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The unsaturated fatty acids in menhaden body oil: the C_{18}, C_{20}, and C_{22} series*

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SUMMARY

Complete structural characterization of the C_{18}, C_{20}, and C_{22} unsaturated acids of menhaden body oil is described. The present report brings to 23 the number of chemically defined unsaturated acids in this oil, accounting for 95 per cent of the total weight of unsaturated acids. Of these 23 acids, 7 have not previously been reported. Pure acids (or mixtures of positional isomers) were isolated by fractional crystallization, fractional distillation, countercurrent distribution, rubber column chromatography, and preparative gas-liquid chromatography. Acids which were homogeneous in respect to chain length and number of double bonds were degraded by oxidative and reductive ozonolysis; fragments were identified by gas-liquid chromatography of the carboxylic acids and as addition-products of the aldehydes. Our studies of this complex oil, which is composed of more than 44 different acids, provide convincing evidence of the great usefulness of gas-liquid chromatography as a preparative and analytical procedure. Chemical characterization studies are now feasible on a microscale.

The present report marks the completion of a study of the fatty acid composition of menhaden body oil. Originally undertaken to complement an investigation of the nutritional effects of this marine oil in man (1), this study was aimed at the isolation and complete definition of the chemical structure of its unsaturated fatty acids. A previous report (2) described the C_{16} acids, and the present report the C_{18}, C_{20}, and C_{22} acids. We can account now for more than 95 per cent of all the unsaturated acids in this oil, based on our total quantitative analysis (1) by gas-liquid chromatography (GLC). Only the C_{14} monoene, traces of odd-numbered acids, and traces of three C_{22} acids remain uncharacterized. In the course of this work it was advantageous to determine the GLC characteristics of the acids isolated in pure form; their retention characteristics and stability on polar and nonpolar GLC columns are described elsewhere (3, 4).

Our isolation and identification procedures have been improved and adapted more to a microscale since the previous report (2). Figure 1 shows the present flow sheet. The starting material was especially prepared from fresh menhaden bodies and was not contaminated with head, liver, or visceral fats; a complete description of this preparation is given elsewhere, as well as a variety of chemical characteristics of the oil (1). Low temperature crystallization of the free fatty acids yielded the most highly unsaturated acids, which were then methylated and separated into chain-length groups (C_{18}, C_{20}, and C_{22}) by repeated fractional distillation (4). Only those fractions which were homogeneous in respect to chain length (verified by GLC after complete hydrogenation of a small sample of each) were used for further steps. The various unsaturated esters of each chain length were then separated into groups varying in number of double bonds (mono-, dienes, etc.), using a rubber column for the methyl esters (6) or countercurrent distribution (CCD) (7) of the free fatty acids. The homogeneity of each of these fractions in respect to number of double bonds was checked in three ways: (*a*) superimposition of theoretical and experimental CCD curves; (*b*) ultraviolet spectroscopy after isomerization by alkali (8); and (*c*) GLC on a polar stationary phase.

Having separated the original mixture of most highly unsaturated acids into chain-length groups and each of these into subfractions which were homogeneous in respect to number of double bonds, the final step consisted of identifying the double-bond structure of the acids in each of the subfractions. This was accomplished by oxidative ozonolysis with identification of all fragments by GLC. The fragment from the car-

