Gas Chromatographic-Mass Spectrometric Analysis of Isoprenoid Hydrocarbons and Fatty Acids in Shark Liver Oil Products

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Abstract

The liver oil from the South American Basking shark has been fractionated by silica gel chromatography and analyzed by the new method of combined gas-chromatography mass spectrometry. The major compounds of the nonsaponifiable fraction are pristane and squalene, which account for 7.6 and 31.3% of the oil. The saponifiable fraction contains normal fatty acids from C_{14} to C_{22}; the four major components are palmitic, oleic, and the monounsaturated eicosenoic and docosenoic acids. No correlation was observed between the hydrocarbons (essentially all isoprenoid derivatives) and the fatty acids (essentially all normal) of this oil.

The same treatment was applied to a sample of commercial pristane which was obtained from Basking shark liver oil. It was found to contain about 1% phytane and small amounts of octadecane, nonadecane, and methyl and ethyl palmitates. Mass spectral data for squalene are presented for the first time along with the low electron energy-mass spectra for pristane and phytane.

Introduction

FISH LIVER OILS, in general, have a low content of unsaponifiable matter (1–2%) and relatively large amounts of unsaturated fatty acids (≅50%), of which the main components are highly unsaturated eicosenoic and docosenoic acids (14).

The unsaponifiable fraction in the marine animal oil extracted from the liver fats of Elasmobranchii species ranges from 0.3 up to 80%; the lipid content is of marked monoethenoid character. The higher content of unsaponifiable matter is found within the squalidae family, where it ranges from about 33 to 80% (13,16).

Although it is not always present (5,12) and is randomly distributed among shark families, squalene, whenever found, seems to be the major component of the hydrocarbon fraction (5,6,11,12,30). It has been reported that the liver oil of a Formosan shark contains as much as 87.5% of unsaponifiable matter, of which 84% was mainly squalene (11). Smaller proportions of pristane (4,27,29,32) and zamene (3,4,7,27,31) have also been reported.

Phytane has been shown to be essentially absent from living organisms. There is only one report of its isolation from a living system (8) and none from marine sources. It should be noted that the absence of phytane in living organisms which contain pristane is in contrast with the fact that the C_{20} isoprenoid is usually found together with pristane in petroleum crudes, sediments, and other samples (9,19,21–23).

Because there is current interest in the distribution and formation of isoprenoid hydrocarbons and since there are no mass spectrometric data on the composition of shark liver oil, the hydrocarbons and fatty acids from this oil have been analyzed by the new technique of combined gas capillary chromatography-mass spectrometry (22,26). Furthermore, because of the relatively high concentration of pristane found in this oil and the failure to detect phytane, it became of interest to extend the search for the C_{20} isoprenoid to the analysis of pristane derived by distillation from Basking shark liver oil.

Experimental Procedures

One gallon of raw Basking shark liver oil from the South American coast was obtained from the Aristo Oil Products Company, and a few milligrams of the sample were taken for analysis. The sample was fractionated by means of silica gel-column chromatography according to the techniques described in detail elsewhere (18,19,21,22). Three fractions were collected, of which the methanol fraction, containing the fatty acids and other lipids, and the *n*-pentane fraction, containing the aliphatic hydrocarbons, were analyzed by both gas chromatography and gas chromatography-mass spectrometry (22,26). The methyl esters of the total fatty acids liberated by alkaline hydrolysis (20) were prepared for gas-chromatographic analysis according to a method described previously (15). The benzene fraction was analyzed by gas chromatography for its aromatic content, but no compounds other than squalene were found in appreciable concentrations.

It was not possible to collect all of the unsaponifiable matter in only one fraction. In order to show all of the aliphatic hydrocarbons in one single chromatogram, a portion of the shark oil was analyzed without any previous fractionation.

