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The Origin and Metabolism of Marine Fatty Acids: The Effect of Diet on the Depot Fats of Mugil Cephalus (The Common Mullet)[1]

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THERE IS STRIKING EVIDENCE in favor of the view that a common dietary origin is necessary to explain the distribution of the characteristically highly unsaturated fatty acids found throughout the marine ecological system. The fact that teleostian (bony) fish, elasmobranchs, marine mammals, and sea birds show a general similarity of depot fat not found in terrestial or fresh-water forms, indicates that common origin is at least very probable. This cannot however exclude the possibility that, in the absence or deficiency of this typical marine fat, these forms can synthesize fats of the same general character. In support of this possibility there is evidence by Gunther (2) to indicate that phytoplankton, the plant cells which are the first trophic level in the marine food chain, are deficient in the typical marine fat. A similar condition exists among land plants and animals. Land plants may contain large amounts of dienoic or trienoic acids but be without those of a higher degree of unsaturation. Land mammals appear unable to synthesize significant amounts of the dienes or trienes but do convert them to more highly unsaturated acids. It is very possible that the difference between marine animals and land and fresh-water animals is just one of degree. Lovern (4, 6) and others have shown that hydrogenation-dehydrogenation and alteration of chain length take place in fish.

Quite a number of feeding experiments have been performed by investigators in the field of marine fats to determine the source of the typical marine fat; however these have been performed by utilizing natural diets or modified natural foods. Lovern (3) performed an experiment with eels fed a diet of herring flesh. The result was that when eels, a eurylhaline organism, were maintained in fresh water and fed a typical marine fish such as herring, the eel fats were modified so that they showed characteristics about half-way between normal eel and normal herring fats. In experiments of this nature many factors must be considered. There is wide variation in the quality of the ingested food. Further, such factors as age, season, temperature, feeding, breeding, and general activity have been reported to modify fish fat.

To obtain further evidence on the origin of these typical marine fats, a series of experiments were conducted, using *Mugil cephalus*, the common mullet. The mullet were chosen because of their euryhaline habits and because of their high viability in aquaria and on a synthetic diet.

Experimental

In general, the marine fats are usually free-flowing, low melting oils. The saturated fatty acids rarely contribute more than 20% of the total fatty acids. The predominating chain lengths lie between 16 and 22 carbon atoms although chain lengths of as few as 14 and as many as 26 carbons are known to occur. There may be from none to six unsaturations per molecule. These quite pronounced variations would have led to excessive difficulties had complete separations and analyses been attempted on the small quantities of fats obtained from these experiments. However a qualitative and rough quantitative, comparative classification of fat types was possible on even a micro scale when sufficient unsaturation existed. The method used was spectrophotometric, based on the American Oil Chemists' Society Method Cd 7-48. Although not designed for the quantitative determination of the fatty acids of marine fats, this method is useful in following changes in the polyunsaturated fatty acid composition of fish oil.

As applied in these experiments, the procedure was as follows. The fish were scaled, gutted, and cut into small pieces. The pieces were then placed in a Waring Blendor, covered with chloroform, and agitated at top speed for 60 seconds. The resultant slurry was then vacuum-filtered over a bed of anhydrous sodium sulfate and, if necessary, dried over more of the anhydrous salt. The chloroform was then removed by heating over a steam bath under aspirator vacuum. The resultant clear oil was then separated into two fractions.

The first fraction was dissolved in purified iso-octane, and the conjugated double bonds were ascertained by obtaining the optical density at designated wavelengths in a Beckman DU Spectrophotometer, using iso-octane as a blank.

The second fraction was hydrolyzed and isomerized to the conjugated form by heating to 180°C. with a 21% solution of potassium hydroxide in ethylene glycol under a nitrogen atmosphere. This solution was cooled and diluted with spectral methanol. The optical density was read as before in the spectrophotometer. The blank was prepared exactly like the sample. The conjugated unsaturations appeared as peaks in the curve obtained by plotting wave length *vs.* optical density or extinction coefficient $E^{1\%}_{1\,cm.}$. These peaks can be noted for two to six unsaturations per molecule and occurred, respectively, at wavelengths of 233, 268, 315, 347, and 374 millimicrons. The com-

parative heights of the peaks are a rough measure of the quantities of the respective materials.

