The Isolation and Characterization of *trans*-Octadec-16-enoic Acid from Butterfat

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trans-Octadec-16-enoic acid was first reported as a fatty acid constituent of butterfat by Backderf (1956) and Backderf & Brown (1958), who deduced its presence from infrared-spectrophotometric analyses supported by the conclusive identification of thapsic acid (hexadecanedioic acid) as the main dicarboxylic acid product from the oxidation of appropriate *trans* C₁₈ mono-unsaturated fractions with potassium permanganate and acetic acid. They did not, however, actually isolate *trans*-octadec-16-enoic acid. Formerly its presence in a natural fat had not been recorded although experimental evidence indicated that *trans* isomers of *n*-octadecenoic acid including 'vaccenic' acid were contained in cow milk fat (Bertram, 1928; Gupta, Hilditch, Paul & Shrivastava, 1950; Cornwell, Backderf, Wilson & Brown, 1953; Smith, Freeman & Jack, 1954; Scott, Herb, Magidman & Riemenschneider, 1959; Patton, McCarthy, Evans & Lynn, 1960), in sheep milk fat (Gupta *et al.* 1950), in ox body fat (Bertram, 1928; Rao & Daubert, 1948; Gupta *et al.* 1950; Swern, Knight & Eddy, 1952), and in sheep body fat (Bertram, 1928; Gupta *et al.* 1950).

The isolation and characterization of *trans*-octadec-16-enoic acid is reported in the present paper, which results from an investigation of the trace and minor fatty acid constituents of butterfat. The techniques employed included fractional distillation *in vacuo*, low-temperature crystallization, and purification by elution through a chromatographic column of activated silicic acid. Both the characterization of *trans*-octadec-16-enoic acid and the identification of thapsic acid resulting from its potassium permanganate oxidation have been supported by gas-liquid-chromatographic analyses.

EXPERIMENTAL AND RESULTS

The butterfat sample used in this investigation was prepared from fresh summer butter supplied by the Awahuri Dairy Co., Palmerston North, New Zealand. From this sample *cis*-heptadec-9-enoic acid was recently isolated (Hansen, Shorland & Cooke, 1960).

The gas-liquid chromatogram used in this work was constructed in the Fats Research Laboratory and incorporates the ⁹⁰Sr ionization detector first described by

Lovelock, James & Piper (1959). The glass column employed was 8 ft. long and ¼ in. in diameter and was packed with Celite (Chromosorb; Johns-Manville, U.S.A.) impregnated with 20% (w/w) polydiethylene glycol adipate. A second column impregnated with 5% (w/w) Apiezon L was used for the characterization of the *trans* acid isolated. The carrier gas was argon and the operating temperature 207°. All *R_F* values reported in this paper were calculated relative to methyl stearate (methyl *n*-octadecanoate, *R_F* 1.00) and peak areas represented the moles % of the components present.

X-ray measurements were made with a Phillips Geiger X-ray spectrometer with manganese-filtered iron K α radiation. Samples were melted on a glass slide and cooled.

Combustion and oxygen analyses were determined by Dr A. D. Campbell, Chemistry Department, University of Otago, Dunedin, New Zealand.

Melting points are uncorrected and were determined in closed capillaries for temperatures below 100°, whereas for temperatures above 100° determinations were made with the Hoover Uni-melt with capillaries open at one end.

Hydrolysis of 3502.5 g. of butterfat by the conventional method of Hilditch (1956), followed by steam-distillation, yielded 3099.0 g. of steam-non-volatile acids, which were separated into 'liquids' and 'solids' by crystallizing three times from acetone at -31°, once from 8 vol., once from 14 vol. and once from 15 vol. The 'solid' acids (1673.0 g.) were converted into methyl esters (1746.5 g.), by the method described by Hilditch (1956), of which 1738.5 g. was fractionally distilled *in vacuo* in a glass Vigreux column (460 cm. $\times$ 5 cm.) and yielded 23 fractions and a residue. The twenty-third (wt. 68.30 g.; m.p. 36.4-37.3°; saponification equiv. 298.5; iodine value 9.2) is the main fraction pertaining to this paper. This fraction (65.6 g.) was then submitted to repeated low-temperature crystallization from acetone (and once from ether) at -40°. Data for the first eight crystallizations are recorded in Table 1. In each case shown, the soluble fraction was removed and the insoluble fraction recrystallized.

