extremely high fat content give an excellent opportunity of detecting even slight differences in growth stimulation between various fats. At the levels normally used in human diets these differences will be much smaller, or even vanish (cf. table 1).

A number of other oils and fats are still being investigated. The results of these tests will be known in September 1960.

Hydrogenation. In addition to the treatments outlined above, hydrogenation methods are also applied in the edible oil industry in order to meet the requirement that for satisfactory usability many fats must have a solid consistency. Of course it is possible to use solid fats, but these are often in short supply. Moreover such products frequently do not have the required melting

TABLE 1. *Growth on Crude and Refined Fats in Rat Tests*

	Growth of Animals				
	1 Wk.	2 Wk.	3 Wk.	4 Wk.	5 and 6 Wk.
Crude olive oil, 50 cal.%	26.3	64.9	101.4	135.3	
Refined olive oil, 50 cal.%	26.9	65.8	102.9	140.7	
Butterfat, 70 cal.%	15.1	41.3	78.8	119.2	160.2
Refined butterfat, 70 cal.%	15.6	40.1	72.1	109.9	148.9

behaviour and consistency to come up to the climatic conditions of the country in which they are used. For this reason, it is frequently necessary to harden oils, and sometimes, although to a smaller extent, also solid fats, in order to impart the desired consistency to them. In addition, fish oil and whale oil, which as such are unsuitable for human consumption, must be hardened. In this process the highly unsaturated fatty acids which can give an unpleasant fishy taste, are converted into dietary fats containing fatty acids of satisfactory taste. Of the total quantity of oils and fats used in the edible oil industry about 30% is subjected to this treatment.

Before they can be hardened, the crude oils and fats are first neutralised to remove fatty acids and mucilage which unfavourably affect the hardening. The mucilage and other catalyst poisons, possibly sulphur compounds, could inactivate the catalyst, while fatty acids are able to dissolve the nickel present in the catalyst.

After the addition of an amount of suitable nickel catalyst, the product is hardened at temperatures from 100° to 200°C, while hydrogen is passed through, until it has required the desired properties. By the nature of the catalyst and the hardening method, the properties of the final product can, within certain limits, be controlled. The height of the melting point of the fat and its dilatation values at different temperatures are generally taken as criteria for the final result.

As a result of this hardening, which in the case of edible oils and fats only removes part of the unsaturation, various changes take place in the fatty acid molecules. A part of the double bonds in the fatty acids is saturated. At the same time a shifting of double bonds in the molecule occurs, accompanied by a transformation of a part of these double bonds from the cis- into the trans-form.

Hardened fats have been investigated in order to find out in what way their nutritive value is related to that of unhardened fats.

During hardening a minute amount of nickel is dissolved; but by careful refining (neutralisation, bleaching, deodorisation) of the hardened fat, the dissolved nickel is again removed. As a result, hardened fats contain extremely small amounts of nickel (1–4 μg/kg), much lower than are found in many natural products (100–300 μg/kg).

From investigations by Aaes Jörgensen *et al.* (1–5) and by Thomasson (11, p. 446 *et seq.*) it has appeared that on feeding rats diets containing a high percentage of hardened fats, there is some slight decrease in growth rate as compared with the unhardened products. However, Thomasson has further shown that diets containing hardened oils are completely satisfactory and support normal growth subject to the presence of 3–4% of linoleic acid in the diet. He has further shown that for satisfactory growth it is necessary to have a proper balance between saturated fatty acids with 12–18 carbon atoms and linoleic acid.

It has been investigated whether the trans-fatty acids formed from natural cis-fatty acids have identical nutritive values as the original acids. Sherman *et al.* (9) have published in 1949 some work on the influence of elaidic acid compared with oleic acid and petroselinelaidic acid compared with petroselinic acid. The cis-acids gave some better growth and a higher arachidonic acid content in the body fat. Different results were obtained by Thomasson (11, pp. 471, 472), who has compared a fatty acid having a cis-double bond (oleic acid) and the corresponding fatty acid having a trans-double bond (elaidic acid). It was found that even a very high dosage of elaidic acid gave normal growth, and no indications were obtained that the depotfat-formation and the combustion proceeded differently from those of normal oleic acid. In addition the growth on feeding elaidic acid was not changed by the addition of linoleic acid. As is generally known, trans-fatty acids are also to be found in butter and beef fat.

Thomasson established that on admixture of unhardened and hardened oils, a growth was obtained which in most cases exceeded that of the original non-hardened oil. This assures us that no harmful substances are formed during the hardening process.

