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Highly Unsaturated Fatty Acids. III. Isolation of Methyl Eicosapentaenoate, Ethyl Docosapentaenoate, and Ethyl Docosahexaenoate from Cod Liver Oil Esters by Chromatography[1,2]

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INFORMATION on the role of polyunsaturated fatty acids in metabolism is very meager largely because methods for their analysis have not been available. The advent of the spectrophotometric methods offers some possibility of an adequate means of analysis. However, until pure substances can be obtained for spectral standards, spectrophotometric methods can at most be used to obtain relative data. In an effort to obtain 4, 5, and 6 double bond acids for use as spectral standards, attempts were made to isolate these substances as their esters by chromatographic methods. These, in combination with urea fractionation, were chosen as mild treatments potentially capable of achieving the desired separations. Cod liver oil was chosen as starting material because it contains relatively high concentrations of 5 and 6 double bond acids. Even though its polyunsaturated acids may be different from those found in mammalian tissues, experience gained could be useful in planning isolations from more difficult sources.

In a recent paper (1) methods of concentrating the highly unsaturated acids from natural sources by the urea inclusion compounds have been described. In this paper attempts at isolation of some individual highly unsaturated fatty acids from these concentrates will be discussed, and some of their properties will be described. The success with which chromatography has been applied to separations in various fields of chemistry and biochemistry, its inherent advantage of operation at room temperature, and its high selectivity suggested its use.

In the isolation of unsaturated acids it is desirable to segregate by means of chain length as well as by unsaturation. However fractional distillation results in partial conjugation of the double bonds in the unsaturated acids (2). This is true even with modern, very efficient fractional distillation equipment. When cod liver oil methyl esters were distilled at 0.5 mm. Hg. in a whirling band Podbielniak column, the conjugation appearing in distillate fractions increased as the distillation proceeded. This is shown in Figure 1. It is clear from these data that when distillation is used as a step in isolation of polyunsaturated esters, some conjugated isomers can be expected in the preparation. Its use may be indicated as a preparative step, but distillation as a final purification of polyunsaturated acids leads to considerable conjugation in the product.

A great deal of work on separation of fish oil fatty acids has been done by Japanese invesigators (3-6). They used fractional distillation under pressures as high as 15 mm., fractional crystallization of the acids or their soaps, and bromination-debromination procedures. As a result of these and other (7, 8) investigations fish oils have been shown to contain, in contrast to land animal oils, a highly complex mixture of unsaturated fatty acids of varying degrees of unsaturation. In the light of present-day understanding of the effects of bromination-debromination and prolonged distillation at high temperatures, it is questionable if the presumably pure acids obtained by these methods were wholly in their natural form.

The preparation of methyl docosahexaenoate from cod liver oil by molecular distillation was reported by Farmer and Vandenheuvel (18). This method of segregation is far more gentle than fractional distillation, but separation by degree of unsaturation is very difficult. These workers stated that docosapentaenoate was not present in cod liver oil, but its presence has now been demonstrated by chromatographic techniques.

The first use of chromatography for isolation of highly pure polyunsaturated esters was by White and Brown (9), who isolated arachidonate from beef adrenal lipid and demonstrated the presence of eicosapentaenoate in that lipid. Riemenschneider and coworkers isolated pure natural linoleic and linolenic acids (10) and 90% pure arachidonic acid (11) as their methyl esters by adsorption on silicic acid columns. During the progress of this present work they also reported isolation of eicosapentaenoic and docosapentaenoic acids from beef adrenal lipid by adsorption (12).

Experimental

The chromatographic separations using displacement techniques were performed by using a modified Tiselius-Claesson adsorption analysis apparatus equipped with coupled filters. The apparatus and the details of its use have been described previously (13). In the separations reported here the adsorbent was

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Darco G60 charcoal-Hyflo Supercel (1:2), the solvent was isopropanol, and the displacer was 1.0% methyl behenate. The flow rate was usually 30-35 ml. per hour, and interferometric observations were made at 30°C. Appearance of the displacer was verified by the first appearance of a precipitate in the effluent cooled to −15°C. A plot of micrometer reading against volume of effluent gave a continuous record of the emerging zones, and fraction cuts were made according to this record. The fractions were characterized by iodine values (Wijs 0.5 hr.), alkaline isomerization, and in some cases by other properties.

