- Jackson, S. F. & Fell, H. B. (1963). *Developmental Biol.* **7**, 394.
- Lucy, J. A. & Dingle, J. T. (1962). *Biochem. J.* **84**, 76 p.
- Lucy, J. A., Dingle, J. T. & Fell, H. B. (1961). *Biochem. J.* **79**, 500.
- Lucy, J. A., Luscombe, M. & Dingle, J. T. (1963). *Biochem. J.* **89**, 419.
- Luzzati, V. & Husson, F. (1962). *J. Cell Biol.* **12**, 207.
- Pethica, B. A. (1958). *J. gen. Microbiol.* **18**, 473.
- Rideal, E. K. & Taylor, F. H. (1957). *Proc. Roy. Soc. B*, **146**, 225.
- Rideal, E. K. & Taylor, F. H. (1958). *Proc. Roy. Soc. B*, **148**, 450.
- Schulman, J. H. & Rideal, E. K. (1937a). *Proc. Roy. Soc. B*, **122**, 29.
- Schulman, J. H. & Rideal, E. K. (1937b). *Proc. Roy. Soc. B*, **122**, 46.
- Skou, J. C. (1961). *J. Pharm., Lond.*, **13**, 204.
- van Deenen, L. L. M., Houtsmuller, U. M. T., de Haas, G. H. & Mulder, E. (1962). *J. Pharm., Lond.*, **14**, 429.
- Weitzel, G., Fretzdorff, A. & Heller, S. (1952). *Hoppe-Seyl. Z.* **290**, 32.

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The Analysis of the Fatty Acids of Normal Human Depot Fat by Gas-Liquid Chromatography

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Kingsbury, Paul, Crossley & Morgan (1961) reported the use of gas-liquid chromatography, alkaline isomerization and chemical methods in a pilot study of the composition of human fat. These methods, however, were unable to analyse the variety of trace components present. Since that time analytical methods have improved (James, 1960; Landowne & Lipsky, 1961; Lipsky & Landowne, 1958; Lovelock, 1958; Hornstein, Elliott & Crowe, 1959; Farquhar, Insull, Rosen, Stoffel & Ahrens, 1959; Holman & Hayes, 1958), and this paper reports a procedure for the complete analysis of the C_{10} - C_{24} fatty acids of human depot fat by gas-liquid chromatography.

EXPERIMENTAL

All solvents were of analytical reagent grade.

Sampling. The samples of human fat were obtained by surgical incision from patients undergoing operations, or by a biopsy procedure (Kingsbury *et al.* 1962a) from out-patients and normal subjects.

Extraction of lipids. This was done by the methods described by Kingsbury *et al.* (1962a).

Saponifications were performed by the method advised by the Analytical Methods Committee (1959), since this was found to cause less fatty acid changes than the 'weak'-alkali method of Bottcher, Woodford, Boelsma-Van Houte & van Gent (1959). The propan-2-ol solutions of the fat were added to the reaction mixture.

Hydrogenation was carried out at room temperature, with hydrogen at atmospheric pressure and palladium as catalyst. The methyl esters of the fatty acids were prepared

according to Schlenk & Gellerman (1960). Ether and diazomethane were removed by evaporation in a jet of nitrogen; the last traces of water and methanol were removed by evaporation several times with chloroform by using the nitrogen jet. The samples of methyl esters were warmed to 50° and run on to the columns as a liquid from micro-pipettes. In our hands methylation with diazomethane did not produce the volatile by-products, giving rise to peaks on the chromatogram in the C_{12} region, which were reported by Morrison, Lawrie & Blades (1961).

Gas-liquid chromatography. Two types of instrument were used. (1) A catharometer instrument equipped with a hot-platinum-wire catharometer detector, maintained at room temperature. The column used was 366 cm. long, 0.4 cm. diam. and consisted of 20% ethylene glycol-glutarate polyester supported on 100-120 mesh acid- and alkali-washed Celite, at a temperature of 185°. The column eluent was nitrogen. (2) A Pye argon chromatograph equipped with a radium D detector and the standard 4 ft. columns. Two stationary phases, 5% polyvinyl acetate and 5% Apiezon L grease (non-polar), supported on acid- and alkali-washed Celite, 100-120 mesh, were used. Analyses on the polyvinyl acetate column were carried out at 175-180° at an argon flow rate of 40 ml./min., and at 195° and 40 ml./min. on the Apiezon L column.

In the catharometer instrument all components were detected as carbon dioxide; hence the instrument was intrinsically linear but not of extreme sensitivity, and thus unsuitable for the analysis of components of chain length greater than C_{18} .

