The Isolation and Identification of 2,6,10,14-Tetramethylpentadecanoic Acid from Butterfat

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Butterfat has been shown to contain small amounts of branched-chain fatty acids of the iso and anteiso series (Hansen & Shorland, 1951a; Shorland & Hansen, 1957; Magidman, Herb, Barford & Riemenschneider, 1962; Herb, Magidman, Luddy & Riemenschneider, 1962) as well as a C_{20} multibranched-chain acid (Hansen & Shorland, 1951b, 1953, and unpublished work) isolated and identified as 3,7,11,15-tetramethylhexadecanoic acid (N. Bjurstam, B. Hallgren, R. Ryhage & S. Stållberg-Stenhagen, referred to by Stenhagen, 1961; Sonneveld, Haverkamp Begemann, van Beers, Keuning & Schogt, 1962). This acid has also been isolated from ox plasma (Lough, 1963, 1964) and from the blood of humans with the rare disease of Refsum syndrome (Kahlke, 1963; Klenk & Kahlke, 1963). Continued investigations in the Fats Research Laboratory on trace fatty acid constituents of fats have now revealed the presence in butterfat of a C_{19} saturated fatty acid which contains four methyl side chains (Hansen, 1964). This component of butterfat, of which trace amounts only have been isolated, has not previously been identified in natural sources.

EXPERIMENTAL

Combustion analysis and *C*-methyl determinations were carried out by Dr A. D. Campbell, Chemistry Department, University of Otago, New Zealand.

The gas-liquid chromatograph used in this investigation was constructed in the Fats Research Laboratory and is fitted with a ^{90}Sr -ionization detector (Lovelock, James & Piper, 1959). The glass columns employed were 2.4 m. in length and 6.5 mm. in internal diameter and were packed with Celite (Chromosorb; Johns-Manville Co., U.S.A.) impregnated with 20% (w/w) polydiethylene glycol adipate or 5% (w/w) Apiezon L. The operating temperature in all cases was 207°, and argon was used as carrier gas. Samples were applied to the chromatograph as methyl esters and the relative retention volumes, V_R , have been calculated relative to methyl stearate (V_R 1.00).

The mass spectrometer used was a 60° sector, 30 cm. radius, single-focusing instrument. The sample was introduced by means of an all-glass greaseless inlet system at 200°. The mass spectrum (see Fig. 1) was recorded by using magnetic scanning of the mass scale.

The infrared analysis was made on thin films of the methyl ester by using a Perkin-Elmer model 21 spectrophotometer.

The butterfat sample (N2) used in this investigation was that described by Hansen, Shorland & Cooke (1960). Summer butterfat (3502.5 g.) was hydrolysed and steam-distilled, and the non-steam-volatile fatty acids (3099.0 g.) were crystallized three times from acetone at -31°. The combined 'liquid' acids were converted into methyl esters and fractionally distilled *in vacuo* (0.08 mm. Hg) in a glass Vigreux column (460 cm. $\times$ 5 cm.). The fourteenth fraction (wt. 38.71 g.; m.p. -18.9° to -16.3°; saponification equiv. 288.8; iodine value 77.0) was refractionated *in vacuo* (0.08 mm. Hg) in a column (50 cm. $\times$ 1.8 cm.) fitted with a coiled spring (column E; Shorland, 1952), and of the resulting fractions the third to the eighth, which had saponification equivalents ranging between 281.1 and 285.4, were combined, denoted G6, converted into fatty acids (12.59 g.) and crystallized from 40 vol. of acetone at -40°, yielding a soluble fraction G6L (wt. 7.30 g.; m.p. -2.3° to -1.2°). Fraction G6L was crystallized from 40 vol. of acetone at -40° and the soluble part was recrystallized from 10 vol. of acetone at -50° to yield another soluble fraction G6L2L (wt. 2.73 g.; m.p. -27.0° to -16.5°) and an insoluble fraction G6L2S (wt. 4.43 g.; m.p. 4.3° to 5.6°). Three further crystallizations of G6L2L from 10 vol. of solvent, one from acetone at -50° and two from light petroleum (b.p. 50-60°) (one at -40° and the other at -70°), yielded a fraction denoted G16L2L (wt. 2.18 g.; m.p. -27.3° to -25.4°; saponification equiv. 263.8; iodine value 67.8). Crystallization at -70° of the methyl esters of G16L2L, once from 10 vol. of acetone, once from 10 vol. of ethanol and once from 5 vol. of ethanol, gave a soluble fraction G16L5L which was liquid at -70° (wt. 1.89 g.; iodine value 31.7). This fraction was chromatographed in light petroleum on a column (approx. 40 cm. $\times$ 2 cm. internal diam.) containing 113 g. of activated silicic acid (cf. Hirsch & Ahrens, 1958). Four fractions eluted between 50 and 150 ml. of solvent had identical gas-liquid chromatograms and were combined and denoted G172 (wt. 0.29 g.). Final purification was effected by chromatographing 0.10 g. through silicic acid-AgNO₃ adsorbent (10 g.) with 10 ml. charges of light petroleum-benzene mixture (9:1, v/v) as eluent (de Vries, 1962), and 0.06 g. was recovered and denoted G173. A further fraction (wt. 0.08 g.) identical with fraction G173 was also isolated in this investigation.

