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The Occurrence of Unusual Fatty Acids in Faecal Lipids from Human Beings with Normal and Abnormal Fat Absorption

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Little information has been published on the detailed fatty acid composition of human faecal lipids, though it has long been a vexed question whether or not the microbial flora of the intestinal tract play a major part in the production of the large amounts of fat excreted in diseases such as steatorrhoea or sprue. Studies relating the composition of faecal lipids to that of the ingested fat should provide some information on this problem.

The earlier methods of fatty acid analysis used in such studies, e.g. fractional distillation (Edwards & Cook, 1951) and reversed-phase liquid–liquid chromatography (Van de Kamer, Pikaan, Bolssens-Frankena, Couvée-Ploeg & van Ginkel, 1955), are not capable of providing sufficiently detailed information. We have used gas–liquid chromatography both for analysis (James & Martin, 1956) and for structure determination (James & Webb, 1957; James, 1959).

EXPERIMENTAL

Faeces were collected and either extracted immediately with the medium described by Dole (1956) or stored at 2° until required. The extracted lipids were divided into free and bound acids by the titration technique described by Dole (1956).

The free acids were methylated directly with anhydrous methanolic HCl (James, Lovelock, Webb & Trotter, 1957) and the bound acids were methylated after saponification and extraction. Analyses of the methyl esters of the fatty acids were carried out on 80–100 μg. samples on 4 ft. glass columns with stationary phases of Apiezon L grease (Shell Chemicals Ltd.) at 200° with a column efficiency of 4000–5000 theoretical plates, and also polyethylene glycol adipate at 180° with an efficiency of 3000–3500 theoretical plates. In both cases the argon ionization monitor was used as detector (Lovelock, 1958; Lovelock, James & Piper, 1959; and Pye Scientific Instruments Ltd.). Isolation of individual acids on the milligram scale was carried out on similar columns with the gas-density meter as

detector (James & Webb, 1957). Identification of individual acids was carried out by measurement of log relative retention volumes on the two types of column described by James (1959). Determinations of structure of unsaturated and hydroxy acids were done by the permanganate-oxidation method with gas–liquid chromatography to identify the mono- and di-carboxylic acids produced (James & Webb, 1957).

The u.v. absorptions of isolated acids were determined at a concentration of 1 mg./ml. in purified *n*-hexane at a wavelength range of 250–315 mμ. Infrared spectra were determined by the KCl-disk technique. *Trans*-acid contents were determined in CS_2 solution by the method described by Hartman, Shorland & Cleverley (1958). A standard curve was constructed with known concentrations of methyl elaidate; solutions of methyl stearate at similar concentrations were used as the solvent blank.

Faecal lipids were separated into different classes with the silicic acid chromatogram described by Hirsch & Ahrens (1958).

RESULTS

Fig. 1 shows a typical chromatogram of the faecal fatty acids of a human subject with steatorrhoea (a heavier-than-normal load being used to demonstrate the minor components). A qualitatively similar picture is given by a normal subject. Two major peaks can be seen in unusual positions: (*a*) in a position expected for positional and configurational isomers of oleic acid and (*b*) in a position expected for a C_{20} monoenoic acid.

Identification of the substances responsible for these peaks was carried out as described below.

Peak (a). On running the same mixture as in Fig. 1 on a polyethylene glycol adipate column, peak (*a*) moved into the oleic acid peak, behaviour expected from isomeric octadecenoic acids on this type of column. A sample of the components in