Because of its thermal stability at the high temperatures necessary for the elution of long-chain components (Hornstein, *et al.* 1959), polyvinyl acetate was used in the Pye argon chromatograph, which was much more sensitive, although its response was not as linear as the

catharometer instrument. Polyvinyl acetate has the additional, although perhaps minor, advantage of giving carbon numbers with the branched-chain acids at about 178° similar to Apiezon L at about 197°. In our experience, the C₁₆ figure was exaggerated, on the average, by about 2.5% at the expense of the C₁₈ figure by the Pye argon chromatograph. This type of behaviour is illustrated in Table 1, which summarizes 11 analyses of a known mixture of C₁₄, C₁₆ and C₁₈ fatty acids. The results also show that this instrument gave reasonably consistent results.

Infrared analyses were performed by the method of Hartman, Shorland & Cleverley (1958), methyl elaidate being used as the standard.

The usual fatty acid notation is employed, where *x* in C_{*x*} is number of carbon atoms; C_{*x*:*z*} etc. shows number of double

bonds and C_{*x*:*z*} etc. (1, 4, 7 etc.) shows the position of double bonds numbered from the carboxyl carbon atom.

Three or four different amounts (0.025–0.1 μl.) of the methyl esters of each sample were run at various sensitivities (× 1 ↔ × 10) at (1000–1250 v) on Apiezon and polyvinyl acetate to obtain complete analyses of the major and minor components in hydrogenated and non-hydrogenated samples.

Presentation of retention data. Retention volumes were expressed as carbon numbers (Woodford & van Gent, 1960) and logarithmic retention volumes relative to methyl stearate. Carbon numbers for each run were averaged to give the mean carbon number for each component.

Identification of peaks. The straight- and branched-chain saturated acids of samples of normal human depot fat were established in runs of completely hydrogenated fat and by using authentic samples of straight-chain, even-numbered fatty acids (C₈–C₂₄) and C₁₇ and C₁₉ straight-chain acids as markers. No branched-chain markers were available.

Pure myristoleic acid, palmitoleic acid, oleic acid, gadoleic acid and erucic acid formed the monoenoic, and linoleic acid and C_{20:2} and C_{22:2} acids (with double bonds after C-9 and C-12, from the methyl end, in both) formed the dienoic standards, and linolenic acid the trienoic standard. Tetraenoic standards were C_{16:4} (6, 9, 12 and 15) and C_{18:4} (4, 7, 10 and 13) fatty acids (both supplied by Professor E. Klenk), a C_{20:4} (synthetic arachidonic acid supplied by Roche Products Ltd.), and a C_{20:5} (5, 8, 11, 14 and 17) and C_{22:6} (4, 7, 10, 13, 16 and 19) fatty acid [three samples of each: 1, supplied by Professor Klenk, 2, pre-

Table 1. *Summary of deviation in results from 11 analyses of a known mixture of C₁₄ (myristic), C₁₆ (palmitic) and C₁₈ (stearic) fatty acid methyl esters*

Composition, by weight, estimated by the percentage of peak areas is shown.

Fatty acids	Percentage composition (by wt.) of known mixtures	Average of 11 analyses (% of peak area)	S.D.
C ₁₄	10.5	10.0	±1.29
C ₁₆	29.5	32.0	±1.98
C ₁₈	60.0	58.0	±2.92

Table 2. *Fatty acids of completely hydrogenated human depot fat*

Percentage composition is by weight and is calculated from the areas under the separated peaks.

Polyvinyl acetate		Apiezon L		Identity of fatty acid
Carbon no.	Percentage composition of fatty acids	Carbon no.	Percentage composition of fatty acids	
8.35?	0.03	9.50?	0.003	Caprylic acid?
10.00	0.002	10.65?	0.004	Capric acid?
12.00	0.5	12.00	0.4	Lauric acid
—	—	12.30	0.03	C ₁₃ neo
13.00	0.02	13.00	0.02	Tridecanoic acid
13.50	0.01	13.50	0.01	C ₁₄ iso
14.00	3.8	14.00	3.7	Myristic acid
14.55	0.2	14.60	0.2	C ₁₅ iso
15.00	0.4	15.00	0.3	Pentadecanoic acid
15.50	0.1	15.50	0.1	C ₁₆ iso
16.00	23.2	16.00	24.5	Palmitic acid
—	—	16.30	0.3	C ₁₇ neo
16.60	0.4	16.65	0.6	C ₁₇ iso
17.00	0.8	17.00	0.7	Heptadecanoic acid
—	—	17.35	0.03	C ₁₈ neo
—	—	17.55	0.04	C ₁₈ iso
18.00	66.8	18.00	66.0	Stearic acid
—	—	18.60	0.05	C ₁₉ iso
19.00	0.4	19.00	0.3	Nonadecanoic acid
19.50	0.01	19.55	0.01	C ₂₀ iso
20.00	2.3	20.00	2.3	Arachidic acid
—	—	20.15	0.02	C ₂₁ neo?
21.00	0.05	21.00	0.03	Henicosanoic acid
—	—	21.40	0.03	C ₂₂ neo
22.00	0.8	22.00	0.45	Behenic acid
23.00	0.03	—	—	Tricosanoic acid
23.30	0.02	—	—	C ₂₄ neo

