Thalictrum polycarpum Fatty Acids—A New Class of Fatty Acids from Vegetable Seed Oils*

M. O. BAGBY, C. R. SMITH, JR., K. L. MIKOLAJCZAK, AND I. A. WOLFF

From the Northern Regional Research Laboratory,† Peoria, Illinois

Received January 30, 1962

The principal fatty acid of *Thalictrum polycarpum* seed oil is the previously unknown *trans*-5,*cis*-9,*cis*-12-octadecatrienoic acid (35%). The oil also contains *cis*-9-octadecenoic (oleic) acid; *trans*-5-octadecenoic acid, not previously demonstrated in plant material; and two major components which were not characterized, a C_{18}-dienoic acid (18%) and an unknown C_{18}-acid (8%).

Thalictrum polycarpum or Sierra Meadowrue (fam. Ranunculaceae) is an uncultivated perennial herb native to the coast ranges of California and north to the Columbia River (Bailey, 1939). Earle *et al.* (1959) reported that *T. polycarpum* seed oil is unusual in that alkali isomerization methods that normally conjugate methylene-interrupted double bonds are not applicable to *T. polycarpum* seed oil.

sequent countercurrent distributions (Fig. 2 and 3).

Isolation of the Octadecenoic Acids.—The combined low-temperature crystallization fraction (H and K, Fig. 1) was shown by gas-liquid chromatography to be 68.1% C_{18}-monoenoic acid (Table I) consisting of more than one isomer. A 790-transfer countercurrent distribution (Fig. 2) of the methyl esters of the concentrate in an acetoni-

TABLE I

GAS-LIQUID CHROMATOGRAPHIC ANALYSES OF METHYL ESTERS OF FRACTIONS DERIVED FROM *Thalictrum polycarpum* FATTY ACIDS

Type of Acid	Equivalent Chain Length		Acid, %				
						Countercurrent Dist.	
	Apiezon-L	LAC-2-R 446	Original Acids	Low-Temp. Fractions H + K	Low-Temp. Fractions E + N	Run I[a] (402–461)	Run II[b] (404–479)
C_{14} Saturated	14.0	14.0	0.3	0.2	---	—	—
C_{16} Saturated	16.0	16.0	4.6	15.6	—	18.3	—
C_{16} Monoene	15.7	16.4	3.4	7.5	0.4	—	—
C_{18} Saturated	18.0	18.0	4.0	5.5	—	trace	—
C_{18} Monoene	17.7	18.4	23.6	68.1	0.8	81.2	—
C_{18} Diene	17.6	19.0	18.2	1.5	10.6	—	0.5
C_{18} Unknown	17.7	18.7	8.3	—	11.8	—	—
C_{18} Unusual triene	17.4	19.3	35.2	1.3	76.4	—	99.4
C_{20} Monoene	19.7	20.4	2.4	0.6	—	0.5	—

[a] C_{18} Monene concentrate. [b] C_{18} Triene concentrate.

Gas-liquid chromatographic analyses of the methyl esters of the mixed acids from *T. polycarpum* seed oil indicated that the principal components were a C_{18} monoene, C_{18} diene, and two C_{18} "unknowns" (Table I). When the mixed acids were hydrogenated, these unsaturated acids were converted to stearic acid.

RESULTS

Low-Temperature Fractionation. Concentrates of the fatty acids were obtained by a sequence of low-temperature crystallizations (Fig. 1) and sub-

trile-hexane (Meade, 1957, and Scholfield *et al.*, 1960) system effected an enrichment of the C_{18}-monoene components; however, gas-liquid chromatographic analyses indicated that the palmitic acid was not resolved from the mixture. Partial resolution of the two C_{18}-monoenoic acids was achieved, as evidenced by the shoulder on the main peak (Fig. 2). The C_{18}-monoene concentrate, of about 81% purity, was obtained by combining transfers 402 through 461 (Table I). The presence of one double bond was established by iodine value (Wijs) and quantitative hydrogenation. Infrared spectral analysis indicated 75% isolated *trans*-unsaturation. The C_{18}-monoene isomers were reacted with mercuric acetate in methanol (Jantzen and Andreas, 1959), and the addition compounds were resolved from the palmitic acid by solvent partitioning; however, no attempt was

* Presented at the fall meeting of the American Oil Chemists' Society, Chicago, October 30–November 1, 1961.

† Northern Utilization Research and Development Division, Agricultural Research Service, U. S. Department of Agriculture. Article not copyrighted.

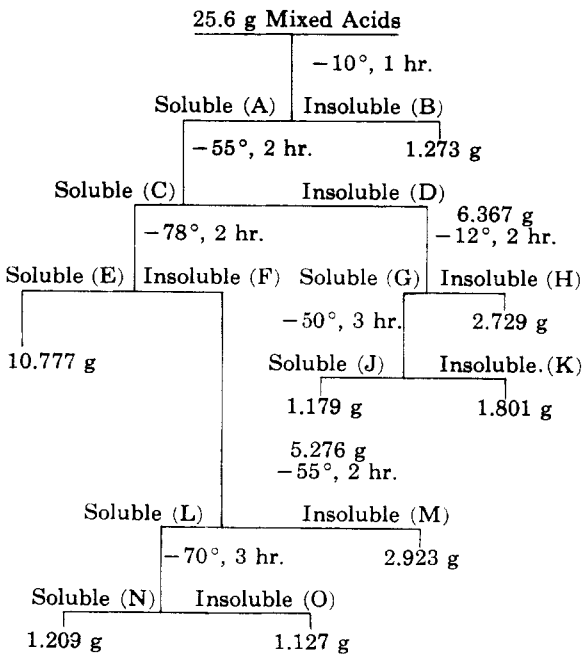


FIG. 1.—Low-temperature crystallization of mixed fatty acids of *Thalictrum polycarpum* seed oil (20 ml of acetone/g of free acid).