The elution chromatographic system used was a modification of that used by Riemenschneider *et al.* (10). Pyrex columns were fitted at the lower end with male plugs having perforated or fritted faces. The adsorbent was Mallinckrodt's silicic acid AR mixed with Hyflo (4:1 by weight) to speed the flow. The column was packed dry immediately after heating the mixed adsorbent to 120° for 1 hour. The top of the column was fitted with a 2-hole stopper carrying a 6-liter separatory funnel and a pressure equalizer tube connected to the top of the funnel and to a source of oxygen-free nitrogen. Before adding the sample to the column, oxygen-free nitrogen was passed through the dry adsorbent, and then the column was wetted with Skelly F. The sample was added (roughly 1 g. sample per 20 g. of adsorbent) as a concentrated solution, and this was followed by Skelly F containing increasing proportions of ethyl ether or chloroform. Two means were used to aid in collecting fractions. A fraction collector was employed in some cases, and an automatic evaporating device was used on the large column. This periodically admitted percolate from the column through a solenoid valve (glass) to an evacuated three-neck flask. The second neck carried a thermometer inserted to the bottom of the flask, and the third carried a vacuum take-off and a capillary inlet leaking oxygen-free nitrogen to the system. The flask was warmed sufficiently by a mantle to evaporate the percolate but not enough to raise the temperature above room temperature. Flow rates varied between 300 and 500 ml. per hour, and cuts were made arbitrarily. Fractions were characterized by their iodine values (Wijs ½ hr.) and by alkaline isomerization.

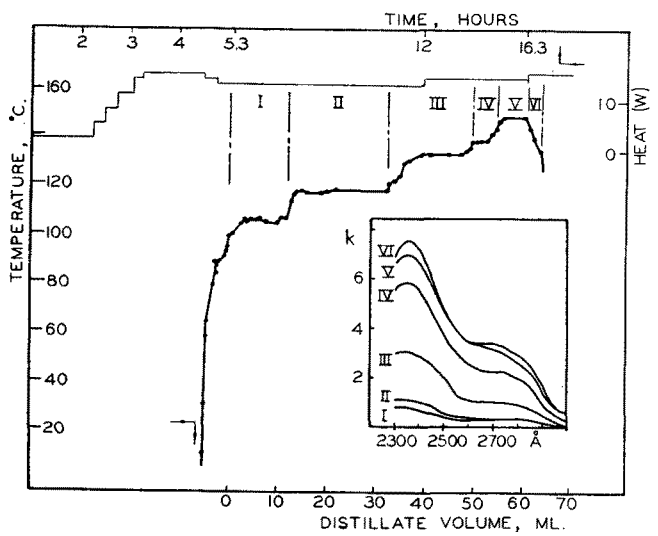

FIG. 1. Fractional distillation of cod liver oil methyl esters in a whirling band fractionation column at 0.5 mm. Hg. Insert shows ultraviolet spectra of successive fractions.

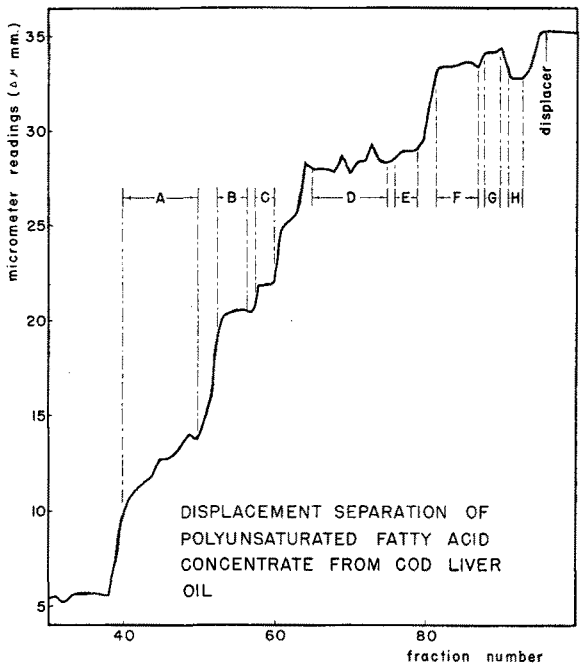

FIG. 2. Displacement separation of unsaturated fatty acid concentrate from cod liver oil.

