a hydrolysate of the DNP derivative of fraction A, showing that the only glycine residues are *N*-terminal.

The results with carboxypeptidase support the conclusion that asparagine is the *C*-terminal residue of the glycy chain. In the case of acetylinsulin the liberation was approximately quantitative, assuming that it is present in both the glycy chains, if one assumes that the molecular weight is 12000.

SUMMARY

1. Fraction A of oxidized insulin was hydrolysed with pepsin and chymotrypsin. The resulting

peptides were fractionated and their structure investigated.

2. It is concluded that the amino-acid sequence in the glycy chain of insulin is Gly. Ileu. Val. Glu. Glu. CyS. CyS. Ala. Ser. Val. CyS. Ser. Leu. Tyr. Glu. Leu. Glu. Asp. Tyr. CyS. Asp.

We wish to express our thanks to Mr J. Lightbown for the chymotrypsin and to Dr B. C. Saunders for the diisopropyl fluorophosphate.

One of us (E. O. P. T.) wishes to thank the Commonwealth Government of Australia for a Commonwealth Scientific and Industrial Research Organization studentship.

Part of the work described in this paper was carried out during the tenure by F.S. of a Beit Memorial Fellowship.

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The Branched-chain Fatty Acids of Mutton Fat

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

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(Received 13 June 1952)

Following the isolation of branched-chain fatty acids from butterfat (Hansen & Shorland, 1950, 1951, 1952; Hansen, Shorland & Cooke, 1951) and from ox fat (Hansen, Shorland & Cooke, 1952a), a detailed examination was made of the lipids extracted from the external fatty tissue of old ewes. The occurrence of (+)-14-methylhexadecanoic acid as a constituent of this fat was recently established (Hansen, Shorland & Cooke, 1952b, c). In this present paper are reported the isolation from mutton fat of two isomeric C_{15} branched-chain saturated fatty acids, (+)-12-methyltetradecanoic acid and 13-methyltetradecanoic acid.

Although the optically active *ante-iso*-acid, (+)-12-methyltetradecanoic acid, has been isolated

from wool grease (Weitkamp, 1945), the *iso* acid, 13-methyltetradecanoic acid, has not formerly been found in natural fats.

EXPERIMENTAL

The long crystal spacings reported in this work were determined by means of a Philips Geiger X-ray spectrometer. Except where otherwise indicated, nickel-filtered copper $K\alpha$ radiation was used. Samples were melted on a glass slide and quickly cooled.

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

Analyses were by Weiler and Strauss, Oxford.

The fat used in this investigation was obtained by mincing and then steam-rendering the external fatty tissues

of old overweight ewes (Hansen *et al.* 1952c). The extracted glycerides (sap. equiv. 286.9, iodine value 46.6, unsaponifiable matter 0.56%, free fatty acids 0.4%) were converted to methyl esters (7.69 kg.) and hydrogenated at 180°. Ni supported on kieselguhr being used as a catalyst. The hydrogenated methyl esters (iodine value 3.0) were then repeatedly crystallized from 10 vol. acetone at -30° and finally yielded 332.6 g. of 'liquid' methyl esters (iodine

-11.8°, sap. equiv. 257.6, iodine value nil); H2SL2S4L (1.04 g., m.p. -0.2°); and H2SL2S4S (0.67 g., m.p. 1.5°).

Fraction H2SL2S2L2L was converted to fatty acids and denoted H25. Repeated low-temperature fractional crystallization of H25 acids from ether and from methanol yielded fraction H25S2LS (Table 3).

Further purification was effected by eluting the methyl esters of this fraction (renamed H35) with light petroleum (b.p. 50-60°) through an Al₂O₃ column (see Table 4).

