Further Studies on the Isomerization of Polyunsaturated Fatty Acids by Potassium Tertiary Butoxide[3]

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IN THE COURSE of a previous investigation (1) on the use of potassium-t-butoxide to isomerize polyunsaturated acids it was observed that an increase in the boiling point of the reagent because of an increase in concentration of active reactant was accompanied by an increase in total conjugation. It is therefore to be expected that at still higher temperatures further and possibly complete conjugation of the polyunsaturated acids can occur. To use a low boiling reagent such as potassium-t-butoxide in t-butanol (the one used in the previous investigation) at higher temperatures we have resorted to the classical organic chemist's procedure of conducting the reaction in a sealed tube. By using this technique we have been successful in arriving at the conditions which result in the highest degree of conjugation of linoleic, linolenic, arachidonic, and higher polyunsaturated fatty acids so far reported.

Experimental

Materials Used

Linoleic Acid Concentrate. This was prepared by low temperature crystallization of cottonseed oil fatty acids from pentane: I.V., 152.0; about 70.0% linoleic acid. Linolenic acid concentrate, methyl arachidonate II, pure linoleic and linolenic acids, and the samples of oil examined were the same as those used previously (1). Alpha- and beta-eleostearic acids were supplied by F. G. Dollear of Southern Regional Research Laboratory, New Orleans, La. U.v. data on a cyclohexane solution of these acids follow:

Wave length in mμ	276.8	271.5	269
Alpha acid	124.4, 124.6	176.5, 178.7	156.9, 159.0
Beta acid	123.4, 124.0	168.0, 166.8	202.0, 203.0

Apparatus

Isomerization Tube. A Pyrex glass vial of about 30-ml. bulb capacity (length 9 cm., diameter 2.5 cm.) and a stem 12 cm. long with 7-mm. bore was used for this purpose.

Heating Bath. A two-liter beaker filled to about 50 mm. (2 in.) from the top with peanut oil was used. When a large number of determinations were made, a four-liter beaker was employed. The heating bath was provided with a mechanical stirrer of adjustable speed. It was heated by a burner. The temperature was noted by means of a thermometer suspended in the bath and was maintained within ± 2°C. of the desired one.

Reagent. This was of the same strength (5 g. of potassium in 100 ml. of tertiary butanol) and was prepared in the same way as previously (1).

General Procedure. The isomerization tube was flushed out with nitrogen and about 100 mg. of the sample were introduced through a 2-mm.-bore glass tube. Any sample left accidentally on the walls of the vial stem was washed down, after weighing, with 0.5 ml. of tertiary butanol added dropwise along the sides with the help of a medicine dropper. Five ml. of the reagent were added. The stem was then washed again with 0.5 ml. of tertiary butanol as before. The tube was flushed gently with nitrogen and sealed about two inches above the bulb. In sealing, the tube at the selected portion was drawn out into a capillary, the end of which was fused and bent to form a hook so that the tube could be suspended into the bath from another metallic hook, which was attached to a metallic ring. When a number of tubes have been mounted thus, the whole assembly was lowered into the bath which has been brought to the desired temperature (140°C. in the finally adopted procedure). The time of reaction was noted from here on. After about 5 min. of heating, each tube was lifted from the metallic hook and was swirled gently while still being immersed in the bath. After heating for the desired period (2 hours in the finally adopted procedure), the whole assembly was lifted out of the oil bath and was transferred to a water bath so as to be cooled quickly to room temperature. The sealed tube was broken, and the contents were transferred to a 100-ml. standard flask with methanol in the usual manner. After requisite dilutions the optical density readings were taken with a Beckman DU spectrophotometer.

Temperature Study. All the three concentrates were isomerized at temperatures between 120–170°, the times of isomerizations being 2 hrs. for linoleic