Results and Discussion

Displacement separation of a cod liver oil fatty acid concentrate. The starting material was a concentrate of cod liver oil acids, iodine value 350, obtained by urea fractionation (1). The chromatographic column was 340 ml., the sample 2.0 g., and the displacer 1.0% methyl behenate in isopropanol. To allow continuous running overnight 5-ml. fractions were collected by fraction collector, and these were later injected into the cuvette for measurement. The displacement diagram is shown in Figure 2, and characterization of the eight cuts is summarized in Table I. It is obvious that segregation has been accomplished by displacement chromatography beyond that achieved by urea fractionation. Fraction F (approximately 80 mg.) having an iodine value of 446, represents a docosahexaenoic acid of estimated purity 78.5 to 96.5%, assuming the impurity to be a C_{22} fatty acid ranging from 5 to 0 double bonds. Theoretical iodine values for docosapentaenoic and docosahexaenoic acids are 384 and 463, respectively. Analyses indicated that

TABLE I

Analysis of Fractions from Displacement Separation of Polyunsaturated Fatty Acid Concentrate from Cod Liver Oil

Fraction	I.V.	Wt. of Sample mg.	Extinction coefficient (k)[a] 3750	3475	3000	2700	2340Å
Original mixture	350.0						
A	78.4	25.3	0.37	1.01	2.33	3.52	12.7
B	280.0	28.6	1.56	5.21	49.1	40.0	53.4
C	281.0	11.5	3.25	9.40	39.6	35.7	39.8
D	386.0		not analyzed				
E	372.0	23.3	4.90	59.4	58.1	50.0	46.5
F	446.0	31.4	27.1	43.9	45.4	52.1	50.7
G	414.0	25.2	27.8	43.0	44.0	51.0	49.5
H	362.0	27.6	28.0	43.6	42.3	43.3	44.9

[a] After alkali-isomerization with 23% KOH-ethylene glycol for 8 minutes (15).

fraction A consists mainly of monounsaturated acids, B and C may contain dienoic, trienoic, and tetraenoic acids, D and E contain pentaenoic acids, whereas F, G, and H contain hexaenoic acids. The low iodine values of G and H suggest possible blending with displacer. The hexaene absorptions for F, G, and H are not in proportion to their iodine values. The size of the sample (16) or the proportions of pentaene and hexaene acids in these samples may be factors in this discrepancy. The limited size of the fractions did not allow further characterization.

Isolation of ethyl docosahexaenoate by displacement chromatography. The polyunsaturated ethyl esters of cod liver oil were concentrated by urea fractionation to an iodine value of 231 (1). Two ml. of this preparation were placed on a 173-ml. coupled filter column and displaced with 1.0% methyl behenate in isopropanol. Figure 3 shows the displacement diagram. Flooding the column (shown by the spike at dead volume), which usually results in poor separation, was applied purposely in this case. It was known that the hexaene acid appears last in a chromatogram. By flooding, one can place more of the desired constituent on the column, and excess lesser unsaturated substances appear in the elution peak at the end of the dead volume. However the most adsorbed constituents are well separated by the time they emerge. Two distinct zones appeared just ahead of the displacer. Fractions A and B had iodine values 337.5 and 423, respectively. Hydrogenation of A, using platinum catalyst, yielded a product melting between 42 and 46°, suggesting it was a mixture of ethyl arachidate and ethyl behenate. Hydrogenation of B yielded a product which after recrystallization from acetone melted at 47.5°. The free acid obtained from this melted at 79.0° (reported m.p. for behenic acid is 79.95°). The neutralization equivalent was 339.0 (theory for behenic acid is 340.6). These characterizations indicated that fraction B (approximately 200 mg.) consisted of ethyl docosahexaenoate of purity between 95.2% and 99.4%, based on iodine value, assuming a C_{22} ethyl ester contaminant having 5 to 0 double bonds. The chief contaminant probably was the displacer methyl behenate. The ultraviolet absorption spectrum after isomerization of 56.6 mg. of the sample with 11.0 g. 21% KOH in ethylene glycol for 15 minutes at 180° (12, 14) was determined and is shown in Figure 4. The measurements above 3,200 Å were made with the ultraviolet hydrogen arc lamp, and slit widths of 0.3 to 0.4 mm. were used. Extinction coefficients (k) at the characteristic wavelengths were calculated for the free acid and are given in Table II.