pared with British Cod Liver Oil Ltd. by urea fractionation and molecular distillation (Dr K. de Witt and Dr K. Reed) and 3, prepared with Dr P. Ridgeway-Watt (Vitamins Ltd.) by chromatography and multiple-stage molecular distillation] were also available in over 95% purity and were used as the most-unsaturated markers. Multiple-stage molecular distillation (also by Dr Ridgeway-Watt of Vitamins Ltd.) provided $C_{20:3}$, $C_{22:3}$, $C_{22:4}$ and $C_{22:5}$ acids in 85-95% purity from urea concentrates of cod-liver oil. The positions of the double bonds in these acids have been established by Klenk (1961). The position of the double bonds in the fatty acids of the various peaks were therefore assumed by comparison with the above known markers, or with markers in which the double bond positions were already established.

The scatter of markers from C_8 to $C_{22:5}$ and the positional changes between polar and non-polar columns placed the main peaks and established their degree of unsaturation, which was checked by alkaline-isomerization analyses (Holman & Hayes, 1958) and determination of iodine values. The overlaps of linoleic acid and linolenic acid, and of $C_{20:4}$ and $C_{20:5}$ acids, which occurred on the Apiezon L columns, were resolved on the polar columns, on which, however, a partial overlap of C_{22} saturated and $C_{20:5}$ acids occurred. On Apiezon L columns most peaks of the 'iso' branched-chain even-numbered acids were hidden within the large peaks of the corresponding straight-chain mono- and di-enoic acids, whereas on the polyvinyl acetate columns the odd-numbered 'iso' branched-chain acids were obscured by the mono- and di-enoic acids of one less

Table 3. Designation, retention volume relative to methyl stearate, carbon number and percentage composition of the commonly occurring fatty acids of normal human depot fat

br, Branched acid. neo and iso refer to the type of isomer.

(a) Results with Apiezon L columns

Name	Length of carbon chain	No. of double bonds	Position of unsaturated bonds counting from carboxyl (assumed by comparison with known markers)	Retention volume relative to methyl stearate	Carbon no.	Concn. (%)	
						> 1%	< 1%
Lauric	12:0		—	0.075	12.00	—	0.5
	13:0 br		(neo)	0.085	12.30	—	0.03
	13:0 br		(iso)	0.095	12.60	—	0.02
<i>n</i> -Tridecanoic	13:0		—	0.116	13.00	—	0.02
	14:2		(5, 8)	0.143	13.50	—	0.03
Myristoleic	14:1		(5)	0.157	13.70	—	0.3
Myristic	14:0		—	0.175	14.00	4.7	—
	15:0 br		(iso)	0.232	14.55	—	0.2
<i>n</i> -Pentadecanoic	15:0		—	0.272	15.00	—	0.5
Palmitoleic	16:1		(9)	0.362	15.65	6.0	—
Palmitic	16:0		—	0.420	16.00	24.0	—
	17:0 br		(neo)	0.487	16.35	—	0.3
	17:0 br		(iso)	0.550	16.60	1.0	—
<i>n</i> -Heptadecanoic	17:0		—	0.649	17.00	—	0.7
Linolenic	18:3		(9, 12, 15)	0.792	17.45	7.0	—
Linoleic	18:2		(9, 12)				
Oleic	18:1		(9)	0.859	17.60	44.0	—
Stearic	18:0		—	1.000	18.00	7.0	—
	19:0 br		(neo)	1.18	18.40	—	0.2
	19:0 br		(iso)	1.31	18.60	—	0.3
	20:5		(5, 8, 11, 14, 17)	1.47	18.90	—	0.4
	20:4		(5, 8, 11, 14)				
Arachidonic	19:0		—	1.58	19.00	—	0.02
<i>n</i> -Nonadecanoic	20:3		(5, 8, 11 or 8, 11, 14)	1.65	19.20	—	0.2
	20:2		(8, 11)	1.78	19.30	—	0.09
	20:2		(11, 14)	1.86	19.50	—	0.2
	20:1		(11)	2.00	19.60	—	0.3
Gadoleic	20:0 br		(iso)	2.06	19.70	—	0.01
	20:0		—	2.36	20.00	—	0.2
Arachidic	21:0 br		(neo)?	2.41	20.15	—	0.1
	22:6		(4, 7, 10, 13, 16, 19)	3.02	20.55	—	0.1
	22:5		(7, 10, 13, 16, 19)	3.29	20.75	—	0.2
Henicosanoic	21:0		—	3.60	21.00	—	0.4
	22:4		(7, 10, 13, 16)				
	22:3		(7, 10, 13)				
	22:2		(10, 13)	3.96	21.20	—	0.03
	22:0 br		(neo)	4.36	21.40	—	0.03
Behenic	22:1		(6)	4.47	21.50	—	0.03
	22:1		(9)	4.74	21.65	—	0.01
	22:0		—	5.55	22.00	—	0.02