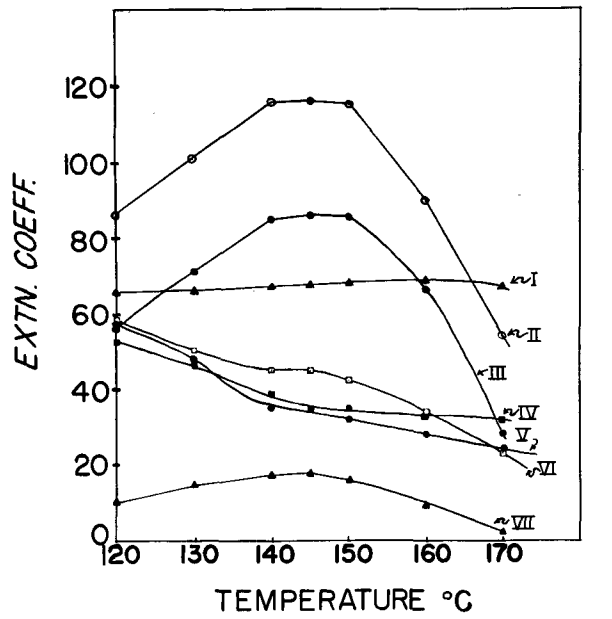

FIG. 1. Temperature study of isomerization by Potassium-t-butoxide.

I k_{233} Linoleic acid concentrate.
II k_{268} Linolenic acid concentrate.
III k_{315} Methyl arachidonate concentrate.
IV k_{233} Linolenic acid concentrate.
V k_{233} Methyl arachidonate concentrate.
VI k_{268} Methyl arachidonate concentrate.
VII k_{346} Pentaenoate.

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and linolenic acid concentrates and 1 hr. for the arachidonate as polymerization is likely to be dominant at higher temperatures and longer periods of reaction with this acid. The results are shown in Figure 1.

Time Study. A time study of isomerization was conducted for all the concentrates at temperatures 140°, 145°, and 150°, respectively, and the results are represented in Figures 2, 3, and 4. Extinction coeffi-

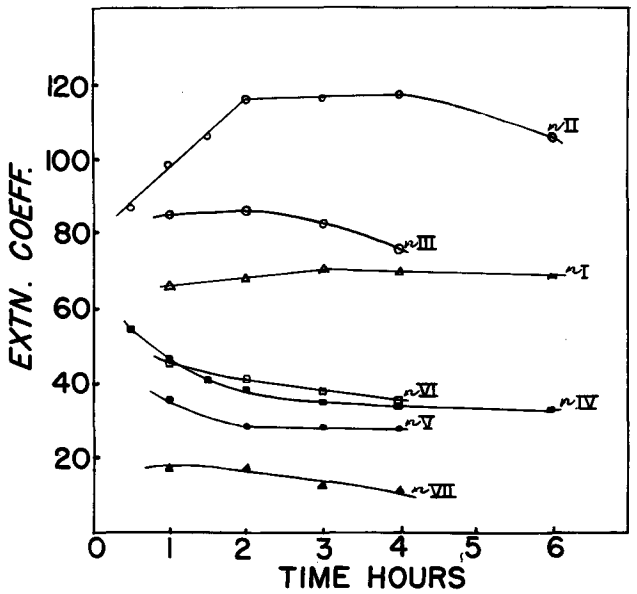

FIG. 2. Time study of isomerization by potassium-t-butoxide at 140°C.

I k_{233} Linoleic acid concentrate.
II k_{268} Linolenic acid concentrate.
III k_{315} Methyl arachidonate concentrate.
IV k_{233} Linolenic acid concentrate.
V k_{233} Methyl arachidonate concentrate.
VI k_{268} Methyl arachidonate concentrate.
VII k_{346} Pentaenoate.

cients at wavelengths of maxima for the different concentrates at the best times of isomerizations at these temperatures are recorded in Table I. Pure

TABLE I

Extinction Coefficients for the Polyunsaturated Acid Concentrates at the Optimum Times of Isomerization at Different Temperatures

Concentrate	Wavelength of maxima, mμ	140° 2 hrs.	145° 1.5 hrs.	150° 1 hr.
Linoleic acid	233	67.8	67.9	67.9
	268	1.8	1.8	1.8
Linolenic acid	233	39.0	38.0	37.8
	268	115.7	113.5	114.3
	315	4.8	4.9	4.9
Methyl arachidonate II	233	28.8	31.4	32.0
	268	40.8	41.6	42.5
	315	86.2	85.5	85.6
	346	16.4	15.8	15.8

linoleic and linolenic acids and methyl arachidonate II were isomerized for 2 hrs. at 140°, and the extinction coefficients at characteristic wavelengths of peaks are recorded in Table II. The absorption spectra for these acids over the range 220–360 mμ are shown in Figure 5.

