attributed to three causes, namely, non-ideal boundary formation, concentration dependence of the diffusion constant and polydispersity, all factors being operative in this case. In the case of B substance it was found experimentally impossible to calculate the coefficient for the main component only, the diffusion constant determined represents an average as defined by Quensel (1942). The calculated molecular weight of B substance stands therefore only as an approximation. Both B substances are of greater molecular size and asymmetry than the other blood-group substances (Kekwick, 1950, 1952*a, b*), but very similar from the standpoint of comparative homogeneity. The B substances both show considerable asymmetry with axial ratios of 200 and 100, respectively, or alternatively may be highly hydrated.

SUMMARY

1. The B substances have been shown to give single peaks in an electrophoresis apparatus at pH's 8 and 4.

2. The molecular weight calculated from sedimentation and diffusion data is 460 000 for B' and approximately 1 800 000 for B substance, with frictional ratios 3.9 and 5.7, respectively.

3. The B substances are both somewhat polydisperse and probably highly asymmetrical in shape.

I wish to acknowledge the technical assistance of Mr H. Murray.

REFERENCES

- Gosting, L. J., Hanson, E. M., Kegeles, G. & Morris, M. S. (1949). *Rev. Sci. Instrum.* **20**, 209.
Grlen, N. (1941). *Kolloid Z.* **95**, 188.
Kahn, D. S. & Polson, A. (1947). *J. phys. Chem.* **51**, 816.
Kegeles, G. & Gosting, L. J. (1947). *J. Amer. chem. Soc.* **69**, 2516.
Kekwick, R. A. (1950). *Biochem. J.* **46**, 438.
Kekwick, R. A. (1952*a*). *Biochem. J.* **50**, 471.
Kekwick, R. A. (1952*b*). *Biochem. J.* **52**, 259.
Philpot, J. St L. (1938). *Nature, Lond.*, **141**, 283.
Quensel, O. (1942). Dissertation, Uppsala. Almquist & Wiksells Boktryckeri A.-B.
Svedberg, T. & Pedersen, K. O. (1940). *The Ultracentrifuge*, Oxford University Press.

The Branched-chain Fatty Acids of Butterfat

4. THE ISOLATION OF (+)-12-METHYLTETRADECANOIC ACID AND OF 13-METHYLTETRADECANOIC ACID

By R. P. HANSEN, F. B. SHORLAND AND N. JUNE COOKE
*Fats Research Laboratory, Department of Scientific and Industrial Research,
Wellington, New Zealand*

(Received 30 November 1953)

Branched-chain fatty acids have been shown to be present in trace quantities in butterfat (Hansen & Shorland, 1951*a, b*, 1953; Hansen, Shorland & Cooke, 1951), in ox fat (Hansen, Shorland & Cooke, 1952*a*), in mutton fat (Hansen, Shorland & Cooke, 1952*b*, 1953), and in shark liver oil (Morice & Shorland, 1953). Two isomeric C₁₅ branched-chain acids, namely (+)-12-methyltetradecanoic acid and 13-methyltetradecanoic acid, which were reported as occurring in mutton fat (Hansen *et al.* 1953), have now been isolated from butterfat.

EXPERIMENTAL

The butterfat used in this investigation was prepared by washing and centrifuging fresh butter supplied by the Rangitaiki Plains Dairy Co. Ltd., Whakatane, New Zealand. The butter had been churned in the first week of May 1951, from a bulked sample of cream representative of numerous dairy herds in the district.

Butterfat glycerides (sample G/43: 17.85 kg.; sap. equiv. 250.0; iodine value 40.1) were hydrogenated at 180° and at atmospheric pressure using Ni supported on kieselguhr as catalyst. The resulting glycerides (iodine value 1.1) were saponified in the cold with KOH and the soaps converted into fatty acids with H₂SO₄. Steam distillation removed part of the steam-volatile acids, after which the remaining mixed acids were washed with water and then repeatedly crystallized from 10 vol. acetone at -33°. The resulting 'liquid' acids were converted into methyl esters (1876 g.; sap. equiv. 223.8; iodine value 4.7) and were fractionated *in vacuo* in a 490 × 3.8 cm. stainless steel column packed with 3-4 mm. diameter single-turn glass helices.