The basic fat-free diet was:

Major constituents	
Sucrose or starch	68%
Albumin (egg)	25%
Salt mixture No. 4	5%
Glycine	1.7%
Choline hydrochloride	0.25%
Inositol	0.125%
Water-soluble vitamins	
Thiamin	6 mg./kg.
Calcium pantothenate	25 mg./kg.
Pyridoxine hydrochloride	8 mg./kg.
Niacin	70 mg./kg.
Folic acid	2 5 mg./kg.
Para-amino benzoic acid	3 mg./kg.
Lederle's extract for vitamin B_{12}	6 mg./kg.
Oil-soluble vitamins	
Mixed tocopherols	1 mg./kg.
Menadione	1.5 mg./kg.
Carotene	33 mg./kg.
Vitamin D_3	0.08 mg./kg.

The major constituents and the water-soluble vitamins were mixed thoroughly with an equal weight of water. The fat-soluble vitamins were mixed with an equal volume of Tween 20 (Atlas Powder Company) and emulsified with a small quantity of water. This was then added to the major constituents, and the whole was agitated thoroughly in a Waring Blendor. The resultant suspension or solution was then cooked in a double boiler until solidified, cooled, and stored in a refrigerator until used. Large batches were kept in a deep freeze.

Three groups of fish were fed three diets, respectively: a) a fat-free diet containing carbohydrate, protein, and all necessary known dietary supplements, such as vitamins and minerals, but devoid of fat or fatty acids; b) a marine diet composed of the fat-free diet with the addition of 10% emulsified menhaden oil, a marine fish oil; and c) a fresh-water diet composed of the fat-free diet with the addition of 10% emulsified "Wesson Oil," a commercial cooking oil made of refined cottonseed oil. This oil contains fatty acids with no more than two unsaturations per molecule.

The use of a fat-free diet served multiple purposes. Failure to live would indicate a requirement for essential fatty acids. Secondly, a fat-free diet should reduce the polyunsaturated fatty acids of fish to minimum levels and thus accentuate later changes on special fat diets. Finally, if the characteristics of the depot fats of a freshly caught fish are similar to those found in fish on a fat-free diet, it would indicate that this fat type is typical for the species in question and that synthesis or tenacious retention of polyunsaturated fatty acids had occurred.

The use of simulated fresh water and simulated marine type of diets was prompted by the following considerations. Should a marine fish, fed the fresh-water type of diet, show on analysis a fat typical of marine fish, it would indicate that the fish synthesized the marine fats from more saturated fat or from non-fat precursors. The simulated marine type of diet served as a control since menhaden oil is very similar to the oil obtained from freshly caught mullet.

The experiments were conducted as follows. A representative sample of young mullet 3 to 6 in. in length was placed in a 45-gal. aquarium containing filtered, natural sea water which had a salinity of 34 to 36 parts per thousand. Each tank was equipped with a continuous filter which recycled the water through glass wool and charcoal at a rate between 50 and 100 gal. per hour. The tanks were also provided with an air supply of approximately one liter per minute. The fish were fed sufficient food once daily to provide approximately a fifth of an ounce per specimen. The food was added in a finely divided form. In the initial experiment which lasted 102 days, no fish died under these conditions. In this experiment three aquaria outfitted as above were placed in an air-conditioned room and arranged so that environmental conditions were duplicated. All the fish were fed the fat-free diet for 12 days. At the end of this period one tank of fish was continued on the fat-free diet, a second was placed on the simulated fresh-water diet, and the third on the simulated marine diet. At the end of the prescribed feeding-time the fish were killed and analyzed for muscle and subcutaneous fat.

Results

The results of the first experiment are shown in the graphs of Figure 1, A through F. The curve A is a plot of wavelength *vs.* extinction coefficient for the menhaden oil used in the diet, and B is the extinction coefficient curve of the commercial cooking oil.

Plots C, D, E, and F are the extinction coefficient $E^{1\%}_{1\,cm.}$ curves of mullet fed the natural, fat-free, simulated marine and simulated fresh water diets, respectively.

