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The Alkali Isomerization of a Methyl Docosahexaenoate and the Spectral Properties of Conjugated Fatty Acids[1]

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THE preparation of a quite pure methyl docosahexaenoate from hog brain lipids is reported in another paper from this laboratory (1). In the present paper the ultraviolet spectral characteristics of this compound, following alkali-isomerization under various conditions, will be considered in relation to alkali-conjugation-spectrophotometric techniques of analysis of polyunsaturated fatty acids and esters (2, 3, 4).

Also to be described are some general relationships in the ultraviolet spectral characteristics of fatty acids with conjugated double bond systems, whether natural or alkali-induced, which were developed on the basis of previous work by Lewis and Calvin (5).

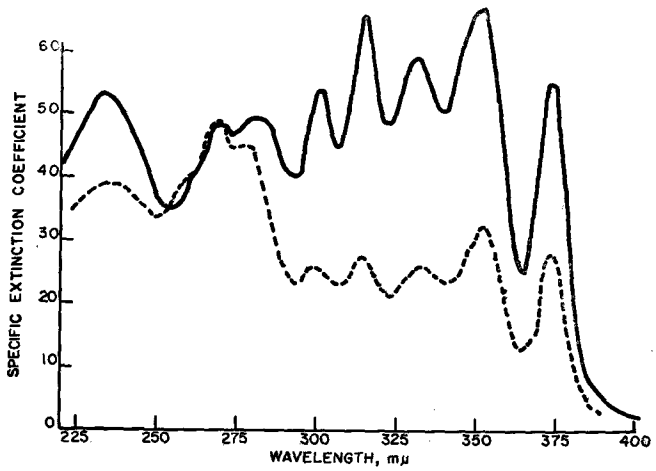

FIG. 1. Specific extinction coefficient vs. wavelength for small samples of methyl docosahexaenoate isomerized in 21% KOH-glycol at 180°C. for 4 min. (solid line) and 15 min. (broken line) by the micro procedure.

Ultraviolet Spectral Absorption Following Alkali Isomerization

Small samples of the methyl docosahexaenoate isomerized at 180°C. in 21% KOH-ethylene glycol for 4 and 15 minutes by the micro procedure of Herb and Riemenschneider (6) gave the absorption spectra represented in Figure 1. The data were obtained with a Beckman DU spectrophotometer. Measurements at wavelengths above 320 mμ were made with an incandescent light source. A slit width of 0.15 mm. was used for readings made at 374 mμ. (The importance of specifying slit width at this wavelength will be discussed below.) Absorption peaks are found at 233, 268, 279, 301, 315, 333, 352.5, and 374 mμ.

As with the fatty acids of lower unsaturation, the spectral properties following isomerization depend markedly on time of heating, alkali concentration, and temperature. In addition, two other factors, the spectrophotometer slit width and the size of the sample isomerized, are important in working with hexaenoate.

Effect of time of heating and alkali concentration. Figure 2 illustrates the effects of time of heating in alkali isomerization. The fixed conditions were the same as those used in obtaining the data for Figure 1. Evidently the maximal concentrations of conjugated materials are attained very rapidly in the case of hexaenoate. The heating times to reach a maximum for the various conjugated forms of alkali isomerized hexaenoate were found to increase in the following order as might be expected: a) di-, tri-, and tetraene, b) pentaene, and c) hexaene. Presumably if times shorter than 2 minutes were used, the maxima for the first three would be reached in the order listed.