The following fractions soluble in acetone at -40° (see Table 1) were combined: wt. 1.19 g., m.p. 24.0-24.6°; wt. 0.77 g., m.p. 23.3-24.2°; wt. 0.42 g., m.p. 27.0-27.7°. After conversion into fatty acids (wt. 1.97 g., m.p. 58.2-59.7°) this fraction was crystallized five times from acetone at -13°, the first two crystallizations being from 20 vol., the second two from 40 vol. and the final from 60 vol. The final insoluble fraction (wt. 1.23 g.; m.p. 62.7-63.1°; iodine value 67.0) was further purified by elution of its methyl esters through a column (60 cm. $\times$ 2.5 cm.) of activated silicic acid (75 g.) prepared according to the method of Hirsch & Ahrens (1958). Light petroleum (b.p. 40-60°) was used as eluent for the first 15 fractions, light petroleum

containing 1% of ether for the next four fractions and light petroleum containing 5% of ether for the final fraction (see Table 2). The nineteenth fraction (wt. 0.73 g., m.p. 28.3–28.9°) when converted into fatty acids possessed the following chemical and physical properties, which identified it as *trans*-octadec-16-enoic acid: m.p. 65.6–66.2° [Kapp & Knoll (1943) reported m.p. 62.8–63.5° for synthetic *trans*-octadec-16-enoic acid]; saponification equiv. 281.8 (calc. for $C_{18}H_{34}O_2$: 282.5); combustion analysis (Found: C, 76.8; H, 12.5. Calc. for $C_{18}H_{34}O_2$: C, 76.5; H, 12.1%); oxygen analysis (Found: O, 11.25%. Calc. for $C_{18}H_{34}O_2$: 11.4%); iodine value 86.8 (calc. for *n*-octadecenoic acid: 89.8); X-ray long spacing, 44.6 Å, when standardized against pure stearic acid 40.1 Å [Swern, Witnauer, Fusari & Brown (1955) reported 44.9 Å for *trans*-octadec-6-enoic and -8-enoic acid and 45.5 Å for *trans*-octadec-10- and -12-enoic acid: for each of the odd-numbered *trans*-octadec-7-, -9- and -11-enoic acids they reported 49.0 Å]. Methyl esters: m.p. 28.3–28.9°; hydrogenation of 0.10 g. of methyl esters with platinum oxide as catalyst, followed by hydrolysis, yielded

0.09 g. of fatty acids with m.p. 68.0–69.0° and when mixed in equal proportions with pure stearic acid (m.p. 69.9–70.2°) gave m.p. 69.0–69.6°.

Gas-liquid-chromatographic analysis of methyl esters. With the 20% polydiethylene glycol adipate column, one major peak was recorded with R_F 1.27, representing approx. 99% of the total peak areas, and two minor peaks of similar area, one with R_F 1.00 (corresponding to methyl stearate or methyl elaidate) and one with R_F 1.35. With the 5% Apiezon L column, with added methyl stearate only one peak appeared.

Infrared analysis. With a Perkin-Elmer model 21 spectrophotometer, a solution containing 15 mg. of methyl esters/ml. of carbon disulphide solution was examined in a 0.0846 cm. cell and the *trans* peak was found to absorb at 966 cm^{-1} . The *trans* peak of a sample of methyl elaidate absorbed at 964 cm^{-1} . On comparing solutions of the same concentration, the intensity of the *trans* peak was 1.16 times the intensity of the corresponding peak of methyl elaidate.