Table 3 (cont.)
(b) Results with polyvinyl acetate columns

Fatty acids			Position of unsaturated bonds counting from carboxyl (assumed by comparison with known markers)	Retention volume relative to methyl stearate	Carbon no.	Concn. (%)	
Name	Length of carbon chain :	No. of double bonds				>1 %	<1 %
Lauric	12:0		—	0.123	12.00	—	0.5
	13:0 br		(neo)	0.136	12.30	—	0.03
	13:0 br		(iso)	0.151	12.50	—	0.02
<i>n</i> -Tridecanoic	13:0		—	0.170	13.00	—	0.02
	14:0 br		(neo)	0.199	13.35	—	Trace
	14:0 br		(iso)	0.218	13.50	—	0.01
Myristic	14:0		—	0.251	14.00	4.7	—
Myristoleic	14:1		(5)	0.298	14.50	2.0	—
	14:2		(5, 8)	0.314	14.70	—	0.03
<i>n</i> -Pentadecanoic	15:0		—	0.353	15.00	—	0.5
	16:0 br		(neo)	0.398	15.35	—	Trace
	16:0 br		(iso)	0.416	15.50	—	0.1
Palmitic	16:0		—	0.499	16.00	24.0	—
Palmitoleic	16:1		(9)	0.577	16.45	7.0	—
<i>n</i> -Heptadecanoic	17:0		—	0.703	17.0	—	0.7
	18:0 br		(neo)	0.790	17.35	—	0.5
	18:0 br		(iso)	0.841	17.50	—	Trace
Stearic	18:0		—	1.000	18.00	7.0	—
Oleic	18:1		(9)	1.12	18.35	45.0	—
Linoleic	18:2		(9, 12)	1.34	18.90	6.5	—
<i>n</i> -Nonadecanoic	19:0		—	1.41	19.00	—	0.02
	20:0 br		(neo)	1.59	19.35	—	0.04
	20:0 br		(iso)	1.71	19.55	—	0.04
Linolenic	18:3		(9, 12, 15)	1.84	19.75	—	0.5
Arachidic	20:0		—	2.00	20.00	—	0.2
Gadoleic	20:1		(11)	2.20	20.35	—	0.7
	20:2		(8, 11)	2.69	20.90	—	0.06
	21:0		—	2.82	21.00	—	—
Henicosanoic	20:3		(5, 8, 11 or 8, 11, 14)	3.00	21.10	—	0.5
	20:4		(5, 8, 11, 14)	3.55	21.60	—	0.2
	22:0		—	4.08	22.00	—	0.04
Behenic	20:5		(5, 8, 11, 14, 17)	4.21	22.10	—	0.2
	22:1		(11)	4.68	22.35	—	0.2
	22:2		(10, 13)	5.75	22.90	—	0.05
Tricosanoic	23:0		—	5.90	23.00	—	0.03
	24:0 br		(neo)	6.16	23.30	—	0.02
	22:3		(7, 10, 13)	6.76	23.40	—	Trace
	22:4		(7, 10, 13, 16)	7.39	23.60	—	0.03
	22:5		(7, 10, 13, 16, 19)	8.43	24.05	—	0.3
	22:6		(4, 7, 10, 13, 16, 19)	9.61	24.40	—	0.14

carbon atom. Thus most of the overlaps of the depot fatty acids could be resolved by using the two types of column.

By this means the number of carbon atoms and degree of unsaturation of the fatty acid peaks were defined. The absence of more than one methylene group between double bonds was established by the agreement between analysis by alkaline isomerization and gas-liquid chromatography.

RESULTS

The carbon numbers, identities and percentage composition of the saturated acids in completely hydrogenated human depot fat are shown in Table 2.

Straight-chain acids from C₈ to C₂₃ were present, and branched-chain acids from C₁₃ to C₂₄. Two

species of branched-chain acids were detected with carbon numbers about C_{x-1+0.2-0.4} and C_{x-1+0.5-0.65}. Comparison with published retention volumes of standard markers (Farquhar *et al.* 1959; Hawke, Hansen & Shorland, 1959) indicated these to be 'neo' and 'iso' isomers respectively. The 'iso' form was found to be the most common type of chain branching in human depot fat, whereas the anti-'iso' form did not appear to be present in the hydrogenated samples studied.