Combustion analyses on the methyl ester were carried out (Found: C, 76.5; H, 12.8. Calc. for $C_{20}H_{40}O_2$: C, 76.8; H,

12.9%). The iodine value was 5.3 (Wijs's solution, 1 hr.). The m.p. was below -70° . The *C*-methyl value was 11.4%.

Gas-liquid-chromatographic analyses. With the adipate column, fraction G 173 gave one main peak and two minor ones, the main one, V_R 0.50, occupying approx. 98% of the total peak areas. With the Apiezon L column only one peak, V_R 0.52, was present.

DISCUSSION

Mass-spectrometric analysis (see Fig. 1). The parent ion occurred at m/e 312 and the relative abundances of the isotope peaks suggested that it contained 20 or 21 carbon atoms. Since the sample was known to be the methyl ester of a fatty acid, with m/e 312, its molecular formula was $C_{20}H_{40}O_2$. As would be expected for a methyl ester, a prominent *M*-31 ion was present. The structure of the acid chain was deduced by using the rules derived by Ryhage & Stenhagen (1960*a, b*). The base peak and most abundant fragment was at m/e 88; this was a rearrangement ion, and was conclusive evidence for a methyl group at C-2 in the fatty acid chain. In methyl esters of this type, rupture was found by the above authors to be especially probable on either side of carbon atoms where branching occurred. By applying this rule to the present spectrum, the peaks at m/e 129 and 157, 199 and 227, and 269 and

297, were noticeably larger than those at m/e 143, 213 and 283 respectively. This suggested that there were methyl groups attached at C-6, C-10 and C-14. Rearrangement ions of the type:

are also to be expected, and were found at m/e 125, 195 and 265, in agreement with the postulated methyl substitution at C-6, C-10 and C-14. The presence of a small *M*-65 ion supported the conclusion that the end of the chain was an isopropyl group. A marked feature of the spectrum was the rearrangement peak at m/e 222 corresponding to *M*-90. One other reported case of an *M*-90 rearrangement fragment ion is in methyl mycocerosate (see Ryhage & Stenhagen, 1960*a*) and there it is not nearly so prominent. It has been observed by these authors that methyl substitution at C-6 gives rise to a prominent *M*-76 ion, and, as this C_{19} fatty acid ester had a methyl group substituted at C-2 also, the assignment of a methyl group to C-6 was consistent with this observation. The mass-spectrometric examination suggested therefore that the structure was (I).

Infrared analyses. The infrared-absorption spectrum of fatty acid fraction G 173 was characterized by the following absorptions (see Fig. 2):

(I)

Fig. 1. Mass spectrum of the methyl ester of fatty acid fraction G 173 from butterfat.

(1) A strong band at 1170 cm.⁻¹ with a shoulder at 1150 cm.⁻¹ which is attributed to skeletal vibrations of a terminal isopropyl group (Simpson & Sutherland, 1949; Pliva & Sørensen, 1950; Bendoraitis, Brown & Hepner, 1962).

(2) A doublet at 1370 and 1380 cm.⁻¹ which is interpreted as being due to C-H deformation modes of an isopropyl group (Thompson & Torkington, 1945; Bendoraitis *et al.* 1962).

(3) A strong band at 736 cm.⁻¹ (unaccompanied by a prominent shoulder at 727 cm.⁻¹) which may be assigned to the methylene rocking vibrations in the grouping (R·[CH₂]₃·R')⁺ which is most significant in establishing the regular isoprene chain sequence (Pliva & Sørensen, 1950; McMurry & Thornton, 1952; Bendoraitis *et al.* 1962). The presence of one irregular R·[CH₂]₄·R' sequence would be detected readily by a prominent shoulder at 727 cm.⁻¹, as was observed by Pliva & Sørensen (1950) to be present in crocetane (2,6,11,15-tetramethylpentadecane) and to be absent from pristane (2,6,10,14-tetramethylpentadecane).

Had this acid possessed a terminal ethyl grouping, a moderately strong band characteristic of this grouping would have appeared at 770 cm.⁻¹ (Thompson, 1948; Pliva & Sørensen, 1950), but no such absorption was observed.