A sample of pristane, isolated as a by-product from Basking shark liver oil (24) with the commercial name of Robuoy, was obtained from Robeco Chemicals Inc., New York. In this case a fractionation by distillation was carried out. A spinning band column was used for the distillation of about 600 ml of the original product, and a total of nine cuts was collected within the temperature range of the initial boiling point and 171.5C.

The chromatographic analyses were carried out on an F&M Model 810 and a Barber-Colman Series 5000 Gas Chromatograph, both equipped with flame ionization detectors.

The following stainless steel open tubular columns were used in this work: a) a column of 90 m × 0.076 cm I.D., coated with Polysev (m-bis-m-(phenoxy-phenoxy)-phenoxy benzene); b) a 300 m × 0.075 cm I.D. column, coated with Polysev; c) a column of 30 m × 0.025 cm I.D., coated with 10% Apiezon L (a high-temperature grease); d) a 150 m × 0.05 cm I.D. column, coated with OV-17 (methyl phenyl silicone); e) a 150 m × 0.076 cm I.D. column, coated with Igepal (CO 990) (nonyl-phenoxy polyoxethylene ethanol). Two short 1.7 m × 0.3 cm glass columns,

packed with SE-30 (methyl silicone) and OV-1 (methyl silicone fluid), were also used.

The combined gas chromatographic-mass spectrometric analyses were performed on an LKB 9000 gas chromatograph-mass spectrometer (26). The sample of squalene used as a standard was obtained from Eastman Organic Chemicals.

Thin-layer chromatography was run on glass plates, spread with Silica Gel G (Stahl) and heat-activated at 100C for 1 hr. The plates were developed in unlined tanks by the ascending method. Petroleum ether-glacial acetic acid (90:10:1) was used as solvent. Components were identified by their R_f values.

Results

Monoethenoid fatty acids of even carbon number from C_{18} to C_{22}, together with the saturated n-C_{16} fatty acid, were observed as the major components of the lipid fraction of Basking shark oil. The relative percentage composition was close to 18.5 for each of the monounsaturated C_{18}, C_{20}, and C_{22} acids and was 20.3 for palmitic acid. Myristic, palmitoleic, and stearic acids were present in relative concentrations of 5.5, 6.8, and 3.1 respectively. Some polyunsaturated C_{18} fatty acids were detected in much lower percentages (<1%). The identities of these fatty acids were based on their GC retention times and on the GC-mass spectrometric analysis. Their mass spectra were identical to those described in the literature (25,28). All components of the methanol fraction identified by these techniques were normal unbranched fatty acids. The total fatty acid content of the shark liver oil was determined as 33.8%.

The qualitative and quantitative results agree quite well with those previously reported for the same shark species from the South African coast (16) except for the fact that, in the present work, the C_{20} saturated and the C_{24} unsaturated fatty acids have not been found and there seems to be an appreciable deviation on the concentration of the saturated C_{16} and the unsaturated C_{18} fatty acids. It may be pointed out that the distribution found closely resembles that reported for the small spotted dogfish (17).

The nonsaponifiable hydrocarbon fraction consists of only two major peaks, which account for about 38.6% of the total oil. Quantitative data on this fraction were obtained from 18.0 mg of whole Basking shark liver oil by analyzing directly a small aliquot on a stainless steel column, coated with OV-17 (Fig. 1). The first peak in the chromatogram, which represents 7.6% of the total amount of oil, has the same retention time as pristane. Its identity was verified by means of the retention data on four different stationary phases (Polysev, Apiezon L, OV-17, and OV-1) and by use of an internal standard of known concentration. Final proof of the structure was obtained from its mass spectrum, which corresponds to that of pristane, as shown on Fig. 2 (1,10).

It is important to mention at this point that the application of low electron energy-mass spectrometry (12–20 eV) provides a remarkable enhancement in the intensities of the characteristic fragment ions at m/e 113 (base peak) and m/e 183 as well as for that of the parent ion (m/e 268). Although, at high electron energies, the parent ion of pristane can have a relative intensity as low as 0.2% of the base peak (m/e 57) (1,10), it is shown with an intensity close to 2.5% of the base peak at 20 eV.