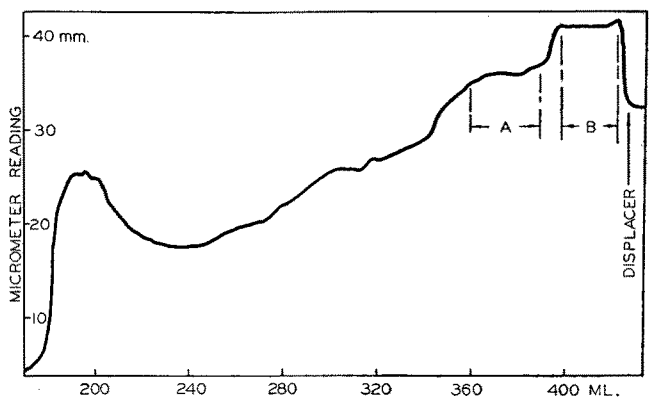

FIG. 3. Displacement separation of unsaturated ethyl esters of cod liver oil. First appearance of displacer shown by arrow.

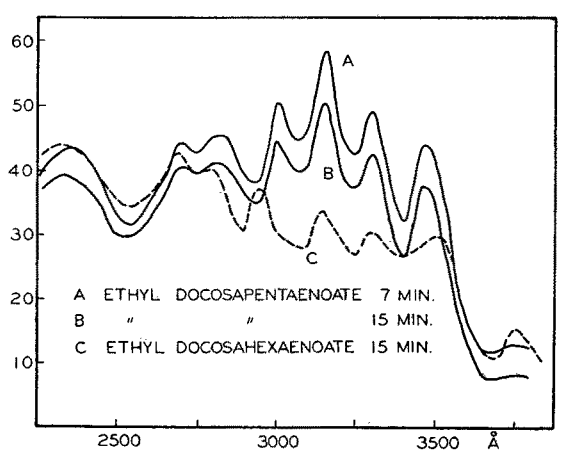

FIG. 4. Ultraviolet absorption spectra of isomerized ethyl docosapentaenoate and ethyl docosahexaenoate.

TABLE II

Extinction Coefficients (k) at Characteristic Maxima for Polyunsaturated Acids after Alkaline Isomerization

	3750 Å	*3460 Å*	*3150 Å*	*2680 Å*	*2330 Å*
21% KOH-glycol, 15 minutes, 180°C. (14)					
Docosahexaenoic acid	16.8	30.4	36.9	46.4	48.0
Docosapentaenoic acid	8.7[a]	40.8	54.7	42.8	42.7
21% KOH-glycol, 7 minutes, 180°C.					
Docosapentaenoic acid	14.1[a]	48.7	64.2	47.2	47.0
23% KOH-glycol, 8 minutes, 180°C. (15)					
	3750 Å	*3475 Å*	*3000 Å*	*2700 Å*	*2340 Å*
Docosahexaenoic acid	27.1	43.9	45.1	52.1	50.7
Docosapentaenoic acid	11.5[a]	42.7	52.4	55.6	51.3

[a] Principally end absorption of the 3,460 Å chromophore.