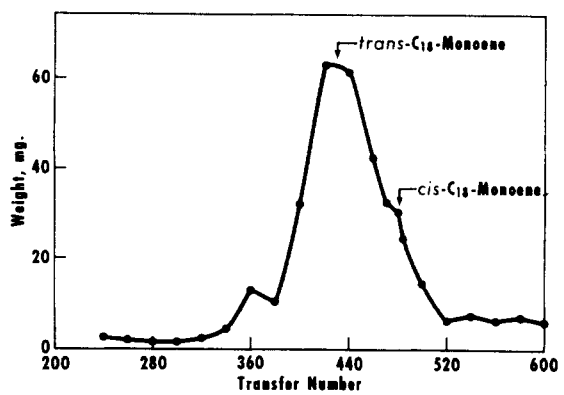


FIG. 2.—Countercurrent distribution of methyl esters of combined low-temperature crystallization fractions H and K from *Thalictrum polycarpum* seed oil, with the solvent system hexane-acetonitrile.

made to resolve the *cis* and *trans* isomers at this stage.

Characterization of the Octadecenoic Acids.—The purified octadecenoic acid mixture (I*a* and I*b*, Scheme A) was subjected to oxidative cleavage by permanganate-periodate (Lemieux and von Rudloff, 1955). The monobasic acids (II*a* and II*b*) and dibasic acids (III*a* and III*b*) were separated by solvent partitioning. The dibasic acid mixture was resolved by a combination of solvent partition and recrystallization. The two acids were characterized as glutaric (pentanedioic) and azelaic (nonanedioic) acids by mixed m.p. determinations

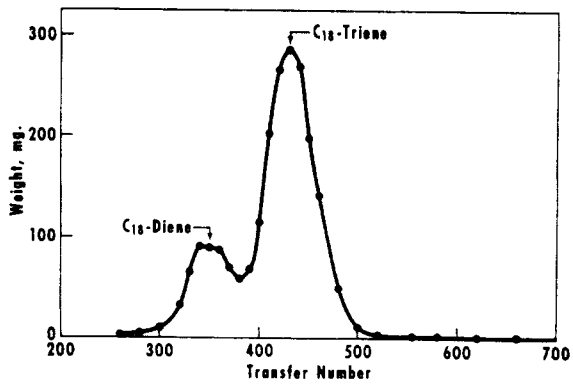
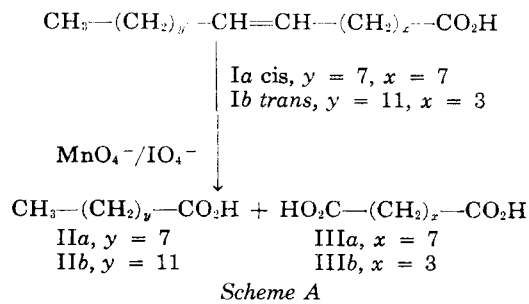


FIG. 3.—Countercurrent distribution of methyl esters of combined low-temperature crystallization fractions E and N from *Thalictrum polycarpum* seed oil, with the solvent system hexane-acetonitrile.

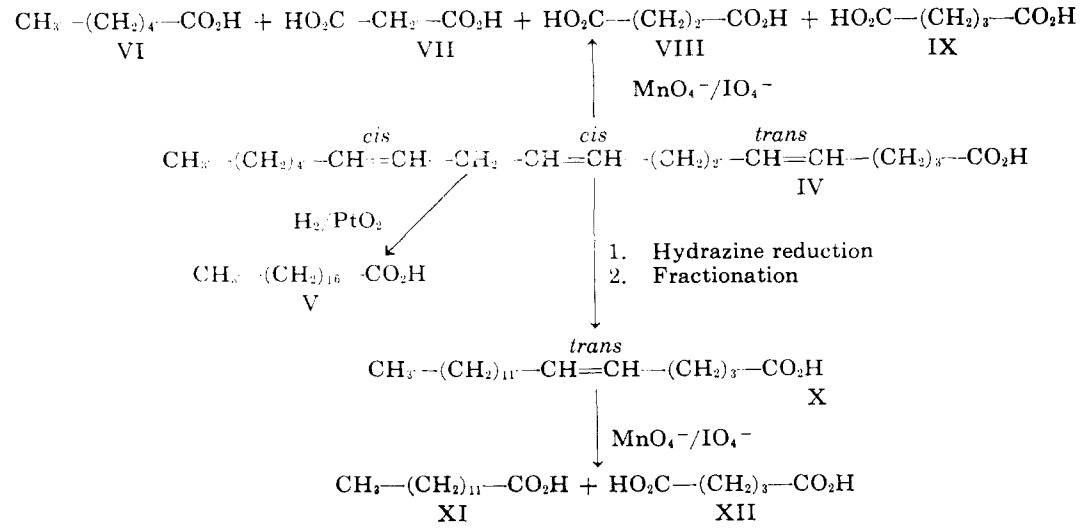


Scheme A

and as bis-*p*-bromophenacyl esters. The [illegible]-decanoic acid, corresponding to the glutaric acid, was identified by mixed m.p. determination of the acid and as its *p*-bromophenacyl ester. Pelargonic (nonanoic) acid, corresponding to the azelaic acid, was not identified at this time. For further confirmation and to determine any effects resulting from the action of mercuric acetate, a portion of the unadducted monoene mixture from the countercurrent distribution was oxidized, and the products were identified by gas-liquid chromatography. The five components found were glutaric, azelaic, tridecanoic, pelargonic, and the original palmitic acid. The most abundant cleavage pair, which resulted from the oxidation of the *trans*-acid, were glutaric and tridecanoic acids. This analysis establishes the structure of the *trans*-C_{18}-monoene to be *trans*-5-octadecenoic and the *cis*-C_{18}-monoene to be *cis*-9-octadecenoic (oleic) acid.