In these plots the peaks corresponding to the various unsaturated fatty acids are readily seen. The curves shown were obtained by plotting only pertinent wavelengths. That no materials in the formu-

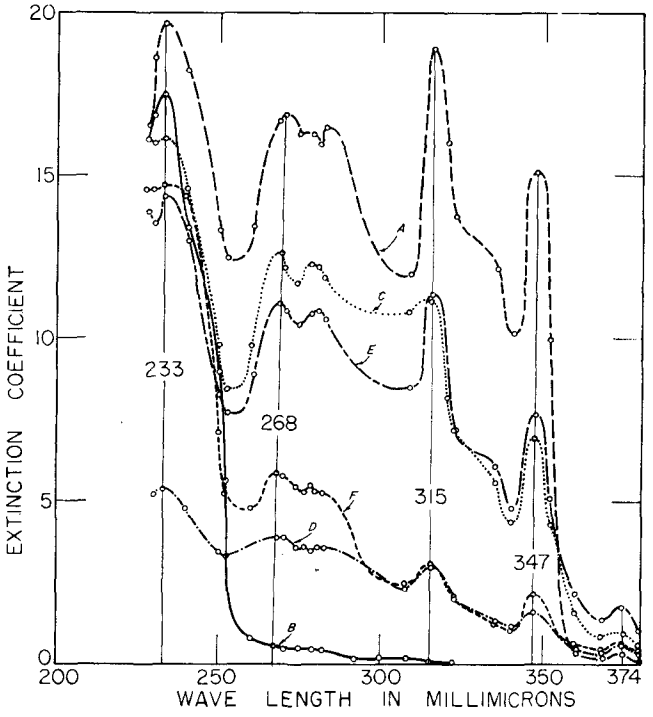

FIG. 1. First experiment—a) spectrum exhibited by the menhaden oil used in the simulated marine diet; b) spectrum of commercial cooking oil; c) spectrum of oil of mullet on natural diet; d) spectrum of oil of mullet on fat-free diet; e) spectrum of oil of mullet on simulated marine diet; f) spectrum of oil of mullet on simulated fresh-water diet.

lated diets interfere is inferred from the lack of any peaks in the wavelength *versus* optical density curve obtained by analyzing a chloroform extract of the fat-free diet. This curve is shown in Figure 2, plot E.

It can be seen by examining these graphs that the depot fats of the fish under test reflected to a high degree the nature of the ingested fats. Further, although the amount of polyunsaturated fatty acids of the fish on the fat-free diet was reduced, there was no indication of selective utilization. The presence of these fatty acids after 102 days may indicate synthesis, but they also may be the residue remaining at the end of the depletion period. In appearance the fish on the fat-free diet were just as plump, sleek, and active as those on the other diets.

In the second experiment the feeding program was essentially reversed. Thus, instead of maintaining the fish on the fat-free diet for two weeks and then placing them for 90 days on another diet, these fish were kept 90 days on the fat-free diet and then placed on another diet for two weeks. In all other respects the conditions of the first experiment were duplicated except for a basic dietary change. The carbohydrate used in the first food-batch was sucrose. This material however was so soluble that the food in the tanks caused high bacterial growth. Consequently in the second experiment sugar was replaced with corn starch. This food also seemed to be preferred by the fish. It was realized that corn starch contains traces of essential fatty acid. It was decided however that these small amounts could not affect significantly the composition of the body fatty acids.

As may be seen from Figure 2, the results of the second experiment confirm, in general, those of the first. That is, the levels of the polyunsaturated fatty acids in the fish decreased pronouncedly on the fat-free diet, and the nature of the body fat was markedly influenced by dietary fat. As before, the comparatively small amounts of polyunsaturated acids in the fish at the end of the experiment could have been the unused residues of those acids present at the beginning of the depletion period, or they could have been produced from nonfatty precursors.