After 5 minutes of heating the various conjugated forms generally undergo destruction more rapidly than they are formed. However the net amount of conjugated diene represented by the curve for 233 mμ decreases rather slowly, and the net amount of conjugated triene represented by the curve for 268

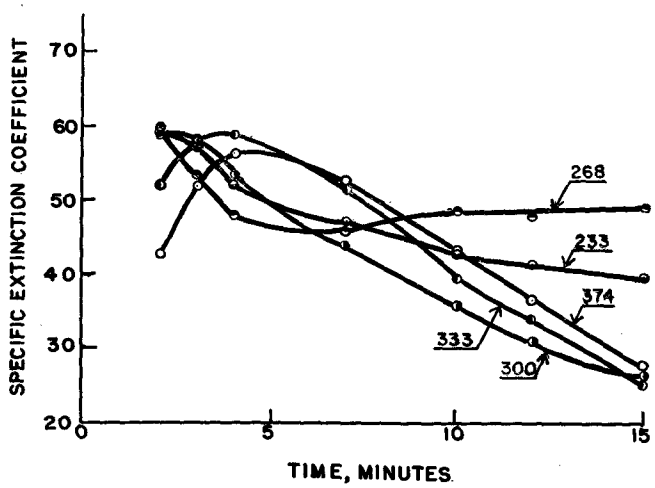

FIG. 2. Specific extinction coefficient at various wavelengths vs. time of alkali isomerization for methyl docosahexaenoate isomerized in 21% KOH-glycol at 180°C. by the micro procedure.

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mμ actually appears to undergo a slight increase. Among factors that may contribute to these effects are: a) the formation of some conjugated diene and triene from higher conjugated polyenes through polymerization or cyclization, b) the formation of some conjugated diene and triene via a shifting apart of double bonds that were originally located toward the center of the unsaturated system; it would be expected that conjugated dienes and trienes so formed could not disappear so readily by conversion to higher conjugated polyenes, and c) a greater reactivity of the higher conjugated forms which would cause them to disappear more rapidly by conversion to other materials.

When 16% KOH was used instead of 21%, the maximum in the hexaene conjugation at 374 mμ occurred at about 10 minutes and the curve was falling again at 15 minutes. However the maximum extinction coefficient was much lower than that obtained with 21% alkali. This behavior is similar to that reported for the conjugated tetraene formed from arachidonic acid (7).

It was decided that a more precise determination of the optimum conditions of time, alkali concentration, and temperature should be delayed until similar studies could be carried on concurrently with pentaenoates. The limited studies that were made strongly indicate that a shorter reaction time, or possibly a lower temperature, would yield better spectral absorption values for hexaenoates.

More complete data on the effects of these factors would probably prove useful in several other ways. If fatty acids that have the same number of double bonds but differ in the positions of the double bonds or in carbon chain length respond differently to changes of isomerization conditions, it may be possible to distinguish them in mixtures in some cases. Also the mechanisms involved in alkali isomerization might be clarified. In this connection a pentaenoate concentrate prepared from hog brain lipids and alkali isomerized under the conditions used in obtaining the data of Figure 2 showed a spectral absorption at 346 mμ that changed little when the heating time was varied between 5 and 15 minutes. On the basis of its chromatographic behavior this was believed to be the ester of a 22-carbon acid. Klenk and Bongard also found no pentaenoate in their 20-carbon fraction from brain tissue (8).

Effect of spectrophotometer slit width. As shown in Figure 3, the extinction coefficient at 374 mμ varies markedly with the slit width. Such variation is contrary to experience with conjugated forms involving fewer double bonds arising from the hexaenoate or from other less unsaturated acids. Because, in general, lipid materials contain at most a relatively small proportion of hexaenoate, it is important for the sake of accuracy to use a narrow slit to obtain high values of the conjugated hexaene absorption compared to background. The reason for the sharpness of the absorption by this form as compared with the lower conjugated forms is not known.

Effect of sample size. With the pure methyl docosahexaenoate the size of the sample used in alkali-isomerization had a marked effect on the extinction coefficients obtained. This effect was in no way related to the concentrations of any reagents or solvents present in the solutions used in making the spectral readings.