Of the fractions distilled over, the 32nd (KL32; 48.00 g.; sap. equiv. 275.6; iodine value 7.9) was selected for the investigation reported in this paper. When freed of unsaponifiable matter this methyl ester fraction had sap. equiv. 250.4; iodine value 0.9; m.p. -4.1 to -2.0°. Fractional distillation of KL32 was then repeated at 0.05 mm. in a 50 × 1.8 cm. column fitted with a closely coiled spring as packing (column E, Shorland, 1952). Results of fractionation are shown in Table 1.

Table 1. *Fractional distillation of KL32 methyl esters (38.48 g.)*

Fraction	Wt. (g.)	Sap. equiv.	Iodine value (Wijs)	M.p. (°)
KL32L1	1.48	240.5	1.0	16.9 to 17.3
KL32L2	3.32	244.0	0.9	13.0 to 14.0
KL32L3	1.38	244.2	0.8	12.5 to 13.2
KL32L4	2.66	244.5	0.8	12.8 to 13.8
KL32L5	2.77	247.1	0.2	11.0 to 12.0
KL32L6	3.15	250.1	Nil	-0.5 to +1.8
KL32L7	4.80	254.0	Nil	-8.0 to -7.0
KL32L8	2.55	253.5	Nil	-10.5 to -9.0
KL32L9	3.75	256.0	Nil	-10.8 to -9.8
KL32L10	2.44	256.1	Nil	-11.0 to -10.0
KL32L11	3.95	254.7	Nil	-11.0 to -10.0
KL32L12	1.40	255.1	0.9	-12.8 to -12.0
KL32LR	4.53	256.9	4.0	2.0 to 2.5

K8

Table 2. *Fractional distillation of K8 methyl esters (16.93 g.)*

Fraction	Wt. (g.)	Sap. equiv.	Iodine value (Wijs)	M.p. (°)
K8L1	1.45	252.0	0.6	2.5
K8L2	2.77	255.4	0.4	-8.0
K8L3	2.05	256.4	0.3	-10.8
K8L4	2.91	256.6	0.3	-11.5
K8L5	2.12	256.8	0.2	-11.3
K8L6	2.50	256.0	0.3	-11.2
K8L7	0.80	254.9	0.6	-10.8
K8LR*	0.48	274.0	11.5	—

K14

* K8LR when freed of unsaponifiable matter gave sap. equiv. 250.0 (acids).

Fractions KL32L7 to KL32L12 (Table 1) were bulked (K8) and refractionated as methyl esters (wt. 16.93 g.; vac. 0.05 mm.) in column *E* (Shorland, 1952). See Table 2.

Fractions K8L2 to K8L7 were combined (K14), converted into fatty acids (10.51 g.; m.p. 33.2°) and submitted to repeated low-temperature crystallization from light petroleum (b.p. 50–60°) and finally from methanol (Table 3) to yield the purified fraction K14S17LS3S with the following properties. Acid (1.80 g.) m.p. 52.0°; sap. equiv. 242.2 (calc. for $C_{15}H_{30}O_2$, 242.4); found: C, 74.4; H, 12.5% (calc. for $C_{15}H_{30}O_2$: C, 74.4; H, 12.5%); iodine value (Wijs) 0.5; *C*-methyl, 10.7% (equiv. to 1.72 mol. acetic acid); X-ray long spacing, 30.1 Å; optical rotation, nil; refractive index, n_D^{20} 1.4312. Methyl ester, m.p. 9.4°; n_D^{20} 1.4384. A mixed m.p. made with equal weights of this acid and synthetic 13-methyltetradecanoic acid supplied by the courtesy of Prof. E. Stenhagen (Arosenius, Stållberg, Stenhagen & Tägtström-Eketorp, 1949) gave m.p. 51.9°. Similarly, fraction K14S17LS3S and the C_{15} iso acid H29S2LS isolated from mutton fat (Hansen *et al.* 1953) gave no depression in melting point when mixed in equal proportions.

As is illustrated in Table 3, crystallization from 40 vol. light petroleum at -40° was the basis of separation of *iso* from *ante-iso* acids.