Table 1. Fractional distillation of HL6 methyl esters

Fraction no.	Wt. (g.)	Sap. equiv.
HL6L1	0.96	247.0
HL6L2	1.23	245.3
HL6L3	2.77	249.1
HL6L4	3.07	252.7
HL6L5	7.53	258.0
HL6L6	2.18	258.5
HL6L7	2.64	259.8
HL6LR	0.87	278.7

value 7.0). Fractional distillation at approx. 0.2 mm. in a 22-plate Vigreux column provided a series of thirteen fractions and a residue. (See Table 1, Hansen *et al.* 1952c.) Fraction HL6 of this series (wt. 21.48 g., sap. equiv. 256.0, iodine value 1.1) was then refractionated at approx. 0.1 mm. in a 60 cm. electrically heated and packed column which possessed about twenty-one plates (Table 1). As fractions HL6L5, HL6L6 and HL6L7 had similar molecular weights, they were bulked together (12.0 g.) and denoted H2. Repeated low-temperature crystallization of H2 esters from different solvents effected separation into three main fractions as shown in Table 2: H2SL2S2L2L (1.55 g., m.p.

Table 4. Chromatographic separation of the methyl esters of fraction H35

(Eluant, light petroleum, b.p. 50-60°)

Fraction no.	Vol. of eluant (ml.)	Wt. (g.)	n_D^{20}	Sap. equiv. (methyl ester)
H35a	25	0.23	1.4385	249.2
b	25	0.30	1.4383	254.0
c	25	0.09	1.4377	—
d	25	0.03	1.4377	—
e	50	0.02	1.4385	—
f	75	Trace	—	—

Fraction H35b (wt. 0.30 g., sap. equiv. 254.0, m.p. 27.0 (acid) X-ray long spacing 30.1 Å. (acid), $[\alpha]_D^{25} + 3.7^\circ$ (acid) in CHCl₃) was then submitted in the form of acids to low-temperature crystallization from methanol and from acetone (Table 5), H35bL2S finally separating out as a white crystalline substance: wt. 0.11 g., m.p. 25.8°, sap. equiv. 241.6, iodine value (Wijs) 1.1. The chemical and physical characteristics of fractions H35b and H35bL2S are shown in Table 6.

Table 2. Low-temperature crystallization of methyl esters (H2) (12.0 g.)

Fraction	Conditions of crystallization: solvent; temp. (°)	Soluble			Insoluble		
		Fraction	Wt. (g.)	M.p. (°)	Fraction	Wt. (g.)	M.p. (°)
H2	Ether, 4 vol. -68	H2L	1.13	—	H2S	10.87	—
H2S	Light petroleum, 40 vol. -70	H2SL	8.24	-13	H2SS	2.52	-14
H2SL	Ether, 40 vol. -70	H2SLL	8.08	—	H2SLS	0.11	—
H2SLL	Acetone, 20 vol. -70	H2SL2L	2.53	—	H2SL2S	5.52	—
H2SL2S	Acetone, 20 vol. -60	H2SL2SL	0.57	—	H2SL2SS	4.87	—
H2SL2SS	Acetone, 20 vol. -50	H2SL2S2L	1.65	—	H2SL2S2S	3.21	—
H2SL2S2L	Ether, 20 vol. -55	H2SL2S2LL	1.61	—	H2SL2S2LS	0.03	—
H2SL2S2LL	Ether, 40 vol. -70	H2SL2S2L2L	1.55	-11.8	H2SL2S2L2S	0.04	—
		(Sap. equiv. 257.6; iodine value Nil)					
		(Converted to acids, H25)					
H2SL2S2S	Acetone, 20 vol. -40	H2SL2S3L	1.44	—	H2SL2S3S	1.76	—
H2SL2S3S	Acetone, 20 vol. -30	H2SL2S4L	1.04	-0.2	H2SL2S4S	0.67	1.5
		(Converted to acids, H28)			(Converted to acids, H29)		

Table 3. Low-temperature crystallization of fatty acids (H25) (1.39 g.)

