Composition of the diacyl glyceryl ethers and triglycerides of the flesh and liver of the dogfish (*Squalus acanthias*)

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SUMMARY The major lipids of the dogfish (*Squalus acanthias*) are the diacyl glyceryl ethers and triglycerides. These classes of compounds in the flesh (dorsal section) and liver were separated by thin-layer chromatography. The glyceryl ethers and/or fatty acids resulting from saponification of each of these fractions were analyzed by gas–liquid chromatography as their isopropylidene and methyl ester derivatives, respectively.

Few significant differences were apparent 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 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 C_{22} monoenoic acids. Although the triglycerides of the flesh contained more of the polyenoic acids than the liver, the differences in this fraction between the flesh and liver were less striking. It appears that the over-all unsaturation of the dogfish lipids is largely governed by the relative amounts of monoenoic and polyenoic acids in the C_{20} and C_{22} series.

KEY WORDS diacyl glyceryl ethers · fatty acids · triglycerides · dogfish (*Squalus acanthias*) · flesh of dogfish · liver of dogfish · acetonation of glyceryl ethers · thin-layer chromatography · gas–liquid chromatography

THE GLYCERYL ETHERS found in fish usually occur together with triglycerides as diacyl glyceryl ethers in which the hydroxyl groups on the 1- and 2-positions of the glycerol moiety are esterified with fatty acids (1). Almost two decades ago, Karnovsky et al. (2, 3) reported the presence of large quantities of glyceryl ethers in the liver and body oils of a number of elasmobranch fish and other marine animals. Lovern (1, 4) also investigated the liver oils of these fish and reported an unusual fatty acid composition. Several workers, using gas–liquid chromatography (GLC), recently studied the glyceryl ethers (5–7) and fatty acids (6) liberated by saponification of the liver oils of several elasmobranch fish. The liver lipids were characterized by large quantities of monoenoic acids and small amounts of polyenoic acids of C_{20} and C_{22} chain lengths. In recent studies of the dogfish (*Squalus acanthias*), however, it was shown that the percentage of polyenoic acids was unusually high in the lipids of the flesh.[1]

The question still remained whether the C_{20} and C_{22} polyenoic acids, so characteristic of the body oils of fish, are typical of only certain lipid classes. It was of interest, therefore, to investigate the distribution of these acids between the diacyl glyceryl ethers and triglycerides of the flesh and to compare the distribution with that of the liver. In order to extend present knowledge in the glyceryl ether composition of elasmobranch fish, a similar comparison was made between the ether chains of the flesh with those of the liver. Accordingly, the lipids were extracted from the flesh (dorsal section) and liver of the dogfish (*Squalus acanthias*) and analyzed by thin-layer chromatography (TLC) and GLC.

MATERIALS AND METHODS

Samples

Four dogfish (*Squalus acanthias*) were caught by line and tackle in Puget Sound in December. Flesh samples were obtained by excising a square section immediately

[1] J. Olley and W. H. R. Duncan, manuscript in preparation.

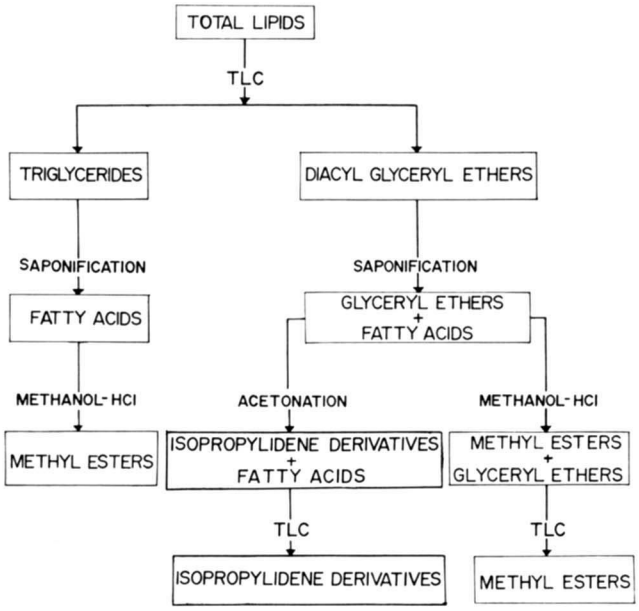

FIG. 1. Flow diagram illustrating methods for the separation and analysis of diacyl glyceryl ethers and triglycerides of *Squalus acanthias*.