Fractions K14L to K14S9L (see Table 3) were bulked (K20) and after repeated low-temperature crystallization had failed to effect satisfactory separation, were applied to

a column (2.6 cm. diameter) packed with 133 g. of activated Al_2O_3 and eluted with light petroleum (b.p. 50–60°) and finally with ether.

Of the fifteen resulting fractions, K20c to K20k had similar melting points (range -14.0 to -13.4°) and refractive indices (range 1.4398 to 1.4401). Accordingly they were combined (K21; 6.33 g.; sap. equiv. 257.3, methyl esters); and hydrolysed, and the acids (5.85 g., m.p. 23.0°) submitted to low-temperature crystallization as shown in Table 4, to yield a purified fraction K21S10LS4S.

The properties of fraction K21S10LS4S were as follows: Acid (3.96 g.) m.p. 23.7°; sap. equiv. 242.2 (calc. for $C_{15}H_{30}O_2$, 242.4); found: C, 74.3; H, 12.5% (calc. for $C_{15}H_{30}O_2$: C, 74.4; H, 12.5%); iodine value (Wijs) 0.0; *C*-methyl, 10.1% (equiv. to 1.65 mol. acetic acid) X-ray long spacing, 30.1 Å; $[\alpha]_D^{25} + 4.8^\circ$ in $CHCl_3$ (c, 20.5; 0.5 dm. tube); refractive index, n_D^{20} 1.4327. Methyl ester, m.p. -12.3°; n_D^{20} 1.4398.

The two acids reported in this paper were derived from the original 'liquid' methyl ester fraction KL32. Investigation of the succeeding fraction (KL33) by low-temperature crystallization (but not by further high-vacuum distillation) yielded an acid (K13) whose properties were similar to but not identical with those of the C_{15} *ante-iso* acid K21S10LS4S. The properties of fraction K13 are as follows: 3.34 g.; sap. equiv. 243.8; m.p. 27.6°; iodine value 0.0; $[\alpha]_D^{25} + 3.9^\circ$; X-ray long spacing 30.1 Å. When mixed in equal proportions with the C_{15} *ante-iso* acid from mutton fat (H2S4LS, m.p. 25.5°) it gave m.p. 27.3°. In an attempt at further purification, K13 was repeatedly crystallized at low temperatures, submitted to chromatographic separation on activated Al_2O_3 , and those fractions with corresponding properties were combined (K26) and crystallized a total of 39 times, firstly as acids and then as ethyl esters. The final product K30LS16S had m.p. 27.8–29.0°, and when mixed in equal quantity with K21S10LS4S (m.p. 23.7°) gave m.p. 26.0–27.0°.

The X-ray long crystal spacings reported in this work were made with a Philips Geiger X-ray spectrometer using nickel-filtered copper $K\alpha$ radiation. Samples were melted on a glass slide and cooled.

All melting points were determined in closed capillaries and are uncorrected.

Combustion analyses and *C*-methyl determinations were made by Weiler and Strauss, Oxford.

Table 3. *Low temperature crystallization of fatty acid fraction K14 (10-51 g.)*

The insoluble fraction (S) from one crystallization was used for the next crystallization except in the case of fraction K14S17L.

Fraction crystallized	Conditions of crystallization	No. of crystallizations	Soluble			Insoluble		
			Fraction	Wt. (g.)	M.p. (°)	Fraction	Wt. (g.)	M.p. (°)
K14	Light petroleum, 40 vol. -40°	10	K14L to K14S9L (combined as K20, contains <i>ante-iso</i> acid)	7.88	20.6 to 26.8	K14S9S	2.56	50.5
K14S9S	Light petroleum 40 vol. -40°	7	K14S10L to K14S16L	0.36	30.0 to 46.0	K14S16S	2.17	51.9
K14S16S	Light petroleum, 100 vol. -5°	1	K14S17L	2.12	51.6	K14S17S	0.05	51.3
K14S17L	Methanol, 40 vol. -40°	4	K14S17LL to K14S17LS3L	0.35	47.2 to 49.6	K14S17LS3S (<i>iso</i> acid)	1.80	52.0

Table 4. *Low temperature crystallization of fatty acid fraction K21 (5.85 g.)*

The insoluble fraction (S) from one crystallization was used for the next crystallization except in the case of fraction K21S10L.