Isolation of the Octadecatrienoic Acid.—The combined low-temperature crystallization fraction (*E* and *N*, Fig. 1) was shown by gas-liquid chromatography to contain 76.4% of unusual C_{18}-trienoic acid (Table I). A 686-transfer countercurrent distribution (Fig. 3) of the methyl esters of the concentrate, in the acetonitrile-hexane system, yielded nearly pure methyl octadecatrienoate. The material for characterization work was obtained by combining transfers 404 through 479 (composition, Table I).

Characterization of the Octadecatrienoic Acid



Scheme B

(*IV*).—The presence of three double bonds in the octadecatrienoic acid (IV) was confirmed by iodine value (Wijs) and quantitative hydrogenation. The presence of one *trans* double bond was shown by infrared spectroscopy. Alkali isomerization conjugated two of the double bonds; only about 2% of conjugated triene was noted. Treatment with lipoxidase (MacGee, 1959) also produced conjugated diene. Since this enzyme is known to be specific for *cis,cis*-methylene-interrupted double bonds (Holman, 1960), the presence of this grouping in the unknown trienoic acid was established.

The isomerization data indicate that the one *trans* double bond must be separated from the *cis* double bonds by more than one methylene group. (See Bagby *et al.*, 1961, for discussion and leading references.) The octadecatrienoic acid (IV, Scheme B) was oxidized with permanganate-periodate; three of the cleavage products were characterized as caproic (hexanoic), succinic (butanedioic), and glutaric acids by chemical methods and gas-liquid chromatography (malonic acid would have decomposed). To establish the position of the *trans* double bond and, as a result, establish the position of the *cis,cis*-methylene-interrupted group, the C_{18}-trienoic acid (IV) was partially reduced with hydrazine hydrate. This reduction has been shown to proceed slowly and to reduce double bonds in a polyunsaturated acid in a random fashion without causing isomerization (Aylward and Rao, 1956; Rao, 1959; Schilling, 1961; Scholfield *et al.*, 1961). From the mixture of reduction products was isolated in "pure" form a monounsaturated acid shown to be *trans*-5-octadecenoic acid (X). This information establishes the C_{18}-trienoic acid to be the previously unknown *trans*-5,*cis*-9,*cis*-12-octadecatrienoic acid.

The remaining C_{18} acids have been partially purified; however, they have not been characterized.

Discussion

The occurrence of a considerable quantity of acids with isolated *trans*-unsaturation in *Thalictrum* seed oil is indeed novel in that most natural fatty acids have *cis*-unsaturation.

It has been suggested that acids containing isolated *trans*-unsaturation, which occur in animal fat, arise from more highly unsaturated acids through the operation of a biohydrogenation process in animal fat metabolism (Meara, 1957, and Hartmann and Shorland, 1959). James *et al.* (1961) found in human fecal lipids a number of monoethenoid acids, of which many have *trans*-configuration, and Backderf and Brown (1958) report the presence of *trans*-16-octadecenoic acid in butter fat.

The *cis*-5-eicosenoic acid and *cis*-5-docosenoic acid from *Limnanthes douglasii* seed oil (Smith *et al.*, 1960), 5-tetradecenoic acid from sperm head oil (Meara, 1957) and free acids of human hair (Weitkamp *et al.*, 1947), and 5-octadecenoic acid from human fecal lipids (James *et al.*, 1961) and possibly from milk fat (James and Webb, 1957) are other sources of monoethenoid acids with the 5,6-double bond. Oleic acid, *cis*-11-octadecenoic acid from *Asclepias syriaca* seed oil (Chisholm and Hopkins, 1960), and petroselinic acid (Hilditch, 1956) are the only previously known C_{18}-monoenoid acids from plant sources.

Probably other *Thalictrum* species contain the same acids as *T. polycarpum*. Earle *et al.* (1960) have suggested that *T. revolutum* seed oil, which has much the same properties as *T. polycarpum*, contains acid with double bonds which are too isolated to be conjugated by alkali.

The discovery of fatty acids from *Podocarpus nagi* (eicosatrienoic acid) by Koyama and Toyama (1957), *Limnanthes douglasii* (docosadienoic acid), and now *Thalictrum polycarpum* (octadecatrienoic acid) (members of different plant families), in which all the double bonds do not

conjugate on treatment with alkali, seems to indicate that fatty acids with double bonds more widely separated than methylene-interruption are more than a biogenetic oddity. Earle *et al.* in 1960 (and in unpublished notes) have infrared, ultraviolet, and gas-liquid chromatographic analyses of seed oils from the genera *Aquilegia*, *Calea*, *Aster*, and *Arctium*, which indicate the presence of acids that appear to be similar to the *trans*-5,-*cis*-9,*cis*-12-octadecatrienoic acid from *T. polycarpum*. Thus a new class of acids containing wide separation of double bonds has been discovered in several families within the plant kingdom, and at least some members have large amounts of isolated *trans*-unsaturation.

The acids do not appear to fit any biogenetic patterns as proposed by Lovern (1958) and by Klenk and Debuch (1959).