ahead of the dorsal fin down to the lateral line. The livers and the flesh samples were then homogenized in a blender, and the lipids from a 100 g sample of each homogenate were extracted according to the procedure of Bligh and Dyer (8). The oils were then immediately sealed in bottles under nitrogen and frozen prior to analysis.

Thin-Layer Chromatography

Although diacyl glyceryl ethers and triglycerides are not easily resolved on columns of adsorbents, discrete separations of milligram amounts are readily obtained on thin layers of silica gel (5, 9).

The flow diagram shown in Fig. 1 illustrates the methods used in the separation and analysis of the diacyl glyceryl ethers and triglycerides. TLC was carried out on Silica Gel G (E. Merck AG, Darmstadt, Germany) (10). The samples (2–8 mg), dissolved in diethyl ether, were applied 2–3 cm from the edge of the plate as a series of closely spaced spots and chromatographed for 30–40 min in a tank that was lined on three sides with filter paper, the lower edge of which was beneath the solvent: petroleum ether (bp 30–60°)–diethyl ether–acetic acid 90:10:1 (v/v/v). Chimyl diolein (a gift from Dr. H. K. Mangold, Hormel Institute, Austin, Minn.) and triolein were run as standards on the same plate. The lipids were detected by *lightly* spraying the plate with 2′,7′-dichlorofluorescein in 95% ethanol and viewing it briefly under ultraviolet light (9). The silica gel containing the desired fraction was scraped off the plate and the lipids were eluted with diethyl ether.

Figure 2 shows the fractionation of 85 μg of the liver (*A*) and flesh (*D*) lipids of *Squalus acanthias*. It is noteworthy that a greater separation was obtained between the diacyl glyceryl ethers and triglycerides from the liver than from the flesh. On the basis of the later analyses by GLC, this difference in separation can be attributed to the increased polarity imparted to the diacyl glyceryl ether fraction of the flesh by large amounts of polyunsaturated fatty acids. Also demonstrated in Fig. 2 are reruns of the respective fractions isolated for analysis by GLC. These fractions had been obtained by TLC of 2.5 mg of sample (30 spots of 85 μg each) on a single 20 × 20 cm plate.

By use of techniques similar to those described by Krell and Hashim (11), the presence of ester groups in the triglycerides and diacyl glyceryl ethers was unequivocally established by examination of the 1742 cm^{-1} band of the ester carbonyl stretching vibration. The diacyl glyceryl ethers were also characterized by an additional band at 1125 cm^{-1} attributable to the absorption of the alkyl ether group.

The over-all composition of the classes of lipids in the flesh and liver is given in Table 1. The percentage com-