The first analysis that was carried out on this fraction showed a small peak eluted after pristane,

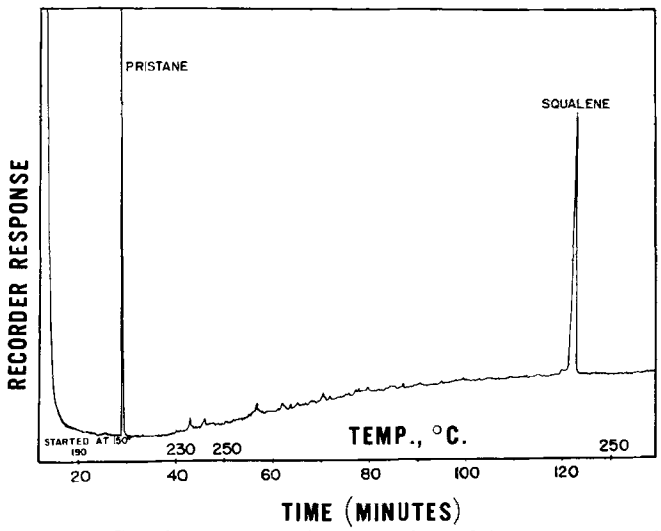

FIG. 1. Gas-chromatographic separation of hydrocarbons of Basking shark liver on a 150 m × 0.05 cm I.D. stainless steel tubing, coated with OV-17. Nitrogen pressure 1050 gr/cm², no split. Barber-Colman series 5000 gas chromatograph, equipped with a flame ionization detector. Range ×5; attenuation, 10. Temperature programmed at 2C per minute from 150 to 250C; 1/1000 of the sample injected. Pristane: 2,6,10,14-tetramethyl pentadecane. Squalene: 2,6,10,15,19,23-hexamethyl tetracosa-2,6,10,14,18,22-hexaene.

which appeared to be one of the isomeric C_{19} olefins, but the mass spectrum is somewhat unreliable in this case because of the small amount in which it was present. Its presence would not seem surprising especially since the isomeric C_{19} olefins, known by the collective name of "Zamene," have been found in several marine organisms including the Basking shark (3,4). The fact that, upon standing, this peak disappeared would also be in favor of the tentative mass spectrometric identification since, by reduction, it would be transformed into pristane (29,31). It is known that these oils have a great tendency towards saturation or hydrogenation (17).

The gas-chromatographic retention data (Fig. 1), together with the mass spectrum (Fig. 3) obtained from the second major component of Figure 1, confirmed the presence of squalene in Basking shark liver oil. This component represents 31.5% of the oil.

In agreement with these results, the thin-layer chromatograms show compounds with R_f values corresponding to hydrocarbons and glycerides (the sample does not contain free fatty acids). In addition, they indicate the possible presence of cholesterol in low amounts and some fatty alcohols as previously reported (27).

Phytane was not detected in this oil, but direct gas-chromatographic analysis of the sample of commercial pristane proved the presence of phytane in a concentration of the order of 1% of the total amount of pristane (Fig. 4), which in turn accounts for almost 99% of the whole product.

The first cuts obtained from the distillation process represented fractions enriched in the light-weight

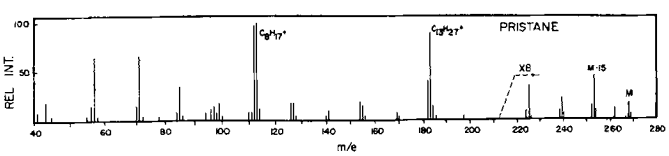

FIG. 2. Mass spectrum of Basking shark liver oil pristane. It was taken as it was eluted from a 1.7 m × 0.3 cm I.D. glass column, coated with SE-30, and it was ionized by electron impact at 20 eV when it entered the ion source of the LKB 9000 gas chromatograph-mass spectrometer.