The fatty acid composition of normal human depot fat, shown in Table 3 (*a* and *b*), was compiled from the results of replicate analyses in which each sample was run on both polyvinyl acetate and Apiezon L columns at several sensitivities, to-

gether with analyses of fully hydrogenated samples and the results from spectrophotometry and mass spectroscopy. A tracing demonstrating the complexity of the polyunsaturated fatty acids is shown in Fig. 1.

Examples of most of the common unsaturated acids were present, except the C_{16} and C_{18} tetraenoic acids and the C_{16} trienoic acids. The dienoic acids of C_{16} and C_{14} have been found infrequently and then only in concentrations below 0.08 %. Only traces of the isomeric forms of linoleic acid and linolenic acid were found on either polyvinyl acetate or Apiezon L columns (Farquhar *et al.* 1959). We have no evidence from the peaks on our Apiezon tracings of the presence of the reasonably large amounts of oleic isomers reported by McLaren, Read & Chimbe (1962).

Infrared analysis showed the presence of 1–8 % of *trans* acids.

With the column conditions as described, a change of one carbon number equalled a change in the logarithmic retention volumes of the fatty acids relative to methyl stearate of 0.19 and 0.15 on Apiezon L and polyvinyl acetate columns respectively.

Fig. 1. Gas-liquid-chromatographic tracings of the fatty acids of normal non-hydrogenated depot fat on 5 % polyvinyl acetate columns for the $C_{18:2}$ to $C_{22:6}$ regions. Sample 0.1 μ l. $\times$ 1. 1750 v. 175°.

DISCUSSION

The authors agree with the advantages of the carbon-number system described by Woodford (1961) for comparing quantitative changes in tissue fatty acid composition, especially for the recognition of homologous series with dissimilar retention values but similar carbon numbers, such as the branched-chain acids, and because small changes in column conditions during a run can cause large variations in retention volumes relative to methyl stearate but less change in carbon numbers.

The retention distances of the fatty acid relative to methyl stearate on polar and non-polar columns were almost identical with published data of runs performed at similar temperatures (Farquhar *et al.* 1959). When the carbon numbers on polar columns were plotted against those on non-polar columns it was found that, as shown by James (1959), the saturated, mono-, di-, tri-, tetra-, penta- and hexa-enoic acids were separated from each other, each type being regularly positioned according to chain length along predictable lines parallel to those drawn through the plots of the saturated acids.

To check the gas-liquid chromatographic analyses of the predominant saturated and mono-unsaturated fatty acids, two samples were also analysed by mass spectroscopy. Table 4 shows that good correlation was obtained.

The monoenoic and various polyunsaturated acids of each chain length, as estimated by gas-liquid chromatography, were summed to give the total mono-, di-, tri-, tetra-, penta- and hexa-enoic acids and these figures compared with equivalent values obtained by alkaline conjugation, and were a useful check on the definition of the chromatographic peaks. The similarity in the results from these two types of analysis has been mentioned previously (Kingsbury *et al.* 1962a) and showed that no marked oxidation of fatty acids had occurred, that it was justifiable to calculate the $E_{1\text{cm}}^{0.1\%}$ values after conjugation from weights of esterified fatty acid

Table 4. Comparison of percentage predominant fatty acid composition of two samples of human depot fat by gas-liquid chromatography and by mass spectroscopy

Fatty acid	Analysis (% by wt., of fatty acids)			
	Sample 1		Sample 2	
	Gas-liquid chromatography	Mass spectroscopy	Gas-liquid chromatography	Mass spectroscopy
Myristic	4.9	4.7	4.3	5.1
Palmitic	24.0	20.5	24.0	27.4
Stearic	6.5	6.5	5.4	5.1
Oleic	45.0	48.0	45.0	42.9
Linoleic	6.6	8.6	7.0	8.7

estimated by colorimetric analysis, and that gas-liquid chromatography of the extracted fatty acids gave similar results to those obtained by alkaline-conjugation analyses of the whole depot-fat extract. In our experience the quantitative measurement of very low concentrations of highly unsaturated fatty acids (less than 0.05 %) is difficult by gas-liquid chromatography and discrepancies frequently occur between the chromatographic and isomerization results. This is because of the limitation in the size of charge that can be applied to a gas-liquid chromatograph without overloading it. This was also the experience of Herb, Magidman & Riemenschneider (1960).