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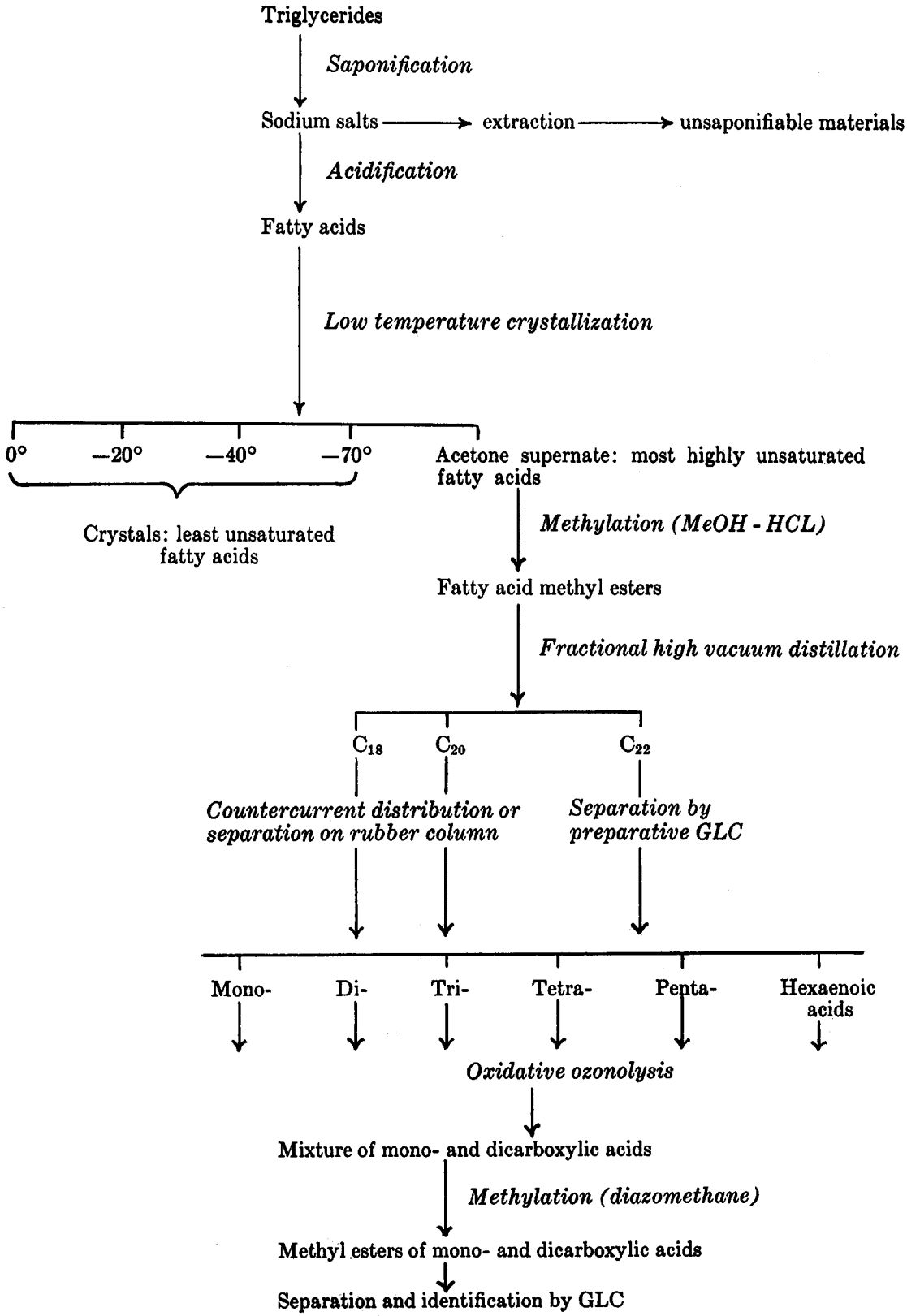
FIGURE 1. FLOW SHEET



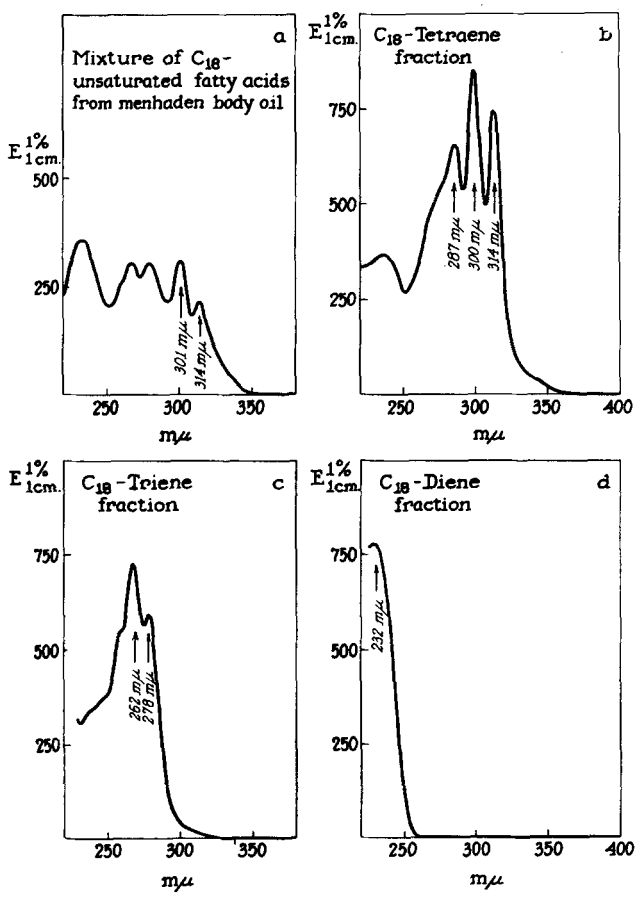


FIG. 2. C_{18} Acids—ultraviolet spectra after isomerization with alkali (8): (*a*) the C_{18} acid mixture; (*b*) the C_{18} tetraene (CCD peak D); (*c*) the C_{18} trienes (CCD peak C); (*d*) the C_{18} dienes (CCD peak B). Positions of peaks are italicized.