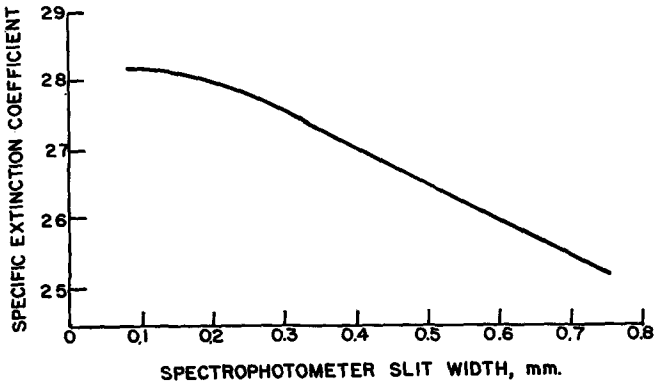

FIG. 3. Specific extinction coefficient vs. slit width of the spectrophotometer for the 374 mμ band of methyl docosahexaenoate alkali-isomerized in 21% KOH-glycol at 180°C. for 15 minutes.

Other observations suggested that the effect of sample size was related to the time required completely to dissolve the docosahexaenoate in the isomerization reagent. Employing the macro isomerization method described below and a slit width of 0.15 mm., the extinction coefficients at 374 mμ increased with sample size when a heating time of 15 minutes was used, being 28.7, 29.5, and 31.4 for sample weights of 19, 38, and 58 mg., respectively. On the other hand, with a heating time of 6 minutes, the extinction coefficients showed decreasing values with increasing sample size, being 49.1, 49.0, 45.5, and 44.1 for sample weights of 5, 32, 56, and 73 mg., respectively.

That these results are consistent with a greater solution time for the larger samples is evident from Figure 2. Large samples would have a lesser average time of contact with the isomerization reagent than small samples. With the apparent heating time at 6 minutes the appreciably lower effective heating time for large samples would result in lower extinction coefficients. On the other hand, with an apparent heating time of 15 minutes the progressive lowering of effective heating time with increasing sample size would yield higher extinction coefficients. An examination of the effect of sample size on the extinction coefficients at wavelengths other than 374 mμ is also completely in accord with this interpretation.

Further evidence relating the effect of sample size to solution time was found by the isomerization technique of Vandenheuvel and Richardson (9). In this procedure the samples are partly or wholly dissolved in the reagent by a preliminary heat treatment at 80°C. The extinction coefficients at 374 mμ of two 5 mg. samples and two 55 mg. samples so treated and then isomerized all agreed within 1%.

The nature of the solution effect was more clearly established in still another experiment. Small samples of docosahexaenoate were diluted with methyl oleate or with methyl esters of corn oil fatty acids, and larger samples of the resulting solution, 60 to 90 mg., were analyzed by the macro method. The extinction coefficients agreed well with those previously obtained with small samples of docosahexaenoate alone. When similar treatment was given to small samples of docosahexaenoate diluted with methyl erucate, the erucate samples failed to dissolve completely. Moreover the extinction coefficients for conjugated hexane and pentaene were appreciably lower and those for conjugated tetraene, triene, and diene

appreciably higher than normal for small samples, indicating that the effective heating time was much less than the apparent heating time of 15 minutes. Thus it appears that the effect of sample size is to be attributed to the relatively longer time required for the solution of esters of 22-carbon acids as compared with 18-carbon acids.

These results suggest also that previously published spectral constants obtained by the macro method for arachidonate, eicosapentaenoate, and docosapentaenoate may require reevaluation. The marked differences reported for the spectra of alkali-isomerized eicosapentaenoate and docosapentaenoate (10) may be due as much to solubility differences as to other causes that have been proposed (7). If the two compounds have the same pentaene structure and differ only by two carbons in one of the end groups (or one carbon in each of the two end groups), one would expect that the spectra following equal alkali-isomerization treatment would be more nearly alike. A solution effect may also account for the apparent differences mentioned earlier between the eicosapentaenoate isolated by Herb *et al.* (10) and that isolated from fish oil by the present authors (1). However it has not been established whether solubility factors are important in the isomerization of C_{20} acids with the KOH-glycol reagent.