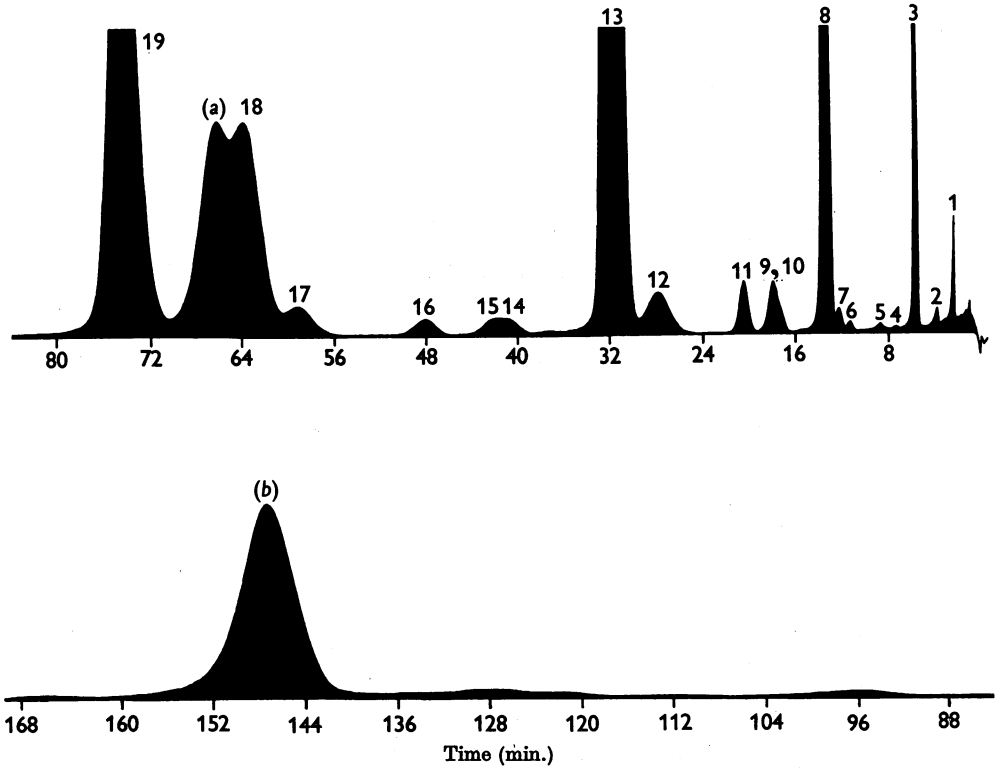

Fig. 1. Gas chromatogram of methyl esters of the fatty acids isolated from human faecal lipids, a heavy load being used to demonstrate the minor components. Conditions: column load, 250 μg.; temperature, 197°; argon inlet pressure, 15 lb./in.² above atmosphere, outlet at atmospheric pressure; stationary phase, 15% (w/w) Apiezon L vacuum grease; support, Celite (100–120 mesh); column length, 4 ft. Apparatus by W. G. Pye Ltd., Cambridge. Detector, argon ionization monitor. Peaks in order of appearance: (1) methyl *n*-decanoate; (2) methyl *n*-undecanoate; (3) methyl *n*-decanoate; (4) branched tridecanoate; (5) methyl *n*-tridecanoate; (6) highly branched tetradecanoate; (7) methyl myristoleate; (8) methyl *n*-tetradecanoate; (9), (10) branched pentadecanoates; (11) methyl *n*-pentadecanoate; (12) methyl palmitoleate; (13) methyl *n*-hexadecanoate; (14), (15) branched heptadecanoates; (16) methyl *n*-heptadecanoate; (17) methyl linoleate; (18) methyl oleate; (*a*) unknown; (19) methyl *n*-octadecanoate, (*b*) unknown.

Table 1. *Fragments isolated from oxidative degradation of the oleic acid-isomer peak*

Monocarboxylic acid	Dicarboxylic acid	Parent unsaturated acid	Approximate amount as percentage of total peak
C_6	C_{12}	Δ^{12}-Octadecenoate	3·5
C_7	C_{11}	Δ^{11}-Octadecenoate	45·5
C_8	C_{10}	Δ^{10}-Octadecenoate	18·1
C_9	C_9	Δ^9-Octadecenoate	17·0
C_{10}	C_8	Δ^8-Octadecenoate	6·6
Masked by C_{11} carrier	C_7	Δ^7-Octadecenoate	3·6
C_{12}	C_6	Δ^6-Octadecenoate	2·8
C_{13}	C_5	Δ^5-Octadecenoate	1·4
Trace of C_{14}	C_4	Δ^4-Octadecenoate	0·9

Table 2. Trans-*acid content of faecal lipids*

Sample	Percentage of *trans*-acid determined spectroscopically	Percentage of oleic acid isomers in sample
Mixed faecal acids	10·1	7·6
Equimolar mixture of isolated oleic acid and oleic acid isomers	26	50

peak (*a*) was isolated with an Apiezon L column, and oxidative degradation followed by identification of the mono- and di-carboxylic acids produced gave the results shown in Table 1. The components of peak (*a*) therefore consist of a mixture of Δ^4-, Δ^5-, Δ^6-, Δ^7-, Δ^8-, Δ^9-, Δ^{10}-, Δ^{11}- and Δ^{12}-octadecenoic acids. The relative amounts of the different acids are also given in Table 1, the major components being the Δ^8-, Δ^9-, Δ^{10}- and Δ^{11}-octadecenoic acids, the last-named representing 45% of the mixture.