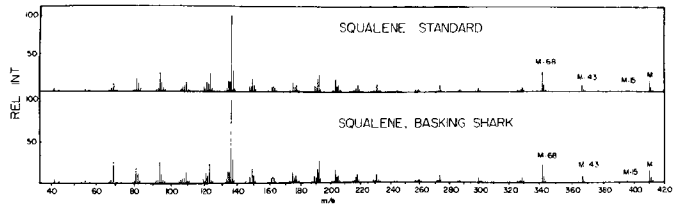

FIG. 3. Squalene standard: spectrum taken as the sample was eluted at 270C from a short glass column, packed with SE-30. It was ionized by electron impact in the ion source of an LKB 9000 gas chromatograph-mass spectrometer at 70 eV. The ionizing current was set at 65 μA and the accelerating voltage at 3.5 KV. The electron multiplier voltage was 2.5 KV.

Basking shark liver oil squalene: spectrum taken under exactly the same conditions as those described for the standard. About 2/1000 of the sample, at a concentration of 14.1 mg/ml, was injected.

materials; pristane was the major component, as shown by gas chromatographic analysis. For instance, the gas chromatograph of the first cut showed only two peaks; retention times corresponded to pentadecane and pristane.

The cuts of most interest were centered among the last fractions. Two of them in particular (No. 7 and 8) showed a significant increase in the relative concentration of phytane, which allowed its mass spectrometric identification as well as a preliminary measurement of its optical activity.

One of these distillation cuts (No. 8) was analyzed by gas chromatography-mass spectrometry (26). It was found to contain 28.6% of pristane and 55.4% of phytane plus small amounts of octadecane, nonadecane, methyl hexadecanoate, and ethyl hexadecanoate. Of the total amount of phytane in the commercial product, 0.17% was present in this particular distillation cut. The mass spectrum of phytane is given in Fig. 5.

As in the case of pristane, this spectrum shows too the enhanced intensities of the parent and characteristic fragment ions typical in low energy mass-spectrometry.

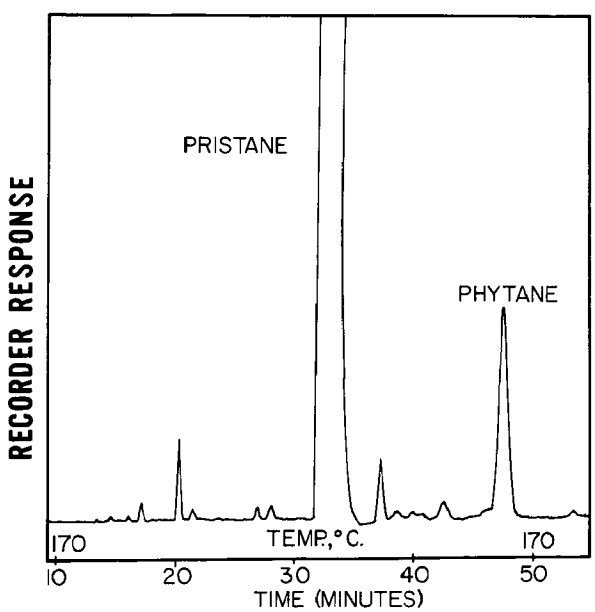

FIG. 4. Gas-chromatographic separation of the hydrocarbons of "Robuoy Pristane" on a 300 m × 0.076 cm I.D. stainless steel capillary column, coated with Polysev (m-bis-m(phenoxy-phenoxy)-phenoxybenzene) by using a Barber-Colman 5000 apparatus, equipped with a flame ionization detector. Hydrogen pressure 1400 g/cm^2. Isothermal at 170C. About 0.2 μl of the original sample was injected.