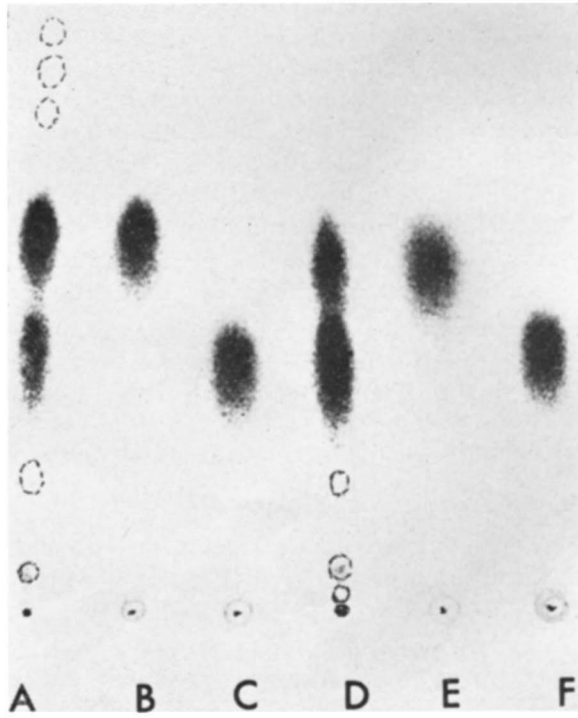

FIG. 2. Thin-layer chromatography of the neutral lipids of *Squalus acanthias* on Silica Gel G. Solvent: petroleum ether (bp 30–60°)–diethyl ether–glacial acetic acid, 90:10:1 (v/v/v). Indicator: sulfuric acid spray followed by charring. *A*, dogfish liver oil (85 μg); *B*, diacyl glyceryl ether fraction from the liver oil (50 μg); *C*, triglyceride fraction from the liver oil (50 μg); *D*, flesh oil (85 μg); *E*, diacyl glyceryl ether fraction from the flesh oil (50 μg); and *F*, triglycerides from the flesh oil (50 μg).

TABLE 1 THE COMPOSITION OF THE LIPIDS FROM THE FLESH AND LIVER OF *Squalus acanthias*

Class	Flesh	Liver
	wt %	
Cholesterol	tr.	1.8
Diacyl glyceryl ethers	35.2	45.1
Triglycerides	49.0	46.6
Phospholipids	8.5	1.3
Free fatty acids	5.3	3.8
Unknown	1.8	1.3

position of the "neutral lipids" (including free fatty acids) was determined by densitometry of the charred spots according to the method of Privett and Blank (12). The phospholipid content was obtained by column chromatography using the technique described by Dhopeshwarkar and Mead (13).

Preparation of Derivatives

Triglycerides and diacyl glyceryl ethers, isolated by TLC, were saponified under an atmosphere of nitrogen in 0.5 ml of 10% KOH for 30 min. After acidification, the mixtures were examined by TLC using petroleum ether–diethyl ether–acetic acid 90:10:1 and 60:40:1. Fatty acids from the triglycerides, and fatty acids and glyceryl ethers from the diacyl glyceryl ethers, were the only classes of compounds detected.

Methyl esters were obtained from fatty acids by reaction with methanol–HCl, and isopropylidene derivatives of glyceryl ethers were formed by acetonation (7). In the preparation of both derivatives, the reactions were conducted on the crude mixtures and the final products were obtained by TLC (Fig. 1). A rapid acetonation reaction was achieved at room temperature by dissolving several milligrams of crude glyceryl ethers in 5 ml of acetone and adding one drop of perchloric acid. After the reaction had proceeded for 30 min, it was terminated by adding water. The mixture was extracted with diethyl ether, the organic phase was washed several times with water and then dried with sodium sulfate. Yields in excess of 90% of isopropylidene glyceryl ethers were obtained.

Gas–Liquid Chromatography

Analyses by GLC were carried out with a Research Specialties instrument (Series 600) equipped with a 6 ft × 1/4 in. stainless steel column packed with 5% diethylene glycol succinate polymer (DEGS) (Wilkens Instruments, Walnut Creek, Calif.) on Anakrom ABS (70–80 mesh) (Analabs, Incorporated, Hamden, Conn.). The methyl ester and isopropylidene derivatives were analyzed on the same column, before and after hydrogenation, at a column temperature of 170° and a flow rate of 40 ml of argon per minute. Peaks were identified by use of standards and logarithmic plots of relative retention times vs. chain lengths or degrees of unsaturation. The percentage of each component was determined by triangulation.