Location of the double bond. By the method of Armstrong

Table 1. *Low-temperature crystallization of methyl ester fraction*

All crystallizations were at -40°.

Wt. (g.)	Conditions of crystallization	Soluble		Insoluble	
		Wt. (g.)	M.p.	Wt. (g.)	M.p.
65.60	Acetone, 40 vol.	3.90	10.8–12.8°	61.70	30.0–38.8°
61.70	Acetone, 40 vol.	1.39	17.2–18.7	60.31	37.7–38.5
59.80	Acetone, 60 vol.	1.19*	24.0–24.6	58.61	37.9–38.8
58.61	Acetone, 60 vol.	0.77*	23.3–24.2	57.84	37.6–38.6
57.84	Acetone, 100 vol.	0.69	28.8–29.3	57.15	37.8–39.0
52.12	Ether, 40 vol.	2.40	34.4–35.3	49.72	37.8–38.7
49.72	Acetone, 100 vol.	0.42*	27.0–27.7	49.30	38.1–38.9
49.30	Acetone, 100 vol.	0.32	34.0–34.6	48.98	38.0–39.0

* These fractions were combined.

Table 2. *Chromatographic separation of methyl esters (1.25 g.) on silicic acid column*

Fraction	Wt. (mg.)	Eluent		M.p.
		Light petroleum (ml.)	Ether (%)	
1	10	100	—	22.0–40.0°
2	—	100	—	—
3	1	100	—	—
4	5	100	—	35.0
5	10	100	—	34.0–35.3
6	60	200	—	33.4–34.3
7	70	200	—	35.0–35.5
8	70	200	—	35.2–35.5
9	40	200	—	35.2–35.5
10	30	200	—	33.2–33.6
11	30	200	—	32.0–32.5
12	20	200	—	30.1–31.6
13	20	200	—	30.6–30.9
14	50	500	—	29.2–29.6
15	30	250	—	28.8–29.4
16	20	200	1	28.8–29.4
17	20	200	1	28.8–29.4
18	40	200	1	28.3–29.0
19	730	300	1	28.3–28.9
20	Trace	300	5	—

& Hilditch (1925), as recommended by Haverkamp Bege-mann, Keppler & Boekenooen (1950), 0.10 g. of fatty acids was dissolved in 2 ml. of acetic acid and oxidized with 0.38 g. of finely powdered potassium permanganate. After decolorization with sodium metabisulphite in a few drops of 2*N*-sulphuric acid, the solution was extracted with ether and washed with water. Ether from the ethereal extract was removed by distillation and the solid residue was dried *in vacuo*, giving 0.09 g. of solid material, m.p. 117.0–119.0°, of which a sample of 0.02 g. was converted into methyl esters, and on analysis by gas-liquid chromatography showed one major peak with R_F 4.50 (for purified thapsic acid, observed R_F 4.50), representing 90.3% of the combined peak areas. The remaining 0.07 g. was crystallized successively at room temperature as follows: twice from 50 vol. of ethylene dichloride, once from 50 vol. of light petroleum (b.p. 40–60°), once from 50 vol. of water and once from 100 vol. of water, removing in all only a trace (0.01 g.) of soluble material. Purification of the remaining 0.06 g. of residue was effected by two crystallizations each from 50 vol. of acetic acid at room temperature, yielding a white crystalline insoluble fraction (wt. 0.04 g.; m.p. 123.2–124.0°) (Found: C, 67.3; H, 10.5. Calc. for $C_{18}H_{30}O_4$: C, 67.1; H, 10.6%). When this 0.04 g. fraction was mixed in equal proportions with purified commercial synthetic thapsic (hexadecanedioic) acid (m.p. 123.3–124.0°; saponification equiv. 142.3) it gave a m.p. of 123.1–124.0° and when converted into the dimethyl ester (m.p. 50.9–51.3°) and mixed in equal proportions with the corresponding ester of purified synthetic thapsic acid (m.p. 51.2–51.7°) gave m.p. 51.1–51.3°. Gas-liquid analysis showed it to have one major peak with R_F 4.54 (observed for purified dimethyl ester of synthetic thapsic acid, R_F 4.50) representing 98.3% of the combined peak areas. The soluble fractions from this series of crystallizations were combined (wt. 0.03 g.; m.p. 117.0–119.3°) and when converted into dimethyl esters and analysed by gas-liquid chromatography the fraction was shown to be approximately 88.3% of thapsic acid with R_F 4.53.

