The Origin of the Marine Polyunsaturated Fatty Acids. Composition of Some Marine Plankton[1]

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IN PREVIOUS COMMUNICATIONS from this laboratory (8, 9) it was reported that although both fresh water and marine teleost fish appear able to synthesize some polyunsaturated fatty acids, or at least, tenaciously to retain them in fat-free diets, the high levels of these acids common to marine teleosts are probably ingested. The dietary source of these adventitious fatty acids however remains to be determined. The food of fish is largely phytoplankton and zooplankton. The phytoplankton are algae, upon which the zooplankton feed.

Because of uncertainties in reported analyses of phytoplankton and zooplankton one cannot decide from published data alone which are the ultimate source of the highly unsaturated acids of marine origin. According to Fogg (2), the fatty acids of algae are little different from those of higher plants. Gunther (3) also found that marine algae are deficient in typical marine fatty acids. Paschke and Wheeler (16) found that Chlorella, a fresh-water alga, contained comparatively small amounts of long-chain fatty acids although it did contain hexadecatetrenoic and octadecatetrenoic acids.

Lovern (12) has stated that fatty acids of the green and brown algae resemble those of fresh-water animals except that the C_{16} and C_{18} acids are of a higher degree of unsaturation. The red algae, as concluded from data of a single analysis, are the only ones with significant quantities of unsaturated C_{20} and C_{22} acids, with the C_{16} again of a high degree of unsaturation. All published analyses of zooplankton and crustacea are of organisms from their natural habitat and not grown under controlled conditions on a fat-free diet. Such analyses are useless for the problem at hand since their constituent polyunsaturated acids may have had their origin in their diet.

In order to obtain additional information on the subject, shrimp *(Penius sp.)*, crabs *(Callinectes sapidus)*, *Nitzschia closterium*, *Chlorella pyrenoidosa*, and *Platymonas sp.* were cultured in the laboratory and analyzed for their constituent polyunsaturated acids, as described previously (8, 9). Phytoplankton and zooplankton catches from the Gulf of Mexico were similarly analyzed, and the results were compared to the studies with teleost fish and the laboratory cultures.

Experimental

Shrimp. A catch of brown shrimp was made from inlets along the Gulf Coast from the vicinity of Galveston, Tex. The animals were 1 to 3 in. in length. They were kept in the same tanks under the same conditions as previously reported (8, 9) for the fish. The water was normal Gulf of Mexico sea water collected from the same location as the organisms.

The shrimp were placed in three aquaria and fed the low fat, the 10% cottonseed oil terrestrial type of fat, and 10% menhaden oil marine type of fat diets previously described (8, 9). This experiment was terminated after the shrimp were on the diets for only 30 days because of the cannibalistic nature of the shrimp.

Blue Crabs. A dozen blue crabs were obtained, which were 4 to 5 in. between the lateral tips of the shells. They were maintained at first exactly like the shrimp. Their method of feeding however precluded using the same diet. After a few attempts at holding the material in their pincers, largely unsuccessful, they subsequently refused the food. Consequently a new series of diets was prepared. For the marine fat diet they were fed fillets of red ocean perch, a rather oily fish whose fatty acids are of the typical marine form. The terrestrial fat diet was represented by lean beef heart.

The fat-free diet was formulated as follows. First, one hundred g. of chopped heart were added to 100 g. of chopped perch fillet. This mixture was placed in the Waring blendor and agitated 15 min. at top speed with enough chloroform to cover the mix. It was then filtered and the treatment repeated. The filtered material was successively washed with chloroform until the washings were clear. Next, it was then rolled into half-inch balls and heated in an 80°-C. oven until solid and until all chloroform odor was removed. This produced a hard food, which gradually softened on immersion and the crabs ate it readily. Like the shrimp the crabs were maintained on the three diets for 30 days. Some 10% cottonseed or menhaden oil was added for the simulated land or marine fat diets.

Algae. Three species of algae, *Platymonas sp.*, a marine green alga, *Nitzschia closterium*, a marine diatom, and *Chlorella pyrenoidosa*, a fresh water alga, each a pure culture, were grown in the laboratory. They were cultured in eight-liter conical flasks at light intensities of 500 to 1,000 foot candles and temperatures of 20° to 25°C. The nutrient solution compositions are found in Tables I and II. The algae which settled to the bottom of the flasks were withdrawn daily and immediately frozen. The cultures were maintained until growth slowed markedly. Thus each species was represented by a mixed-age group.

