CHEMICAL AND NUTRITIONAL STUDIES ON FISH OILS[1]

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ABSTRACT

This paper reports progress on the following four projects: (1) determination of the structure of fish-oil fatty acids and development of analytical techniques for determination of the fatty-acid composition of fish oils, (2) study of the chemistry of odor-producing compounds in fish oils, (3) study of the chemical reactions of fish-oil fatty acids, and (4) study of the nutritional effects of fish oils.

INTRODUCTION

The Hormel Institute, a research branch of the University of Minnesota Graduate School, currently is conducting four projects in the research program established by the U. S. Bureau of Commercial Fisheries with funds made available by the Saltonstall-Kennedy Act.

The objectives and the early stages of the work in three of these projects were described previously (Lundberg 1957). They are concerned with (1) determination of the structure of fish-oil fatty acids and development of analytical techniques for the determination of the fatty-acid composition of fish oils, (2) study of the chemistry of odor-producing compounds in fish oils, and (3) study of chemical reactions of fish-oil fatty acids.

Since our last report was made, a fourth project, which is concerned with the nutritional effects of fish oils, has been started. Progress in each of these four projects since the time of the last publication in this journal is summarized in the following paragraphs.

STRUCTURE AND ANALYSIS OF FISH-OIL FATTY ACIDS

Work on the structure and analysis of fish-oil fatty acids in the past year has involved two main lines of endeavor: (1) the concentration and purification of the principal polyunsaturated fatty acids of tuna and menhaden oils and (2) the complete determination of structure of the main polyunsaturated fatty acids.

CONCENTRATION AND PURIFICATION: The more tedious task, the isolation of pure individual polyunsaturated fatty acids, is nearing completion. Methyl esters of tuna and menhaden fatty acids have been subjected repeatedly to fractional distillation, urea fractionation, and low-temperature crystallization to obtain concentrates of relatively pure samples of individual acids. Separation into various chain lengths has been achieved mainly by distillation through a spinning-band column. Fractionation of each chain length into concentrates of the more important polyunsaturated acids has been achieved by repeated urea fractionations and fractional crystallizations. By these means, concentrates or relatively pure samples of C_{22} hexaenoic, C_{20} pentaenoic, and C_{18} and C_{16} tetraenoic acids have been prepared. In the course of the separation of these acids, the materials have been characterized by alkali-isomerization and chromatographic analyses.

Although C_{22} pentaenoic acid appears to be another important polyunsaturated constituent of some fish oils, a suitable concentrate of this acid from either menhaden or tuna oil has not been obtained. Other fish oils, therefore, are being investigated as possible sources of this acid.

STRUCTURE ANALYSIS: Considerable effort has been devoted to developing a technique for determining the locations of double bonds in polyunsaturated fish-oil

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[1] This study was supported by funds made available under the Saltonstall-Kennedy Act through a contract with the U. S. Fish and Wildlife Service, Bureau of Commercial Fisheries.

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fatty acids. A permanganate-periodate oxidation technique, developed in another laboratory for less unsaturated fatty acids, has been modified satisfactorily for the structure analysis of highly unsaturated fatty acids of fish oils.

A portion of quite pure methyl docosahexaenoate prepared from tuna oil was subjected to such structure analysis. The iodine value of this sample was 441.5 (theoretical 444.7). The purity of the material was established further by chromatographic analysis. By means of the permanganate-periodate oxidation technique, it was demonstrated unequivocally that this is a 4, 7, 10, 13, 16, 19, docosahexaenoate.

For the first time, it recently was reported by another laboratory that a docosahexaenoate of this structure is present in pilchard oil. The fact that this acid now has been shown also to be present in tuna oil is of interest from two points of view: first, apparently this is the only docosahexaenoic acid that occurs in appreciable quantities in ordinary fish oils; and second, the acid found in fish oils evidently is identical with that obtained from land animals.

Only a small amount of work was done on the phase of the project concerned with developing analytical techniques for the analysis of fatty acid composition of fish oils. It is planned, however, that in the near future, when additional quantities of the pure polyunsaturated fatty acids become available, the characteristics of the individual acids in alkali isomerization will be determined, and spectral constants for fish-oil analysis will be established. On the basis of work done during the past year, another possible technique for analysis of the fatty acid composition of oils has come to light, involving a combination of distillation, ozonolysis, and paper chromatography of the oxidation productions of ozonolysis. This method of analysis will also be investigated because, if successful, it will yield more complete results than does the alkali-isomerization method.