Results of the analyses by GLC of standard mixtures of methyl esters and isopropylidene glyceryl ethers agreed with the known composition within 2%. The following standards of methyl esters were used: 16:1, 18:1, 20:1, and 22:1 (Hormel Foundation model mixture); 14:0, 16:0, 16:1, 18:0, and 18:1 (National Heart Institute Standard "D"); and 16:0, 18:0, 18:1, 18:2, 18:3, and 20:5 (Bureau of Commercial Fisheries Technological Laboratory standard). Various loadings of standards of isopropylidene glyceryl ethers (e.g., 16:0, 18:0, and 18:1) confirmed a previous report (7) that β-ionization detectors give a linear response to these compounds.

The column resolution (14) of isopropylidene derivatives of glyceryl ethers on commonly used polar phases, such as DEGS, is comparable to that obtained with methyl esters on the same column (Fig. 3). Under the conditions of GLC described, saturated isopropylidene derivatives were eluted from the column before the corresponding unsaturated compounds. Figure 3 illustrates a typical separation of isopropylidene derivatives obtained from the flesh lipids.

Both the methyl esters and isopropylidene glyceryl ethers were dissolved in ethanol and hydrogenated over platinum oxide. The resulting saturated compounds were then analyzed by GLC. This procedure, aside from providing a check on the chain lengths of the unsaturated derivatives, was particularly valuable for detecting branched-chain compounds that overlapped unsaturated straight-chain compounds. The presence of significant amounts of these isomers, both saturated and unsaturated, in the liver oil of *Squalus acanthias* was reported previously by Hallgren and Larsson (6).

TABLE 2 GLYCERYL ETHERS IN THE FLESH AND LIVER OF *Squalus acanthias*

Sample	Composition of Long-Chain Ether Group															
	14:0	15br	15:0	16br	16:0	16:1	17br	17:0	17:1	18br	18:0	18:1	18:2	19*	20:0(?)	20:1
	wt %															
Flesh	4.8	0.9	1.0	0.8	22.9	13.2	0.5	0.8	0.8	0.3	2.8	47.3	0.7	0.3	0.1	1.9
Liver	2.6	0.6	0.7	0.7	13.4	10.2	0.9	0.3	1.1	0.7	3.9	61.4	tr.	0.8	0.2	2.3

* Includes both saturated and unsaturated compounds.

RESULTS

Glyceryl Ethers

Diacyl glyceryl ethers were found in large amounts in the lipids from both the flesh and liver of the dogfish from Puget Sound (Table 1).

No qualitative differences were apparent between the ether moieties of the glyceryl ethers from the flesh and liver (Table 2). There was a higher percentage of chimyl alcohol (16:0 ether) in the flesh than in the liver. The flesh, however, contained significantly less selachyl alcohol (18:1 ether). No evidence was obtained from the analyses of the hydrogenated isopropylidene derivatives by GLC to indicate the presence of more than a possible trace of C_{22} glyceryl ethers in either the flesh or liver samples. The docosenyl glyceryl ether reported by Hallgren and Larsson (6) to be present in the livers of *Squalus acanthias*, as well as in several other elasmobranch fish from Scandinavian waters, was not detected in the present investigation (Table 2). It is possible, however, that trace amounts (<0.1%) would escape detection by the method of analysis employed. It is noteworthy that a docosenyl glyceryl ether was found in only trace amounts by Guyer et al. (15) in their studies on the liver oil of *Squalus acanthias* from Puget Sound. Pronounced differences in the composition of glyceryl ethers of fish of