After a suitable amount of each species had been collected, the samples were dried by the freeze-drying technique. The dry material was then covered with chloroform and agitated at top speed in the Waring blendor for 30 min. The mixture was then filtered. This chloroform solution was so highly colored it was deemed essential to remove most of the nonsaponifi-

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able pigments. To accomplish this the chloroform was removed by vacuum distillation, and the resultant oil was refluxed for two hours with 150 ml. of 1 normal aqueous KOH. This solution was cooled, filtered, and washed successively with three 50-ml. portions of 30°–60° B.P. petroleum ether and three 50-ml. portions of chloroform. These washings were discarded. The KOH solution was then acidified with 6N HCl and extracted with three 50-ml. samples of chloroform. The chloroform extracts were combined and washed with 6N HCl until the washings were colorless, then with water to remove the acid. The chloroform solution was successively shaken with 5 g. of fine mesh silica, 5 g. of sucrose, and finally with 5 g. of anhydrous sodium sulfate. A filtration followed each of these treatments. The chloroform was removed as before, and the oil was analyzed as in previous experiments (8, 9).[4] Any emulsions which formed during the washing procedures were broken by centrifuging at 1500 × g. for 10 min.

Three plankton samples from the Gulf of Mexico were also analyzed. The first of these was collected by bucket from a dense bloom. It appeared dead and smelled as if decomposing. These algae were unidentified but appeared under microscopic examination to be composed of filamentous strings of cells. The material was brown in color. The two other samples were a composite catch made by towing a one-meter tandem net at two knots. This net was constructed so that a number 20 mesh net was towed inside a number 6 mesh net. The number 6 mesh had openings large enough to pass nearly all phytoplankton but small enough to catch the zooplankton. The number 20 mesh net caught most of the phytoplankton. The plankton catches were extracted and treated like the pure algae cultures. Microscopic examination of these catches showed that the zooplankton catch was almost exclusively crustacean, with a few jellyfish-like organisms dispersed throughout. The phytoplankton catch consisted of the same filamentous algae as found in the bloom, diatoms and flagellates and some jelly-like cells. Little could be said of the proportions because of clumping of the organisms.

TABLE I

Culture Media for Marine Algae

Constituent	Amount per 5 gal. of reconstitutted autoclaved sea water
Sodium silicate	46.6
Soil extract[a]	16.0 ml.
Arnon's solution (1938)	8.0 ml.
Ketchum and Redfield's "A" (1938)	32.0 ml.
Ketchum and Redfield's "B" (1938)	16.0 ml.
Sodium molybdate dihydrate	0.054 mg.

[a] *Soil extract:* autoclave at 15 lbs. 250°F. for 30 min. equal parts of soil and water. Decant, filter clear, and re-autoclave.

Results

The results of this study are presented in Table III. With one exception, that of shrimp ingesting cottonseed oil, the response of both crabs and shrimp to the diets was the same as that of marine fish. The results of studies with mullet and bullhead from previous studies are given for comparison (8, 9). On a low-fat diet all polyunsaturated fatty acids were appreciably reduced but responded quickly to fat added to the diet. The apparent lesser degree of response than that of fish results from a shorter period on the diets.

TABLE II

Culture Media for Fresh Water Algae

Constituent	Amount per liter of distilled water
Magnesium sulfate heptahydrate	4.94 g.
Monobasic potassium phosphate	2.47 g.
Potassium nitrate	2.53 g.
Ferric sulfate heptahydrate	1.33 mg.
Soil extract	2.0 ml.
Arnon's solution	0.5 ml.

The shrimp on the low-fat diet however responded to cottonseed oil with increase in 3,4,5, and 6 as well as the 2 double bond acids although none of these are present in cottonseed oil. Thus shrimp possess to a greater degree than land animals, teleost fish, or even blue crab the ability to readily convert acids with a low degree of unsaturation to acids with a high degree of unsaturation.

The fatty acid composition of the phytoplankton, both native and laboratory-reared, and of zooplankton catch present some interesting and enlightening data. It is especially noteworthy that the zooplankton catch contained larger amounts of all the acids, especially the 5 and 6 double bonds, than any other organism so far examined in this study. If one may interpolate from the studies with shrimp and crabs, these acids must have had their origin in the phytoplankton. It might appear that there are some phytoplankton with more of these acids than *Nitzschia closterium* and that the zooplankton must have derived their acids from these. It may also be that the natural plankton contained attached organisms, such as bacteria or nannoplankton, which were responsible for the higher values. On the other hand, there is the possibility that the zooplankton preferentially store the more highly unsaturated acids and thus concentrate them in their tissues.