Experimental

General Methods.—Gas-liquid chromatographic analyses were carried out with a Burrell Kromotog K-5,[1] and the retention values were treated as described by Miwa *et al.* (1960). The operating conditions and description of the columns are the same as those mentioned in other communications, *e.g.*, Smith *et al.* (1960) and Bagby *et al.* (1961), and, except where noted, methyl esters were prepared from methanol with acid catalyst (*loc. cit*). When desired for characterization work, fractionated esters were saponified by refluxing 0.5 hour with 2 N ethanolic potassium hydroxide.

Melting points were determined with a Fisher-Johns block and are uncorrected.

Infrared spectra were measured in a 1-mm cell with a Perkin-Elmer model 137-0001 recording spectrophotometer. Quantitative values were obtained in carbon disulfide by the baseline technique (O'Connor, 1959). A baseline is constructed between about 10.02 μ and 10.59 μ on the infrared spectra. The quantitative values are obtained by comparing the extinction coefficient of the "unknown" with that of a standard.

Preparation of Mixed Fatty Acids.—Coarsely ground seed of *Thalictrum polycarpum* was extracted overnight in a Soxhlet apparatus with petroleum ether (b.p. 33–57°). The bulk of the solvent was evaporated on a steam bath under nitrogen, and the remainder was removed *in vacuo* with a rotating evaporator.

T. polycarpum seed oil (28.00 g) was refluxed under nitrogen for 0.5 hour with 175 ml of 2 N ethanolic potassium hydroxide. The unsaponifiable material was removed, and the free fatty acids (27.6 g) were obtained in the usual manner.

Low-Temperature Crystallization.—Mixed fatty acids (25.6 g) were fractionated by low-temperature crystallization from acetone (20 ml of acetone/g of free acids). After standing as indicated in Figure 1, the mixtures were separated with a filter stick. Fraction H was a solid, m.p. 31–40°, and fraction K was a semisolid, m.p. range from below 20° up to 34°.

Compositions of the low-temperature crystallization fractions were determined by gas-liquid chromatographic analyses of the methyl esters prepared by esterification with diazomethane (Arndt, 1943). The presence of more than one C_{18}-monoenoic acid in K (Fig. 1) was evidenced by a shoulder on the monoene peak obtained by gas-liquid chromatography carried out on the nonpolar liquid phase. Attempts to resolve artificial mixtures of methyl oleate and methyl elaidate and of methyl oleate and methyl petroselinate on this column failed.

Isolation of Octadecenoic Acids by Countercurrent Distribution. — Low - temperature crystallization fractions H and K (shown to be similar by gas-liquid chromatographic analyses) were combined to give an "octadecenoic acid" concentrate (Table I). Mixed methyl esters (4.298 g) of the concentrate were dissolved in mutually saturated hexane (10 ml) and acetonitrile (80 ml) and subjected to a 790-transfer countercurrent distribution in a 200-tube automatic Craig-Post apparatus. The methyl esters were divided evenly between the first two tubes, and 40 ml (full in the decant position) of hypophase was placed in each of the remaining tubes. The automatic operation of the instrument introduced 5 ml of equilibrated hexane (hyperphase) to tube 0 at each transfer stage. As hyperphase progressed past tube 200, it was decanted into an automatic fraction collector: two transfers per tube were combined and successively collected until 295 fractions had been obtained. The solvent was evaporated, under reduced pressure, from the contents of selected tubes. The weight-distribution plot obtained from these data is shown in Figure 2. Gas-liquid chromatographic analyses of some of the fractions indicated the presence of more than one C_{18} monoene, as evidenced by the presence of a shoulder on the peak in Apiezon L. Transfer number 484 contained nearly equal quantities of the two components. Combination of transfer numbers 402 through 461, selected on the basis of the gas-liquid chromatograms and of the distribution plot to obtain the *trans*-isomer, provided 1.483 g of material. It was primarily methyl octadecenoate (81.2%) and methyl palmitate (18.3%) (Table I). Infrared spectral analyses indicated 75% isolated *trans*-unsaturation (as methyl elaidate); and the concentrate absorbed 0.93 moles of hydrogen (corrected on the basis of the gas-liquid chromatogram) and had an iodine value (Wijs) of 72. Theoretical iodine value, based on the gas-liquid chromatogram, is 71.

Isolation of the Octadecenoic Acids as Mercury Adducts.—The C_{18}-monoene concentrate (0.903 g) from the countercurrent distribution was adducted with 2.00 g of mercuric acetate (Jantzen and

[1] Mention of firm names or trade products does not imply that they are endorsed or recommended by the U. S. Department of Agriculture over other firms or similar products not mentioned.

Andreas, 1959) in 150 ml of hexane-equilibrated methanol containing 5% water and 0.3% acetic acid. The mixture was swirled occasionally until a homogeneous solution was obtained, which was allowed to stand for 27 hours. The resulting esters were fractioned between the methanolic solution and hexane by Gunstone's (1954) scheme of isolating dihydroxy acids. The resulting fractions were shaken with 6 N hydrochloric acid, diluted with water, and extracted with pentane-hexane (b.p. 33–57°). The petroleum ether extracts were washed with water and then dried over sodium sulfate. The solvent was removed under reduced pressure, and the major fraction (0.403 g), from the polar solvent, was shown by gas-liquid chromatography to be 98.1% C_{18}-monoene. Saponification of this mixture yielded 0.367 g of a solid (a mixture of oleic acid, I*a*, and *trans*-5-octadecenoic acid, I*b*; m.p. 36–42°). Recrystallization of this acid (0.020 g) from 65% ethanol and then from 60% ethanol yielded 0.008 g of *trans*-5-octadecenoic acid (I*b*) melting at 43–44°; Posternak (1916) gave as m.p. 47.5°; $\lambda_{max}^{CS_2}$ 10.36 μ (K 0.455).