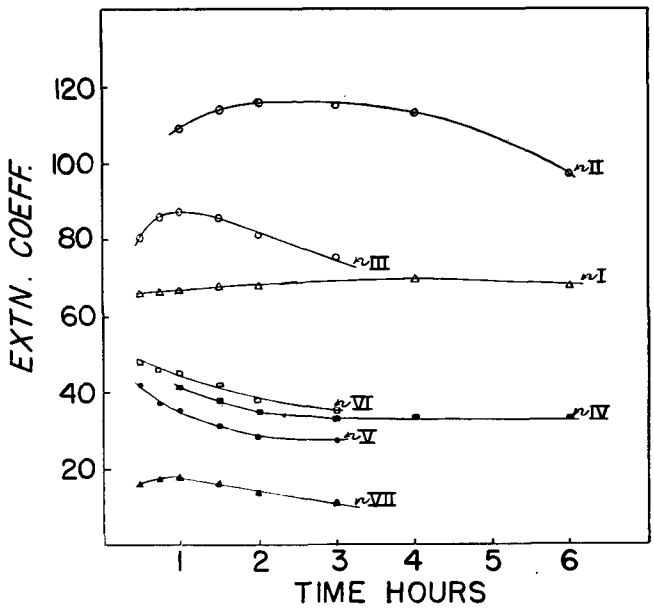

FIG. 3. Time study of isomerization by potassium-t-butoxide at 145°C.

I k_{233} Linoleic acid concentrate.
II k_{268} Linolenic acid concentrate.
III k_{315} Methyl arachidonate concentrate.
IV k_{233} Linolenic acid concentrate.
V k_{233} Methyl arachidonate concentrate.
VI k_{268} Methyl arachidonate concentrate.
VII k_{346} Pentaenoate.

Calculations. From Table II the average k_{232} value for linoleic acid is 98.0, and k_{232} and k_{268} values of linolenic acid are 30.5 and 138.4, respectively. Using these values, the following relationships are derived for calculating linoleic and linolenic acids in mixtures:

$$\% \text{ linoleic acid} = 1.020\, k_{232} - 0.225\, k_{268}$$
$$\% \text{ linolenic acid} = 0.723\, k_{268}$$

Samples of oils were examined by this sealed-tube method, and the results are recorded in Table IV.

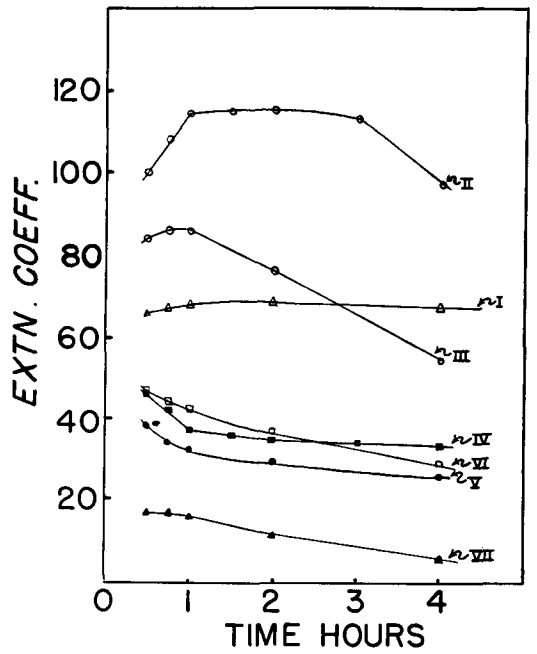

FIG. 4. Time study of isomerization by potassium-t-butoxide at 150°C.

I k_{233} Linoleic acid concentrate.
II k_{268} Linolenic acid concentrate.
III k_{315} Methyl arachidonate concentrate.
IV k_{233} Linolenic acid concentrate.
V k_{233} Methyl arachidonate concentrate.
VI k_{268} Methyl arachidonate concentrate.
VII k_{346} Pentaenoate.