An extremely important aspect of the problem of the origin of the marine fatty acids is that of their structure. As was discussed in a previous paper (9), animals have the ability to add double bonds and chain length to dienoic and possibly monoenoic acids (13, 14, 18, 19). Herbivorous land animals have also been shown to synthesize and concentrate reasonably large amounts of these acids in selected tissues. In addition to numerous reports of indirect evidence, Herb, Witnauer, and Riemenschneider (6) have isolated an eicosa- and a docosapentaenoic acid from adrenal lipides. Hammond and Lundberg (4) have isolated a docosahexaenoic acid from pig brain lipides. Holman and Greenberg (7) have shown that lamb testes may have as high as 15.6% hexaenoic acid and 10.3% tetraenoic. Montag *et al.* (15) have very recently isolated 5,8,11- and 8,11,14-eicosatrienoic and 5,8,11,14,17-eicosapentaenoic acids from beef liver lipides.

Fish may have the ability to effect this synthesis to a greater degree than land animals. If the mechanism described by Mead does operate to a great degree in fish and other aquatic animals, oleic, linoleic, and linolenic acids may be converted to com-

[4] The extinction coefficient, $E^{1\%/1cm.}$, was used in these studies because the structure of the acids with three, four, five, and six double bonds are not known and may well be mixtures. Since only comparative values are necessary, the $E^{1\%/1cm.}$ values of the oils were considered at least as accurate as absolute values for fatty acids of uncertain coefficient constants.

TABLE III

The Extinction Coefficients ($E^{1\%/1cm.}$) at the Wavelengths of Maximum Absorption of the Fatty Acids of a Typical Marine and Fresh Water Teleost, of Typical Marine Crustacea, and of Laboratory Grown and Natural Algae[e]

Fish and Plankton	Natural diet double bonds					Low-fat diet double bonds					Cottonseed oil diet double bonds					Menhaden oil diet double bonds				
	2	3	4	5	6	2	3	4	5	6	2	3	4	5	6	2	3	4	5	6
Mullet[a]	16	13	11	7.0	1.0	5.4	3.9	3.0	1.6	0.6	15	5.9	3.0	2.1	0.6	14	11	11	7.7	1.8
Bullhead[b]	9.4	7.0	2.3	0.8	0.0	8.7	6.1	1.9	0.8	0.0	9.4	5.8	1.0	0.2	0.0	11	15	3.3	0.8	0.3
Shrimp	11	12	7.5	4.0	2.5	4.5	2.5	1.7	1.2	0.9	11	5.1	5.3	4.8	1.9	11	9	7.5	5.0	1.8
Crabs	11	13	9.0	5.3	2.0	8.6	5.3	3.7	2.0	0.6	16	7.9	4.0	2.7	0.4	14	15	6.5	3.0	0.8
Nitzschia closterium[c]	19	14	11	4.7	0.8															
Chlorella pyrenoidosa[d]	16	10	5.5	1.0	0.0															
Phytoplankton catch	16	12	13	10	5.0															
Zooplankton catch	20	15	16	12	5.5															

[a] *Mugul cephalus* (marine). [b] *Ictalurus natilis* (fresh water). [c] Diatom, a marine alga. [d] A fresh-water alga. [e] The mullet were on the experimental diets 90 days, the bullhead for 43 days, and the shrimp and crabs for 30 days.

plicated mixtures of long-chain polyunsaturated acids with no value as essential fatty acids.

There is another interesting possibility. Although the 1,4 or methylene system of double bonds common to land fats is known to exist in marine oils, the evidence until recently indicated that most of the double bond systems in typical marine oils are the 1,5 or ethylene-interrupted system (17). If this were so, it is unlikely that the acids are derivatives of oleic, linoleic, and linolenic acids. Actually the 1,5-diene system is reminiscent of squalene, a hydrocarbon found in large amounts in some marine forms, which has a straight chain of 24 carbon atoms, 6 side chain methyl groups, and five 1,5-diene groups. If the 1,5-diene groups were to be proved correct, one would be tempted to look to a synthetic mechanism similar to that of squalene and other so-called isoprene derivatives. As has been discussed by Herb (5) however, the isomerization behavior of these acids toward alkali resembles that of the 1,4 rather than 1,5 dienes. Furthermore a recent careful study of Pilchard oil, in which the acids were isolated by molecular distillation and chromatography and oxidized by ozonolysis so as not to cause double bond migration, demonstrated that the eicosapentoenoic acid present had the 5,8,11,14,17 system (20). Similar conclusions have been reached by Klenk (10, 11).