Anal. Calcd. for $C_{18}H_{34}O_2$: C,76.54: H, 12.13. Found: C, 76.58; H, 12.17.

The major fraction (0.123 g) from the nonpolar phase was 81.4% methyl palmitate. After this ester was saponified, two recrystallizations of the free acid from ethanol yielded a solid melting at 58–60°. Mixed melting point with authentic palmitic acid (m.p. 61–2°) was 59–61°.

Permanganate-Periodate Oxidation (Lemieux and von Rudloff, 1955) of the Octadecenoic Acids.—The C_{18}-monoenoic acid mixture (I*a* and I*b*) (0.280 g) obtained from the mercury adducts was stirred for 74 hours with potassium carbonate (0.416 g), sodium periodate (1.769 g), potassium permanganate (0.020 g), and water (400 ml). The reaction was stopped by chilling the solution and acidifying it with 6 N hydrochloric acid. Excess reagents were reduced by adding sodium bisulfite. The solution was extracted continuously (45 hours) with ethyl ether. The bulk of the ether was removed by cautious distillation (maximum vapor temperature 38°).

The residue (*ca.* 20 ml) was diluted with water (40 ml) and ethanol (2 ml), and the aqueous solution was extracted with five 25-ml portions of pentane-hexane (b.p. 33–57°). The combined petroleum ether extract was reserved for the identification of the monocarboxylic acids.

The aqueous phase was extracted with ethyl ether (5 × 30 ml), and the combined ether extract was fractionated by washing with water. The combined aqueous phase was chilled, saturated with sodium chloride, and extracted with ethyl ether. The ether was removed from the two solutions of dicarboxylic acids to yield 0.035 g of crude azelaic acid (III*a*), m.p. 65–95°, and 0.074 g of crude glutaric acid (III*b*) (semisolid). Crude azelaic acid (III*a*) was recrystallized once from each of mixed solvents pentane–hexane–chloroform and pentane–hexane–ethyl ether to yield 0.006 g of a solid melting at 101–104.5°; an admixture with authentic azelaic acid (m.p. 105–106.5°) melted at 104–106.5°. The bis-*p*-bromophenacyl ester (Shriner *et al.*, 1956) of III*a* (azelaic acid) was prepared, m.p. 129.5–130.5°. The mixed melting point with authentic bis-*p*-bromophenacyl azelate (m.p. 130.5–131.5°) was 130–131°. Crude III*b* (glutaric acid; 0.074 g) was recrystallized from chloroform–pentane–hexane and then from ethyl ether–benzene to yield 0.028 g of a solid, m.p. 95–97°. An admixture with authentic glutaric acid (m.p. 96.5–97.5°) melted at 95.5–97°. The bis-*p*-bromophenacyl ester of III*b* (glutaric acid) melted at 136–137°; mixed melting point with authentic bis-*p*-bromophenacyl glutarate (m.p. 137–8°) was 137–8°.

The combined petroleum ether extract was concentrated by distillation (maximum vapor temperature 42°), and the residue was steam distilled for about 10 minutes in an effort to resolve the mixture of pelargonic acid (II*a*) and tridecanoic acid (II*b*). The two fractions were taken up in ethyl ether. The solvent was removed from the undistilled acid to yield 0.198 g of a semisolid, m.p. range below 25° up to 36°. Crystallization from 2.5 ml of 75% aqueous acetone allowed the removal of a yellowish-brown semisolid, and the filtrate solvent was evaporated to leave a solid residue (0.188 g) melting at 30–6°. A portion (0.095 g) of this residue was chromatographed on a 2.5-g column of alumina to yield 0.057 g of a solid melting at 34–8°. A single crystallization from pentane-hexane (1 ml) yielded 0.016 g melting at 38.0–39.5°. An admixture with authentic tridecanoic acid (m.p. 40–1°) melted at 39.0–40.5°. The *p*-bromophenacyl ester of II*b* (tridecanoic acid) and an admixture with authentic *p*-bromophenacyl tridecanoate (m.p. 71.5–72°) melted at 71–2°. The steam-volatile acid was converted to the *p*-bromophenacyl ester; however, a satisfactory derivative of the more volatile acid was not obtained.

Permanganate-Periodate Oxidation of the Octadecenoic Acid Concentrate from the Countercurrent Distribution.—A portion (0.071 g) of combined countercurrent distribution transfer numbers 402–461 (Table I) was oxidized with permanganate-periodate as above, except the reaction was terminated after 48 hours. The cleavage products were esterified with diazomethane and the methyl esters were identified by gas-liquid chromatography. Five components were present: glutarate, azelate, pelargonate, tridecanoate, and palmitate. The area percentages were 12.8, 6.6, 4.6, 53.1, and 22.9 respectively. Glutarate and tridecanoate were the most abundant cleavage pair.

Isolation of the Octadecatrienoic Acid by Countercurrent Distribution.—Methyl esters (12.298 g) of the combined low-temperature crystallization fractions E and N (composition, Table I) were

subjected to a 686-transfer countercurrent distribution as described previously, except that the esters were divided equally among the first five tubes and 10 ml of hyperphase was added at each transfer stage. The weight distribution plot is shown in Figure 3. Transfer numbers 404 through 479 were combined on the basis of gas-liquid chromatograms of selected fractions, to give 7.269 g of nearly pure methyl octadecatrienoate (Table I). Infrared spectral analysis indicated one isolated *trans* double bond (10.35 μ, 93.1% isolated *trans*-unsaturation as methyl elaidate). Free acid was obtained by saponification during a 2-hour reflux under nitrogen with N ethanolic potassium hydroxide followed by acidification.