Fraction	Conditions of crystallization: solvent; temp. (°)	Soluble			Insoluble		
		Fraction	Wt. (g.)	M.p. (°)	Fraction	Wt. (g.)	M.p. (°)
H25	Ether, 10 vol. -70	H25L	0.10	15.0	H25S	1.28	26.8
H25S	Ether, 10 vol. -65	H25SL	0.15	23.2	H25SS	1.12	26.0
H25SS	Methanol, 100 vol. -40	H25S2L	1.04	26.5	H25S2S	0.08	27.2
H25S2L	Methanol, 40 vol. -40	H25S2LL	0.29	24.0	H25S2LS	0.74	27.5
					(Converted to methyl esters, H35)		

Table 5. *Purification of fatty acid fraction H35b (0.21 g., m.p. 27.0°)*

Fraction	Conditions of crystallization: solvent; temp. (°)	Soluble			Insoluble		
		Fraction	Wt. (g.)	M.p. (°)	Fraction	Wt. (g.)	M.p. (°)
H35b	Methanol, 100 vol. -30	H35bL	0.20	26.8	H35bS	0.01	—
H35bL	Methanol, 100 vol. -35	H35bLL	0.17	26.5	H35bLS	0.02	27.2
H35bLL	Acetone, 20 vol. -40	H35bL2L	0.05	24.0	H35bL2S	0.11	25.8
					(Sap. equiv. 241.6)		

Table 6

(a) *Characteristics of fatty acid fraction H35bL2S and of H35b from which it was isolated*

Fraction no.	Wt. (g.)	M.p. (°)	Sap. equiv.	Combustion analysis (%)	Iodine value (Wijs)	C-Methyl	X-ray long spacing	Specific rotation
H35b	0.21	27.0	254.0 (Me ester)	—	—	—	30.1 Å.	$[\alpha]_D^{25} + 3.7^\circ$ (CHCl ₃)
H35bL2S	0.11	25.8	241.6	C, 74.7 H, 12.3	1.1	8.38% (equiv. to 1.35 mols. acetic acid)	—	—
Calc. for C ₁₅ H ₃₀ O ₂ :			242.4	C, 74.4 H, 12.5				

(b) *Characteristics of fatty acid fraction H29S2L2S*

Fraction no.	Wt. (g.)	M.p. (°)	Sap. equiv.	Combustion analysis (%)	Iodine value (Wijs)	C-Methyl	X-ray long spacing
H29S2L2S	0.25	50.2	241.7	C, 74.4 H, 12.4	0.9	10.7% (equiv. to 1.72 mols. acetic acid)	30.05 Å.
Theor. for C ₁₅ H ₃₀ O ₂ :			242.4	C, 74.4 H, 12.5			

Table 7. *Low-temperature crystallization of fatty acids (H29) (0.60 g.)*

Fraction	Conditions of crystallization: solvent; temp. (°)	Soluble			Insoluble		
		Fraction	Wt. (g.)	M.p. (°)	Fraction	Wt. (g.)	M.p. (°)
H29	Ether, 40 vol. -50	H29L	0.23	45.0	H29S	0.37	50.0
H29S	Ether, 40 vol. -50	H29SL	0.06	45.2	H29SS	0.31	50.2
H29SS	Methanol, 100 vol. room temp.	H29S2L	0.30	—	H29S2S	0.01	—
H29S2L	Methanol, 100 vol. -7	H29S2LL	0.28	50.0	H29S2LS	0.02	49.2
H29S2LL	Methanol, 40 vol. -40	H29S2L2L	0.02	42.0	H29S2L2S	0.25	50.2
					(Sap. equiv. 241.7)		

Subsequent to the isolation of H35bL2S four further fractions with properties corresponding to those of H35bL2S were independently extracted by means of fractional distillation and repeated crystallization from bulked fractions HL7, HL8 and HL9. (See Table 1, Hansen *et al.* 1952c.) The characteristics of these four fractions were as follows:

H51S4LS2S, wt. 0.25 g., m.p. 26.5°, sap. equiv. 244.3, X-ray long spacing 29.97 Å. (Fe K α).