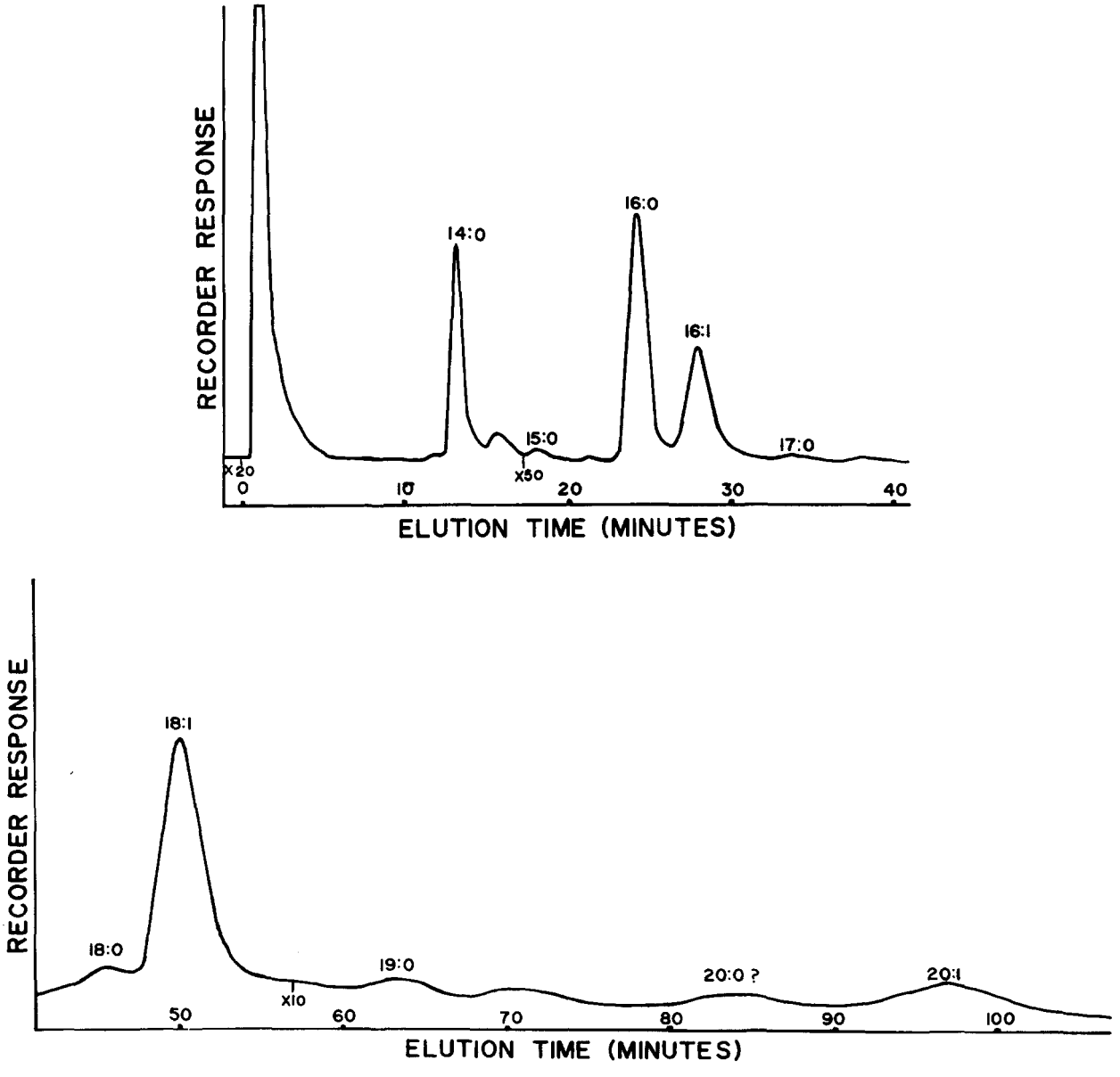

FIG. 3. A representative gas–liquid chromatogram of isopropylidene derivatives of glyceryl ethers derived from the flesh of *Squalus acanthias*. Compounds that have long retention times were measured after increasing the column load.