Anal. Calcd. for $C_{18}H_{30}O_2$: neutral equivalent, 278.4, iodine value 273.5. Found: neutral equivalent, 276.7, iodine value (Wijs) 283.

The acid absorbed 2.8 moles of hydrogen, and the hydrogenated acid melted separately and in admixture with stearic acid (m.p. 68.5–69.5°) at 68–9°.

Isomerization Studies of the Octadecatrienoic Acid.—The ultraviolet absorption spectrum of the octadecatrienoic acid (IV) indicated no preformed conjugated diene (American Oil Chemists' Society, 1959), λ_{max}^{EtOH} 233 mμ (E 41.3). Alkali isomerization indicated 94.7% dienoic and 2.2% trienoic acids; λ_{max}^{EtOH} 233 mμ (E 936), 268 mμ (E 14.1). Treatment of the acid with lipoxidase (MacGee, 1959) showed 99.5% polyunsaturated acid.

Permanganate-Periodate Oxidation of the Octadecatrienoic Acid.—A portion (0.557 g) of the octadecatrienoic acid (IV) was oxidized with permanganate-periodate as above, except the reagents were increased to take into account the presence of three double bonds and the reaction was terminated after 24 hours. The resulting free acids were continuously extracted with ethyl ether.

A portion (11.5%) of the ether extract was set aside for gas-liquid chromatographic analysis. The remainder of the ether extract was concentrated by distillation.

Characterization of the Octadecatrienoic Acid Oxidation Products.—The concentrate of the cleavage products was steam distilled to yield 1.15 mmoles (by titration with 0.100 N sodium hydroxide) of steam-volatile acid (caproic acid, VI). The *p*-bromophenacyl ester was prepared from about 0.63 mmoles of VI (caproic acid). The product was recrystallized two times from aqueous ethanol to yield 0.032 g of material melting at 69.5–70.5°; an admixture with authentic *p*-bromophenacyl caproate (m.p. 70.5–71°) melted at 70–71°. The dicarboxylic acids were taken up in ethyl ether to yield 0.312 g of material melting over a wide range. A portion (0.119 g) was fractionated by a series of crystallizations from benzene, ethyl acetate–pentane–hexane, and chloroform. The least soluble portion was a high melting compound (> 150°), and the combined filtrate residues melted at less than 130°. A solid (0.013 g), m.p. 181–184°, was obtained from the least soluble fractions. The m.p. of an admixture with authentic succinic acid (m.p. 190–190.5°) was 188.5–190°. The bis-*p*-bromophenacyl ester melted separately and in admixture with authentic bis-*p*-bromophenacyl succinate (m.p. 215–216°) at 214–215°. A solid (0.021 g), m.p. 90.5–93.5°, was obtained from the more soluble portions. The m.p. of an admixture with authentic glutaric acid (m.p. 96.5–97.5°) was 92–96°. The bis-*p*-bromophenacyl ester melted at 137–137.5°, an admixture with authentic bis-*p*-bromophenacyl glutarate (m.p. 137–8°) melted at 137–138°.

The ethereal extract reserved for gas-liquid chromatography was esterified with diazomethane. The products found were the esters of caproic, succinic, and glutaric acids. The area percentages were 27.5, 33.0, and 41.2 respectively.

Partial Reduction of the Octadecatrienoic Acid.—A portion (0.253 g) of the octadecatrienoic acid (IV) was dissolved in 19.5 ml of ethanol, and the solution was stirred vigorously at 50° with 0.25 ml of hydrazine hydrate (5:1 hydrazine-to-acid ratio) (Aylward and Rao, 1956). At hourly intervals, a 2-ml aliquot of the reaction mixture was added to 10 ml of water, and the aqueous solution was acidified with N hydrochloric acid. The product was taken up in ethyl ether and dried over sodium sulfate. The ether was removed under reduced pressure, and the residue was esterified with diazomethane. Gas-liquid chromatographic analyses of the products are shown in Table II. Only partial resolution of the resulting monoenes and dienes by gas-liquid chromatography make it necessary to report them as mixtures (Fig. 4). The product reported to be C_{18} diene may also be a mixture; however, this product had retention values comparable to methyl linoleate. The 5-hour sample showed 70% isolated *trans*-unsaturation and had an iodine value of 182.

A portion (1.241 g) of the octadecatrienoic acid (IV) was reacted for 4.5 hours in a manner similar to that described above except that air was blown

TABLE II
GAS-LIQUID CHROMATOGRAPHIC ANALYSES OF METHYL ESTERS OF HYDRAZINE REDUCTION FRACTIONS DERIVED FROM THE OCTADECATRIENOIC ACID

Type of Acid	Time (hr.)				
	1	2	3	4	5
	% of Acid				
C_{18} Saturated	0.3	1.0	1.6	1.6	1.5
C_{18} Monene and diene	28.1	34.2	37.7	38.7	42.6
C_{18} Diene	12.8	14.7	15.4	14.7	15.6
C_{18} Unusual triene	58.8	50.0	44.8	41.3	40.2
C_{18} Unknown	—	—	0.5	—	0.1
C_{18} Unknown	—	—	—	3.6	Trace