From the results of numerous investigations it is at present assumed that a high content of unsaturated compounds may have a blood-cholesterol lowering effect. This is actually a fact which has to be taken into account. For this reason it would seem advisable to see to it that during the hardening the content of unsaturated fatty acids decreases as little as possible. The theory that in this respect the essential fatty acids are important, now appears to be incorrect. Various oils (whale oil, fish oils and rapeseed oil) which have a low content of essential fatty acids are, according to Ahrens, Brock, Malmros and Thomasson, more effective in bringing about a decrease in the blood-cholesterol content than oils having a very high content of essential fatty acids.

Moreover Thomasson is investigating the influence of various fats and fatty acids on the blood-cholesterol content by means of a rat-screening test. He has found that by proper hardening of various oils and fats, marine oils, cottonseed oil, soybean oil, etc., provided this process is not continued to too high melting points (not in excess of 35°C), there is very little effect on the cholesterol-lowering action. In addition it was found that not only the degree and the nature of unsaturation is important, but other factors may play a role as well. According to Thomasson it is probable that the longer the chain length of the unsaturated fatty acid, the greater the effect will be on blood-cholesterol lowering. These experiments are only in the initial stage and will need confirmation on human panels.

In all the discussions on the influence of specific fats on growth, blood-cholesterol, etc., it must, however, be kept in mind that man seldom consumes a single fat. Indeed, we eat much fat in such products as fish, meat, cereals, etc. This invisible fat comprises 40% of the total fat intake in some Western countries and up to 70% of the fat intake in certain underdeveloped coun-

tries. Thus the human diet generally contains a mixture of many kinds of fat, which individually may have a specific action. This, however, will be modified when in admixture with other fats.

It is perhaps useful in conclusion to consider the contribution that hardening has made to the fat industry. As I have observed already, the advantage of hardening is that with the prevailing shortage of solid fats, a product suitable for certain climatic conditions can also readily be made from oils. Next, the decrease in the degree of unsaturation imparts better keeping qualities to the product. When the degree of unsaturation is higher, the fat is usually more susceptible to oxidation. This means a shorter shelf-life and increase in the chance that oxidised fatty materials which are not always harmless will be consumed. It is important, therefore, to find a compromise between these various factors.

Esterification. To improve the consistency, and to impart other desired properties to margarine, shortenings, and similar products, interesterification is frequently applied. In this process a fat or a mixture of fats is treated with a catalyst which is able to rearrange the fatty acid-radicals in the glyceride molecules via a transition stage, in such a way that a random esterified product is obtained. In addition, by application of so-called directed interesterification, high-melting triglycerides formed at low temperature can be made to crystallise, thus effecting a fractionation at the same time. In this operation catalysts are employed (sodium, sodium glycerate, sodium methylate and sodium ethylate) at temperatures varying from 50° to 150°C. Conditional for this operation is that it is carried out with a completely dry fat, as otherwise the catalyst is split by the water present, before the interesterification reaction has taken place. In this operation, the fatty acids undergo no change whatever. Only their position on the glycerin molecules changes. These conversions are comparable with the processes going on in the animal body during fat metabolism, when partial splitting off of fatty acids and subsequent re-synthesis to fats of frequently quite different compositions take place.

In addition to interesterification it is also possible to re-esterify fatty acids as such or a mixture of fatty acids and glycerides to a triglyceride. If in this process the starting products are fatty acids originating from natural fats either of sufficient purity or which have been purified, for instance by distillation *in vacuo* of the relevant fatty acids, no objections can be raised to this procedure on physiological grounds. These acids can then be re-esterified with glycerin in various ways, under the influence of catalysts. This process was formerly carried out at high temperatures, while using, inter alia, tin as a catalyst. Now it is, however, also possible to carry out this esterification at much lower temperatures.

The interesterification process does not affect the nutritive value of a fat. This is especially clear from the results of relevant experiments. Thomasson carried out tests with rats; the growth of the animals was determined after administration of diets containing high percentages of triglycerides in their original state by the side of the fatty acids made from these triglycerides and then re-esterified. The results can be seen in table 2. No significant growth difference could be found.