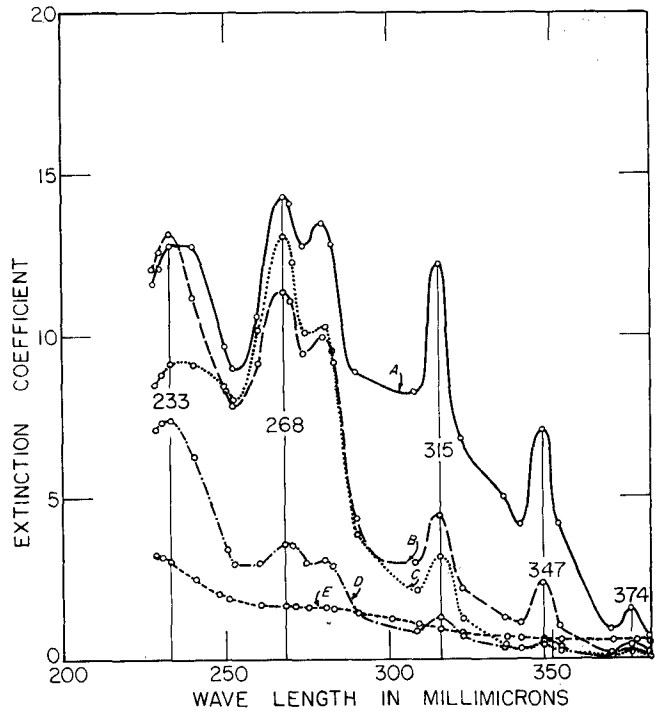

FIG. 2. Second experiment—a) spectrum exhibited by the menhaden oil used in simulated marine diet; b) spectrum of oil of mullet on simulated fresh-water diet; c) spectrum of the oil of mullet on simulated marine diet; d) spectrum of oil of mullet on fat-free diet; e) spectrum of chloroform-extracted material from the fat-free diet. Spectrum of cooking oil used in these experiments and of mullet oil on the natural diet are shown in Figure 1, graphs b and c.

There are several considerations which make it impossible to exclude the synthesis hypothesis. Although some statements by Lovern (4) may be interpreted to mean that he considers it possible that all fish fat is derived from dietary fat and none from carbohydrate, this can hardly be likely. If one may assume that the production of fat from carbohydrate does occur and, further, that fish can dehydrogenate and hydrogenate fatty acids, the required basic conditions are present for the synthesis by fish of the marine type of fatty acids. The limiting factor is the degree of ability of the fish to hydrogenate, dehydrogenate, and lengthen the carbon chain. Lovern has discussed at length the dehydrogenation of fatty acids by fish (6).

The ability of animals to synthesize long-chain polyunsaturated fatty acids takes on new significance with the recent work of Mead and his co-workers. These authors have demonstrated that unsaturated acids alternately add a double bond at the methylene interrupted position toward the carboxyl end, then two carbon atoms (7, 8, 9, 10).

By this procedure linoleic (△9,12 octadecadienoic) acid is converted to arachidonic (△5,8,11,14 eicosatetraenoic) acid, and linolenic acid to an eicosapentaenoic, probably the △5,8,11,14,17. By the same mechanism the △5,8,11 eicosatrienoic acid which has been found to increase in animal tissues on essential fatty acid deficient diets (11, 12) can be the product of oleic acid.

The fact that laying hens continue to lay down polyunsaturated fatty acids in eggs after 52 weeks on a rigidly fat-free diet (13) is further evidence that animals can synthesize polyunsaturated fatty acids.

Montag, Klenk, Hayes, and Holman have recently demonstrated the presence of △5,8,11 and △8,11,14 eicosatrienoic and △5,8,11,14,17 eicosapentaenoic acids in beef-liver phosphatides. Since these acids are not present in vegetable matter, they must have been synthesized by the animals (14).

The large amounts of polyunsaturated fatty acids in marine fish may thus express only a quantitative difference between them and fresh-water and land forms.

Conclusions

It appears that young mullet make excellent experimental fish for dietary studies. They are hardy, easily caught, resistant to disease, and take well to aquaria conditions. Of particular interest, they may be fed synthetic food and they resist changes in salinity well. These experiments indicate that, on a fat-free diet, the mullet does not synthesize the large amounts of polyunsaturated fatty acids normally found in its body fat. However the mullet, like land animals, can apparently convert dienoic acid from cottonseed oil into small amounts of tetraenoic, pentaenoic, and hexaenoic acids. Unlike land animals, the mullet appears to be capable of the conversion of linoleic acid to a trienoic acid. Finally, when fed a diet containing a typical marine fat such as menhaden oil, the mullet stores it almost unchanged.

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Thiol Esters of Long-Chain Acids and Long-Chain Alkanethiols[1]

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THIOL ESTERS of long-chain acids were believed to be unstable until Ralston and co-workers (5) demonstrated that methyl thiol esters through *n*-butyl thiol esters of lauric, myristic, palmitic, and stearic acids can be prepared in good yield by the action of an acyl halide on the appropriate mercaptan. The stability of the thiol esters was demonstrated by the fact that they can be purified by distillation under diminished pressure without decomposition.