boxyl group to the nearest double bond ("carboxyl fragment") and the fragments between double bonds ("intermediate fragments") were identified as dicarboxylic acids, while the "terminal fragment" from the terminal methyl group to the nearest double bond was identified as a monocarboxylic acid. In a few critical cases, terminal fragments were identified and quantified as the 2,4-dinitrophenylhydrazones of the aldehydes produced by reductive ozonolysis (9). On the basis of these pieces of evidence the double-bond structures of the acids in each of the subfractions were assigned and their relative proportions calculated.

In the case of the C_{22} acids, it proved difficult to separate the mixture into cleanly resolved peaks by CCD. However, it was possible to use GLC as a preparative method because of the wide separation between pentaenes and hexaenes. By micro-ozonolysis of these fractions[1] and GLC of the mono- and dicarboxylic acids formed, their double-bond structures were defined.

[1] W. Stoffel, F. Chu, and E. H. Ahrens, Jr. Manuscript in preparation.

Table 1 lists the fragments determined by oxidative and reductive ozonolysis of the C_{18}, C_{20}, and C_{22} unsaturated fatty acids in menhaden body oil and the proposed structures of the fatty acids in these groups. The C_{16} acids previously described (2) are included for sake of completeness. Many of the acids in Table 1 have been characterized previously by other workers, having been isolated from a wide variety of biological sources; references to this literature are included. However, seven of the acids listed have not been described before.

EXPERIMENTAL

Separation by Chain Length. Fractional high vacuum distillation (column length 750 mm., 10^{-4} mm. Hg pressure at the distillation head) (5) was carried out on about 50 g. of mixed esters. Small samples of distillate were hydrogenated completely with Adams catalyst (PtO_2) and the homogeneity of chain length checked by GLC. The fractions shown to be exclusively C_{18} and C_{20}, respectively, were used for further investigation. The esters were colorless oils. The C_{18} fraction had an iodine value (I.V.) of 201, the C_{20} fraction 290. Ultraviolet spectroscopy showed absence of conjugated acids, and infrared spectroscopy absence of *trans* acids. After isomerization with alkali, tetraenoic C_{18} and pentaenoic C_{20} acids were shown to be the most unsaturated components in their respective fractions (Figs. 2 and 3).

Separation by Degree of Unsaturation. Mixed C_{18} esters (5.20 g.) and mixed C_{20} esters (5.15 g.) were saponified, and the free fatty acids were separated by CCD (7) into four groups of acids in each case, differing from each other only in the number of double bonds. A 200-tube all-glass fully automatic machine with 10 ml. lower phase volume was used with a solvent system of n-heptane : methanol : acetonitrile : acetic acid (3 : 1 : 1 : 1). After 680 transfers in the C_{18} distribution, alkali isomerization of aliquots of each peak gave evidence that *D* in Figure 4 was the tetraene, *C* the triene, and *B* the diene group. That peak *A* (tubes 220 to 250) contained the monoene was established by the I.V. of 88; in addition, its GLC retention time on a polar stationary phase (ethylene glycol adipate polyester, EGA) was identical to that of methyl oleate.

In the case of the C_{20} distribution, the dienes and trienes (Fig. 5, peaks *C* and *B*) were well separated in 400 transfers and were removed; after 660 transfers of the remaining material the tetraenes (A_2) and pentaenes (A_1) were separated. The tetraene peak was removed and the distribution of the pentaene continued

TABLE 1. Structures of Unsaturated Fatty Acids in Menhaden Body Oil. Table Demonstrates Evidence on Which Are Based the Structures of 23 Acids, Comprising 95% of the Total Weight of Unsaturated Acids in This Oil.