H63L2S, wt. 0.19 g., m.p. 26.2°, sap. equiv. 240.5, X-ray long spacing 29.97 Å. (Fe K α).

H52S3LSS, wt. 0.52 g., m.p. 25.0°, sap. equiv. 244.1.

H52S4LS, wt. 0.90 g., m.p. 25.5°, sap. equiv. 241.0, $[\alpha]_D^{18} + 3.9^\circ$ (CHCl₃).

By means of low-temperature crystallization of fraction H2SL2S4S (renamed H29) from ethyl ether and from methanol (see Table 7), fraction H29S2L2S was isolated:

0.25 g., m.p. 50.2°, sap. equiv. 241.7. The chemical and physical characteristics of fraction H29S2L2S are recorded in Table 6b.

When a further acid fraction, H2SL2S4L (Table 2), was independently submitted to a series of low-temperature crystallizations similar to those employed for H2SL2S4S, it yielded a final product H28S5LS with the following properties: wt. 0.21 g., m.p. 51.2°, sap. equiv. 242.5; C-Me, 10.0; C, 74.7; H, 12.4; iodine value 8.7; X-ray long spacing 30.07 Å. Both H28S5LS and H29S2L2S had common origin in fraction H2SL2S2S (Table 2). A third fraction, H67SLSLSS, with properties corresponding closely with those of H29S2L2S, was derived from bulked fractions HL7, HL8 and HL9 (Table 1, Hansen *et al.* 1952c). Purification of this fraction was effected by distillation (0.1 mm.) in a 29-plate column (column C; Shorland, 1952) followed by selective bulking of fractions, redistillation (0.05 mm.) in a

30-plate column (column E; Shorland, 1952), and repeated low-temperature fractional crystallization. Properties of H67SLSLSS are as follows: wt. 0.56 g., m.p. 50.5°, sap. equiv. 240.4.

DISCUSSION

The two fractions H35bL2S and H29S2L2S isolated from the external body fat of ewes are both characterized by an absence of appreciable iodine value and both possess saponification equivalents and combustion analyses in close accord with the requirements for a saturated fatty acid with the empirical formula $C_{15}H_{30}O_2$ (see Table 6). The *C*-methyl values represent the liberation during chromic acid oxidation, by the Kuhn-Roth (1933) method, of acetic acid in amounts equivalent to 1.35 and 1.72 mols. respectively, and, when interpreted according to the evidence of Ginger (1944), indicate the presence of one side-chain methyl group in each substance. X-ray diffraction analyses indicate long spacings of 30.1 and 30.05 Å. for fractions H35bL2S and H29S2L2S respectively.

These values are in close agreement with recorded spacings of 29.2 Å. for the amide derived from 12-methyltetradecanoic acid (Velick, 1947) and of 29.8 Å. for 13-methyltetradecanoic acid (Arosenius, Stållberg, Stenhagen & Tagtström-Eketorp, 1949). *n*-Pentadecanoic acid, by contrast, has an X-ray long spacing of 35.75 Å. (Slagle & Ott, 1933).

The melting point of H35bL2S further distinguishes it from *n*-pentadecanoic acid which has been accredited with melting points of 52.4° (Weitkamp, 1945) and 52.26° (Meyer & Reid, 1933). A melting point of 25.8° for H35bL2S is, however, consistent with this substance being a C_{15} *ante-iso*-acid (branching on the antepenultimate carbon) the value approximating that of 23.0° obtained by Weitkamp (1945) for the 12-methyltetradecanoic acid isolated from wool grease.

The optical rotation of fraction H35bL2S, $[\alpha]_D^{25} + 3.7^\circ$ (in chloroform), further correlates it with Weitkamp's (1945) (+)-12-methyltetradecanoic acid $[\alpha]_D^{25} + 4.7^\circ$ (pure substance).