CHEMISTRY OF THE ODOR-PRODUCING COMPOUNDS IN FISH OILS

Much of the work done on odoriferous materials in fish oil has been concentrated on the isolation, separation, and identification of carbonyl compounds because such compounds previously have been found to be involved in the oxidative deterioration of fish oils and because they also have been reported to be the cause, at least in part, for the "reverted" odors of vegetable oils. The distillates obtained by vacuum-steam deodorization at 100°-120° C. (212°-248° F.) and also by flushing the oxidized oil with nitrogen at 35°-40° C. (95°-104° F.) have been examined. Column and paper chromatography have been used extensively in the separation and identification of the 2,4-dinitrophenylhydrazine derivatives of the carbonyl compounds. These methods have been supplemented by ultraviolet and infra-red spectrometry.

MONOCARBONYL COMPOUNDS: Monocarbonyl compounds that have been shown to be present in the steam-volatile distillates are formaldehyde, acetaldehyde, propionaldehyde, butyraldehyde, valeraldehyde, and hexaldehyde. Unsaturated aldehydes also have been detected.

UNKNOWN 2,4-DINITROPHENYLHYDRAZONE DERIVATIVES: The monocarbonyl derivatives, however, represent a relatively small proportion of the total 2,4-dinitrophenylhydrazones obtained. Most of the hydrazones could not be chromatographed on paper by any of a number of methods that were tried. Most of the nonmigrating compounds gave blue to violet colors when treated with strong alkali, suggesting that they are derivatives of the Greek letter "ALPHA" α-dicarbonyl compounds, but some of the nonmigrating fractions remain unchanged on treatment with alkali.

From ultraviolet spectra, it has been determined that the unknown 2,4-dinitrophenylhydrazone derivatives are quite different, in some cases, from those of known mono- and dicarbonyl derivatives. Efforts therefore are being made to separate and identify such carbonyl derivatives in a different manner. The volatile compounds from fish oils have been neutralized with sodium hydroxide and subjected to steam distillation to remove nonacidic compounds. These, in turn, have been treated with silver oxide to oxidize the aldehydes to acids, which again have been separated from the rest of the compounds by neutralization and steam distillation. Preliminary examination by paper chromatography has indicated the probable presence of acetic, propionic, and suberic or azelaic acids in the acidic fraction. Some mono- and dibasic acids that have not been identified have been detected in the acid fraction formed by oxidation with silver oxide.

These studies show that a variety of volatile, saturated and unsaturated, monocarbonyl and dicarbonyl compounds contribute to the odor of oxidized fish oils. Earlier studies showed that nitrogen compounds also contribute to the odor. Although the steam distillates from the oxidized oils have strong, pungent, disagreeable odors, they do not resemble the odor of the oxidized oil, and attempts to reproduce the odor of fish oil by diluting the distillates with bland mineral oil have not been successful. Thus, the compounds causing the characteristic "fishy" odor are altered or destroyed during steam deodorization.

The volatile compounds removed from oxidized fish oil by bubbling nitrogen through the oil at room temperature have therefore been collected by various devices, including passage of the nitrogen and accompanying volatile compounds through cold traps, 2,4-dinitrophenylhydrazine solutions, and charcoal. It has not been possible to deodorize completely fish oils in this manner, and only small amounts of volatile compounds have been collected. The eluate from the charcoal trap, however, has been found to contain the characteristic fishy odor. The fact that not all of the odor is removed by passage of the gases through 2,4-dinitrophenylhydrazine suggests that other compounds may be as important as are carbonyl compounds in the characteristic odor of fish oil.

A major part of the characteristic fishy odor produced by oxidation, resides, in extremely small amount, in the more volatile fractions. Our future efforts will be directed toward the fractionation, isolation, and identification of this material.

CHEMICAL REACTIONS OF FISH-OIL FATTY ACIDS

The aim in the study of the chemical reactions of fish-oil fatty acids is to prepare new derivatives from the fatty acids occurring in fish oils, mainly by taking advantage of their unsaturation. The work has involved a study of thiourea reactions, halogen reactions, and fractionation by means of sulfur dioxide.

THIOUREA REACTIONS: The incidental finding mentioned in the previous report in this journal--that thiourea reacts with hydroperoxides of autoxidized fatty esters--has been investigated further. In menhaden oil, peroxide values up to 500 can be reduced to about 20 by treatment with a solution of thiourea in methanol at 0° C. (32° F.) or at room temperature. By this simple procedure peroxides are converted into unsaturated hydroxy acids, while unsaturated components that have not been autoxidized remain unchanged.