hain ngth	Fatty acids fraction	I.V. (22)	Degradation products after oxidative ozonolysis: Mono-carboxylic acids	Degradation products after oxidative ozonolysis: Di-carboxylic acids	Aldehydes obtained after reductive ozonolysis	Position of double bonds (numbered from carboxyl carbon)	Approximate relative amounts of isomers	Percentage in original menhaden body oil *	Characterized previously
6 V. 30–35)	tetraenoic	405		adipic malonic succinic		6,9,12,15 4,7,10,13	20 1	1.9 0.1	South African pilchard oil (10) herring oil †
	trienoic	302		pimelic adipic malonic	butyraldehyde propionaldehyde acetaldehyde	6,9,12 7,10,13	5 1	1.1 0.2	rape seed oil (11) ‡
	dienoic	202	butyric heptanoic	azelaic adipic malonic	butyraldehyde heptylic aldehyde	6,9 9,12	1 4	0.4 1.6	‡ ‡
	monoenoic	103	heptanoic caprylic	azelaic suberic		8 9	1 6	1.4 8.4	‡ numerous sources (12)
8 V. 01)	tetraenoic	354	propionic	malonic adipic	propionaldehyde	6,9,12,15		3.2	herring oil (13)
	trienoic	266	propionic caproic	malonic adipic azelaic	propionaldehyde capronaldehyde	9,12,15 6,9,12	4 1	1.0 0.3	linseed oil (12), herring oil (9) ‡
	dienoic	176	caproic pelargonic	malonic azelaic adipic		9,12 6,9	4 1	2.2 0.5	herring oil (9) ‡
	monoenoic	88	pelargonic	azelaic		9		14.5	numerous sources (12)
0 V. 90)	pentaenoic	405	propionic	malonic glutaric	propionaldehyde	5,8,11,14,17		12.5	cod liver oil (14) ox liver phosphatides (15)
	tetraenoic	326	propionic caproic	malonic glutaric suberic	propionaldehyde capronaldehyde	5,8,11,14 8,11,14,17	2 3	0.2 0.4	ox liver phosphatides (15) ‡
	trienoic	245	caproic pelargonic	malonic glutaric suberic		5,8,11 8,11,14	1 1	1.0 1.0	ox liver phosphatides (15) ox liver phosphatides (15), rat tissues (16)
	dienoic	163	caproic pelargonic	malonic suberic undecane-dicarb.		8,11 11,14	1 1	0.6 0.6	ox liver phosphatides (15)
2	hexaenoic		propionic § oxalic	malonic succinic		4,7,10,13,16,19		8.9	herring oil (19), pilchard oil (17), cod liver oil (18), ox liver phosphatides (19)
	pentaenoic		propionic	malonic pimelic		7,10,13,16,19		2.0	cod liver oil (18), pilchard oil (20), herring oil (9), ox brain phosphatides (21), ox liver phosphatides (19)

* Calculations based on the total analysis given elsewhere (1) and on the ratios in the column to the left. For example, in Reference (1), C_{18} tetraenoic acid is reported to constitute 2.0% of total fatty acids, and since this fraction is stated in the present study to be made up of two isomers in proportions of 20/1 (approximate), the per cent of each acid in the whole oil is 1.9 and 0.1% respectively.

† E. Klenk, personal communication. ‡ Acids not previously characterized. § Artifact, see text.