Because of the variation in spectral characteristics in relation to sample size, a question arises concerning the accuracy that may be expected in quantitative determinations of hexaenoate by the alkali-isomerization spectrophotometric techniques that have been employed here. On the macro scale, samples of pure docosahexaenoate up to 30 mg. can be used with no significant change in the alkali-isomerization constants. On the micro scale, samples as large as 8 mg. have been used with no significant changes. Since the amount of C_{22} acids in lipid materials other than marine oils is likely to be small and since the solution effect is not encountered if alkyl esters of 18-carbon acids predominate (probably also true for glyceryl esters), it appears that the spectral constants obtained with small samples of methyl docosahexaenoate may be used in the analysis of some lipids by the usual procedure. For accurate analysis of mixtures containing acids of predominantly longer chain lengths smaller samples should be used.

Because hexaene conjugation changes more rapidly with time of heating than the conjugated forms of less unsaturated acids, it is all the more important that the sample be quickly and reproducibly brought into solution in the isomerization reagent. This is not possible if the fatty acids are combined as phospholipid or cholesterol esters (11). From this point of view it is unfortunate that the more unsaturated fatty acids occur predominantly in combinations of this type. Little attention has been given to these factors in previously published data on the content of highly unsaturated fatty acids in lipid materials, and hence such data in general may be unreliable, even in cases where only qualitative comparisons have been attempted.

Spectral absorption constants. Spectral constants obtained from pure methyl docosahexaenoate by macro and micro isomerization methods, using 21% KOH-ethylene glycol, a temperature of 180°, and a heating time of 15 minutes, are given in Table I. Although it has been previously indicated that these are probably not optimal conditions for the determination of highly unsaturated fatty acids, the values are given to permit tentative analysis on the basis of conditions that have been employed for less unsaturated fatty acids and that have received relatively wide acceptance. For studies giving special attention to hexaenoates, methods employing other conditions of isomerization should probably be developed.

TABLE I

Specific Extinction Coefficients for Methyl Docosahexaenoate Isomerized 15 Minutes in 21% KOH Glycol at 180°C.[a]

Wavelength mμ	Micro scale Sample weight: < 10 mg.	Macro scale Sample weight: < 30 mg.
374.0[b]	28.1	28.1
352.5	32.2	32.8
346.0	26.2	26.6
333.0	27.3	27.0
315.0	27.8	28.4
300.0	26.7	26.8
278.5	45.7	46.4
268.0	49.4	50.4
233.0	40.0	40.4

[a] Readings above 315 made with visible light source.
[b] Slit width 0.15 mm.

The constants in Table I for the macro determination were obtained with the following modifications of the A.O.C.S. official method (12). The KOH-glycol was made up to 21 ± 0.1%. It was measured into the reaction tubes by means of a hypodermic syringe instead of by weight; 8.4 ml. of the reagent was found to equal 11 g. within permissible limits of accuracy. A tube of reagent was heated at 180°C. for 3 minutes, shaken in the bath for 15 seconds, and allowed to stay in the bath for a total of 10 minutes before the sample was added. Following introduction of the sample, the tube was shaken for 45 seconds with the lower portion still in the bath. Fifteen minutes after the sample was added, the tube was plunged into cold water. The reaction mixture was protected with oxygen-free nitrogen at all times.

The method of stirring described in the foregoing paragraph is recommended because of the effect of sample size previously described and because of greater reproducibility. The method has been compared experimentally with that of Herb and Riemenschneider (7), and the results were found to be essentially the same if the sample was small.

The constants for the micro procedure were obtained by the method of Herb and Riemenschneider (6) except for modifications identical with those that have been described above for the macro method.

If these isomerization conditions are employed, the equations that follow may be used in calculating the amounts of polyunsaturated fatty acids in appropriate cases. These equations have been derived from data obtained by the micro method, but they may be applied with almost negligible error to data obtained by the macro method. It is not yet known whether they may be used for fish oils; the latter may be composed of mixtures of isomeric polyene acids with differing alkali-conjugation characteristics. Likewise the equations cannot be used if the highly unsaturated acids are present as cholesterol esters or as other forms which are dissolved with difficulty in the isomerization reagent.