Preliminary concentration of the trace polyunsaturated components by low-temperature crystallization from acetone (10 ml./g. at -50° for 30 min.) did not provide complete separation in milligram samples, even with careful and time-consuming techniques (A. Crossley, unpublished work). These methods therefore have not seemed suitable for the quantitative study of changes in composition of depot fat. Consequently, the authors prefer always to employ both gas-liquid chromatography and isomerization on each sample. This is adequate for most purposes as the majority of the di-, tetra-, penta- and hexa-enoic acids are linoleic acid, arachidonic acid, $C_{20:5}$ and $C_{22:6}$ acids respectively. Only about half the total trienoic acids are linolenic acid, however, the remaining half being mainly $C_{20:3}$ acids which appear to possess different biological properties (Mead & Slatton, 1956). Hence, improved methods of estimating the various trienoic acids in milligram samples of human depot fat would be an advantage.

Samples of one extract of depot fat were evaporated on an air bath (sample *a*) and by the method described previously (sample *b*) to study the effects of oxidation. Gas-liquid chromatography on polyvinyl acetate columns then showed in sample (*a*) severe decreases in the peaks attributed to $C_{14:0}$, $C_{14:1}$, C_{15} , $C_{16(b)}$, C_{16} and $C_{16:1}$ with increases in those due to the $C_{18:0}$ and $C_{18:1}$ acids. Amongst the minor components the C_{18} di- and tri-, C_{20} and C_{22} di-, tri-, tetra-, penta- and hexa-enoic acids were reduced. However, marked increases occurred in peaks of carbon numbers 19.8, 20.00, 21.2, 21.85 and 22.45, and new peaks appeared at 19.10, 23.10 and 23.55. Hence, unusual increases in the former peaks and the presence of the latter have been regarded as signs of faulty methods, unless these unusual features were confirmed by safe methods. Peaks due to oxidation with retention volumes of 7.40, 7.0, 0.945, 2.82 and 3.92 relative to methyl stearate on adipate columns, and one with a retention time relative to methyl stearate of 0.050 on Apiezon L columns, have been noted by Hirsch, Farquhar, Ahrens, Peterson & Stoffel (1960).

A slow-moving, unknown component (carbon number 24.75) present in some of our samples, and which may itself be a product of mild oxidation, disappeared during this severe method of evaporation.

It can be adequate for most comparative purposes to analyse each sample several times on polar columns (polyvinyl acetate) alone, different amounts of sample being used for each run. On such columns, with the conditions as described, the only consistently occurring fatty acids of human fat that were hidden beneath peak overlaps were the C_{17} and C_{19} 'iso' branched acids. Trace components in the C_{18} - C_{22} region were well separated with the exception of the partial overlap of the C_{22} and $C_{20:5}$ fatty acids.

Although the analyses confirm that over 90 % of the depot fatty acids consist of only six or seven types (Cramer & Brown, 1943; Moore & Cooke, 1959; Hirsch *et al.* 1960; Gellhorn & Marks, 1961; McLaren *et al.* 1962), the extent and complexity of the trace components (those present in concentrations of less than 1 %) have not been shown previously.

It seems from the similarity in the results of the above authors, particularly those of McLaren *et al.* (1962), that the composition of adult human depot fat is reasonably constant and thus demonstrates the importance of phylogenetic determinants (Kingsbury *et al.* 1961). However, marked fluctuations of both major and minor components can occur in normal tissues, and this seems to involve groups of fatty acids which are apparently metabolically dissimilar (Kingsbury *et al.* 1962*a*). Linoleic acid and palmitoleic acid (measured by gas-liquid chromatography), and total di-, tri- and tetra-enoic acids, measured by alkaline isomerization, showed less tendency to change.

Consequently, the authors feel unable to quote normal ranges at present for the majority of the fatty acids when so little is known about the exact influence of the diet and of endogenous metabolism.

Unusual changes have been shown by McLaren & Read (1961), who found 7 % of a *n*-tridecanoic acid in human depot fat; and Hirsch *et al.* (1960) found 26 % of linoleic acid in the subcutaneous fat of a patient who had taken corn oil daily for 2 years, and 14 % of linolenic acid after linseed oil daily for 1 year. We also have found marked variations in hospital patients. Thus a patient with obesity had 12 % of lauric acid, and a case of sprue showed only 13 % of palmitic acid (unpublished results). The variations that can occur in atheromatous patients have been published elsewhere (Kingsbury *et al.* 1962*b*). Therefore it seems that man's fat composition, like that of other mammals, is determined phylogenetically and is reasonably constant under steady conditions of diet and habit,

but large fluctuations can occur in normal people and in patients as a result of marked and persistent alterations in the diet or owing to mechanisms at present unknown.