Determination of the *trans*-acid content (see Experimental section) of the total mixed faecal acids gave a value of 10% (Table 2), the same order as the amount of oleic acid isomers in the sample (7·6%). However, estimation of the *trans*-acid content of an approximately equimolar mixture of oleic acid and its isomers isolated from the mixed faecal acids gave a value of only 26%, suggesting that only about 50% of the oleic acid isomers possess the *trans*-configuration, and that other acids in the mixed sample are also *trans*-acids.

Peak (b). Although the change in position of peak (*a*) on the polyethylene glycol adipate column was readily observed, no peak could be detected in the position expected for a C_{20} monoenoic acid (the suggested structure for peak *b*). Only after prolonging the time of analysis was a peak found agreeing in area with the expected for peak (*b*); its retention relative to methyl stearate was 9·0. The substance responsible for this peak was isolated and run again on the Apiezon L column and had a retention volume relative to methyl stearate of 1·96.

When log relative retention volumes of a variety of acids in the Apiezon L column are plotted against the values obtained in the polyethylene glycol adipate column, a family of parallel lines can be drawn through the points belonging to different classes of fatty acids, i.e. saturated, mono-, di-, tri-, tetra-, penta- and hexa-enoic acids (James, 1959). The values obtained for peak (*b*) did not fall on any of these lines and suggested an impossible number of double bonds in the molecule. Examination of the u.v.-absorption spectrum of a sample of the unknown acid dissolved in spectroscopic-quality *n*-hexane failed to reveal any absorption at the wavelengths expected for unsaturated acids (250–315 mμ). An attempt was made to reduce catalytically a sample of the acid under pressure, but only the original material could be isolated. Thus the unknown acid possessed some unusual group or groups not found in the common straight-chain saturated or unsaturated acids. The presence of a ring structure in the unknown acid was possible, so lactobacillic acid was tried on the two columns but did not show a large enough relative retention in the polar column (Table 3). This idea was not further pursued. Since the London dispersion forces between solute and solvent are of the same order in both the polyester and Apiezon L columns, the large retention of the unknown acid in the polyester column must be due to some very polar group in the molecule. The effect of an hydroxyl group on chromatographic behaviour was not known so ricinoleic acid (12-hydroxy-Δ^9-octadecenoic acid) was chosen as a model substance. This compound has a relative retention volume of 9·05 in the Apiezon L column whereas oleic acid (Δ^9-octadecenoic acid) had a relative retention of 4·75. The ratio of these two figures gave a relative retention of 1·9 for the hydroxyl group alone (Table 3). A pure sample of methyl ricinoleate was isolated by means of the gas chromatogram and catalytically reduced to give 12-hydroxystearic acid. This

Table 3. *Relative retention volumes (methyl myristate = 1) of methyl esters of saturated, unsaturated and hydroxy acids in two stationary phases*

Acid	Relative retention volumes: Apiezon L at 197°	Relative retention volumes: Polyethylene glycol adipate at 180°
Oleic (*cis*-Δ^9-octadecenoic)	4·75	4·42
Elaidic (*trans*-Δ^9-octadecenoic)	4·95	4·42
cis-Δ^4-Octadecenoic	4·95	—
trans-Δ^4-Octadecenoic	5·15	—
Oleic isomers (from faeces)	4·95	4·42
Lactobacillic	7·22	8·60
Ricinoleic (12-hydroxy-Δ^9-octadecenoic)	9·05	—
12-Hydroxystearic	11·0	36·0
10-Hydroxystearic (from faeces)	11·0	35·0
DL-10-Hydroxystearic (synthetic)	11·0	35·7
9:10-Dihydroxystearic	21·2	Too slow to measure
Stearic	5·5	4·0
Branched pentadecanoic (possibly *iso*-)	1·3	1·19
Branched pentadecanoic (possibly *anteiso*)	1·35	1·27
Branched heptadecanoic (possibly *iso*-)	3·14	2·55

Table 4. *Acids obtained from oxidative degradation of the hydroxystearic acid*

Dicarboxylic acids	Monocarboxylic acids	Corresponding hydroxy acid
C_4	C_4 (trace)	6-Hydroxystearic acid
C_5	C_{13} (trace)	7-Hydroxystearic acid
C_6	C_{12}	8-Hydroxystearic acid
C_7	C_{11}	9-Hydroxystearic acid
C_8	C_{10}	
C_9*	C_9*	10-Hydroxystearic acid
C_{10}*	C_8*	

* Major components.

