# Lipid excretion

## 2.* Fractionation of human faecal lipids†

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The routine clinical laboratory methods used in metabolic studies on lipids normally provide information about a very limited number of fractions, such as free and bound fatty acids, total unsaponifiable matter and sterols precipitable by digitonin or giving a colour reaction with the Liebermann–Burchard or other reagent. The newer chromatographic and allied techniques applied to blood and various tissues have revealed the complexity of lipid mixtures and the inadequacies of the classical methods which have necessarily formed the basis of most clinical investigations, including many on the relationship of diet to composition of plasma lipids.

In recent years considerable progress has been made in the separation and characterization of lipids of plasma and other tissues by, for example, chromatography on silicic acid columns and gas–liquid chromatography, and these methods have been used in many recent studies (e.g. Böttcher, Woodford, Boelsma-van Houte & van Gent, 1959). However, comparatively little work has been done on faecal lipids by these newer techniques and in particular there is a lack of detailed information on steroid fractions.

In our own investigations we have used colorimetric and digitonin precipitation methods for sterols (Aylward & Wills, 1962) recognizing that the information given is limited because of the difficulty in determining specific sterols (such as cholesterol) in mixtures, but we have shown also (Aylward & Wills, 1961) that chromatographic methods can be applied to faecal lipids and that it is possible to identify, for example, sterol esters.

It became clear during these studies and from a survey of older and recent papers that faecal lipid mixtures were in many respects more complex than those from any other source and that it would be difficult to devise improved routine methods for nutritional and general metabolic studies until the different components of faecal lipids were more thoroughly examined. It has become evident also that the importance of balance studies on human subjects is now accepted by an increasing number of workers (e.g. Aylward, 1958; Hellman & Rosenfeld, 1959).

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This paper describes the fractionation of faecal lipids and related compounds by chromatographic and other techniques. The application of these methods in metabolism experiments will be considered in subsequent publications.

## EXPERIMENTAL AND RESULTS

### *Extraction of faeces and preliminary fractionation*

Samples of faeces were obtained from normal subjects and treated in the way shown in Fig. 1.

*Extraction of lipid.* In our experiments special attention was given to the initial extraction techniques; methods involving the air drying of faeces had to be avoided because of oxidation not only of fatty acids but of other unsaturated compounds, including sterols. Methods involving the preliminary saponification of lipids were also ruled out because they result in the breakdown of esters so that the fatty acids in the different fractions cannot be identified. Fractions were stored at $-35°$ and operations were carried out whenever practicable in an atmosphere of oxygen-free nitrogen.

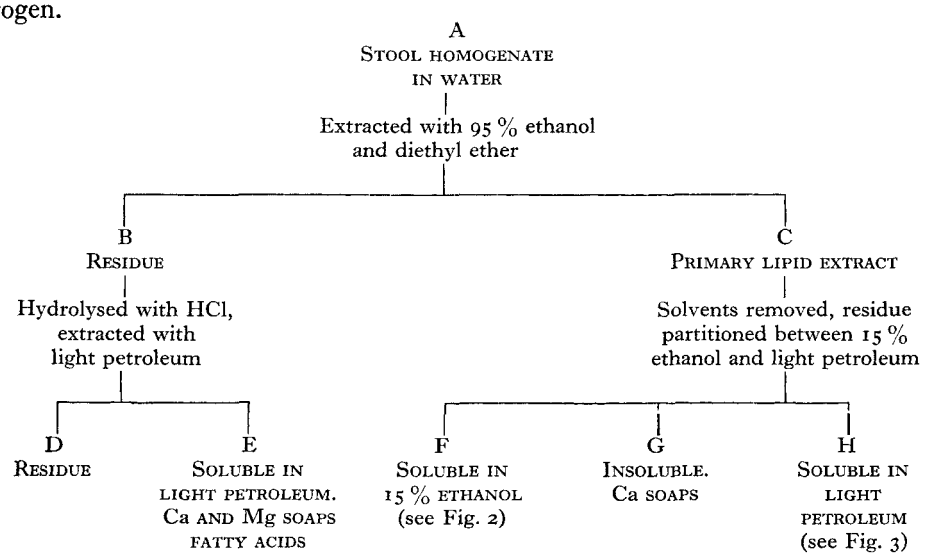


Fig. 1. Scheme of extraction and preliminary fractionation of human faecal lipids.