The opinion had been expressed that composition of depot fat throughout the body was similar (Kingsbury *et al.* 1961; Hirsch *et al.* 1960). However, McLaren *et al.* (1962) found that fat from the abdominal wall of Africans had a lower concentration of palmitoleic acid but was higher in stearic acid (3.8% and 4.9% respectively) than fat from their legs (8.5% and 3.3% respectively). Our experience is that the sum of the palmitoleic acid and stearic acid concentrations in different individuals has varied between 11% and 16.5% in both buttock and abdominal wall fat, even though the stearic acid content has ranged from 1.4% to 8.8% and the palmitoleic content from 5.3% to 13.8%. The main difference between our results and those of other workers is that we have never found a concentration of palmitoleic acid lower than 5% in any body site. Whether this difference results from differences in the clothing habits or ambient temperatures of the subjects remains to be seen.

The composition of the body fat is such that the peaks of the C_{15} , 17, 19 and 21 branched acids are overlapped by the monoenoic and saturated peaks of one less carbon atom on polar columns, whereas the C_{14} , 16, 18, 20, 22 and 24 branched acids tend to be obscured on the Apiezon L columns. The origin and metabolism of these branched forms in human fat are unknown, although they probably arise mostly from the diet (James, Webb & Kellock, 1961; Hawke *et al.* 1959), but it is interesting that C_{24} branched-, but not straight-chain, acids are found, and that there is a regular double series of 'neo' and 'iso' forms classifiable by the retention volumes on the gas-liquid-chromatographic columns. This indicates a regular and not haphazard mechanism of branching, for the presence of irregular branching would give very different retention volumes, as mentioned by Farquhar *et al.* (1959).

The presence of anteiso acids in several samples of depot fat has been indicated by similar carbon numbers of $C_{x-1+0.70-0.85}$ on both columns for both the C_{13} and C_{15} acids, particularly after diets containing fish oils or much butter (unpublished results). The C_{17} , 18, 19, 20 and 21 anteiso acid peaks would anyway be obscured on both polyvinyl acetate and Apiezon L column runs of human depot fats by other larger peaks, so that, although the hydrogenated samples of human depot fat showed no anteiso acids, the authors believe them to be an irregular and dietary-induced constituent of human fat.

Finally, the occurrence of 1-5% of *trans* acids is within the range 2-14% found by Johnston,

Johnston & Kummerow (1957) in samples from normal people on usual diets. It would appear from the work of Johnston, Kummerow & Walton (1958) that these originate mostly in the diet: this has been discussed by James *et al.* (1961). In completion, it may be said that ultraviolet spectroscopy has shown only negligible amounts of conjugated acids to be present.

SUMMARY

1. The sample preparation and gas-liquid-chromatographic analysis of the fatty acids in fully hydrogenated and non-hydrogenated human depot fat are described and discussed.

2. Exact scale copies of tracings of representative column runs are shown, together with Tables in which the logarithmic relation of retention volumes of the 40-45 commonly occurring fatty acids to that of a standard, their carbon numbers on both polar and non-polar columns and their percentage concentrations are given.

3. A thorough analysis of the trace fatty acids is presented, including for the first time the series of branched-chain acids from C_{13} to C_{24} .

4. The difficulties in the gas-liquid-chromatographic analysis of human tissue fat are discussed.

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REFERENCES

- Analytical Methods Committee (1959). *Analyst*, **84**, 356.
- Botcher, C. J. F., Woodford, F. P., Boelsma-Van Houte, E. & van Gent, C. M. (1959). *Rec. Trav. chim. Pays-Bas*, **78**, 794.
- Cramer, D. L. & Brown, J. B. (1943). *J. biol. Chem.* **154**, 427.
- Farquhar, J. W., Insull, W., Rosen, P., Stoffel, W. & Ahrens, E. H. (1959). *Nutr. Rev.* **17**, suppl. 8.
- Gellhorn, A. & Marks, P. A. (1961). *J. clin. Invest.* **40**, 925.
- Hartman, L., Shorland, F. B. & Cleverley, B. (1958). *Biochem. J.* **69**, 1.
- Hawke, J. C., Hansen, R. P. & Shorland, F. B. (1959). *J. Chromat.* **2**, 547.
- Herb, S. F., Magidman, P. & Riemenschneider, R. W. (1960). *J. Amer. Oil Chem. Soc.* **37**, 127.
- Hirsch, J., Farquhar, J. W., Ahrens, E. H., Peterson, M. L. & Stoffel, W. (1960). *Amer. J. clin. Nutr.* **8**, 499.
- Holman, R. T. & Hayes, H. (1958). *Analyt. Chem.* **30**, 1422.
- Hornstein, I., Elliott, L. E. & Crowe, P. F. (1959). *Nature, Lond.*, **184**, 1710.
- James, A. T. (1959). *Proc. Conf. Chemistry & Technology of Edible Oils & Fats, Unilever, Port Sunlight. J. Chromat.* **2**, 552.
- James, A. T. (1960). *Meth. biochem. Anal.* **8**, 1.
- James, A. T., Webb, J. P. W. & Kellock, T. D. (1961). *Biochem. J.* **78**, 333.
- Johnston, P. V., Johnston, O. C. & Kummerow, F. A. (1957). *Science*, **126**, 698.
- Johnston, P. V., Kummerow, F. A. & Walton, C. H. (1958). *Proc. Soc. exp. Biol., N.Y.*, **99**, 735.