The extinction coefficient of 16.75 for docosahexaenoic acid as determined by the method of Herb *et al.* (14) is very low compared to that determined by the method of Holman and Burr (15) on Fraction F, Table I, which was assumed to be docosahexaenoic acid of 78.5% minimum purity. The former conditions thus are not optimum conditions for maximum hexaene conjugation. Although alkali concentrations differ slightly in the two methods and sample size may influence the degree of isomerization (16), the conditions of isomerization differ mainly in the time of treatment. An experiment to learn the effect of time on hexaene conjugation was performed, and because the amount of hexaene acid available was limited, the study was performed on shark liver oil and a concentrate of unsaturated methyl esters of beef testes. The 21%-KOH reagent was used, and only hexaene conjugation was measured at 3,750 Å, involving the least interference from other polyenes. The times of treatment for maximum yields of conjugated hexaene from these samples were 6 and 4 minutes, respectively, when 21% KOH glycol was used as reagent and 70- to 85-mg. samples were used. Obviously the 15-min. heating period is not optimum for hexaene conjugation. The difference in optimum treatment for the two samples may arise from differing proportions of 5 and 6 double bond acids in the two samples or from

true differences in the hexaene acids themselves. Optimum conditions must be defined for each purified hexaene acid before firm conclusions can be drawn.

Methyl eicosapentaenoate by combined elution and displacement chromatography. A concentrate of the methyl esters of cod liver oil fatty acids (I.V. 226) prepared by urea fractionation was used as starting material. For the elution separation a 30-g. sample of this concentrate was chromatographed on a Pyrex glass column 105 cm. x 6.0 cm. packed with 675 g. dry silicic acid Hyflo mixture, 4:1. Data pertaining to the fractionation are presented in Table III. The

TABLE III

Elution Fractionation by Silicic Acid-Skelly F System of Cod Liver Oil Methyl Ester Concentrate

Fraction No.	Volume of Eluate ml.	Ethyl Ether Content in Skelly F % by volume	Weight of Fraction g.	Iodine Value (Wijs)
Original	.....	.....	30.0	226.0
1	7,200	0.25	.....	.....
2	7,000	0.25	.....	.....
3	14,800	0.25	1.65	83.2
4	5,000	0.50	7.36	108.3
5	7,000	0.50	2.71	362.0
6	8,000	1.00	2.94	411.0
7	7,000	1.00	trace	—
8	2,000	2.00	trace	—
9	4,000	4.00	trace	—
10	5,200	10.00	0.70	248.5

recovery was rather low, hardly above 50%, and the experiment was stopped when the iodine value of the fractions began decreasing. The melting points of the completely hydrogenated fractions were 44 to 50°C. for fraction 5 and 45.3 to 46.9°C. for fraction 6. The melting points of methyl arachidate as reported in the literature are 45.8 and 46.6°C. Fraction 5 was probably a mixture of methyl esters of C_{20} and C_{22} acids with five or less double bonds because extinction coefficients did not show any maximum at 3,750 Å and fraction 6 (approximately 210 mg.) consisted mainly of methyl eicosapentaenoate. Fraction 6 also contained an ester with hexaene unsaturation as indicated by the extinction coefficient at 3,750 Å and by its iodine value 411, which is about 2.5% higher than the theoretical value of 401 for methyl eicosapentaenoate. The saponification equivalent of the hydrogenated material was 332 (theoretical 326.6). Thus the impurity was probably methyl docosahexaenoate.

In an attempt to purify this preparation (fraction 6) further, displacement chromatography was applied. A sample of 1.0 ml. was put into a 173-cc. column packed with Darco G-60 charcoal-Hyflo (1:2), and a 1% methyl behenate in isopropanol was used as displacer. The plot of micrometer reading against volume of effluent is shown in Figure 5. Two major and approximately equal steps, A and B, were obtained with iodine values 347.5 and 405, respectively. The fact that the starting material had an iodine value of 411 and that both fractions obtained by displacement chromatography had lower iodine values suggests that fraction A contains material altered in some way or that the most unsaturated constituent was not displaced adequately. The extinction coefficients (k) of fractions A and B after isomerization for 15 min. with 21% KOH-glycol were:

	3750 Å	3460 Å	3150 Å	2680 Å	2330 Å
A	7.52[a]	31.7	36.9	46.8	46.2
B	10.29	48.0	47.4	45.7	43.0

[a] No maximum was indicated.