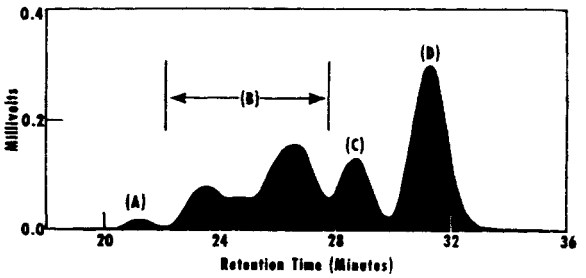


FIG. 4.—Significant portion of a typical gas-liquid chromatogram of methyl esters of fatty acids from hydrazine partial reduction of the octadecatrienoic acid. Stationary phase: Resoflex-446 on celite. Compounds presumed to be present: (A) methyl stearate; (B) methyl octadecenoates and methyl octadecadienoates; (C) methyl linoleate and possibly other methyl octadecadienoates; and (D) methyl-*trans*-5,*cis*-9,*cis*-12-octadecatrienoate.

over the reaction solution for 1.25 hours. Air is reported to increase the rate of reduction (Aylward and Rao, 1956; Aylward and Sawistowska, 1961; and Scholfield *et al.*, 1961). The product contained 57% of the octadecatrienoic acid, and it was fractionated in acetone (20 ml/g of acid) at −50° to give an insoluble fraction, m.p. range below 20° up to 41°. The filtrate residue (0.943 g) was reduced as above, except that air was blown over the reaction solution for 4 hours. The products (0.842 g) had an iodine value of 181, and the infrared spectra indicated 71% of isolated *trans*-unsaturation. The gas-liquid chromatogram showed considerable C_{18}-diene and 24.2% C_{18}-triene.

The product (0.800 g) was fractionated from acetone as above to yield 0.131 g of insoluble product, m.p. range below 20° up to 55°. The filtrate residue (0.663 g) was reduced as previously for 3 hours under a continuous stream of air. The gas-liquid chromatographic analyses of the recovered product (0.630 g) showed 4.3% C_{18}-triene. This final product (0.572 g) was fractionated in acetone as before to yield 0.232 g of insoluble acids. Methyl esters of the combined acetone-insoluble fractions had an iodine value of 77, and the infrared spectra indicated 30% isolated *trans*-unsaturation. Gas-liquid chromatographic analyses showed 30% stearic acid, 63.3% C_{18}-monoene acids plus C_{18}-diene acids, and 5.1% C_{18}-triene acid.

Isolation of the C_{18}-Trans-Unsaturated Monoene (X) Derived from the Octadecatrienoic Acid.—An initial fractionation of the above combined acetone-insoluble esters (0.364 g) was effected by adduction with mercuric acetate in methanol and subsequent solvent partitioning as described.

A concentrate (0.235 g) of the unsaturated esters, obtained by combining the fractions from the polar phase, was fractionated in acetone at −15° to yield 0.005 g of a solid. The filtrate residue was further fractionated from acetone at −45°. The esters (0.095 g), soluble in acetone at −15° but insoluble at −45°, were crystallized from hexane at −60° to yield 0.071 g of a precipitate (50% isolated *trans*-unsaturation). Gas-liquid chromatographic analysis of the concentrate indicated 96% of the product consisted of two major components (C_{18}-monoenes) of about equal amounts. The *trans*-unsaturated acid concentrate (0.065 g) was saponified under reflux over a period of 1 hour with N alcoholic potassium hydroxide. The free acid was crystallized two times from aqueous acetone at 8° to yield 0.016 g of solid melting at 41.5–43°; an admixture with Ib (*trans*-5-octadecenoic acid, m.p. 43–4°) melted at 42–4°. This acid was shown to be about 90% *trans*-monoene. An additional 0.002 g of this acid was obtained from the aqueous acetone filtrate residues.

Permanganate-Periodate Oxidation of the C_{18}-Trans-Octadecenoic Acid (X) Derived from the Octadecatrienoic Acid.—The *trans*-octadecenoic acid (X) (0.018 g) was oxidized by the procedure described above. The reaction was terminated at the end of 23.5 hours, and the solution was extracted with pentane-hexane (b.p. 33–57°) to obtain XI (tridecanoic acid). The aqueous phase was then extracted with ethyl ether to obtain XII (glutaric acid). The two fractions were dried over sodium sulfate, and the solvents were removed under reduced pressure to yield 0.014 g and 0.013 g respectively. The methyl esters of the two products, prepared by reaction with diazomethane, were submitted to gas-liquid chromatography, and the oxidation products, XI and XII, were shown to be tridecanoic and glutaric acids respectively.

ACKNOWLEDGMENT

The authors wish to express their appreciation to Mr. C. R. Scholfield and Dr. H. J. Dutton for assistance with the countercurrent distributions; Mrs. Clara McGrew for microanalyses; and Dr. Quentin Jones, Crops Research Division, Agricultural Research Service, U. S. Department of Agriculture, for making available the *Thalictrum polycarpum* seed.

REFERENCES

American Oil Chemists' Society (1959), Official and Tentative Methods, ed. 2, revised 1959, Cd 7-58.
Arndt, F. (1943), *Org. Syntheses, Col. Vol. 2*, 165.
Aylward, F., and Rao, C. V. N. (1956), *J. Appl. Chem. (London) 6*, 248.
Aylward, F., and Sawistowska, M. (1961), *Chem. & Ind. (London)*, 433.
Backderf, R. H., and Brown, J. B. (1958), *Arch. Biochem. Biophys. 76*, 15.
Bagby, M. O., Smith, C. R., Jr., Miwa, T. K., Lohmar, R. L., and Wolff, I. A. (1961), *J. Org. Chem. 26*, 1261.
Bailey, L. H. (1939), The Standard Cyclopedia of Horticulture, vol. III, New York, The Macmillan Company, p. 3327.
Chisholm, M. J., and Hopkins, C. Y. (1960), *Can. J. Chem. 38*, 805.