TABLE II

Extinction Coefficients for Pure Linoleic and Linolenic Acids and Methyl Arachidonate II Isomerized by Different Methods

Acid	Wavelength mμ	6.6% KOH-glycol[b] 180°, 25 min.	21.0% KOH-glycol[b] 180°, 15 min.	Potassium-t-butoxide, t-butanol[a]	
				Open tube[b] 89–90°, 4 hrs.	Sealed tube 140°, 2 hrs.
Linoleic acid	231	—	—	—	98.6, 97.6, 98.2
	232	—	—	—	98.4, 97.6, 98.2
	233	91.0	91.6	94.0	97.0, 95.9, 96.4
Linolenic acid	232	—	—	—	30.6, 30.4, 30.6
	233	59.3	47.1	63.2	32.5, 32.1, 32.7
	268	50.5	90.6	74.2	138.6, 137.8, 138.9
Methyl arachidonate II	232	—	—	—	29.0, 29.5, 29.7
	233	55.2	39.2	62.4	28.8, 29.8, 30.0
	268	48.0	41.2	68.8	40.8, 39.6, 40.0
	315	24.1	59.2	38.8	86.2, 85.5, 85.8
	346	2.5	9.2	5.4	16.4, 16.0, 16.4

[a] 5 g. potassium dissolved in 100 ml. t-butanol.
[b] Values in these columns are those reported in the previous paper (1) reproduced here for purposes of comparison.

Isolation of Conjugated Trienoic Acids Produced by Potassium t-Butoxide Isomerization of Linolenic Acid. About 2 g. of linolenic acid concentrate were isomerized in 0.5-g. lots with 10 ml. of the tertiary butoxide reagent at 140° for 3 hrs. The isomerized lots were pooled; the fatty acids were recovered in the usual manner and crystallized from 50 ml. of pentane at —70°; and the crystals were recrystallized from 25 ml. of pentane at —70° once and 25 ml. of acetone at —40° once. The final crystal fraction F_1 and acetone filtrate (F_2) were free of dienes and were examined by ultraviolet and infrared spectrophotometry (Table III). The ultraviolet and infrared spectra are shown in Figures 5 and 6. The infrared spectra of the samples were taken in carbondisulfide solutions, using a 2-mm. sodium chloride cell in a Perkin Elmer Model 21, double-beam instrument. The wavelengths of peaks were located by actual scanning on a Beckman I. R. instrument, using a 1-mm. cell with the same solutions which were also used for quantitative measurements. During the isolation of these trienoic acids it was observed that some dark-colored fatty material insoluble in pentane stuck to the walls of the separating funnel and flasks, thereby showing that some polymerization does occur under these conditions.

Results and Discussion

Temperature Study. Potassium tertiary butoxide isomerizes linoleic acid at a rapid rate and practically to a maximum extent even at the lowest temperature studied, 120°; k_{233}, 66.0. Isomerization progresses only slightly with a further rise in temperature, reaching a maximum at about 160° (k_{233}, 68.6) with a negligible tendency towards decomposition at the highest temperature studied 170° (k_{233}, 67.3). The almost flat curve, Figure 1, suggests that

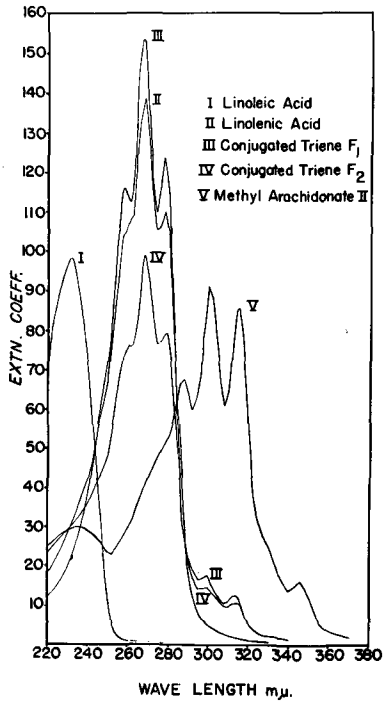

FIG. 5. Absorption curves for pure linoleic and linolenic acids, methyl arachidonate II, conjugated trienes F_1 and F_2.

any one of these temperatures is suitable for isomerizing linoleic acid and that the resulting conjugated dienoic acids are fairly stable thermally. With linolenic and arachidonic acids the isomerization reaches a maximum at about 140°, stays steady up to 150°, and declines rapidly at higher temperatures. With the pentaenoic acid a maximum is attained at 145°, and a further rise in temperature results in a pronounced and progressive loss of conjugation. It is thus obvious that conjugated counterparts of linolenic and higher polyunsaturated acids are not thermally very stable and maximum conjugation is attained in the range of 140°–150°. Therefore a time study of the isomerization of the polyunsaturated acids was carried out at 140°, 145°, and 150°, respectively, to select the optimum conditions of conjugation.