Four other fractions, combined weight approximately 1 g., which were isolated in this work and which were derived from original fractions 18–23 referred to earlier, consisted wholly or largely of *trans*-octadec-16-enoic acid as determined by melting points, mixed melting points, saponification equivalents, iodine values, and gas-liquid-chromatographic and X-ray analyses.

Purified synthetic thapsic acid. In order to provide a standard acid and to establish the identity of the isolated dicarboxylic acid as thapsic acid, a sample of synthetic thapsic acid from L. Light and Co. Ltd., Colnbrook, Bucks., was purified by repeated crystallization from 25 vol. of acetic acid followed by saponification, removal of any unsaponifiable matter, regeneration of the dibasic fatty acids and further crystallization from water. The purified synthetic thapsic acid then possessed the following constants: m.p. 123.3–124.0° [Ralston (1948) records m.p. 125.0–125.2° for thapsic acid; Backderf (1956) 122.8°]; saponification equiv. 142.3 (calc. equiv. for pure thapsic acid: 143.2); combustion analysis (Found: C, 67.6; H, 10.2. Calc. for $C_{18}H_{30}O_4$: C, 67.1; H, 10.6%); dimethyl ester: m.p. 51.2–51.7° [Ralston (1948) records m.p. 51.6° for dimethyl ester of thapsic acid]. Gas-liquid-chromatographic analysis of this purified synthetic thapsic acid recorded one peak only with R_F 4.50.

DISCUSSION

The chemical and physical properties of the fraction reported in this paper, supported by identification of thapsic acid as the dicarboxylic acid product of oxidative fission, conclusively establish its identity as *trans*-octadec-16-enoic acid. Although the molecular weight as determined by the saponification equivalent and confirmed by the C, H and O analyses indicates a C_{18} monocarboxylic fatty acid, the iodine value (86.8), which closely corresponds with the theoretical value (89.8), proves the presence of a single double bond. Further proof of unsaturation is provided by the fact that hydrogenation yielded stearic acid. The melting point (65.3–66.1°) of the fatty acid isolated is similar to that (62.8–63.5°) obtained by Kapp & Knoll (1943) for synthetic *trans*-octadec-16-enoic acid, and its closeness to the melting point of stearic acid (69.9–70.2°) distinguishes it from all other known octadecenoic acids. It has been observed by Huber (1951) that the *cis*- and the *trans*-octadecenoic acid series each exhibits an alternation in melting points just as do the *normal* saturated odd- and even-numbered fatty acids. Although the melting points of all the *cis*- and *trans*-octadecenoic acids are not known, the melting point of the fraction reported in this paper conforms readily to the pattern of the even and not to the odd series. This is demonstrated by comparison of the synthesized even-numbered C_{18} *trans* isomers (-6-, m.p. 52.7–53.4°; -8-, m.p. 51.5–52.3°; -10-, m.p. 52.0–52.6°; -12-, m.p. 52.0–53.0°) with those of the odd-numbered homologues: -7-, m.p. 43.5–44.5°; -9-, m.p. 44.5–45.5°; -11-, m.p. 43.5–44.5°. [Melting points for the above synthetic *trans*-octadec-7-, -8-, -9-, -10-, -11- and -12-enoic acids were reported by Huber (1951), whereas that for *trans*-octadec-6-enoic acid was reported by Swern *et al.* (1955).] Similarly, the melting point of this fatty acid distinguishes it from synthetic *trans*-octadec-17-enoic acid [reported: m.p. 55.0–55.5°, Kapp & Knoll (1943); 55.5–56.1°, Huber (1951)], which has a double bond in the terminal position in the chain. Consistent with this acid's being an even-numbered *trans*-octadecenoic acid is the fact that its melting point contrasts with the considerably lower melting points of the corresponding *cis*-octadecenoic acid homologues, as for example *cis*-octadec-6-enoic acid, m.p. 30.0–30.2° (Fusari, Greenlee & Brown, 1951), *cis*-octadec-9-enoic acid, m.p. 13.3–13.5° (Fusari *et al.* 1951), *cis*-octadec-11-enoic acid, m.p. 13.5–14.5° (Bounds, Linstead & Weedon, 1954), and *cis*-octadec-12-enoic acid, m.p. 26.8–27.6° (Huber, 1951). Positive identification of this fraction as a *trans* acid was provided by infrared-spectrophotometric analysis of its methyl esters. Just as the odd- and even-numbered *trans*-