Earle, F. R., Melvin, E. H., Mason, L. H., Van Etten, C. H., Wolff, I. A., and Jones, Q. (1959), *J. Am. Oil Chemists' Soc. 36*, 304.
Earle, F. R., Glass, C. A., Geisinger, G. C., Wolff, I. A., and Jones, Q. (1960), *J. Am. Oil Chemists' Soc. 37*, 440.
Gunstone, F. D. (1954), *J. Chem. Soc.*, 1611.
Hartmann, L., and Shorland, F. B. (1959), *Nature 184*, 2024.
Hilditch, T. P. (1956), Chemical Constitution of Natural Fats, ed. 3, New York, John Wiley and Sons.
Holman, R. T. (1960), *in* Food Enzymes, Schultz, H. W., editor, Westport, Conn., The Avi Publishing Company, Inc., p. 77.
James, A. T., and Webb, J. (1957), *Biochem. J. 66*, 515.
James, A. T., Webb, J. P. W., and Kellock, T. D. (1961), *Biochem. J. 78*, 333.
Jantzen, E., and Andreas, H. (1959), *Chem. Ber. 92*, 1427.
Klenk, E., and Debuch, H. (1959), *in* Annual Reviews of Biochemistry, Vol. 28, Luck, J. M., *et al.*, editors, Palo Alto, Calif., Annual Reviews, Inc., p. 39.
Koyama, Y., and Toyama, Y. (1957), *J. Chem. Soc. Japan, Pure Chem. Sect., 78*, 1223.
Lemieux, R. U., and von Rudloff, E. (1955), *Can. J. Chem. 33*, 1701.
Lovern, J. A. (1958), *J. Sci. Food Agr. 9*, 773.
MacGee, J. (1959), *Anal. Chem. 31*, 298.
Meade, E. M. (1957), *in* Progress in the Chemistry of Fats and Other Lipids, vol. 4, Holman, R. T., Lundberg, W. O., and Malkin, T., editors, New York, Pergamon Press, p. 56.
Meara, M. L. (1957), *in* Encyclopedia of Plant Physiology, vol. VII, Ruhland, W., editor, Berlin, Springer-Verlag, pp. 14–15.
Miwa, T. K., Mikolajczak, K. L., Earle, F. R., and Wolff, I. A. (1960), *Anal. Chem. 32*, 1739.
O'Connor, R. T. (1959), *J. Am. Oil Chemists' Soc. 36*, 627.
Posternak, M. S. (1916), *Compt. rend. 162*, 944.
Rao, C. V. N. (1959), *J. Sci. Ind. Research (India) 18B*, 131.
Schilling, K. (1961), *Fette, Seifen, Anstrichmittel 63*, 421.
Scholfield, C. R., Nowakowska, J., and Dutton, H. J. (1960), *J. Am. Oil Chemists' Soc. 37*, 27.
Scholfield, C. R., Jones, E. P., Nowakowska, J., Selke, E., and Dutton, H. J. (1961), *J. Am. Oil Chemists' Soc. 38*, 208.
Shriner, R. L., Fuson, R. C., and Curtin, D. Y. (1956), The Systematic Identification of Organic Compounds, ed. 4, New York, John Wiley and Sons, p. 200.
Smith, C. R., Jr., Bagby, M. O., Miwa, T. K., Lohmar, R. L., and Wolff, I. A. (1960), *J. Org. Chem. 25*, 1770.
Weitkamp, A. W., Smiljanic, A. M., and Rothman, S. (1947), *J. Am. Chem. Soc. 69*, 1936.

Studies on the Metabolism of 16α-Hydroxyprogesterone in Humans; Conversion to Urinary 17-Isopregnanolone*

HAROLD I. CALVIN† AND SEYMOUR LIEBERMAN

From the Departments of Biochemistry and of Obstetrics and Gynecology, College of Physicians and Surgeons, Columbia University, New York 32

Received March 23, 1962

The metabolic conversion of 16α-hydroxyprogesterone to 17-isopregnanolone has been demonstrated. The results also indicate that a C_{21}-Δ^{16}-steroid may serve as an intermediate and that its reduction occurs stereospecifically. Evidence has been obtained that 16α-hydroxyprogesterone may be secreted by the normal subject in amounts of 1–2 mg per day.

The isolation of Δ^{16}-androsten-3α-ol from hog testes (Prelog and Ruzicka, 1944) and from the urine of normal humans (Brooksbank and Haslewood, 1949, 1950, 1952) and, more recently, its quantitative estimation in human urine (Brooksbank and Haslewood, 1961) have stimulated interest in its biosynthetic origin. Burstein and Dorfman (1960) showed that this metabolite was formed from both cholesterol and pregnenolone in a woman with a virilizing benign adrenal tumor. They pointed out that such a result is consistent with two general mechanisms for the formation of the 16-olefin. In one of these, it would arise by direct 2-carbon elimination from a C_{21} steroid. Alternatively it could be formed by the dehydration of a 17β-hydroxy-C_{19} steroid such as testosterone. Although it has been demonstrated that rat testes and human liver are apparently both able to convert testosterone to $\Delta^{4,16}$-androstadien-3-one *in vitro* (Stylianou *et al.*, 1961a,b), no *in vivo* evidence exists that testosterone or any other C_{19} steroid can serve as precursor of a Δ^{16}-metabolite. Furthermore, Savard (personal communication, 1962) has recently shown that 50 mg of testosterone, when injected into adult males, does not re-

* Supported in part by U. S. Public Health Service Grant No. A-110. A preliminary report of this work has appeared (Calvin and Lieberman, 1962).

† Predoctoral Research Fellow, National Institutes of Health.