Time Study at 140°, 145°, 150°. From Figures 2, 3, and 4 it is seen that the time curves for linoleic acid at all three temperatures are practically flat, suggesting thereby that conditions chosen for the other acids will be suitable for this acid as well. At 140° isomerization of linolenic acid has reached practically the maximum in 2 hrs. (k_{268}, 115.7). It shows only a small rise up to 4 hrs. (k_{268}, 117.1) and thereafter declines gradually. At 145° the maximum is attained again in 2 hrs. and stays constant for 3

TABLE III

Analysis of Conjugated Trienes and Eleostearic Acids

Sample	m.p. °C.	u.v. Extinction Coefficients[a]				I.R. Extinction Coefficients[b]				
		232 mμ	267 mμ	268 mμ	270 mμ	10.08 μ	10.10 μ	10.13 μ	10.42 μ	10.43 μ
F_1	54.0	22.6	153.4	152.6	134.9	1.46	—	—	0.240	—
F_2	27.5	30.6	96.6	98.9	93.8	0.78	—	—	0.361	—
Alpha-eleostearic acid	48.0	15.8	158.0	168.0	182.0	—	0.78	1.13	—	0.389
Beta-eleostearic acid	70.5	20.5	204.4	203.8	175.9	—	1.72	1.30	—	0.213

[a] About 100 mg. of the sample were mixed with 5 ml. of the terbutoxide reagent, then purified methanol was added to dissolve the soap. The solution was diluted further with the same methanol for optical density measurements.
[b] Carbondisulfide solution—about 60 mg. in 10 ml. for F_2 and alpha-eleostearic acid and about 40 mg. in 10 ml. for F_1 and beta-eleostearic acid.

TABLE IV

Analysis of Samples of Oils by Different Methods [a]

Sample of oil	Component acid	Method: 6.6% KOH-glycol 25 min. 180°	21.0% KOH-glycol 15 min., 180°	Open tube 4 hrs., 90°	Sealed tube 2 hrs., 140°
Olive oil	Linoleic	5.0[b]	5.8[b]	5.5[b]	5.8, 5.8
	Linolenic	0.5[b]	0.7[b]	1.4[b]	0.9, 0.9
Cottonseed oil	Linoleic	47.5[b]	49.8[b]	49.3[b]	47.8, 47.9
Soybean oil	Linoleic	27.8[b]	29.8[b]	30.6[b]	26.6, 26.6
	Linolenic	5.8[b]	5.5[b]	6.5[b]	7.5, 7.5
Linseed oil [e]	Linoleic	13.3, 13.5	13.8, 14.4	14.0, 14.3	15.1, 15.4
	Linolenic	34.8, 35.0	33.6, 33.5	38.4, 38.0	35.4, 35.4
	Tetraenoic	2.8, 2.8[c]	4.1, 4.0[c]	3.5, 3.6[d]	4.3, 4.2[d]

[a] Results expressed as % acid in the sample.
[b] Average of values reported in the previous paper (1) reproduced for comparison.
[c] Calculated as arachidonic.
[d] k_{315} mμ.
[e] This sample of linseed oil has an I.V. 158.2, peroxide value 2.1, and unsaponifiable matter content of 1.1%. The unsaponifiable matter when isomerized and analyzed by the potassium-t-butoxide sealed tube method shows no peak in the tetraene region. The unsaponifiable matter free, fatty acids (distilled) similarly treated shows a peak in the tetraene region with k_{315} mμ 2.8.

hrs. (k_{268}, 115.3) when it starts declining which is pronounced beyond 4 hrs. At 150° the maximum is almost reached in 1 hr., k_{268}, 114.3. It is steady up to 2 hrs. (k_{268}, 115.3), then declines slowly up to 3 hrs., and very rapidly thereafter. Thus at higher temperatures the duration of stability of the conjugated triene decreases and the rate of destruction of conjugation increases, as evidenced by the decreasing length of the flat portion and increasing slope of the declining part of the time curves. With methyl arachidonate the maximum amount of conjugation at 140° is attained in 2 hrs. (k_{315}, 86.2), and further heating produces only a gradual decline. Similar maxima are reached in 1 hr. at 145° (k_{315}, 86.2) and 150° (k_{315}, 85.6), respectively, and the rate of destruction is very rapid thereafter. With the pentaenoic acid there is a decline of conjugation after 1 hr. at all the temperatures, which is more pronounced as the temperature is increased. It may be noted that increasing both time and temperature produces a tremendous destruction of conjugation with both arachidonic and pentaenoic acids, revealing the labile nature of the conjugated isomers of these acids. From the values listed in Table I it is seen that isomerization for 2 hrs. at 140° represents the best condition. A glance at Figure 2 shows that in 2 hrs. the isomerizations of both linoleic and linolenic acids are close to the maximum and that of arachidonic acid has already reached the same. Therefore these conditions were chosen for the isomerization and estimation of polyunsaturated fatty acids.