The equations are based on the use of a slit width of 0.15 mm. for spectrophotometer readings at 374 mμ. If a different slit width is desired, corresponding equations may be obtained by employing the appro-

priate value for the extinction coefficient at 374 mμ given in Figure 3.

EQUATIONS FOR % ACID, MICRO METHOD

(Constants for acids other than hexaenoic were taken from data of Herb and Riemenschneider [6])

If pentaene is C_{22},

Hexaene $= 3.41\ k_{374}$[a]
Pentaene $= 1.98\ k_{346} - 1.84\ k_{374}$
Tetraene $= 1.65\ k_{315} - 1.86\ k_{346} + 0.10\ k_{374}$
Triene $= 1.10\ k_{268} - 0.88\ k_{315} - 0.02\ k_{346} - 0.88\ k_{374}$
Diene $= 1.09\ k_{233} - 0.57\ k_{268} - 0.26\ k_{315} - 0.12\ k_{346} - 0.18\ k_{374}$

If pentaene is C_{20},

Hexaene $= 3.41\ k_{374}$[a]
Pentaene $= 1.14\ k_{346} - 1.06\ k_{374}$
Tetraene $= 1.65\ k_{315} - 1.55\ k_{346} - 0.19\ k_{374}$
Triene $= 1.10\ k_{268} - 0.88\ k_{315} + 0.31\ k_{346} - 1.35\ k_{374}$
Diene $= 1.09\ k_{233} - 0.57\ k_{268} - 0.26\ k_{315} + 0.02\ k_{346} - 0.27\ k_{374}$

The Ultraviolet Absorption Spectra of Conjugated Fatty Acids

There have been numerous attempts in recent years to explain the absorption spectra of various polyenes on the basis of quantum mechanics (13, 14) and by analogy to various types of oscillators (5, 15, 16). Little attention appears to have been given however to fundamental relationships in the absorption spectra of naturally conjugated and alkali-conjugated fatty acids. With other types of compounds, analogies between conjugated systems and various types of coupled oscillators have led to accurate predictions of the wavelengths of absorption maxima in some cases.

Such developments of theoretical relations between spectral absorption and the number of double bonds have in general been concerned only with the lowest frequency absorption peak or band of the several that may occur in the ultraviolet spectrum. With many types of polyenes only a single cusped peak is found in the ultraviolet region that is usually studied (17), but with others there may be more complex patterns, as in the case of pure conjugated fatty acids whose absorption occurs as tricusped peaks. In such cases usually either the most prominent of the longer wavelength cusps or the longest wavelength cusps for a family of compounds are compared.

Lewis and Calvin (5) have derived the relation

$$\lambda^2 = A + B\,n$$

where λ is the wavelength of the absorption maximum for the lowest frequency of absorption, A and B are constants, and n is the number of conjugated double bonds in the system. This relation was obtained by considering the several double bonds in a conjugated system to be simple harmonic oscillators which must vibrate in unison.

Kuhn (15) has derived the more complex formula from a more rigorous treatment of the nature of coupled resonators:

$$\lambda = \frac{\lambda_0}{\left(1 - 2\,a\cos\frac{\pi}{n+1}\right)^{1/2}}$$

λ again is the wavelength of the cusp with the lowest frequency in cases where multi-cusped absorption is found, λ_0 is the corresponding wavelength for a compound with one double bond, a is a constant related to the force constants of the coupled oscillators, and n is the number of double bonds in the conjugated system. Kuhn has also developed mathematical predictions of higher order bands; in the case of fatty acids these would occur in wavelength regions below 200 mμ and hence are out of the range of ordinary ultraviolet spectrophotometers. Although the foregoing relation may be successfully employed in predicting ultraviolet absorption wavelengths for a number of polyenes, it does not predict the wavelengths of the absorption maxima for conjugated fatty acids as accurately as the relation given by Lewis and Calvin.