The infrared-absorption evidence, considered in conjunction with the molecular weight obtained by mass spectrometry, indicates a C₁₉ saturated fatty acid with methyl groups located at C-2, C-6, C-10 and C-14. This assignment of methyl groups accords with the symmetrical arrangement of side-chain methyl groups in the homologous C₂₀ multi-branched-chain fatty acid, 3,7,11,15-tetramethylhexadecanoic acid (N. Bjurstaam, B. Hallgren, R. Ryhage & S. Stållberg-Stenhagen, referred to by Stenhagen, 1961; Sonneveld *et al.* 1962), also found in butterfat.

To confirm the assessment of the methylene chain length in this C₁₉ acid the infrared-absorption spectrum was compared with that of the C₂₀ multibranched-chain acid 3,7,11,15-tetramethylhexadecanoic acid. No indications that the substances differed in this respect were revealed.

James & Martin (1954) established for any homologous series of fatty acids a linear relationship between the logarithm of retention time and the number of carbon atoms. The *n*-saturated, iso, anteiso and neo series not only conform to this pattern but the straight lines are essentially parallel (cf. Farquhar, Insull, Rosen, Stoffel & Ahrens, 1959; Hawke, Hansen & Shorland, 1959). By using this relationship and the relative retention volumes from adipate columns, a straight line was drawn through the plot of the C₂₀ multibranched-chain acid (*V_R* 0.72) parallel to the *n*-saturated series. When the plot for the C₁₉ branched-chain acid was

read from this graph, *V_R* 0.55 was obtained which approximates closely to the *V_R* value 0.50 found experimentally. Similarly, with the Apiezon L column the value read from the graph was *V_R* 0.54 as compared with 0.52 found experimentally. The above evidence is consistent with the C₁₉ acid reported in this paper being homologous with the C₂₀ acid 3,7,11,15-tetramethylhexadecanoic acid.

Independent support for the structure assigned to this acid is provided by its infrared-absorption characteristics relating to both the terminal isopropyl grouping and the regular triple methylene sequence being identical with those of the isoprenoid hydrocarbon pristane (2,6,10,14-tetramethylpentadecane), whose constitution has been conclusively established by mass and infrared spectrometry, nuclear magnetic resonance, gas-liquid chromatography and synthesis (cf. Pliva & Sørensen, 1950; Lederer & Pliva, 1951; Bendoraitis *et al.* 1962; Mold, Stevens, Means & Ruth, 1963; Hallgren & Larsson, 1963).

In the original fractional distillation *in vacuo* of the 'liquid' methyl esters of butterfat, it was found that fraction G 173 distilled over with the C₁₇ components, which were composed mainly of the esters of *n*-heptadecanoic acid and (+)-14-methylhexadecanoic acid together with traces of 15-methylhexadecanoic acid and *n*-heptadecanoic acid. Likewise the C₂₀ branched-chain acid was found (Hansen & Shorland, 1951b, 1953) in the fraction which distilled over with the C₁₇ 'liquid' components. Another property common to both acids is that they are liquid at -70°. It appears to be characteristic of high-molecular-weight fatty acids with more than one methyl side chain that they have extraordinarily low melting points.

Fig. 2. Infrared-absorption spectrum of thin films of the methyl ester of fatty acid fraction G 173 from butterfat: A, 1300-1400 cm.⁻¹; B, 1100-1200 cm.⁻¹; C, 700-800 cm.⁻¹.

The present investigation indicates the content of this C₁₉ fatty acid in butterfat to be approx. 0.01 % of the total weight of fatty acids.

Although 2,6,10,14-tetramethylpentadecanoic acid has not formerly been isolated from natural fat, Smith & Boyack (1948) converted phytol into phytene-1 and then into a fatty acid with formula C₁₉H₃₈O₂ which they named 'apophytoic' acid and which may correspond with the butterfat constituent reported in this paper.

SUMMARY

1. 2,6,10,14-Tetramethylpentadecanoic acid has been isolated from butterfat and identified by mass and infrared spectrometry and gas-liquid chromatography.

2. It was present to the extent of approx. 0.01 % of the total weight of fatty acids.

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Effect of Trace Elements on the Production of Pigments by a *Pseudomonad*

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Several workers (Georgia & Poe, 1931; Burton, Campbell & Eagles, 1948; Grossowicz, Hayat & Halpern, 1957) have demonstrated the essentiality of Mg²⁺ ions for the production of various pigments by different species of *Pseudomonas*, though earlier reports (Jordan, 1899; Sullivan, 1905; Tanner, 1918) were contradictory. The effect of other trace-element ions such as Fe³⁺, Mn²⁺, Zn²⁺, Co²⁺, Cu²⁺

etc. (Burton *et al.* 1948; Grossowicz *et al.* 1957) or the replacement of Mg²⁺ by other metal ions (Thumm, 1895) on the production of pigments in a variety of pseudomonads has also been studied, but not without some contradictory results. However, data on the effects of trace-element ions on the production of the water-soluble fluorescent pigments (fluorescein) by species of the *Pseudomonas*