By using purified hydroperoxides of methyl oleate and linoleate, we found that two peroxidic groups react with one thiourea molecule. In the course of the reaction, the latter is converted into amino-imino-methane-sulfinic acid. Under these mild conditions, between 90 and 95 percent of the peroxidic groups are reduced, but the residual peroxides are not eliminated by repeating the reaction. Apparently, different types of peroxides are present, or rearrangement takes place in the purified hydroperoxides to prevent part of them from reacting with thiourea. The resulting sulfinic acid, and to some extent thiourea itself, is not stable in solvents at 50° to 70° C. (122°-158° F.). When the reaction is forced to completeness at elevated temperatures, sulfurized compounds are formed from the secondary products and from the highly unsaturated esters.

HALOGEN REACTIONS: Although halogenated fatty acids often are used to characterize unsaturated lipides, they have found little practical application. Fish oil would be an excellent source of polyhalogenated lipides. Instead of following the usual procedures by which halogens are introduced into unsaturated lipides, we used reactions of chlorinated or brominated carbon compounds. The reactions of double bonds with dihalocarbenes, which so far had been studied only with olefins of low molecular weight, have been applied to olefins derived from fatty acids and to unsaturated fatty alcohols and fatty acid esters. Chloroform or bromoform, when reacted with potassium t-butylate, yields potassium salt and dichloro- or dibromocarbene. The latter are highly reactive, short-lived reactants that add to double bonds to form derivatives of dichloro- or dibromocyclopropane. Octadecene-1, for example, is converted in good yield into n-hexadecyl-2,2-dichlorocyclopropane. The same reaction can be carried out with oleyl alcohol or oleic acid esters where the double bonds are in the 9,10 position. Both double bonds of methyl linoleate can be reacted, and 50 percent of the double bonds of menhaden-oil fatty acid methyl esters have been converted into dichlorocyclopropane rings. Crude menhaden oil, as received for the purposes of this project, was reacted without drying it or subjecting it to preparatory processing. By varying the conditions, we could regulate the extent of the reaction, and glyceride mixtures were obtained in which 20 to 50 percent of the double bonds were consumed.

The halogen atoms in the cyclopropane ring are remarkably resistant to chemical agents. In accordance with their lowered level of unsaturation, the products either autoxidize much more slowly than do the original oils, or do not autoxidize at all. They are oily liquids like the unsaturated materials from which they have been derived but are heavier than water.

FRACTIONATION IN SO_2: Fish oils contain a considerable amount of saturated fatty acids, and any efficient and economical process of refinement that will eliminate them may enhance the commercial value of the oils. Crystallization, with or without solvent, or liquid-extraction processes are the conventional means for lipide fractionation. Separations are particularly difficult with fish oils because of the great variety of chain lengths occurring in them and the various degrees of unsaturation of their components. For several decades, petroleum chemists have used sulfur dioxide to refine hydrocarbons, achieving thereby separation of the saturated from the unsaturated cyclic and hetero-atomic hydrocarbons. The literature reveals little on fractionation of lipides by means of SO_2, and a study of this method of fractionation therefore has been undertaken.[2]

The curves of solubility versus temperature of the common fatty acids and esters in SO_2 were determined, which then made possible the selection of optimal temperatures for fractional extractions of mixtures. The preparative experiments were carried out with menhaden oil or distilled fractions of it, for which we had detained analyses from previous work. Sulfur dioxide proved to be at least as efficient as were the common organic solvents for such fractionations. Menhaden-oil fatty acid esters, I. V. 151.5, could be separated by a one-step procedure into a saturated fraction, I. V. 1.2, and an unsaturated fraction, I. V. 179. Methyl esters of the C_{18} fraction of menhaden-oil esters, I. V. 105, were split into fractions having I. V. 1.4 (m.p. 36.5°-37° C.) and I. V. 134, respectively. As expected, traces of oleate, linoleate, and linolenate could be demonstrated by paper chromatography in the saturated fraction, but stearate could not be found in the unsaturated one. The amount of stearate had been assayed previously by paper chromatography to be about 25 percent, while in this separation, 22 percent was isolated. In another experiment, crude menhaden oil, I. V. 177, was separated into glyceride fractions having I. V. 112 and 210.

The structures of the lipides are not changed by the treatment with SO_2, and recoveries are virtually 100 percent. Sulfur dioxide is not flammable, and it has a

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[2] A detailed report on this investigation will be published in the near future.

boiling point of -10° C. (14° F.). It can be handled at low temperatures like any organic solvent, and it is easily recovered. For the laboratory investigator, its pungent odor makes efficient ventilation necessary. The use of SO_2 on a large scale in processing and at the same time as a refrigerant has been mastered with hydrocarbons. The use of it with lipides should not present any greater problem.