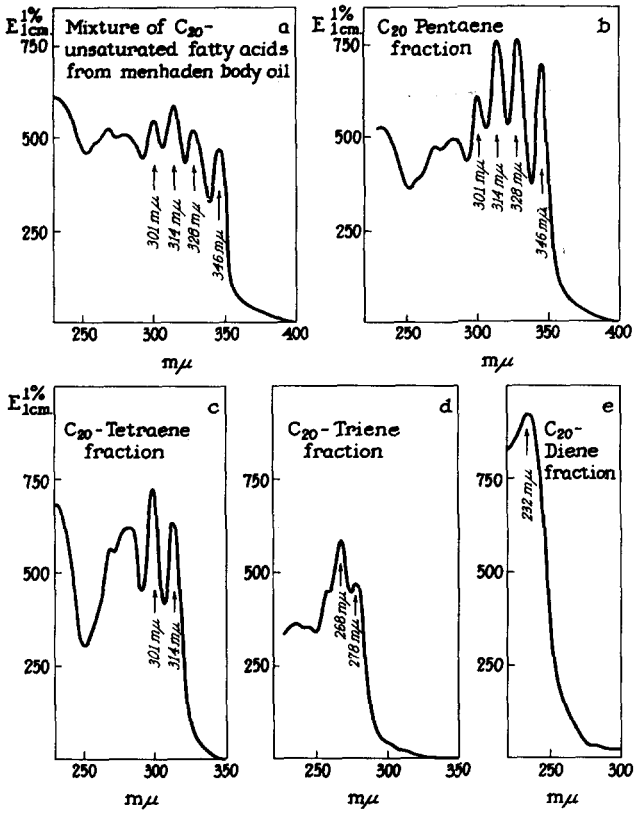


FIG. 3. C_{20} Acids—ultraviolet spectra after isomerization with alkali (8): (*a*) the C_{20} acid mixture; (*b*) the C_{20} pentaene (CCD, tubes 380 to 430); (*c*) the C_{20} tetraenes (CCD, tubes 460 to 500); (*d*) the C_{20} trienes (CCD peak B); (*e*) the C_{20} dienes (CCD peak C). Positions of peaks are italicized.

for 975 transfers without demonstrating any further peaks. The partition coefficient (K) of the pentaene remained constant between 700 and 975 transfers, and the theoretical and experimental CCD curves were superimposable.

The ultraviolet absorption spectra after isomerization with alkali (8) of the total mixture and of the different groups isolated by CCD are given in Figure 2 for the C_{18} series, in Figure 3 for the C_{20} series, and in Figure 6 for the C_{22} series. It is evident that there was no contamination of any fraction by its more highly unsaturated homologue.

The methyl esters of the C_{18} and C_{20} chain-length groups also were separated into groups, homogeneous in respect to numbers of double bonds, by chromatography on columns (1 × 150 cm.) of finely ground natural rubber (6), eluting with 10 and 12 per cent water in acetone, respectively. Fifty-milligram samples of the mixed methyl esters were chromatographed; the chromatograms were monitored by an automatic recording differential refractometer; fractions were collected automatically. The separations obtained were identical to those produced by CCD. Sufficient material was present in the various peaks for definitive degradation studies by micro-ozonolysis.[2]

The residue of the fractional distillation of C_{20} acids was subjected to preparative GLC on an Apiezon-M column at 197°C, utilizing an ionization chamber detector (4). Five samples (about 2 mg. each) were chromatographed. Despite overloading of the column, an excellent separation of C_{22} penta- and hexaenoic acid methyl esters was permitted by the very different retention times of these esters (3, 4). The contaminating C_{20} acids and C_{22} acids present only in trace amounts were eluted well ahead of the two major C_{22} acids. These major acids were separately collected; their chain length was checked by complete hydrogenation (4), followed by GLC of the n-saturated products, while the number of double bonds in each fraction was verified by ultraviolet spectroscopy after

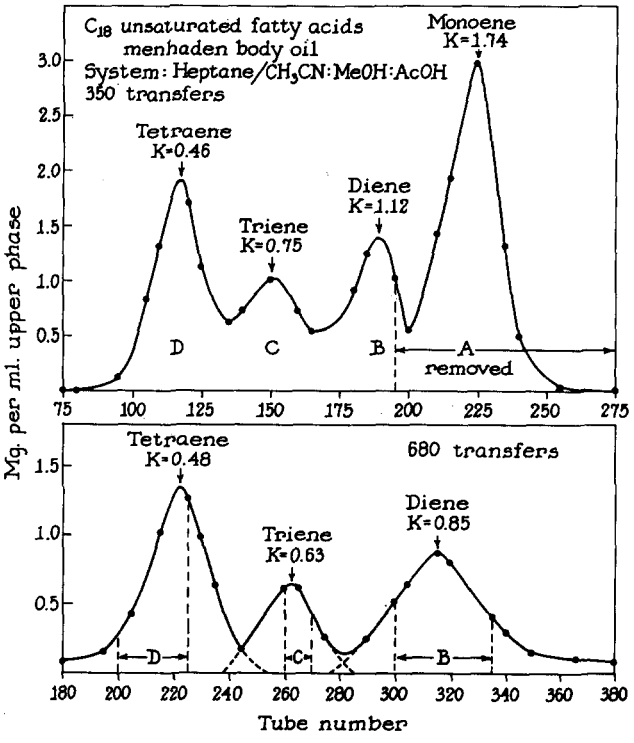


FIG. 4. Countercurrent distribution (CCD) pattern of 5.20 g. of C_{18} unsaturated fatty acids from menhaden body oil. Fractions *B-D* were harvested as shown, fraction *A* from tubes 220 to 250.

isomerization with alkali. Sufficient materials remained for oxidative ozonolysis.

Procedure of Oxidative Ozonolysis and Identification of Fragments. When 20 to 25 mg. of acids were available for ozonolysis, the procedure previously described (2) was used; for 0.5 to 5 mg. samples a micro-

[2] See footnote 1, p. 141.