Fraction crystallized	Conditions of crystallization	No. of crystallizations	Soluble			Insoluble		
			Fraction	Wt. (g.)	M.p. (°)	Fraction	Wt. (g.)	M.p. (°)
K21	Light petroleum, 40 vol. -70°	2	K21L to K21S1L	0.31	4.8 to 16.5	K21SS	5.53	23.3
K21SS	Methanol, 40 vol. -70°	1	K21S2L	0.17	21.2	K21S2S	5.37	23.2
K21S2S	Acetone, 20 vol. -70°	5	K21S3L to K21S7L	0.49	8.2 to 22.2	K21S7S	4.74	23.8
K21S7S	Light petroleum, 40 vol. -70°	1	K21S8L	0.02	22.2	K21S8S	4.70	23.8
K21S8S	Ether, 10 vol. -70°	1	K21S9L	0.24	21.6	K21S9S	4.41	23.6
K21S9S	Ether, 40 vol. -55°	1	K21S10L	4.38	23.7	K21S10S	0.03	22.2
K21S10L	Aq. ethanol, 10 vol. -40°	1	K21S10LL	0.05	18.2	K21S10LS	4.22	23.7
K21S10LS	Aq. ethanol, 10 vol. -35°	1	K21S10LSL	0.04	22.0	K21S10LSS	4.16	23.5
K21S10LSS	Acetone, 20 vol. -70°	3	K21S10LS2L to K21S10LS4L	0.20	17.8 to 22.0	K21S10LS4S (<i>ante-iso</i> acid)	3.96	23.7

DISCUSSION

The saponification equivalents, carbon and hydrogen contents and the iodine values of the two fractions isolated (K14S17LS3S and K21S10LS4S) are in close agreement with the calculated requirements for a C_{15} saturated fatty acid.

The X-ray long spacing of each of the two acids ($30.1\text{\AA}$) corresponds within experimental error ($\pm 0.5\text{\AA}$) with the value of $29.8\text{\AA}$ reported by Arosenius *et al.* (1949) for 13-methyltetradecanoic acid, and contrasts with the value of $35.75\text{\AA}$ for pentadecanoic acid (Slagle & Ott, 1933). (The sample of pure synthetic 13-methyltetradecanoic acid (Arosenius *et al.* 1949), when analysed here under the same conditions as fractions K14S17LS3S and K21S10LS4S gave a value of $29.9\text{\AA}$.) No X-ray long spacing appears in the literature for (+)-12-methyltetradecanoic acid, although values for the amide of the dextro form (Velick, 1947) and the racemic form (Nunn, 1951) of this acid have been reported as 29.2 and $28.0\text{\AA}$, respectively.

Chromic acid oxidation of fractions K14S17LS3S and K21S10LS4S by the Kuhn-Roth (1933) method yielded acetic acid in amounts which represented 10.7 and 10.1 %, respectively, of *C*-methyl. These data, when interpreted in accordance with the work of Ginger (1944), point to the presence of one side-chain alkyl group in each fraction.

The position of branching in the two fractions is established mainly by melting-point evidence. Fraction K21S10LS4S with melting point 23.7° corresponds to the C_{15} *ante-iso* acid (branching on the antepenultimate carbon, $\text{COOH}=1$) with melting point 23.0° isolated from wool grease by Weitkamp (1945) and identified as 12-methyltetradecanoic acid. Consistent with this location of the methyl group in K21S10LS4S is its optical activity $[\alpha]_D^{16.5} + 4.8^\circ$ in chloroform (20.6 % solution). Weitkamp (1945) found $[\alpha]_D^{26} + 4.7^\circ$ (pure substance) for the C_{15} *ante-iso* acid extracted from wool grease.

The molecular rotation of the C_{15} *ante-iso* acid separated in this work is $[M]_D + 11.6^\circ$, and as indicated by Crombie & Harper (1950) and subsequently by Klyne (1953) for other *ante-iso* acids, it is almost certainly an 'L-methyl' substituted acid with the methyl group on the left-hand side of the chain in a Fischer projection.