Table 5. *Comparison of composition of esterified (bound) and non-esterified (free) fatty acids in faecal lipids for a normal subject and a steatorrhoea subject on a general ward diet*

Trace: less than 0·5%.

Fat excretion	Percentage of acids in range C_6–C_{20}: Normal (G)		Steatorrhoea (W.S.)	
In 24 hr. (g.)	< 5		17·7	
Acid	Free	Bound	Free	Bound
10:0	0·6	0	0·8	Trace
12:0	4·3	2·3	8·2	2·4
14:0	8·9	4·4	11·9	6·2
Branched 15:0	0·7	1·1	0·4	0·3
15:0	1·4	1·1	0·5	0·4
16:0	55·2	35·3	31·7	41·1
Branched 17:0	0·9	1·4	0·5	0·6
17:0	0·4	0·8	0·3	14·5
18:0	12·9	31·8	5·9	14·5
10-Hydroxy-18:0	0·7	0·9	17·8	2·7
14:1	0	0	0·5	0·2
16:1	1·4	2·1	1·8	1·5
18:1	6·8	10·7	4·1	12·6
Isomer 18:1	3·5	6·5	7·8	11·7
18:2	1·3	2·8	5·9	3·5
18:3	0	0	0	0
20:3	Trace	Trace	4·7	0·9
20:4	Trace	Trace	0·2	Trace
Other unsat. C_{20} acids	Trace	Trace	1·2	1·2

substance behaved on both columns in a manner almost identical with the unknown acid, confirming that this was an hydroxystearic acid.

Comparison of the infrared spectra of the isolated hydroxy acid and 12-hydroxystearic acid showed them to be almost identical, confirming the assigned structure.

Oxidative degradation (James & Webb, 1957) of an isolated sample of the unknown acid gave as major components almost equimolar amounts of the C_8 and C_9 monocarboxylic acids and the C_9 and C_{10} dicarboxylic acids as well as smaller amounts of other monocarboxylic and dicarboxylic acids (Table 4). It was already known that saturated hydroxy acids on oxidation can split on either side of the carbon atom bearing the hydroxyl group (Shukow & Schestakow, 1903). Thus the major component in the unknown acid was 10-hydroxystearic acid, with 6-, 7-, 8- and 9-hydroxystearic acids as minor components.

The small peaks lying between the major components (Fig. 1; peaks nos. 3, 8, 13, 18) were shown to be saturated acids by running on the two types of column. From their chromatographic positions they were identified as *n*-tridecanoic acid, *n*-pentadecanoic acid, *n*-heptadecanoic acid and incompletely resolved branched-chain odd-numbered acids (Table 3). These branched-chain acids are probably the *iso*- and *anteiso*-acids known to occur in animal fats (Hawke, Hansen & Shorland, 1959). Their occurrence in faecal lipids may be due to ingestion of butter, since they are quantitatively increased on a mixed or butter diet as compared with a corn-oil diet.

A total analysis of faecal fatty acids in a normal subject and in a subject with steatorrhoea is given in Table 5. Separation of the faecal lipids into free and bound fatty acids was not very successful by the Dole (1956) technique since a large part of the faecal lipid consists of calcium soaps of the

Table 6. *Composition of the fatty acid content of faecal (steatorrhoea) lipid fractions extracted before and after acidification*