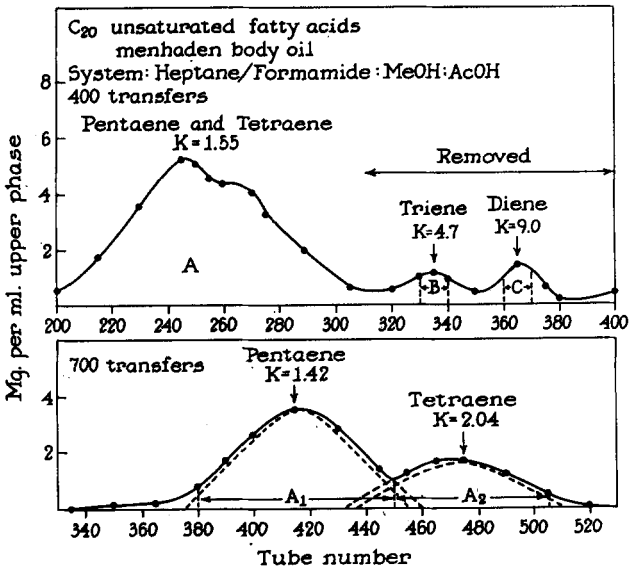


FIG. 5. CCD pattern of 5.18 g. of C_{20} unsaturated fatty acids from menhaden body oil. Fractions *B* and *C* were harvested as shown, fraction A_1 from tubes 380 to 430, fraction A_2 from tubes 460 to 500.

technic was applied.[3] The mixed mono- and dicarboxylic acids produced were dissolved in ethyl ether and methylated with diazomethane in ether in the usual manner (23). After evaporation of solvent, the residue was analyzed by GLC.

GLC was carried out (4) with an ionization chamber detector, argon as moving phase and EGA as stationary phase. Long-chain mono- and C_2-C_9 dicarboxylic acid methyl esters were best resolved at 173°C, the shorter di- and all monocarboxylic acids at 78°C. For identification of fragments produced from polyethenoic acids it was necessary to make analyses at both temperatures. In all cases retention times matched those of standard samples of C_2-C_9 methyl esters of pure mono- and dicarboxylic acids.

It was recognized that the recoveries of C_2-C_5 monocarboxylic acid methyl esters were less than theoretical, due to losses incurred when solvents were evaporated. When it was important to establish molar ratios of the various fragments produced by ozonolysis, the terminal fragments were recovered quantitatively after reduction to the aldehydes and formation of the 2,4-dinitrophenylhydrazones (9).

RESULTS

All fractions were obtained as colorless oils.

C_{18}-tetraenoic acid fraction (fraction *D*, Fig. 4): n_D^{22} 1.4861, I.V. 354 (22) (calculated for $C_{18}H_{28}O_2$, 362); $E^{1\%}_{1\,cm.}$ = 860, ϵ_{max} 23700 at λ_{max} 300 mμ. Oxidative ozonolysis yielded propionic, malonic, and adipic acids. On reductive ozonolysis propionaldehyde was the only aldehyde derived as a terminal fragment. The results indicated a single C_{18}-tetraene with double bonds in the 6,9,12,15 positions (numbered from the carboxyl carbon).

C_{18}-trienoic acid fraction (fraction *C*, Fig. 4): n_D^{22} 1.4665, I.V. 266 (calculated for $C_{18}H_{30}O_2$, 272); $E^{1\%}_{1\,cm.}$ = 725, ϵ_{max} 19900 at λ_{max} 268 mμ. Oxidative ozonolysis yielded propionic, caproic, malonic, adipic, and azelaic acids. On the basis of the ratio of adipic to azelaic acid, the two isomers (9,12,15- and 6,9,12-octadecetrienoic acids) were present in proportions of about 4 : 1. Reductive ozonolysis yielded propionaldehyde and capronaldehyde, also in a ratio of 4 : 1.

C_{18}-dienoic acid fraction (fraction *B*, Fig. 4): n_D^{22} 1.4600, I.V. 176 (calculated for $C_{18}H_{32}O_2$, 180); $E^{1\%}_{1\,cm.}$ = 775, ϵ_{max} 21400 at λ_{max} 232 mμ. Oxidative ozonolysis yielded caproic, pelargonic, malonic, adipic, and azelaic acids, indicating the presence of octadeca-6,9- and octadeca-9,12-dienoic acids in proportions of 1 : 4.