The melting point of K14S17LS3S (52.0°) agrees closely with the melting point of 51.7 – 51.8° reported by Arosenius *et al.* (1949) for the C_{15} *iso* acid 13-methyltetradecanoic acid (and the mixed m.p. of these two acids showed no depression), and at the same time conforms with the observation of Cason & Winans (1950) and of Weitkamp (1945) that *iso* acids melt less than one degree below the melting

point of their corresponding normal acids; penta-decanoic acid has been reported as having melting point 52.4° (Weitkamp, 1945) 52.26° (Meyer & Reid, 1933) and 52.1° (Francis, Piper & Malkin, 1930). K14S17LS3S did not rotate the plane of polarization.

The evidence presented thus establishes fractions K21S10LS4S and K14S17LS3S as (+)-12-methyltetradecanoic acid and 13-methyltetradecanoic acid, respectively.

In both butterfat and mutton fat the C_{15} *iso* and *ante-iso* acids have been found to be closely associated with myristic acid in the fraction soluble in acetone at temperatures about -30° . Furthermore, fractions rich in esters of the C_{15} *iso* and *ante-iso* acids distilled over together immediately after the esters of myristic acid.

The amounts of the two C_{15} branched-chain fatty acids detected in this investigation together constitute about 0.1 % of the total fatty acids present (*iso* acid 0.03, *ante-iso* acid 0.07 %).

Weitkamp (1945) in his investigations on the saponifiable fraction of wool grease identified ten *iso* acids ranging from *isocaproic* (C_{10}) to *isomontanic* (C_{28}), and all of these contained an even number of carbon atoms. Despite this searching examination, *iso* acids with an odd number of carbons were not reported. The present authors in their earlier work on the branched-chain fatty acids of butterfat (Hansen & Shorland, 1951a) isolated two C_{17} acids, one of which was also found in ox fat (Hansen *et al.* 1952a) and was identified as the *ante-iso* acid while the other had properties consistent with its being the *iso* acid. The isolation of the C_{15} *iso* acid from butterfat as reported in this paper thus extends the occurrence of odd-numbered *iso* acids in animal fats, and at the same time suggests that the C_{15} *iso* acid found in mutton fat (but not found in wool wax), is a natural component of the fat and is not of adventitious origin.

Although the two acids reported in this paper were saturated when isolated, the present investigation does not establish whether they exist in nature in that condition, or whether they were formed by hydrogenation of unsaturated constituents during the analysis procedure. However, the results of recent experiments on the tubercle bacillus (Cason, Sumrell, Allen, Gillies & Elberg, 1953) suggest that the branched-chain saturated fatty acid, tuberculo-stearic acid, does not result from the hydrogenation of an unsaturated acid or of an acid containing a cyclopropane ring. Furthermore, evidence that saturated branched-chain fatty acids do occur in butterfat is shown by the isolation of a multi-branched C_{20} saturated acid both by methods which included hydrogenation (Hansen & Shorland, 1951b) and by methods which did not have recourse to hydrogenation (Hansen & Shorland, 1953).

SUMMARY

Two branched-chain fatty acids, (+)-12-methyltetradecanoic acid and 13-methyltetradecanoic, formerly isolated from mutton fat, have been shown to be present in butterfat.

We thank Professor E. Stenhagen of Uppsala University, Sweden, for a synthetic sample of pure 13-methyltetradecanoic acid, and Mr M. Fieldes of the Soil Bureau, Department of Scientific and Industrial Research, Wellington, New Zealand, for the X-ray measurements.