octadecenoic acids display an alternation of melting points, so also do they exhibit an alternation in X-ray long spacings (Lutton & Kolp, 1951). The measurement reported in this paper for the isolated fatty acid is 44.6 Å, and this value is consistent with its being an even-numbered *trans*-octadecenoic acid isomer [Lutton & Kolp (1951) record 45.5 Å for *trans*-octadec-6-, -8-, -10- and -12-enoic acids] and incompatible with its being an odd-numbered isomer [Lutton & Kolp (1951) record 49.5 Å for *trans*-octadec-7-, -9- and -11-enoic acids].

Proof of the position of the double bond in the fatty acid fraction being between the sixteenth and seventeenth carbon atoms was provided by the identification of thapsic acid as the dicarboxylic acid produced on oxidation with potassium permanganate and acetic acid. The oxidation of 0.10 g. of fatty acids yielded 0.09 g. of solid dicarboxylic acids, m.p. 117–119°, and gas-liquid-chromatographic analysis of a sample showed it to contain 90.3% of thapsic acid (i.e. 0.08 g.), assuming the amount detected corresponded with the amount applied to the column. The experimental yield of thapsic acid was thus 80% of theoretical. The presence of a preponderance of thapsic acid in the products resulting from the oxidation of this fatty acid is confirmed, independently of gas-liquid chromatography, by its isolation in the pure state (0.04 g.; m.p. 123.2–124.0°) by crystallization from solvents.

Based on the weight of the fractions isolated in this work together with the amounts estimated to be present in accompanying impure fractions, it is estimated that the content of *trans*-octadec-16:enoic acid in the sample of summer butterfat under investigation represents approximately 0.2% of the total weight of fatty acids. Backderf (1956) and Backderf & Brown (1958), however, believed this acid to be present in their summer sample to the extent of 1–2% of the total butterfat fatty acids.

The occurrence of *trans* acids in butterfat appears to be associated with two significant factors: (1) in ruminants dietary fats are subject to hydrogenation by rumen micro-organisms (Reiser, 1951; Hartman, Shorland & McDonald, 1954, 1955; Shorland, Weenink & Johns, 1955; Reiser & Reddy, 1956; Shorland, Weenink, Johns & McDonald, 1957); (2) partial hydrogenation of unsaturated fatty acids is accompanied by migration of the double bond in either direction and by the formation of positional and geometrical isomers (Allen & Kiess, 1955; Rebello & Daubert, 1951; Feuge & Cousins, 1960). As the diet of ruminants is normally pasture, the fatty acids of which are mainly linolenic acid with lesser amounts of linoleic and oleic acids (Shorland, 1941, 1953; Hilditch & Jasperson, 1945), it is

feasible that a wide range of positional and geometrical isomers of the C₁₈ unsaturated acids should be present in ruminant body and milk fats.