Isomerization of Pure Linoleic and Linolenic Acids and Methyl Arachidonate II. The absorption curve for linoleic acid in Figure 5 is similar to those obtained with the other methods (1), but the peak occurs at 231–232 mμ. This shift to a lower wavelength is likely to result from the production of a different mixture of geometrical isomers of conjugated dienes which are known to have different peak wavelengths (2, 3, 4, 5) under these conditions as compared to those with the other methods. Comparison of the extinction coefficients recorded in Table II shows that the value obtained by the present method is the highest which is obviously due to an increase in the extent of conjugation, thus confirming the point made in the previous paper (1) that these isomerizations are equilibrium reactions.

The absorption curve for linolenic acid in Figure 5 is markedly different from those obtained by the other methods in that there is no peak in the diene region. This can be interpreted as meaning that complete conjugation of linolenic acid has been attained under these conditions. From the time study at 140° for linolenic acid concentrate it is noted that at 2 hrs. the isomerization has not quite reached the maximum. To resolve this contradiction some insight into the nature of products formed by isomerization was considered necessary. This aspect was studied by isomerizing the linolenic acid concentrate, isolating the conjugated trienes and analyzing them by ultraviolet and infrared spectrophotometry. A comparison of the results for fractions F_1 and F_2 with those of alpha- and beta-eleostearic acids as given in Table III and also with the respective infrared spectrums, Figure 6, shows that F_1 is similar to beta-eleostearic acid, and F_2 is similar to alpha-eleostearic acid. Thus in Figure 2 the small increase in the extinction coefficients for the linolenic acid concentrate, 115.7 (2 hrs.) and 117.1 (4 hrs.), is likely to be caused by an isomerization of the alpha-type of acid to the beta-type, which has a higher extinction coefficient. While some general differences between alpha- and beta-eleostearic acids (such as the beta isomer having higher melting point and higher absorption in both ultraviolet and infrared regions) hold also for Fractions F_2 and F_1 (cf. Table III), yet these trienes have much lower values than those of the natural acids. The reason for this difference can only be found by analyzing these trienes for position of the double bonds and especially the exact location of the *cis* bond. This point is studied best by isomerizing pure linolenic acid and isolating and characterizing the resulting conjugated trienoic acids but could not be done because our supply of linolenic acid was exhausted. It is also seen (Figure 5) that the absorp-

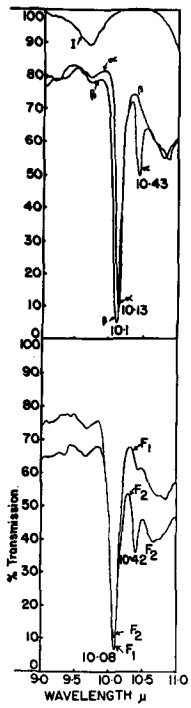

FIG. 6. Infrared spectrum in carbondisulfide I solutions of alpha (α) and beta (β) eleostearic acids and conjugated trienes F_1, F_2.

tion curve for isomerized pure linolenic acid is intermediate between those of F_1 and F_2, which is to be expected for a mixture containing both types of trienes. The presence of the alpha-eleostearic acid type of triene raises the possibility that the isomerization of linolenic acid might have attained completion earlier than 2 hrs. when the extinction coefficient was lower. The extinction coefficient, k_{268}, for pure linolenic acid obtained by the sealed tube method (Table II) is the highest so far reported for this acid. This together with a smaller value in the diene region will render the estimation of linoleic and linolenic acids in mixtures more accurate.

The absorption curve for methyl arachidonate II has peaks in the diene, tetraene, and pentaene regions. The absence of a peak in the triene region shows that complete conjugation of arachidonic acid has also been attained under these conditions. Thus the peaks in the diene and pentaene regions reveal the presence of nonconjugated diene and pentaene in the original sample. The extinction coefficients at 315 mμ and 346 mμ, respectively (Table II, last column), are about the highest as compared to other methods.