TABLE 2. *Growth of Rats Fed on Diets Containing Natural Triglycerides and Re-esterified Glycerides Made From the Same Fatty Acids*

	Caloric Percentage Fat in Diet	Growth
		after 3 wk
Hardened groundnut oil	72	41.5 g
Fatty acids re-esterified	72	39.0 g
Low melting fraction f.a. re-esterified	72	44.5 g
High melting fraction f.a. re-esterified	72	41.0 g
		after 4 wk
Groundnut oil	50	131 g
Groundnut fatty acid distilled re-esterified	50	123 g

Physical treatments. In addition to the methods discussed so far, and which are all to a greater or lesser extent based on chemical reactions, various physical treatments are likewise employed in the edible oil industry. First among these is the mixing of various oils and fats as is current practice in the edible fat industry. In the margarine industry, for instance, oils, solid natural fats and hardened oils and fats are mixed until a composition is obtained which has a satisfactory consistency for the climate and the use for which the product is intended. The same applies to nonwater containing edible fats. This mixing of various fats is not only advantageous from the point of view of use, but it may also favourably affect the nutritive value of the mixture.

From the work by Thomasson we have learnt that on feeding pure fats in diets with a high fat content to rats, a fluctuating growth is observed in the test animals. The greatest growth-action is found in the case of fats such as lard, butterfat, olive oil, cottonseed oil, and sheafat. Beef tallow, maize oil, soybean oil, groundnut oil, and palm oil, at a level of 70 cal.%, give a slightly less marked growth, while other fats such as sunflower-seed oil, coconut fat, sesame oil, whale oil, herring oil, and rapeseed oil, when amounts of 50–60 cal.% are contained in the diets, give even less good growth (10). On mixing various fats these differences can be levelled out, and so give a product which has a more uniform growth action. This brings me to the point I made earlier when I stressed the nutritional advantages of diets containing mixed fats. The range of both visible and invisible fats in our diet normally ensures this.

It is clear that the physical process of mixing introduces no changes at all in the nutritive properties of each of the component fats. The same applies to other physical treatments which are used in industry, such as the emulsification process in the margarine industry where skimmed milk is emulsified in a fat phase, the cooling of fat mixtures on cooling drums or in votators, the sub-

sequent kneading of the cooled fat in various types of kneaders.

ADDITIVES

In the foregoing pages the additives used during the processing of oils and fats and their possible influence on these raw materials have been described in great detail. Now some attention will be paid to those additives which are added to the refined fat or fat mixture.

Vitamins. Low concentrations of fat-soluble vitamins are found in oils and fats. As already mentioned in most vegetable fats vitamin E is present, of which only a small portion is lost during refining. In animal fats only a minute amount of this vitamin is present. Beef tallow contains small amounts of vitamin A; butterfat contains a larger amount of vitamin A; a large amount of carotene is found in palm oil. Vitamin D occurs in butter in very small amounts.

During refining of fats and oils the greater part of the carotene and of the vitamin A is lost. It would be possible to retain part of the carotene in palm oil by application of a special refining technique, but the taste of palm oil so treated becomes poor due to the presence of decomposition products originating from carotene and similar colorants. Therefore if palm oil is used in large amounts, it has to be bleached. It is possible to recover the carotene contained in palm oil, and suitable processes have been worked out. Currently produced synthetic β-carotene, however, is completely pure and its price is not any higher than that obtained from palm oil by applying the methods known at present.

To meet the vitamins A and D requirements of the population, synthetic vitamin A and synthetic vitamin D are added to a number of fat products, inter alia margarine. The amount of added vitamin D must not be too high, for instance *ca.* 3IU/g, to prevent the danger of giving older people an over-dose of this vitamin.

Flavouring substances. Attempts are being made to impart an attractive taste to a number of oils and fat products, notably margarine. This is done by the addition of synthetic flavouring substances. For a long time past diacetyl has been used for this purpose; at present also lactones and other flavouring substances are employed. Physiological tests have shown that these substances, even when added in very large amounts to the diet, are harmless for the animal organism. This has been proved in short-term experiments and in tests covering the entire life-span of rats fed these substances. At a later date it has moreover appeared that these substances are identical to the flavouring substances found in butter.

Anti-oxidants. To improve the shelf-life of edible oils fat-soluble anti-oxidants could be used. As a rule it can be said that these substances have little effect on vegetable oils, but a pronounced action on being added to animal oils and fats. These latter products contain only small amounts of natural anti-oxidants, so that they can be markedly improved by the addition of natural tocopherol or such synthetic anti-oxidants as gallates (propyl, octyl and dodecyl), nordihydroguaiaretic acid (NDGA), butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT). Vegetable oils and fats contain natural antioxidants, inter alia tocopherol. Especially γ-tocopherol is active as such at room temperature. It has appeared that during refining and hardening of vegetable oils only a small part (10–20%) of the tocopherol is converted. Only in those cases when a vegetable fat must be used in edible products which have to be stored for very long periods of time, e.g. biscuits for export, it will be advantageous to add an anti-oxidant to a vegetable fat.