TABLE 3 FATTY ACID COMPOSITION OF THE DIACYL GLYCERYL ETHERS AND THE TRIGLYCERIDES OF *Squalus acanthias*

Fatty Acid	Diacyl Glyceryl Ethers		Triglycerides	
	Flesh	Liver	Flesh	Liver
		wt %		
14:0	4.7	2.1	1.6	2.7
15:0	0.4	0.2	0.2	0.3
16br	0.3	tr.	tr.	tr.
16:0	18.7	24.4	23.5	23.2
16:1	6.9	4.0	5.7	6.8
16:2	0.3	0.6	tr.	0.7
17:0	0.5	1.4	0.9	0.8
18:0	3.7	3.5	3.0	4.0
18:1	21.6	29.6	33.6	35.7
18:2	tr.	0.4	0.6	0.7
18:3	tr.	tr.	0.5	0.5
19	0.3	0.4	0.4	0.2
20:1	4.7	11.3	6.4	7.0
20:4	1.6	0.2	2.8	0.6
20:5	6.2	1.0	6.5	3.7
21:0	0.2	tr.	0.3	tr.
22:1	4.5	12.1	3.5	5.5
22:4	2.8	1.0	0.8	0.4
22:5	3.6	2.4	1.7	1.5
22:6	18.1	2.4	7.0	5.1
24:1(?)	0.4	2.9	0.4	0.4

the same species, caught in different parts of the world, may be related to both the diet and the season in which the fish was caught. Generally, the glyceryl ethers from the flesh and liver of the dogfish were remarkably similar to those found in the livers of other elasmobranch fish such as the Greenland shark (*Somniosus microcephalus*) and ratfish (*Chimaera monstrosa*) (6).

Fatty Acids of the Diacyl Glyceryl Ethers

The fatty acids from the diacyl glyceryl ethers of the liver were characterized by relatively large proportions of C_{20} and C_{22} monoenoic acids and remarkably low percentages of polyenoic acids of the same chain lengths (Table 3, third column). Eicosenoic acid comprised 90% of the C_{20} and docosenoic acid 68% of the C_{22} acids esterifying the diacyl glyceryl ethers of this organ. The polyenoic acids 20:5 and 22:6 represented 8 and 13% of the C_{20} and C_{22} acids, respectively.

The highest proportions of C_{20} and C_{22} polyenoic acids were found in the lipids of the flesh. The diacyl glyceryl ethers of the flesh, in particular, contained relatively large percentages of these acids, notably 20:5 (6.2%) and 22:6 (18.1%), making up 50 and 62% of the C_{20} and C_{22} acids, respectively. The diacyl glyceryl ethers from the flesh, when compared with the other fractions from the flesh and liver, had the lowest percentages of 16 : 0, 18 : 1, and 20 : 1 acids.

Fatty Acids of the Triglycerides

The differences between the C_{20} and C_{22} acids of the triglycerides were less striking (Table 3). It is apparent, however, that there were significantly lower percentages of polyenoic acids in the triglycerides of the liver than in the triglycerides of the flesh. The levels of 20:1 and 22:1 acids were about the same in both triglyceride fractions.

DISCUSSION

Olley and Duncan[1] recently studied the over-all fatty acid composition of the flesh and liver oils from *Squalus acanthias* caught off the coast of Scotland in December. In comparing the fatty acids from these oils, they noted the presence of unusually high percentages of 20:5 acid (8.3%) and 22:6 acid (21.8%) in the neutral lipids of the flesh. Furthermore, the phospholipids of the flesh contained 47% of C_{20} and C_{22} polyenoic acids, primarily docosahexaenoic acid (32.0%). Relatively little 20:1 acid (3.1%) and no detectable amount of 22:1 acid was found in the phospholipid fraction.

In the present investigation, the types of fatty acids in both the triglycerides and the glyceryl ethers were determined. Our results, together with those obtained by Olley and Duncan,[1] indicate that the C_{20} and C_{22} polyenoic acids of the flesh, particularly docosahexaenoic acid, are present in large amounts in the diacyl glyceryl ether and phospholipid fractions. It is also noteworthy that both of these fractions contained relatively small amounts of the C_{20} and C_{22} monoenoic acids compared with corresponding fractions from the liver.