The three cusps of pure conjugated fatty acids, with the exception of conjugated dienoic acid, are easily detected by the spectrophotometers commonly employed for research purposes today. Similarly any alkali conjugated polyene acid might be expected to exhibit three cusps for each of the several classes of conjugated forms present (*e.g.*, diene, triene, etc.). Using data for both pure conjugated materials and alkali-isomerized materials, if the square of the wavelength of the three cusps of each peak is plotted against the number of double bonds associated with that peak, the points for the longest wavelength cusps will fall on one line, those for the second longest wavelength cusps on another, and those of the shortest wavelength cusps on still another, as in Figure 4.

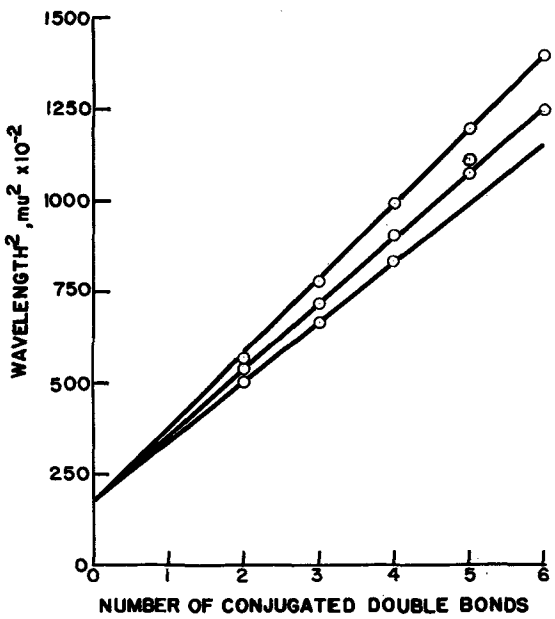

FIG. 4. Wavelength squared of the absorption maxima for various conjugated fatty acids vs. the number of conjugated double bonds associated with the absorption.

This figure is derived from the following data: a) conjugated linoleate, either 9, 11, or 10, 12; 224, 232, and 238 mμ (18) (This material is presumably of *cis-trans* and *trans-cis* configuration. It might be more appropriate, as will be discussed, to use data for a *trans-trans* material. This would substitute 229.5 for 232 mμ (19). The other cusps would be shifted correspondingly. However these differences would not affect the figure appreciably); b) conjugated (allo) linolenate, β-eleostearate, and β-licanate, 258, 268, and 279 mμ (20); c) β-parinarate, 288, 301, 315

[a] The use of this equation will lead to some error, especially in the analysis of mixtures in which the ratio of pentaenoate to hexaenote is high, due to an appreciable absorption by alkali-isomerized pentaenoate at 374 mμ. The same kind of error is encountered at the other wavelength maxima, but to a smaller degree; the effect of such end absorptions by the more unsaturated esters has been noted by other investigations (25).

mμ (20), also alkali isomerized arachidonate, 301 and 315 mμ (7); d) alkali isomerized pentaenoate, 315 (?), 328, and 346 mμ (10); e) alkali isomerized hexaenoate, 333, 352.5, and 374 mμ.

Absorption peaks for all of the various degrees of conjugation developed on alkali isomerization of linolenate, arachidonate, pentaenoate, and hexaenoate fit the curves in the figure well, except in cases where there is overlapping absorption.

The peaks of the beta forms of the eleostearic and parinaric acids coincide more closely with those for the alkali-isomerized acids than do the alpha forms. This strongly indicates that the higher conjugated forms of the more unsaturated alkali isomerized fatty acids are largely in *trans* configurations, at least under the conditions of alkali-isomerization ordinarily employed.

It is evident from Figure 4 that the several cusps yield straight lines with different slopes which appear to converge to a single point. There is some question however about where the point of convergence occurs. There appears to be no theoretical basis for anticipating a convergence at 0 double bonds in preference to a convergence at 0 wavelength, but a convergence at 0 double bonds as shown in Figure 4 appears to fit the available data best.