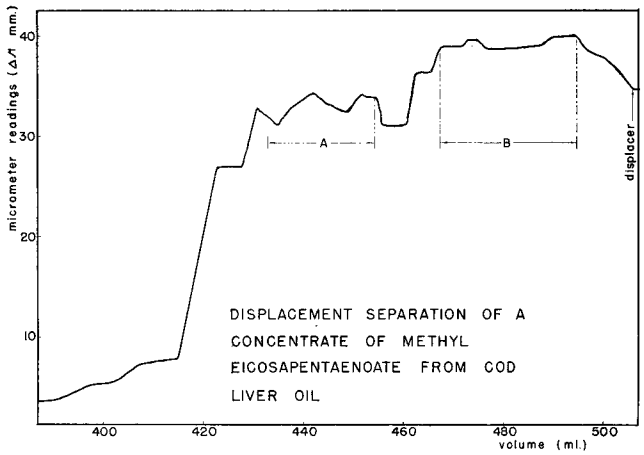

FIG. 5. Displacement separation of partially purified methyl eicosapentaenoate.

Fraction B still contained some hexane contaminant as indicated by the extinction coefficient and the iodine value, 405, which is about 1% higher than the theoretical, 401, for methyl eicosapentaenoate. This fraction is considerably more pure methyl eicosapentaenoate than was the elution fraction 6. The melting point of the completely hydrogenated material was 45.8 to 46.1°C. Saponification equivalent of the latter was 329. Reference values for pure methyl arachidate are 45.8 to 46.6°C. and 326.6. Assuming that the sole impurity was methyl docosahexaenoate, the purity of this methyl eicosapentaenoate preparation is about 91%. Its extinction coefficient at 3,460 Å was found to be about 45% lower than that of a methyl eicosapentaenoate prepared by Riemenschneider and coworkers (12) from adrenal lipids. This may indicate differences in polyunsaturated fatty acids from marine and land animal sources. Closer comparative studies of the composition of polyenoic acids from various sources must be made before firm conclusions may be drawn.

Ethyl docosapentaenoate by elution chromatography. Thirty grams of an ethyl ester concentrate from cod liver oil (I.V. 231) prepared by fractionation with urea was chromatographed on a 105 x 6 cm. column containing 700 g. silicic acid-Hyflo mixture. Results of the fractionation and other data pertaining to it are presented in Table IV. The recovery was rather low as usual. The iodine values began decreasing after fraction 9, and fractions 11 and 12 were deep yellow in color. Since the iodine value of fraction 7 was only 205.5, fractions which were collected before fraction 7 were therefore not characterized. Fraction 9 had an iodine value of 354.9, approximately equal to theoretical iodine value 354 of ethyl docosapentaenoate. The completely hydrogenated product had a melting point of 47.1°C. The melting points of ethyl behenate reported in the literature are 48.25 and 48.7°C. The saponification equivlent was found to be 367.1 (theory for ethyl behenate is 368.6). Fraction 9 was thus ethyl docosapentaenoate about 98.5% pure. The complete ultraviolet absorption spectra after isomerization of approximately 80 mg. samples with 21% KOH-glycol for 7 and 15 minutes are shown in Figure 4. It is obvious from the graphs that the extinction coefficients after a 7-min. treatment are much higher than those after a 15-min. treatment, suggesting that the 15-min. treatment is not