SUMMARY

1. *trans*-Octadec-16:enoic acid has been isolated from butterfat and characterized.

2. The weight of *trans*-octadec-16:enoic acid isolated from the sample of summer butterfat investigated represented approximately 0.2% of the total weight of fatty acids.

3. This acid has not formerly been isolated from a natural fat and characterized, although its presence in butterfat was deduced by Backderf (1956) and Backderf & Brown (1958), who conclusively identified thapsic acid as the main dicarboxylic component when appropriate C₁₈ *trans* concentrates were oxidized with potassium permanganate and acetic acid.

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Metabolism of Nitrate by Cattle

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Nitrate toxicity has been observed frequently in ruminants fed on a variety of plant materials high in nitrate (Bradley, Beath & Eppson, 1939; Grisby & Farwell, 1950; Newson, Stout, Thorp, Barber & Groth, 1937; Savage, 1949; Whitehead, Viets & Moxon, 1948; Whitehead & Moxon, 1952). Davidson, Doughty & Bolton (1941) described the toxicity symptoms in nitrate poisoning in detail. Bradley *et al.* (1939) indicated that the symptoms of nitrate toxicity are associated with the high levels of methaemoglobin and hence the low oxygen-carrying capacity of the blood of affected animals. Lewis (1951) demonstrated that micro-organisms in the rumen of sheep reduce nitrate to ammonium ions with nitrite as an intermediate; enough nitrite was transferred to the blood to convert a considerable fraction of the haemoglobin into methaemoglobin. Sund, Wright & Simon (1957) reported that a non-contagious abortion observed among cattle pastured on marsh land in central Wisconsin apparently was a manifestation of nitrate toxicity.

The work presented here was designed to study the time-course of nitrate reduction in the rumen, the changes induced in the blood by metabolites of nitrate, and the disposition of [^{15}N]nitrate by the animal.

MATERIALS AND METHODS

The experimental animals were 700–800 lb. Hereford cows with permanent rumen fistulas which were fitted with a plug. A known amount of KNO_3 in 500 ml. of water was

added through the rumen fistula each morning before the cow was offered hay, concentrates and water *ad lib.* The conversion of haemoglobin into methaemoglobin in the blood was determined daily about 3 hr. after the administration of KNO_3 . In the experiments with ^{15}N , a solution of K^{15}NO_3 was added to the rumen through the fistula in a cow which showed about 15% methaemoglobin in its daily samples of blood. Throughout the day that K^{15}NO_3 was added, periodic samples were taken of rumen liquor, blood and urine. Blood samples were removed from the jugular vein. Analysis for methaemoglobin was performed immediately on freshly drawn blood, and the remaining blood was treated with sodium oxalate to prevent clotting. Urine samples obtained with a catheter were preserved with toluene in a refrigerator until analysed. The rumen liquor withdrawn through the fistula was strained through 2 layers of cheese cloth; it then was clarified by the method of Lewis (1951), and the clear supernatant solution was used for analysis.

Analyses for nitrate, nitrite, urea and ammonia in the rumen liquor, blood and urine were made by the methods described in the A.O.A.C. handbook (Lepper, 1945). ^{15}N was determined with a Consolidated-Nier isotope ratio mass spectrometer. Methaemoglobin and total haemoglobin were determined by the method of Evelyn & Malloy (1938). The blood sample treated with oxalate was centrifuged, and the cells were washed twice by centrifuging from 0.85% NaCl and then were refrigerated until used. Samples (10 ml.) of the supernatant fluid were stirred while to each were added 3 ml. of 0.5 M-Ba(OH) $_2$ and 3 ml. of 0.5 M-ZnSO $_4$ to precipitate the proteins. The precipitate was removed by centrifuging, and the clear supernatant fluid was used for analysis of ammonium, nitrite and nitrate ions.

Haemin and globin were separated and purified essentially as described by London, Morell & Kassenaar (1958).