In view of the fact that the ether chains are quite similar in the flesh and liver, it appears that the over-all unsaturation of the lipid fatty acids is significantly influenced by the relative amounts of monoenoic and polyenoic acids in the C_{20} and C_{22} series.

Brockerhoff et al. (16, 17) demonstrated that the polyenoic acids in fish body and liver oils are preferentially bound to the 2-position of the glycerol moiety. If this is also true in the diacyl glyceryl ethers of *Squalus acanthias*, the polyenoic acids would be more concentrated in the fatty acids from this fraction than in the triglycerides simply because the proportion of 2-acids is 50% in this mixture as opposed to 33% in the triglyceride fatty acids. Accordingly, the polyenoic acids in the β-position may be given extra prominence in the data on the diacyl glyceryl ethers.

Dietary factors are also instrumental in determining the fatty acid composition of fish. It has been shown that the proportions of C_{20}–C_{22} polyenoic acids in the lipids of planktonic crustaceans, a source of food for many species of fish, are significantly modified by environmental temperature: that is, the relative amounts of polyenoic acids increase with a decrease in water temperature (18). It is most probable therefore that the percentages of polyenoic acids in the lipids of the dog-

fish are indirectly influenced by these temperature-induced changes in the dietary source. There is also evidence to suggest that polyenoic fatty acids in fish result from a series of stepwise alterations of a small number of dietary or biosynthesized acids, such as linolenic acid (19). In view of the probable exogenous origins of the polyenoic acids in fish, the occurrence of high percentages of these acids in the diacyl glyceryl ethers suggests that the fatty acid composition of this fraction is more influenced by diet than are the triglycerides.

The unusual lipid composition of the livers of elasmobranch fish has aroused an interest in the origins of the relatively large amounts of C_{20} and C_{22} monoenoic fatty acids. Lovern viewed their biogenesis in the liver in terms of both hydrogenation (4) and chain elongation processes (1). Recently, Malins and Houle (20) investigated the structures of these acids in the livers of *Squalus acanthias*. They found that Δ^{11}-eicosenoic acid and Δ^{11}- and Δ^{13}-docosenoic acids were the principal isomers. The position of the double bond lends support to the hypothesis that they are formed by chain elongation by addition of acetate units in the following manner:

$$\begin{array}{l} 18:1[9] \rightarrow 20:1[11] \rightarrow 22:1[13] \\ \qquad\quad 20:1[9] \rightarrow 22:1[13] \end{array}$$

Any attempt to determine the origins of the lipids of marine animals is complicated by dietary habits which are often unknown or incompletely understood. *Squalus acanthias* from Puget Sound subsists on relatively large amounts of ratfish (21), in which the lipids have been shown (6, 22) to contain high percentages both of glyceryl ethers (as diacyl derivatives) and of C_{20} and C_{22} monoenoic acids.

Enzymatic cleavage of the ether linkage in glyceryl ethers was shown to take place in the intestinal mucosa of humans (23), rats (24, 25), and rainbow trout (*Salmo gairdneri*).[2] It is possible that ether linkages of dietary glyceryl ethers are cleaved in the intestinal mucosa of the dogfish and that the resulting fatty alcohol moieties are oxidized to acids which, in turn, are deposited primarily as esters of glycerol. If this hypothesis is correct, a portion of the fatty acids in the lipids of the dogfish would be derived from the long-chain ether group of ingested diacyl glyceryl ethers. Perhaps valuable data relating to the origins of the unusual lipids of the dogfish might be obtained from a study of dietary sources.

[2] D. C. Malins, J. C. Wekell, and C. R. Houle. "The metabolic fate of ingested glyceryl ethers in rainbow trout (*Salmo gairdneri*)." Presented at the First World Fat Congress, Hamburg, Germany, October 1964.

The authors are indebted to Richard W. Nelson of the Bureau of Commercial Fisheries Technological Laboratory, Seattle, for providing the fish oil samples.

Manuscript received March 17, 1964; accepted August 3, 1964.

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