	Percentage of acids in range C_6–C_{20}					
	Light-petroleum extract					
		After separation				
Acid	Before separation	Cholesterol	Triglycerides	Diglycerides	Free acids	Ether extract after acidification
n-Decanoic	0·2	0·2	0·2	0·4	0	0·05
n-Dodecanoic	1·9	1·6	1·0	2·7	0·2	0·29
n-Tetradecanoic	9·1	7·4	4·2	11·0	0·6	4·33
n-Hexadecanoic	25·7	44·3	17·8	11·8	2·0	38·9
n-Octadecanoic	10·5	12·6	5·5	2·7	1·0	39·6
Tetradecenoic	0·2	0·2	0·3	0·2	0	0·04
Hexadecenoic	1·8	2·1	1·4	3·8	0·2	0·26
Oleic	20·4	16·2	18·1	21·2	1·5	3·04
Oleic isomers	6·6	6·6	5·8	2·7	1·0	3·68
Linoleic	3·7	3·6	3·6	7·9	1·1	0·76
C_{20} triene	1·4	Trace	Trace	2·6	3·8	0·20
10-Hydroxystearic	14·0	1·4	35·2	25·3	88	7·04
Fraction as percentage of total extract	—	58	17·7	9·9	11·0	—

Table 7. *Some naturally occurring hydroxy acids*

Structure	Trivial name	Source
12-Hydroxy-Δ^9-octadecenoic	Ricinoleic acid	Castor oil
16-Hydroxyoctadecanoic	Junipric acid	Castor oil
11-Hydroxyhexadecanoic	Jalapinolic acid	Jalap resin
11-Hydroxypentadecanoic	Convolvulinic acid	Resin from *Convolvulus scammia* L
12-Hydroxydodecanoic	Sabinic acid	Wax from *Juniperus sabina*

Table 8. *Hexadecenoic acids and octadecenoic acids of milk fat and butterfat*

Acid	Configuration	Source	Authors
Δ^9-Hexadecenoic	20% *trans*-	Butterfat	Backderf & Brown (1958)
Δ^{16}-Octadecenoic	*trans*-	Butterfat	
Δ^{11}-Octadecenoic	Mostly *trans*-	Butterfat	
Δ^4-Octadecenoic	Not determined	Milk fat	James & Webb (1957)
Δ^{11}-Octadecenoic	Not determined	Milk fat	
Δ^{13}-Octadecenoic	Not determined	Milk fat	

Table 9. *Effect of the nature of dietary fat on the output of some faecal acids*

Diet		Percentages												Daily output (g.)
		Palmitic acid		Stearic acid		Oleic acid		Oleic acid isomers		10-Hydroxy-stearic acid		Linoleic acid		
		Bound	Free	Bound	Free	Bound	Free	Bound	Free	Bound	Free	Bound	Free	
Normal subject	Low-fat	34·2	28·5	25·4	19·3	17·2	19·5	8·8	10·0	2·0	3·7	2·2	2·1	0·9
	Butter	37·7	44·2	26·4	13·6	6·8	12·7	8·0	10·5	1·1	—	—	2·1	3·0
	Corn oil	17·0	40·8	36·8	16·3	6·7	4·4	25·0	15·8	3·0	10·6	3·3	2·0	4·2
Subject with steatorrhoea	Low-fat	34·2	30·6	6·6	14·4	5·0	8·9	25·7	29·6	—	1·7	1·3	1·99	18·0
	Butter	26·7	30·8	16·3	17·5	18·69	15·8	9·3	8·0	7·3	4·8	3·8	3·0	50·0
	Corn oil	11·7	15·5	6·3	6·2	32·3	31·2	3·9	5·9	3·2	2·1	37·1	36·6	50·0
Composition of dietary fat	Butter	20·8		16·4		24·2		5·0		0		3·0		—
	Corn oil	10·7		3·2		35·0		0		0		50·2		—

fatty acids. To clarify the nature of the faecal lipids, a 24 hr. collection of faeces from a steatorrhoea patient was extracted first with light petroleum to remove most of the bound non-polar lipids and then, after acidification of the faeces with 5N-H_2SO_4, they were extracted with ether. The ether extract readily deposited crystals but the light-petroleum extract, even after removal of the solvent, remained as a greenish oil.

Each extract was resolved into a series of fractions on a silicic acid chromatogram with the sequence of solvents described by Hirsch & Ahrens (1958). The fractions were saponified, and the acids were extracted, esterified and analysed on the gas chromatogram. The results are shown in Table 6. Titration of each of the fractions showed that the second extract was mainly free fatty acid.