- Kingsbury, K. J., Heyes, T. D., Morgan, D. M., Aylott, C., Burton, P., Emerson, R. & Robinson, P. J. A. (1962a). *Biochem. J.* **84**, 124.
- Kingsbury, K. J., Morgan, D. M., Aylott, C., Burton, P., Emerson, R. & Robinson, P. J. A. (1962b). *Clin. Sci.* **22**, 161.
- Kingsbury, K. J., Paul, S., Crossley, A. & Morgan, D. M. (1961). *Biochem. J.* **78**, 541.
- Klenk, E. (1961). In *Drugs Affecting Lipid Metabolism*, p. 21. Ed. by Garattini, S. & Paoletti, R. London: Elsevier.
- Landowne, R. A. & Lipsky, S. R. (1961). *Biochim. biophys. Acta*, **47**, 589.
- Lipsky, S. R. & Landowne, R. A. (1958). *Biochim. biophys. Acta*, **27**, 666.
- Lovelock, J. E. (1958). *Nature, Lond.*, **182**, 1663.
- McLaren, D. S. & Read, W. W. C. (1961). *Nature, Lond.*, **192**, 265.
- McLaren, D. S., Read, W. W. C. & Chimbe, A. (1962). *Clin. Sci.* **23**, 247.
- Mead, J. F. & Slatton, W. J., jun. (1956). *J. biol. Chem.* **219**, 705.
- Moore, C. H. & Cooke, R. P. (1959). *Biochem. J.* **73**, 43.
- Morrison, W. R., Lawrie, T. D. V. & Blades, J. (1961). *Chem. & Ind.* p. 1534.
- Schlenk, H. & Gellerman, J. L. (1960). *Analyt. Chem.* **32**, 1412.
- Woodford, F. P. (1961). In *Drugs Affecting Lipid Metabolism*, p. 189. Ed. by Garattini, S. & Paoletti, R. London: Elsevier.
- Woodford, F. P. & van Gent, C. M. (1960). *J. Lipid Res.* **1**, 188.

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A Simple Method for the Preparation of ^{32}P -Labelled Adenosine Triphosphate of High Specific Activity

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^{32}P -labelled ATP of high specific activity is not available commercially. Chemical methods for preparing it (see, for example, Lowenstein & Metzenberg, 1960) are tedious and yield a product with the ^{32}P distributed between the β - and γ -phosphate groups in uncertain proportions. Several enzymic methods have been described (see, for example, Racker, 1961), but most of them require the use of biological materials such as mitochondria or chloroplasts, which are not readily available in many laboratories and cannot be stored. An exception is the method developed by Revel and described by Penefsky, Pullman, Datta & Racker (1960), but, though this is moderately satisfactory, in our hands the yields have not been high. A combined chemical and enzymic method described by Tanaka, Mano & Shimazono (1958) is said to give a product with 92% of the radioactivity in the γ -phosphate group, but the method is elaborate and, according to its authors, unsuitable for preparing a product of high specific activity. For these reasons, we thought it worth attempting to develop a method for the preparation of ^{32}P -labelled ATP which, at the same time, was simple, required only enzymes and substrates readily available commercially, and gave a good yield of product labelled in the γ -phosphate group.

The method finally adopted makes use of the exchange reaction between inorganic phosphate and the terminal phosphate group of ATP that occurs in the presence of phosphoglycerate kinase (reaction 1), glyceraldehyde 3-phosphate dehydrogenase (reaction 2) and suitable substrates.

3-Phosphoglycerate + ATP

1,3-Diphosphoglycerate + glyceraldehyde

The equilibrium position of reaction (1) is far to the left, so that ADP will be present only in catalytic amounts. It follows that, even if the enzyme preparations contain a trace of adenylate kinase, there should be little exchange of ^{32}P between inorganic phosphate and the β -phosphate group of ATP.

METHODS

Preparation of labelled adenosine triphosphate. The following were added, in the order given, to a 10 ml. glass-stoppered tube: 5 ml. of water; 0.5 ml. of m-tris ('121 grade' from Sigma Chemical Co., St Louis, Mo., U.S.A.) adjusted to pH 8.0 with HCl; 0.06 ml. of m-MgCl_2 ; 1.02 ml. of 0.1N-NaOH; 20 μmoles of cysteine (Sigma) as the free base; 60 μmoles of crystalline ATP as the disodium salt