C_{18}-monoenoic acid fraction (fraction *A*, tubes 220 to 250, Fig. 4): n 1.4525, I.V. 88 (calculated for $C_{18}H_{34}O_2$, 89). Oxidative ozonolysis yielded only pelargonic and azelaic acids, indicating the structure octadeca-9-enoic (oleic acid) as the sole C_{18}-monoene.

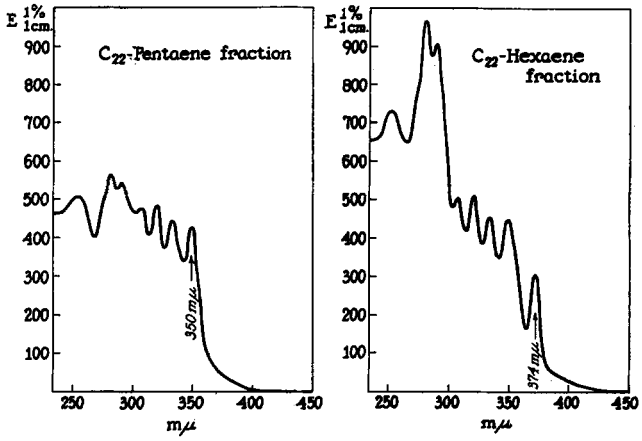


FIG. 6. C_{22} Acids—ultraviolet spectra after isomerization with alkali (8). Left, C_{22} pentaene; right, C_{22} hexaene (both isolated by preparative gas-liquid chromatography). Peak maxima are shown with arrows.

C_{20}-pentaenoic acid fraction (fraction A_1, tubes 380 to 430, Fig. 5): n_D^{22} 1.4890, I.V. 405 (calculated for $C_{20}H_{30}O_2$, 416); $E^{1\%}_{1\,cm.}$ = 695, ϵ_{max} 21400 at λ_{max} 346 mμ. Glutaric, malonic, and propionic acids were obtained by oxidative ozonolysis, and propionaldehyde was obtained by reductive ozonolysis. The results indicated a single pentaene with the 5,8,11,14,17 double-bond structure.

[3] See footnote 1, p. 141.

C_{20}-tetraenoic acid fraction (fraction A_2, tubes 460 to 500, Fig. 5): n_D^{22} 1.4880, I.V. 326 (calculated for $C_{20}H_{32}O_2$, 334); $E^{1\%}_{1\,cm.}$ = 725, ϵ_{max} 22000 at λ_{max} 301 mμ. Oxidative ozonolysis yielded propionic, caproic, malonic, glutaric, and suberic acids. On reductive ozonolysis propionaldehyde and capronaldehyde were obtained. The results indicated 5,8,11,14- (arachidonic) and 8,11,14,17-acids in proportions of about 2 : 3.

C_{20}-trienoic acid fraction (fraction *B*, Fig. 5): n_D^{22} 1.4681, I.V. 245 (calculated for $C_{20}H_{34}O_2$, 248); $E^{1\%}_{1\,cm.}$ = 600, ϵ_{max} 18400 at λ_{max} 268 mμ. Oxidative ozonolysis yielded pelargonic, caproic, malonic, glutaric, and suberic acids. The two dicarboxylic acids were present in about equal amounts. These results indicated the presence of eicosa-8,11,14-trienoic and eicosa-5,8,11-trienoic acids in proportions of 1 : 1.

C_{22}-hexaenoic acid fraction (preparative GLC): $E^{1\%}_{1\,cm.}$ = 300, ϵ_{max} 10000 at λ_{max} 374 mμ. Oxidative ozonolysis yielded oxalic, propionic, malonic, and succinic acids. Oxalic acid was present in only trace amounts and is considered an artifact: the mixed acids soluble in acetone at −70°C contained no preformed conjugation by ultraviolet spectroscopy, but after fractional distillation and GLC, the C_{22}-hexaene fraction showed 8 to 10 per cent conjugation. Discounting the oxalic acid formed by oxidative ozonolysis of the conjugated double bonds, the results indicated the presence of the 4,7,10,13,16,19-acid as the sole component of this fraction.

C_{22}-pentaenoic acid fraction (preparative GLC): $E^{1\%}_{1\,cm.}$ = 510; ϵ_{max} 17600 at λ_{max} 329 mμ. Oxidative ozonolysis yielded malonic, pimelic, and propionic acids, thus indicating that docosa-7,10,13,16,19-pentaenoic acid was the sole component of this fraction.