Types of lipid in human faeces. Extraction of faeces from a steatorrhoea subject with lipid solvents before and after acidification gave two fractions. The first, largely neutral fat, gave rise to a series of subfractions when subjected to chromatography on silica gel. The material eluted in a zone corresponding to cholesterol esters contained very little hydroxystearic acid (Table 6), whereas eluted in a zone corresponding to triglycerides contained 35·2% of hydroxystearic acid. A later fraction assumed to be free acids contained 88% of the hydroxy acid. This evidence suggested that hydroxystearic acid, though present in ester form, was mainly found in the free faecal acids.

Analysis of the material from the second extraction presumably derived from the calcium soaps present in faeces (Sammons & Wiggs, 1960) gave the result shown in Table 6. This material was shown to be almost entirely non-esterified fatty acid by titration but did not contain more hydroxy acids than did the esterified acids from the first light-petroleum extract. The hydroxy acids are thus found in all the faecal lipids of a steatorrhoea patient, the larger part being in the free acids.

DISCUSSION

The presence of large quantities of hydroxy acids in the faecal lipids of humans with steatorrhoea is of some interest since these acids are not present in any of the normal dietary fats and must therefore be synthesized in the gut. This synthesis is more likely to be carried out by micro-organisms in the gut than in the intestinal wall. Long-chain hydroxy acids are limited in their occurrence in oils and fats but are particularly evident in resins and oils used as cathartics. A list of such acids is given in Table 7. Since the physiological action of castor oil is believed to be due to a hydroxy acid (ricinoleic acid), the diarrhoea often associated with steatorrhoea may be due to production of these acids in the intestine.

The close correspondence of the structure of the hydroxystearic acids with the isomeric octadecenoic acids suggests that they may be derived one from another. At this stage it is impossible to state which is the precursor and which the product, though the hydroxy acids could well be intermediates in the formation of the monounsaturated acids.

The monounsaturated C_{18} acids consist of a mixture of isomers with the double bond varying in position from C_4 to C_{12}, about 50% of the mixture having a *trans*-configuration about the double bond. Some, but not all, of these acids occur in milk and butterfat (see Table 8), so that it is unlikely that they all originate from the diet. In any case, these isomeric acids are still excreted when corn oil is fed; this oil contains only one octadecenoic acid (Table 9).

The occurrence of *trans*-acids in faecal lipids from animals was demonstrated by Hartman, Shorland & Morr (1956) and Hartman *et al.* (1958). These workers [see also Hartman & Shorland (1959)] consider that the *trans*-acids arise by bacterial hydrogenation of polyunsaturated acids in the post-absorptive region of the gut. With the human this hypothesis is unlikely since, in all cases of steatorrhoea studied so far, substitution of corn oil (51% of linoleic acid) for butter (2–3% of linoleic acid) in the diet does not cause any marked increase in the faecal content of the isomeric acids. Indeed, one subject after 3 days on a low-fat diet (less than 12 g./day) was excreting 18 g. of fat/day containing 25·7% of oleic acid isomers in the bound lipids (Table 9). In normal subjects substitution of corn oil for butter in the diet does produce an increase in the content of oleic acid isomers in the faecal fat, but there is also an increase in output of stearic acid that may be serving as the precursor of the unsaturated acids. Subjects with steatorrhoea on a butter diet excrete approximately the same amount of linoleic acid as is ingested, together with large amounts of the *trans*-isomers, again suggesting that these isomers are not produced by hydrogenation of dietary linoleic acid.

SUMMARY

1. Human faecal lipids of subjects both with normal and abnormal fat absorption have a fatty acid composition very different from that of the ingested fats.

2. A variety of isomeric octadecenoic acids are present, with the double bond in positions 4, 5, 6, 7, 8, 9, 10, 11 and 12. Approximately 50% of these oleic acid isomers possess the *trans*-configuration about the double bond.

3. Subjects with steatorrhoea do not excrete increased amounts of *trans*-acids on changing the dietary fat from butter (2–3% of linoleic acid) to corn oil (51% of linoleic acid).

4. On a diet of butter, subjects with steatorrhoea appear to excrete linoleic acid in amounts equal to that ingested.

5. No support is found for the theory that *trans*-octadecenoic acids are produced by bacterial hydrogenation of linoleic acid.

6. A new major component of faecal lipids has been shown to be 10-hydroxystearic acid, together with 6-, 7-, 8- and 9-hydroxystearic acids as minor components.

7. These hydroxy acids are suggested as intermediates in the formation of monounsaturated octadecanoic acids from stearic acid.

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