This investigation continues the systematic study of the preparation, properties, and the reactions of thiol esters of long-chain acids. In our first paper (6) it was shown that aromatic as well as branched-chain thiol esters can be prepared by Ralston's procedure. In another paper (7) it was demonstrated that thiol esters of C_{12} to C_{18} saturated acids undergo ester interchange reactions with alkanethiols, arylthiols as well as with alcohols and phenol. Preliminary evaluations of thiol esters of long-chain acids as lube-oil additives indicate that some of these compounds show promise of having anti-wear and extreme pressure properties. They appear to be more promising in mineral oils than in synthetic oils.

In this investigation we extend the reactions of chlorides of long-chain acids to long-chain alkanethiols, forming some thiol esters having molecular weights of more than 500. *n*-Nonyl through *n*-octadecyl thiol esters of lauric, myristic, palmitic, and stearic acids were formed by treating the acyl halide with the appropriate mercaptan.

$$R-\overset{O}{\overset{\|}{C}}-Cl + R'SH \longrightarrow R-\overset{O}{\overset{\|}{C}}-SR' + HCl$$

The results are summarized in Table I.

Most of the alkanethiols were prepared by treating the appropriate alkyl bromide with thiourea (8). *n*-Pentadecanethiol and *n*-heptadecanethiol are believed to be new compounds. Heptadecyl, pentadecyl, and tridecyl bromides were prepared from the silver salts of stearic, palmitic, and myristic acids, respectively, by the silver salt reaction (2, 3).

Experimental

Materials. Undecyl bromide and nonanethiol were obtained from Matheson, Colman, and Bell. *n*-Decanethiol and *n*-dodecanethiol were obtained from the Aldrich Chemical Company. The tetradecyl, hexadecyl, and octadecyl bromides and myristic and palmitic acids as well as the lauroyl, myristoyl, and palmitoyl chlorides were obtained from Eastman. The HumKo Chemical Company supplied S-97, commercial stearic acid. This material was crystallized once from methanol and once from acetone and melted at 69°+. The properties, yields, and analyses of the thiol esters are summarized in Table I. All melting points are corrected.

Silver Myristate, Silver Palmitate, and Silver Stearate. These compounds were prepared from the appropriate ammonium salt dissolved in aqueous alcohol by the addition of silver nitrate (1).

Tridecyl, Pentadecyl, and Heptadecyl Bromide. Tridecyl and pentadecyl bromides (2) and heptadecyl bromide (3) were prepared by treating the anhydrous silver salts of myristic, palmitic, and stearic acids

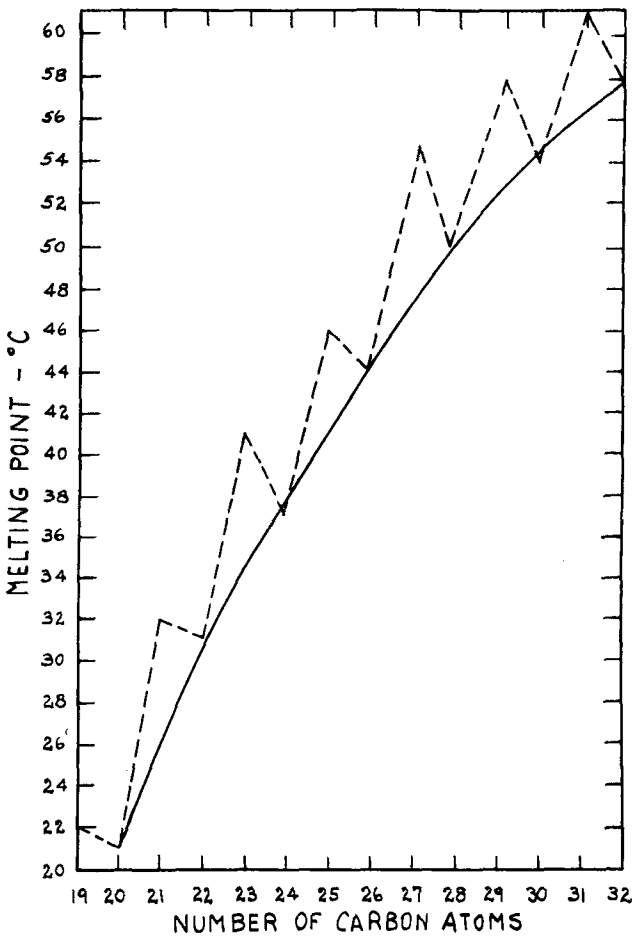

FIG. 1. Melting points of thiomyristates.

[1] This paper was given the 1957 Fatty Acid Producers' Award, administered by the American Oil Chemists' Society.