The above-mentioned anti-oxidants have all been tested biologically, and found to be harmless. It is not necessary here to go into details concerning all the experiments which have been carried out, but they form the basis on which the use of these anti-oxidants has been sanctioned in various countries.

Anti-microbial substances. In some water-containing fat products, such as for instance margarine, the use of preservatives is allowed in many countries. These products have the advantage that even in nonsalted margarine a large amount of milk can be used, without the danger of rapid deterioration. As such especially benzoic acid and sorbic acid are being used. These two acids, as has been proved by biological investigations, can be considered harmless to health. Sorbic acid, however, because it is decomposed in the normal way in the body, is somewhat preferred to benzoic acid.

Colouring matter. The colorants currently used in oils and fats are generally synthetic carotene, palm oil and annatto. In a number of countries fat-soluble aniline-dyes are still allowed, but their use is gradually abandoned, mainly because to fat-soluble aniline-dyes a more unfavourable effect on the animal organism is ascribed than to water-soluble aniline-dyes.

Synthetic carotene which is identical to natural β-carotene (pro-vitamin A) has been discussed higher up. Annatto, the coluring constituent of which, bixine, is present on the outside of the annatto seeds, can be used without any harmful effects on health (6, 7). The annatto seeds are procured in Central America where they are used on a large scale in the preparation of food. The colouring material is dissolved in oil, and this oil can be added to edible fats.

Emulsifiers. The most important emulsifiers used in the oil and fat industry are: MONO/DIGLYCERIDES. At low temperature these substances have the tendency to retain the water phase finely dispersed in the fat phase, which is especially advantageous in the margarine industry. These mono- and diglycerides are preferably prepared from hardened fat, because in that case they have better action than when mono- and diglycerides from lower-melting fats are added. From the work by Frazer and other research-workers it has appeared that in man, on consuming fats and oils, part of the triglycerides is broken down to mono- and diglycerides. Moreover, mono- and diglycerides are present in

natural and also in refined oils and fats. Consequently, they form normal constituents of natural oils and fats and normal degradation products of fat metabolism. Their value as foodstuffs is consequently the same as of triglycerides.

LECITHIN. As already mentioned under deslimming, a phosphatide is obtained from a number of oils, notably soybean oil, during this treatment. After purification, this phosphatide can be used to improve the frying properties of products such as margarine. Moreover, lecithin is a substance known to have favourable physiological effects.

SCIENTIFIC RESEARCH

The oils and fats industry, like many other industries, originally developed almost entirely along empirical lines. Some processes, such as neutralising and deodorising, were originally applied in a comparatively primitive fashion, but grew up through their practical application during the course of years.

The process of hardening, however, had a somewhat different starting point. This process is in fact based on scientific research and was initially due to the work of Sabatier and Senderens who hydrogenated unsaturated compounds in the gaseous state using catalysts. Their method was later applied by Normann to liquid oils and fats. The hardening process and the catalysts employed were further improved, mainly by trial and error methods, but nevertheless with striking results.

It is only in the last few decades that research laboratories in the modern sense of the term have begun to play an important part in the industry. Scientific means were sought to study the effectiveness of various processes and for the development of means to improve them. A desire for perfection ensued and progressive advancement has lead to the present high state of development.

A stage has now been reached at which further improvement of the various processes employed is only likely to be achieved through more fundamental work. The direction in which further improvements can be sought can only be seen when the fundamental processes involved are better understood. In hardening, e.g. x-ray, work is being undertaken to determine the crystal size in nickel catalysts, which in turn should provide a better insight into the mode of action of the catalysts. New techniques, such as spectrophotometry, gas chromatography and mass spectrometry, etc., are being generally applied in the oils and fats field and have already cast new light on many of the problems of the chemistry and physical nature of these substances.

From this account it must be abundantly evident that in addition to chemical and physical research an important position is occupied by biological research. Great attention is being devoted to the possible physiological influence of dietary oils and fats. Much of this research is being undertaken in cooperation with working groups in universities, but it is yet too early to say what the consequences might be. It is certain, however, that in this way industry can make an important contribution to the development of science and to public health.

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