The melting point of 50.2° for H29S2L2S agrees closely with that of 51.7–51.8° for the C_{15} *iso*-acid synthesized by Arosenius *et al.* (1949), and conforms approximately to the observation of Weitkamp (1945) and of Cason & Winans (1950) that *iso*-acids melt less than 1° below the melting point of the normal isomer. From the evidence presented above the two saturated branched-chain acids reported in this paper are identified as (+)-12-methyltetradecanoic acid (H35bL2S) and 13-methyltetradecanoic acid (H29S2L2S).

13-Methyltetradecanoic acid is an example of a saturated branched-chain *iso*-acid with an odd number of carbon atoms. All the *iso*-acids located in wool wax (Weitkamp, 1945) possessed an even number of carbon atoms, but an *iso*-acid with an odd number of carbons has previously been isolated from butterfat (Hansen & Shorland, 1951).

Although the total content of (+)-12-methyltetradecanoic acid has not been determined, those fractions already investigated indicate it to be present in quantities greater than 0.04 % of the total weight of fatty acids. Only minor amounts of 13-methyltetradecanoic acid (about 0.02 % of the total fatty acids) have been isolated, but as the *iso*-acids have melting points and solubilities similar to those of normal acids, it is to be expected that they will be present in greater amount in the 'solids', which were separated by fractional crystallization from acetone at -30°, and which have not as yet been investigated in detail.

From the fatty acids of the coccygeal glands of ducks (Weitzel & Lennert, 1951; Weitzel, 1951) have been isolated an optically active methyl-branched *C*, saturated acid, 4-methylhexanoic acid (contaminated with traces of 2-methylhexanoic acid), together with appreciable amounts of other unidentified alkyl-substituted acids. As the total lipids extracted from these glands contained 47.6 % fatty acids, 48.0 % octadecanol, and only 1.5 % glycerol, the authors concluded that the fatty substances of these glands consist in the main of esters of octadecyl alcohol. Similarly, the branched-chain acids of wool wax are combined mainly with sterols and triterpenoids (Truter, 1951), and those of bacteria appear to be associated not with glycerol, as in true fats, but with some water-soluble compound such as the disaccharide trehalose (Anderson, 1941). As the extracted sample of mutton fat analysed in this present investigation contained only 0.56 % unsaponifiable matter, and as there is no evidence to suggest the presence of alcohols other than glycerol, it appears that these branched-chain fatty acids are present in the fatty tissue as glycerides.

SUMMARY

From the external fatty tissue of sheep two C_{15} branched-chain saturated fatty acids, (+)-12-methyltetradecanoic acid and 13-methyltetradecanoic acid have been isolated.

We are indebted to Mr M. Fieldes and Mr L. D. Swindale of the Soil Bureau, Department of Scientific and Industrial Research, Wellington, for carrying out all the X-ray diffraction analyses reported in this paper.

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ADDENDUM

The Stereochemistry of *ante-iso*-Acids

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(Received 20 September 1952)

The C_{15} , C_{17} and C_{19} *ante-iso*-acids isolated from mutton-fat (Hansen, Shorland & Cooke, 1952, 1953) and wool fat (Weitkamp, 1945) are all dextro-rotatory (Table 1) and the Distance Rule (Entfernungssatz) of Tschugaeff (1898) shows that all of them must have the same configuration at the asymmetric carbon atom. The following evidence indicates that they are almost certainly L-methylalkanoic acids, using the term 'L-methyl' to denote a methyl group on the left-hand side of the chain in a Fischer projection (cf. formula I) following the proposals of Ställberg-Stenhagen (1949) and of Klyne (1951).

The synthesis of (+)-14-methylhexadecanoic acid by Velick & English (1945) correlates its configuration with that of (-)-2-methylbutan-1-ol.