NUTRITIONAL EFFECTS OF FISH OILS

Our study on the nutritional effects of fish oils was started in the summer of 1957. It consists of three phases: (1) a determination of whether fish oils contain significant quantities of essential fatty acids, (2) a study of the effects of the ingestion of fish oils in relation to atherosclerosis, and (3) an investigation of nutritive factors in the minor constituents of fish oils.

ESSENTIAL FATTY ACIDS IN FISH OILS: When rats are fed a fat-free diet, their growth is greatly retarded, and certain other deficiency symptoms develop, including scaly skin and loss of hair. These symptons are relieved by the addition to the diet of certain fatty acids or fatty esters, especially those of linoleic and arachidonic acids. Linolenic acid restores growth but does not alleviate the dermal symptoms. Fatty acids that are effective, partially or wholly, in relieving the symptoms are termed "essential fatty acids" (EFA).

The common belief, apparently based on little if any critical experimental work, is that fish oils contain very little essential fatty acids. The complete absence of essential fatty acid activity in fish oils would be surprising, and wholly unanticipated, on the basis of the results of structure studies of fish-oil fatty acids such as those described above.

Several commercially-important fish oils are being fed to EFA-deficient rats, and the effects of these fish oils on growth and deficiency symptoms are being observed. Concentrates of several individual fatty acids occurring in fish oils are also being fed to EFA-deficient rats, and the effects of these fatty acids are being noted. Should it develop that some of the individual fatty acids exhibit a high degree of EFA activity but that the fish oil themselves are lacking in this quality, an investigation will be made to ascertain what is causing the inhibition of the EFA activity in the natural oils.

In a preliminary study, the methyl esters of tuna-oil acids were prepared by conventional methods and separated into fractions consisting of methyl esters of 16, 18, 20, and 22 carbon atoms. These fractions then were fed to EFA-deficient rats. All of the fractions restored growth, but to date, they have not alleviated the dermal symptoms.

EFFECT OF FISH OILS AND FISH-OIL FATTY ACIDS IN ATHEROSCLEROSIS: Atherosclerosis is the basic cause of more than half of the human deaths occurring in the United States. Recent work has tended to link the degree of saturation of ingested fat with the deposition of cholesterol in the arteries. It has been reported that unsaturated vegetable oils that contain large amounts of linoleic acid lower blood cholesterol in animals and humans when incorporated in the diet. Since the polyunsaturated fatty acids of fish oils are considerably more unsaturated than are those of the common vegetable oils, it appears feasible to investigate their effect on blood levels of cholesterol and on other blood lipides.

Miniature pigs and rats will be used as the experimental animals. The pigs will be fed a special diet designed to elevate the amount of cholesterol in the blood and then will be fed diets containing representative fish oils. The results of such fish-oil supplementation will be compared with the effects produced by vegetable oils. Similar experiments will be conducted in which hypercholesteremic rats will be fed concentrates of individual fish-oil fatty acid esters. The experiments with rats are already under way, but results are not yet available.

<u>NUTRITIVE VALUE OF MINOR CONSTITUENTS OF FISH OILS</u>: It has been suggested that the unsaponifiable fractions of fish oils contain nutritionally important minor constituents in addition to vitamins A and D. In experiments to test this possibility, control rats will be fed synthetic diets in which all the fat consists of resynthesized glycerides from purified fatty acids free from unsaponifiables. This basal diet will be supplemented for experimental groups of rats with fish-oil unsaponifiables. The effects of such diets on growth, reproduction, lactation, and other physiological activity will be noted.

This third phase of the program will not be studied until some time in the second year of the project. It will be looked upon as secondary to the other two phases. Possibly only a preliminary investigation can be made.

LITERATURE CITED

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FISH-OIL RESEARCH AT THE SEATTLE FISHERY TECHNOLOGICAL LABORATORY

By Phillip A. Hart

Fish-oil research at the Seattle Technological Laboratory has been directed primarily toward utilization of the unique features of unsaturation found in fish oils. (This article appeared in Commercial Fisheries Review, April 1958, and is now available as Sep. No. 508.)

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PROGRESS ON STUDIES IN UTILIZATION OF FISH-OIL DERIVATIVES IN ORE FLOTATION

By S. R. B. Cooke

This paper describes the flotation process, the mechanism of collection, and the flotation of iron ore with fish-oil derivatives as collectors. It summarizes experimental findings to date and outlines future work. (This article appeared in Commercial Fisheries Review, January 1958, and is now available as Sep. No. 499.)

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