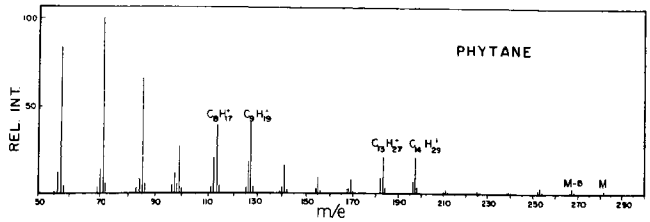

FIG. 5. Mass spectrum of phytane taken as the component was eluted from a Polysev column (Figure 4). Helium pressure 1020 g/cm^2. It was ionized by electron impact at 20 electron volts as it entered the ion source of the LKB 9000 gas chromatograph-mass spectrometer. The ion source temperature was 290C. Ionizing current, 70 μA. Accelerating voltage, 3.5 KV.

The optical activity of cut No. 7 was measured by means of a Rudolf Polarimeter. Two blanks of n-heptane were used as reference. Only a weak specific rotation of about +1.1° was observed at 200–220 mμ and 20C.

Discussion

There seems to be no direct correlation between the fatty acids of the Basking shark liver oil and the hydrocarbons. The identities, nature, and concentrations of the fatty acids as well as those of the unsaponifiable matter are well within the ranged expected for the natural abundance of those compounds in the liver oils of this marine organism.

The relatively small differences found in the concentrations of the fatty acid and unsaponifiable components, when compared with other reported data, can be explained by natural variations between species and by different diets and environmental conditions which affect the formation of these oils.

Phytanic acid, a possible biogenic precursor of pristane, has not been found among the fatty acids, an observation which supports the view that sharks do not synthesize but rather accumulate this hydrocarbon (2,4,27).

It appears that pristane, together with the small amounts of the unsaturated isoprenoid C_{19} isomer, comes from the marine organisms on which the sharks feed (2,3,27), such as the zooplankton. Indeed, it has been reported that copepods are the immediate source of pristane in the liver oils of sharks and whales (2,4). The isomeric monoolefins with the skeleton of pristane are present too in marine zooplankton, fishes, and mammals (3).

The concentration of both isoprenoids, pristane and phytane, in the shark oil products analyzed is remarkably high. Pristane has been found before only as a minor constituent in this type of liver oils (4,27,29), and phytane is considered to be absent both in zooplankton (2–4) and in Basking shark oil (4, this report).

The primary source of pristane is presumed to be the phytyl group of the chlorophyll from marine photo-synthetic microorganisms (2–4,27). Furthermore it is known that phytol can be easily dehydrated to phytenes and phytadienes, both of which by hydrogenation could yield phytane (1,4). For this reason the presence of phytane as a minor component in samples which were obtained from marine animals should not be totally unexpected.

Since the search for phytane has yielded negative results in raw oils, its presence in a commercial distillation product of Basking shark liver oil could be accounted for by various sources of the oils or by the possibility that phytane is generated from certain

precursors in the shark oil during the distillation process used for the preparation of commercial pristane.

Although the necessary precautions were taken, the quantitative data should be considered as a lower limit in all cases owing to the possible losses by volatilization during the preparation and analysis of the sample.

The nonsaponifiable matter and the total fatty acid content of the oil represent 72.7% of the total. This leaves a maximum 27.3% to be accounted for, which most likely is made up by cholesterol fatty alcohols and glyceryl ethers. These compounds are known to be minor constituents of Elasmobranchii liver oils (14,27).

No attempts were made toward a positive identification of all these components by the techniques described herein. Their presence in the oil is considered irrelevant to the main purpose of this report, which, as stated, was directed toward the unequivocal characterization of isoprenoid hydrocarbons and the search for their possible biogenic precursors in shark liver oil.

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Advice and help in various phases of this work provided by D. W. Nooner, T. G. Tornabene, and A. Zlatkis. This work was supported in part by NASA Grants NsG-257 and NGR 44–005–020.

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