Crombie & Harper (1950) have shown that (+)-6-methyloctanoic acid is configurationally related to (-)-2-methylbutanol (II). They point out that the correlation of (-)-2-methylbutanol with L-(-)-glyceraldehyde (Fredga, 1941, 1942, 1944) is rather involved, but it seems sound and is becoming generally accepted (cf. Birch, 1950).

Table 1. Molecular rotations of (+)-*ante-iso*-acids

	Formula I ($n =$)	$[M]_D$	Reference
2L-Methylbutanoic acid	0	+18	a
3L-Methylpentanoic acid	1	+10	b
4L-Methylhexanoic acid	2	+11	c
6L-Methyloctanoic acid	4	+13	d
12L-Methyltetradecanoic acid	10	+10	e, f
14L-Methylhexadecanoic acid	12	+14, +12	e, g, h
16L-Methyloctadecanoic acid	14	+12	e
21L-Methyltricosanoic acid	19	+13	i

a, Marckwald (1899); b, Marckwald & Nolda (1909); c, Welt (1894); d, Crombie & Harper (1950); e, Weitkamp (1945); f, Hansen *et al.* (1953); g, Velick & English (1945); h, Hansen *et al.* (1952); i, Ställberg-Stenhagen (1950).

Stållberg-Stenhagen (1950) has synthesized (–)-21-methyltricosanoic acid ($[M]_D$, –13) by a route which shows that its methyl group is D-oriented in terms of the Fischer convention. The (+)-acid would therefore have $[M]_D$ +13, and would be the 21 L-methyl acid.

The (+)-*ante-iso* acids, the rotations of which are

collected in Table 1, are therefore L-methyl acids as shown by formula I.

SUMMARY

The (+)-*ante-iso*-alkanoic acids are represented by the Fischer projection formula I, and may be termed L-methyl-substituted acids.

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The Relation of Liver Kynureninase to Tryptophan Metabolism in Pyridoxine Deficiency

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(Received 10 September 1952)

The enzyme kynureninase splits alanine from the side chain of kynurenine and hydroxykynurenine to form anthranilic and hydroxyanthranilic acids, respectively (Kotake & Nakayama, 1941; Braunshtein & Goryochenkova, 1949; Wiss & Fuchs, 1950). The enzyme requires pyridoxal phosphate as coenzyme, and its activity in liver is decreased in pyridoxine deficiency (Braunshtein & Goryochenkova, 1949; Dalgliesh, Knox & Neuberger, 1951; Mason & Berg, 1952). The substrates of this enzyme, kynurenine and hydroxykynurenine, and their derivatives which retain the original carbon skeleton of the tryptophan side chain, accumulate in pyridoxine-deficient animals (Dalgliesh, 1951; 1952*a*). Therefore, the lack of pyridoxal phosphate, with its consequent decrease in kynureninase activity and accumulation of compounds usually removed by this enzyme, offers a qualitatively satisfactory explanation of the deranged tryptophan metabolism in pyridoxine-deficient animals.

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It is, however, necessary to show that, in pyridoxine deficiency, the rate of kynurenine formation exceeds that of kynurenine removal, before the observed reduction of kynureninase activity can be accepted as an adequate explanation for the excretion of kynurenine. The potential rates of kynurenine formation by the tryptophan peroxidase of rat liver have already been determined (Knox, 1951). The potential rates of kynurenine removal by kynureninase, in the livers of normal and pyridoxine-deficient rats, have now been determined for comparison. A new method of measuring the kynureninase activity was developed, and the properties of the enzyme were studied to ensure that this assay revealed the full potential activity of the enzyme. Measured under optimal conditions, the kynureninase activity of normal rat liver was higher than that previously determined (Braunshtein & Goryochenkova, 1949; Wiss & Hatz, 1949), and was, in fact, of a magnitude very similar to that of the tryptophan peroxidase. This similarity in the potential rates of kynurenine removal and formation under normal conditions is in keeping with the complete metabolism of tryptophan, without