Lewis and Calvin (5) have indicated in their treatment of diphenylpolyenes that the constant A in their relation is determined by the nature of the phenolic end groups in the oscillating systems, and B is a function of the force constant of the oscillator. However the effect of the end groups has been treated as a quantity to be added to n by Ferguson and Branch (21) in their analysis of the spectra of various polyenes whose conjugated carbon chain was interrupted by nitrogen linkages.

Those constituents of the end groups that are removed by more than 2 or 3 carbons from the oscillating system appear not to affect the wavelength of absorption very appreciably. Thus the predicted peaks for monoene and saturated fatty acids from Figure 4 closely correspond with reported absorption peaks for n-heptene-3 and saturated carbon-carbon linkages (22). However in view of the uncertain significance of the constants in the relation given by Lewis and Calvin and the complexity of the spectra of hydrocarbons in the vacuum ultraviolet as compared to the relatively simple spectra found for conjugated hydrocarbons in the quartz ultraviolet, the apparent approximate correspondence between observed absorption peaks for monoenes and saturated compounds and those predicted from Figure 4 may be fortuitous.

Spectra of Alkali-Isomerized Fatty Acids

Because an alkali-isomerized polyene acid, particularly one that is highly unsaturated, is a rather complex mixture of conjugated polyenes, some of the cusps for the various degrees of conjugation are difficult or impossible to detect. Among other factors involved are the following: a) some types of conjugated chromophores may be present in relatively low concentration; b) isomeric compounds which differ only in the geometric configuration of their double bond systems may be present whose absorption maxima occur at slightly different wavelengths; c) because of the complexity of the mixture, there is some overlapping of absorption by different compounds. A further consideration of Figure 4 leads to explanations for several of the peculiarities of the spectra of the alkali-conjugated fatty acids.

The curves lead to the prediction that the cusps for conjugated dienes will be close together, and the failure to find three cusps is evidently due to a lack of sufficiently good resolution in the spectrophotometers generally used. Kass (18) has published an absorption spectrum for conjugated dienoates which shows evidence of three cusps.

With alkali-isomerized linolenate all three cusps of the conjugated triene are usually seen although the shortest wavelength cusp is quite weak. As a general rule, with all conjugated fatty acids the middle cusp is highest.

In the cases of alkali-isomerized arachidonate or more unsaturated fatty acids, the shortest wavelength cusp of the tetraene conjugation is usually not seen because it is masked by the 279 mμ cusp of the conjugated triene absorption. It is, of course, easily observed in the case of parinaric acid (20). Evidence of it may also be seen in a curve for alkali-isomerized arachidonate published by Herb and Riemenschneider (7).

The pentaene conjugation of alkali-isomerized pentaenoate (7) is represented by two cusps found at 328 and 346 mμ. Figure 4 indicates that a third cusp for conjugated pentaene cannot appear discretely because it virtually coincides with the longest wavelength cusp of conjugated tetraene. This is undoubtedly the reason why the cusp at 315 mμ is higher than that at 301 mμ in the absorption spectra of alkali-isomerized pentaenoate and hexaenoate, unlike the case of alkali-isomerized arachidonate. The 301 mμ cusp is the middle cusp for conjugated tetraene and would normally be expected to be the highest.

With alkali-isomerized hexaenoate the 346 mμ cusp of the pentaene peak is masked by the strong middle cusp of the hexaene peak, which falls at 352.5 mμ (see Figure 1). This may be one cause of the confusion in the literature about the location of this cusp for pentaenoates, and some investigators have measured the absorption at 347.5 mμ (23, 24). In cases where mixtures of pentaenoate and hexaenoate are alkali-isomerized, the position of the absorption in this region will vary according to the proportions present in the original mixture. With some mixtures discrete peaks at 346 and 352.5 mμ may be discerned.

The shortest wavelength cusp of the hexaene peak of isomerized hexaenoate acid would be predicted to fall at about 338 mμ. This cusp is evidently not resolved from the 328 mμ cusp of conjugated pentaene and therefore gives rise to a relatively broad band at about 333 mμ in alkali-isomerized methyl docosahexaenoate (Figure 1). It is for this reason that a point represented by 333 mμ does not fall on one of the lines in Figure 4.