TABLE IV

Elution Fractionation on Silicic Acid of Cod Liver Oil Ethyl Ester Concentrate

Fraction No.	Volume of Eluate ml.	Chloroform Content in Skelly F % (by volume)	Weight of Fraction g.	Iodine Value (Wijs)
Original			30.0	231.0
1	15,500	0.5	.....	.....
2	6,500	1.0	.....	.....
3	6,000	2.0	0.16	.....
4	6,000	5.0	1.65	.....
5	6,000	5.0	1.14	.....
6	7,500	10.0	4.81	.....
7	8,000	10.0	4.00	205.5
8	6,000	10.0	0.94	322.5
9	9,000	15.0	1.67	354.9
10	9,500	20.0	2.02	296.0
11	6,000	20.0	0.27	.....
12	6,000	20.0	0.30	.....

optimum for conjugation of this pentaene. The extinction coefficients of ethyl docosapentaenoate at characteristic wave length maxima are given in Table 2.

From the data included in this report certain limitations of the use of the spectrophotometric analysis of polyunsaturated acids are apparent. It has been supposed that when pure polyunsaturated fatty acids were isolated, contents of hexaene, pentaene, tetraene, triene, and diene could then be calculated simply. It now appears from this and other current work (16) that the case is not so simple. It has been demonstrated that there are quantitative differences between the spectral responses of polyunsaturated fatty acids of different sources and that the optimum conditions which have been so carefully worked out for arachidonic acid (12) are not optimum for pentaene and hexaene acids.

Where the primary interest is analysis of tetraene, pentaene, and hexaene acids, it would seem that the shorter heat treatment, which engenders a greater yield of the conjugated polyenes characteristic of these acids, should be the method of choice. Unfortunately the matters of presaponification and sample size may become a significant factor here, for error due to differing degrees of saponification of samples containing long chain acids may be significant when so short a time is used. No detailed method has been yet worked out for the analysis of the hexaene acids, and it is to be hoped that this will be done.

From our data it is quite clear that calculation of the amounts of all of the polyunsaturated fatty acids must include the use of simultaneous equations. In the calculations of linoleate, linolenate, and arachidonate now commonly used, the end absorption of the less unsaturated acid at the wavelength characteristic of the next more unsaturated acid has not been a significant factor. However the end absorption of isomerized pentaenoic acids is quite large at the characteristic absorption maximum of hexaene, 3,750 Å. This has been shown to be true for all pentaenoic acids thus far isolated (12, 16). This necessitates the use of simultaneous equations for the calculation of pentaenoic and hexaenoic acid contents. Using this method of calculation, and using spectral constants for arachidonate and constants for pentaenoate and hexaenoate from cod liver oil, the polyunsaturated fatty acid contents of a variety of tissue lipids have recently been calculated (17). The values so gained showed surprisingly few anomalous values and are considered valid for comparative purposes or where high precision is not required. This suggests that the constants as applied are not greatly in error, but it is hoped that polyunsaturated acids from mammalian sources can be isolated for use as standards in analysis of mammalian lipids.

Summary

a) Displacement chromatography using a charcoal-isopropanol-methyl behenate system has been successfully applied to the isolation of docosahexaenoic acid and ethyl docosahexaenoate from cod liver oil concentrates.

b) Methyl eicosapentaenoate was isolated from cod liver oil methyl esters by combined elution chromatography on silicic acid and displacement chromatography.

c) Using silicic acid as adsorbent and petroleum ether-chloroform as solvent, ethyl docosapentaenoate was isolated from cod liver oil ethyl esters.

d) With a spinning band fractionation column it was found that fractional distillation of methyl esters of cod liver oil at 0.5 mm. Hg. resulted in increasing conjugation in the distillate as the fractionation proceeded. Fractional distillation is to be avoided as a final step in purification of polyunsaturated esters.

e) The end absorption of conjugated pentaenoic acids is so large at the characteristic maximum for hexaene (3,750 Å) that simultaneous equations are required for the calculation of hexaenoic and pentaenoic acids.

f) Fifteen minutes of treatment with 21% KOH-ethylene glycol at 180° is not optimum for isomerization of ethyl docosapentaenoate from cod liver oil. The optimum isomerization times for hexaenoic acids of various sources are 4 to 6 minutes, considerably different from the 15 minutes established for arachidonate.

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