REFERENCES

- Arosenius, K. E., Stållberg, G., Stenhagen, E. & Tägtström-Eketorp, B. (1949). *Ark. Kemi Min. Geol.* **26**A, no. 19.
- Cason, J., Sumrell, G., Allen, C. F., Gillies, G. A., Elberg, S. (1953). *J. biol. Chem.* **205**, 435.
- Cason, J. & Winans, W. R. (1950). *J. org. Chem.* **15**, 139.
- Crombie, L. & Harper, S. H. (1950). *Chem. & Ind.* p. 757.
- Francis, F., Piper, S. H. & Malkin, T. (1930). *Proc. Roy. Soc. A*, **128**, 214.
- Ginger, L. G. (1944). *J. biol. Chem.* **156**, 453.
- Hansen, R. P. & Shorland, F. B. (1951*a*). *Biochem. J.* **50**, 207.
- Hansen, R. P. & Shorland, F. B. (1951*b*). *Biochem. J.* **50**, 358.
- Hansen, R. P. & Shorland, F. B. (1953). *Biochem. J.* **55**, 662.
- Hansen, R. P., Shorland, F. B. & Cooke, N. J. (1951). *Chem. & Ind.* p. 839.
- Hansen, R. P., Shorland, F. B. & Cooke, N. J. (1952*a*). *Biochem. J.* **50**, 581.
- Hansen, R. P., Shorland, F. B. & Cooke, N. J. (1952*b*). *Biochem. J.* **52**, 203.
- Hansen, R. P., Shorland, F. B. & Cooke, N. J. (1953). *Biochem. J.* **53**, 374.
- Klyne, W. (1953). *Biochem. J.* **53**, 378.
- Kuhn, R. & Roth, H. (1933). *Ber. deutsch. chem. Ges.* **66**, 1274.
- Meyer, J. D. & Reid, E. E. (1933). *J. Amer. chem. Soc.* **55**, 1574.
- Morice, I. M. & Shorland, F. B. (1952). *Chem. & Ind.* p. 1267.
- Nunn, J. R. (1951). *J. chem. Soc.* p. 1740.
- Shorland, F. B. (1952). *J. appl. Chem.* **2**, 438.
- Slagle, F. B. & Ott, E. (1933). *J. Amer. chem. Soc.* **55**, 4396.
- Velick, S. F. (1947). *J. Amer. chem. Soc.* **69**, 2317.
- Weitkamp, A. W. (1945). *J. Amer. chem. Soc.* **67**, 447.

Rapid Determination of Salicylate in Biological Fluids

BY P. TRINDER

Biochemistry Department, Royal Infirmary, Sunderland, Co. Durham

(Received 14 November 1953)

The purple colour given by salicylate ion with ferric salts in weakly acid solution and the blue colour given with the Folin-Ciocalteu reagent in strongly alkaline solution have been most used for the determination of salicylates in serum. Of recent methods employing ferric salts, that of Tarnoky & Brews (1950) is the most convenient. The disadvantages of the method are that the determination occupies 45 min., requires the use of a mechanical shaker and gives a recovery of salicylate added to serum, of only 91–96 %. Moreover oxalated plasma, whole blood or urine cannot be used, and the final coloured solution is not always optically clear. The method of Smith & Talbot (1950), using the Folin-Ciocalteu reagent, has certain advantages. Only 0.2 ml. of sample is required, the determination occupies only 15 min., and either serum or oxalated plasma may be used. However, objections to this method are that 'blank' values on the serum of patients not taking salicylates are high and variable (the average blank being equivalent to 7.5 mg. of salicylate ion/100 ml. of serum, with a range of 4.5–9.5 mg./100 ml.) and that blank values for whole blood and for urine, are very high (about 25 mg./100 ml. for whole blood and 100 mg./100 ml. for urine). The blank values on serum by the method

of Tarnoky & Brews (1950), are stated by the authors to be negligible.

A method which would combine some of the advantages of both methods and which would also be applicable to the analysis of whole blood and urine, would be most useful. The experiments described below were conducted with the object of devising such a method.

METHOD

It was decided to utilize the purple colour given by salicylates with ferric salts. To eliminate any inhibition of this colour by phosphates or oxalates, a reagent was prepared containing a high concentration of ferric nitrate. In order to precipitate the protein in the serum, mercuric chloride and hydrochloric acid were incorporated into the solution. A solution containing 4 g. of ferric nitrate, $\text{Fe}(\text{NO}_3)_3 \cdot 9\text{H}_2\text{O}$, 4 g. of mercuric chloride and 12 ml. of N-HCl /100 ml., was found to precipitate the serum protein and give a purple colour with serum salicylate. The intensity of the purple colour became weaker if more hydrochloric acid was used in the reagent. If the final strength of the acid was much less than 0.12 N, the colour given by serum salicylate was not the same tint as that given by aqueous solutions of salicylate. The effect of the concentration of hydrochloric acid on the colour is shown in Table 1.