DISCUSSION

It is striking that for every polyethenoid acid in this sample of menhaden body oil a double-bond arrangement of the divinyl methane or methylene-interrupted type (=CHCH$_2$CH=) was found. Moreover, we found no evidence of *trans* acids; conjugated double bonds were detected only in the C_{22} hexaenes in small amounts, under circumstances which suggested artifactual changes. In their studies of other marine oils and fats from mammalian sources, Klenk and Bongard (24) have commented on the consistency of divinyl methane rhythm of double bonds in naturally occurring nonconjugated polyenes. Previously, work by Japanese investigators (25, 26) had suggested that some marine polyenes show both methylene- and ethylene-interruption, but this conclusion is currently ascribed to certain laboratory artifacts (27). In the present study, total absence of ethylene-interrupted double bonds in menhaden polyethenoid acids is demonstrated in three ways: (*a*) typical ultraviolet patterns were seen after isomerization by alkali, yet de Surville *et al.* (28) have shown that ethylene-interrupted double bonds prevent the occurrence of isomerization under the conditions used; (*b*) upon oxidative ozonolysis, succinic acid would be produced as an intermediate fragment from a divinyl ethane grouping as well as malonic acid from a divinyl methane grouping, yet Table 1 indicates that succinic acid was found only twice (C_{16} tetraene and C_{22} hexaene) and there as carboxyl fragments; and (*c*) the quantitative results obtained after reductive ozonolysis substantiated in every case the qualitative information obtained by oxidative ozonolysis.

In fatty acids of unknown structure it is possible to define double-bond structure if only three pieces of information are assembled (assuming that conjugated double bonds have been ruled out by ultraviolet spectroscopy): (*a*) the number of double bonds by ultraviolet spectroscopy after isomerization with alkali, (*b*) the chain length of the acid shown by GLC after complete hydrogenation, and (*c*) the identity of the carboxyl fragment after oxidative ozonolysis. Micromethods have been developed which permit the investigator to obtain this information on 3 mg. or less of an unknown acid;[4] the value of GLC in microanalyses of this type cannot be overemphasized. The validity of this shortened procedure for determination of double-bond structure depends, of course, on the assumption that double bonds occur exclusively in divinyl methane rhythm.

It is intriguing to note certain structural similarities among the acids in Table 1. If the double bonds are numbered from the terminal rather than the carboxyl carbon, as suggested by Thomasson (29), it is seen that most of the acids are of three types: oleic (Δ 9,10-terminal), linoleic (Δ 6,9-terminal), and linolenic (Δ 3,6,9-terminal). The most conspicuous exceptions occur among the C_{16} acids, where the unusual positioning of the double bonds suggests the possibility that these acids may have been formed from C_{18} acids by loss of two terminal carbons, or by dehydrogenation of palmitoleic acid as suggested by Fulco and Mead (30).

The total unsaturated acids in menhaden body oil comprise 70 per cent of the whole, yet these acids are by no means evenly distributed in regard to chain length or to degree of unsaturation. For example, the C_{16} unsaturated acids make up 15 per cent; C_{18}, 22

[4] See footnote 1, p. 141.

per cent; C_{20}, 16 per cent; and C_{22}, 11 per cent of the total acids. Moreover, among these chain length groups it is interesting to note that the monoenes predominate in the C_{16} and C_{18} groups, while in the C_{20} and C_{22} groups the penta- and hexaenes are present in highest concentration.

Lacking information on the fatty acid structure of the plankton upon which menhaden feed, it is impossible to know to what extent the fatty acids of menhaden body oil reflect the synthetic or rearrangement processes of the fish or of the algae. Yet it is tempting to speculate that the C_{16} and C_{18} monoenes, both Δ-9 acids, are the progenitors of the more highly unsaturated acids, and that the final pattern of distribution of acids represents the competition between chain elongation by addition of acetate units and progressive desaturation toward both ends of the fatty acid chain, starting from the Δ 9 double bond. If C_{18} monoene is elongated by addition of acetate, the Δ 9 bond in the C_{18} acid becomes Δ 11 in the C_{20} series, and Δ 13 in the C_{22} series. Thereafter, most of the acids shown in Table 1 could have been formed by progressive stepwise dehydrogenation (always in divinyl methane rhythm) on either side of the pivotal double bond.

The very small amounts of "essential fatty acids" in this oil have been noted in a previous publication (1); the total amount of linoleic-type acids was 2.6 per cent, a figure corroborated by the bioassay value of 4 units.

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