Analysis of Samples of Oils. Analysis of the oils for the component polyunsaturated acids by the sealed-tube method compares well with those of the other methods. It should be noted that because of a higher extinction coefficient at all characteristic peaks the estimation of the minor component by this method is likely to be the more reliable. The presence of a tetraene in the linolenic acid concentrate, as noted earlier, prompted a re-examination of linseed oil by all the methods with special reference to this acid. A peak was observed in the tetraene region by all the methods, and no peak was seen in the pentaene region. The contents of tetraenoic acids, calculated as arachidonic acid by the 6.6% KOH-glycol and 21.0% KOH-glycol methods, Table IV, do not agree and only the k_{315} values could be recorded for the potassium t-butoxide methods. Until the nature of this tetraenoic acid (length of the carbon chain and position and geometry of the double bonds) is settled, nothing more than its presence can be shown.

Summary

A temperature and time study revealed that the best conditions for the isomerization and estimation of polyunsaturated acids are heating in a sealed tube for 2 hrs. at 140° with potassium-t-butoxide in t-butanol. Under these conditions conjugation of linolenic and arachidonic acids appears to have attained completion. With linoleic acid a higher degree of conjugation than by other methods and a shift of peak to lower wavelength (231–232 mμ) are observed. Extinction coefficients obtained by this method for isomerized pure linoleic (k_{232}, 98.0) and linolenic (k_{268}, 138.4) acids are the highest so far reported. Ultraviolet and infrared examination of the conjugated trienes isolated from isomerized linolenic acid concentrate showed that, under these conditions, trienes similar to both alpha- and beta-eleostearic acids are produced. Analyses of samples of oils for component polyunsaturated acids, by this method, compare well with those by other methods. The presence of small amounts of a tetraenoic acid in linseed oil is noted.

REFERENCES

1. Sreenivasan, B., and Brown, J. B., J. Am. Oil Chemists' Soc., *33*, 521–526 (1956).
2. Ahlers, N. H. E., Brett, R. A., Metaggart, N. G., J. Appl. Chem., *3*, 433–443 (1953).
3. Allen, R. R., J. Org. Chem., *21*, 143 (1956).
4. Jackson, J. E., Paschke, R. F., Tolberg, Wesley, Boyd, H. M., and Wheeler, D. H., J. Am. Oil Chemists' Soc., *29*, 229–234 (1952).
5. Nichols, P. L., Herb, S. F., and Riemenschneider, R. W., J. Am. Chem. Soc., *73*, 247–252 (1951).

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3-Amino-1-Propanol as a Complexing Agent in the Determination of Total Gossypol

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THE ESTIMATION of total gossypol has proven to be useful for evaluating the influence of processing conditions on the distribution of the gossypol in cottonseed between the meal and oil (10, 14). Recent reports (3, 5), indicating an apparent relationship between total gossypol content and the nutritive value of cottonseed meal for nonruminants, suggest that this constituent may receive increasing attention as a quality factor of cottonseed meal. Although analytical methods currently employed for the estimation of total gossypol (1, 9) are adequate from the view-point of accuracy and precision, they require a minimum 6-hr. hydrolysis period for cleavage of bound gossypol. In the method proposed here total gossypol is completely removed from cottonseed meal in a 30-minute extraction, during which the gossypol is complexed with neutralized 3-amino-1-propanol in dimethylformamide. The difference in absorption of aliquot portions of the extract before and after reaction with aniline serves as a measure of the total gossypol content and allows proper correction for background absorption of the extracts.

The time required for analysis is 2 hrs. as compared to about 7 hrs. for the present methods. For control purposes the time required could be further reduced to 1 hr. by minor modification of the procedure. In addition to meals, the procedure is also applicable to cottonseed meats, crude oils, and soapstocks, offering the advantage of a single total gossypol method for all cottonseed products.

[1] One of the laboratories of the Southern Utilization Research and Development Division, Agricultural Research Service, U. S. Department of Agriculture.

Analytical Method

Reagents

1. Isopropyl alcohol-hexane mixture. Mix 60 volumes of reagent grade isopropyl alcohol and 40 volumes of commercial hexane [A.O.C.S. Specification H 16-56 (1)].

2. Complexing reagent. Pipet 2 ml. of 3-amino-1-propanol (practical grade) and 10 ml. of glacial acetic acid (reagent grade) into a 100-ml. volumetric