This recent work makes suspect all previous conclusions of the structure of marine fatty acids, a suspicion raised a number of years ago by Farmer and Van den Heuvel (1). If marine fatty acids have the 1,4-diene system, they then probably have the same origin as land animal acids, that is, the 5,8,11 and 8,11,14-eicosatrienoic and 5,8,11,14,17-eicosapentaenoic acids isolated from beef liver lipides by Montag *et al.* (15) and the latter also from Pilchard oil by Whitcutt and Sutton (20) were probably derived from oleic, linoleic, and linolenic acids respectively. It then follows, as suggested earlier (8) that the differences in the synthesis of polyunsaturated acids by fish and land animals may be of degree and not of kind.

It is obvious that this problem cannot be resolved until the structure of the marine plant fatty acids are structurally characterized and the fatty acids of marine animals, grown from hatching on fat-free rations, are similarly studied. An alternative procedure for marine animals would be to locate the active carbon of incorporated labelled acetate. If randomly distributed in polyunsaturated acids, the acids could be concluded to be synthesized *de novo*. If incorporated only on the proximate terminus of the long-chain polyunsaturated acids, it could be concluded to be synthesized from pre-existing polyunsaturated acids.

Summary and Conclusions

Shrimp, crabs, the marine diatom *Nitzschia closterium, Platymonas sp.,* and the fresh water alga *Chlorella pyrenoidosa* were maintained or cultured in the laboratory. The crustacea were fed low-fat, cottonseed oil, and menhaden oil rations. The fatty acid composition of all groups, as well as that of native phytoplankton and zooplankton catches, were determined as the extinction coeffcients, ($E^{1\%}_{1cm.}$), at wavelengths of maximum absorption.

It was found that both shrimp and crabs lost much of their polyunsaturated acids on the fat-free diet and regained it again by ingestion, as do fish. The shrimp however appeared to synthesize more highly unsaturated acids from cottonseed oil than did other aquatic animals.

Phytoplankton do produce a high level of polyunsaturated fatty acids.

The importance of the determination of the structure of aquatic plant and animal fatty acids in the problem of the origin of the acids and their mechan ism of synthesis was discussed.

REFERENCES

1. Farmer, E. H., and Van den Heuvel, F. A., J. Soc. Chem. Ind., *57*, 24T (1938) and J. Chem. Soc., *1938*, 427.
2. Fogg, G. E., "Metabolism of Algae," John Wiley and Sons Inc., New York (1953).
3. Gunther, E. R., J. Expt. Biol., *XI*, 173 (1934).
4. Hammond, E. G., and Lundberg, W. O., J. Am. Oil Chemists' Soc., *30*, 438 (1953).
5. Herb, S. F., J. Am. Oil Chemists' Soc., *32*, 153 (1955).
6. Herb, S. F., Witnauer, L. P., and Riemenschneider, R. W., J. Am. Oil Chemists' Soc., *28*, 505 (1951).
7. Holman, R. T., and Greenberg, S. I., J. Am. Oil Chemists' Soc., *30*, 600 (1953).
8. Kelly, Peter B., Reiser, Raymond, and Hood, Donald W., J. Am. Oil Chemists' Soc., *35*, 189 (1958).
9. Kelly, Peter B., Reiser, Raymond, and Hood, Donald W., *ibid.*, *35*, 503 (1958).
10. Klenk, E., "Biochemical Problems of the Lipids," Butterworths Scientific Publications, London, p. 187 (1956).
11. Klenk, E., International Conference on Biochemical Problems of Lipids, Awlsk, Paleis der Academien, Brussels, p. 33 (1953).
12. Lovern, J. A., Biochem. J., *30*, 387 (1936).
13. Mead, J. F., J. Biol. Chem., *227*, 1025 (1957).
14. Mead, J. F., and Howton, D. R., J. Biol. Chem., *229*, 575 (1957).
15. Montag, W., Klenk, E., Hayes, H., and Holman, R. T., J. Biol. Chem., *227*, 53 (1957).
16. Paschke, R. F., and Wheeler, D. H., J. Am. Oil Chemists' Soc., *31*, 81 (1954).
17. Ralston, A. W., "Fatty Acids and Their Derivatives," p. 141 *et seq.*, John Wiley and Sons Inc., New York (1948).
18. Steinberg, G., Slaton, W. H. Jr., Howton, D. R., and Mead, J. F., J. Biol. Chem., *220*, 257 (1956).
19. Steinberg, G., Slaton, W. H. Jr., Howton, D. R., and Mead, J. F., J. Biol. Chem., *224*, 841 (1957).
20. Whitcutt, M. W., and Sutton, D. A., Biochem. J., *63*, 469 (1956).

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