From the foregoing it is clear than in alkali-isomerized docosahexaenoate, or any mixture of fatty acids containing docosahexaenoate, all three cusps of the pentaene conjugation will be overlapped to some extent by conjugated tetraene and hexaene cusps. However this may not introduce serious error into the determination of pentaenoate in a mixture containing hexaenoate since the correction at 346 mμ due to conjugated hexaene is quite reproducible in spite of its location on the side of a steeply sloped absorption band.

Corrections for background based on reading two values on either side of a maximum as suggested in A.O.C.S. Official Method Cd7-48 (9) will not be valid if there is any overlapping of two cusps that represent different degrees of conjugation. This will be true for absorptions at 315, 328, 346, and 352.5 mμ for mixtures containing hexaenoate.

The elucidation of the nature of the absorption spectra of conjugated fatty acids presented by Figure 4 should prove useful also in studies of the geometric configuration of double bonds in conjugated or alkali-isomerized fatty acids. Conjugated systems which differ in geometric configuration of their double bonds seem to show corresponding systematic differences in the position of their absorption maxima. This may also find application in reaching an understanding of the kinetics and mechanisms involved in the alkali isomerization of unsaturated fatty acids.

Summary

The dependence of the ultraviolet absorption spectra for docosahexaenoate upon conditions used in alkali-isomerization has been studied. The extinction coefficients at all wavelengths varied with sample size when 21% KOH-glycol was used as the isomerization reagent at 180°C.; the effect of sample size was due to the solubility characteristics of 22-carbon fatty acid esters in the isomerization reagent.

Spectrophotometer slit width is an important consideration in the measurement of extinction coefficients at the absorption maximum at 374 mμ.

Certain conditions which must be met for accurate analysis of hexaenoate in fatty acid mixtures are described.

An extension of a theory of Lewis and Calvin has been used to clarify the number, position, and to some extent, intensity of the peaks in the ultraviolet absorption spectra of natural conjugated and alkali-isomerized fatty acids.

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A Methyl Docosahexaenoate: Its Isolation and Characterization[1]

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A study of possible relationships between diet and the highly unsaturated fatty acids (4, 5, and 6 double bonds) of human blood lipids has been started in this laboratory. The presence of fatty acids with five and six double bonds in fish oils (1, 2, 3, 4, 5) and in the lipids of land animals (6, 7) has been known for some years, but relatively few investigations of their implication in lipid metabolism have been made (8, 9, 10).

In order to determine if alkali-conjugation spectrophotometric techniques (11, 12, 13) could be applied in accurate quantitative analysis of the highly unsaturated acids in blood lipids, it was necessary to isolate hexaenoate in pure form and to determine its ultraviolet spectral characteristics following alkali isomerization. Two five-double bond acids have previously been isolated and characterized by Herb *et al.* (14). Knowledge of the ultraviolet spectral characteristics of pure pentaenoate and hexaenoate is also necessary for accurate estimates of the less unsaturated acids in mixtures where penta- and hexaenoates are present. No definite evidence of the existence of fatty acids with more than six double bonds in animal lipids has been reported.

This paper reports the preparation (starting with hog brain), some of the properties, and the probable structure of a methyl docosahexaenoate, together with its behavior under various conditions of alkali isomerization. Some general relationships in the ultraviolet spectral characteristics of various alkali isomerized and conjugated fatty acids were encountered, and a general relationship in the light refractions of unconjugated fatty acid esters was also established, but for convenience these findings will be described in separate communications (15, 16).

Isolation

In preliminary work on fish oils that contain both penta- and hexaenoic acids, spectra were obtained for the C_{20} pentaenoic acids after isomerization which were different from those of the pentaenoic acids isolated from beef adrenals by Herb *et al.* (14). It was therefore decided to turn to a higher animal as a source for hexaenoate. Available evidence indicated

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