Several points required attention in the initial extraction of lipids: (*a*) the water content of the faeces was high (about 70%) so that the use initially of a water-miscible solvent was desirable; (*b*) filtration of faecal homogenate–solvent mixtures was unsatisfactory by all methods tried, whereas centrifuging gave clean and rapid separation of solids; (*c*) centrifugal separation of solids to the bottom of suspensions in ethanol or diethyl ether was more convenient than centrifugal separation of solids to the surface of suspensions in chloroform–methanol; (*d*) complete extraction of lipids (other than metal soaps) was required. The following procedure was found to be satisfactory in the above respects.

Fresh samples of faeces were macerated with a little air-free water, 95% (v/v) ethanol

was added and the mixture brought to the boiling point for a few minutes, cooled and centrifuged. The residue was extracted again with boiling 95% ethanol, and then three times with boiling diethyl ether. The combined centrifugates were filtered and the solvents removed on a rotary evaporator under reduced pressure at about 30°, to yield the primary lipid extract (C).

*Extracted residue* (*B*). The pale-coloured residue was treated with 5 N-HCl on a steam bath for 10 min and the diluted mixture extracted with light petroleum (b.p. 40–60°) to yield solid, relatively saturated fatty acids (E), derived mainly from calcium and magnesium soaps. Accompanying dark-coloured material, insoluble in light petroleum but soluble in diethyl ether, may indicate the presence of soaps of bile acids in (B). The fatty acids of faecal calcium soaps have recently been characterized (Sammons & Wiggs, 1960).

*Partition of primary lipid extract* (*C*). The extract was immediately treated with 15% (v/v) ethanol in water (5 vol.) and shaken five times with petroleum (5 × 1 vol.) in a separatory funnel to give three fractions. Fraction (F) was a dark-brown aqueous ethanol solution; Fraction (H) was a bright-yellow light petroleum solution; and Fraction (G) was an insoluble, pale-tan solid associated with the light petroleum solution as a fine suspension, and was separated from it by filtration.

*Fraction* (*G*). The metal soaps (G) were initially soluble in the extracting solvents. Their association with the light petroleum phase as a suspension is in agreement with the observation of Sammons & Wiggs (1960). The acids liberated on hydrolysis were not entirely soluble in light petroleum, suggesting the presence of bile-acid soaps. The soluble fatty acids were solid, with iodine value (Wijs's) 15 and mean equivalent weight (by titration) 279. The phosphorus content of the soaps was very low and indicated the absence of calcium phosphate–fatty acid complexes of the type demonstrated in rat faeces (Richards & Carroll, 1959).

### *Treatment of aqueous ethanol extract*

The aqueous phase (F) contained the pigment stercobilin and the more polar lipids and was treated as shown in Fig. 2. On acidification with hydrochloric acid and extraction with light petroleum, a very small amount of solid (K), mean equivalent weight 529, was obtained, derived in part, perhaps, from sodium and other soluble soaps. On repeated extraction of the acidified solution with diethyl ether, a dark bile acid fraction (M) was slowly removed, with 1·1% nitrogen content and mean equivalent weight 408. The presence of conjugated bile acids probably accounted for part of the nitrogen content. Conversion of the material into ethyl esters and chromatography on alumina yielded several fractions with carbon and hydrogen contents and mean equivalent weights (titration of acids from ethyl esters) appropriate for bile acids of increasing oxygen content. The faecal bile acid mixture is known to be complex, and was not further investigated by us; but the reports of Sjövall, Meloni & Turner (1961) and Blomstrand (1961) suggest that gas–liquid chromatography of the methyl esters may prove invaluable for analysis of faecal bile acids, and Hofmann (1962) has separated several free and conjugated bile acids by thin-layer chromatography on silicic acid.

Even after six extractions with diethyl ether, about 50% of the primary lipid extract (C) still remained in the residue (L), and appeared to consist largely of inorganic salts. It may be noted that a small amount of bile acid was soluble in light petroleum and entered fraction (H).

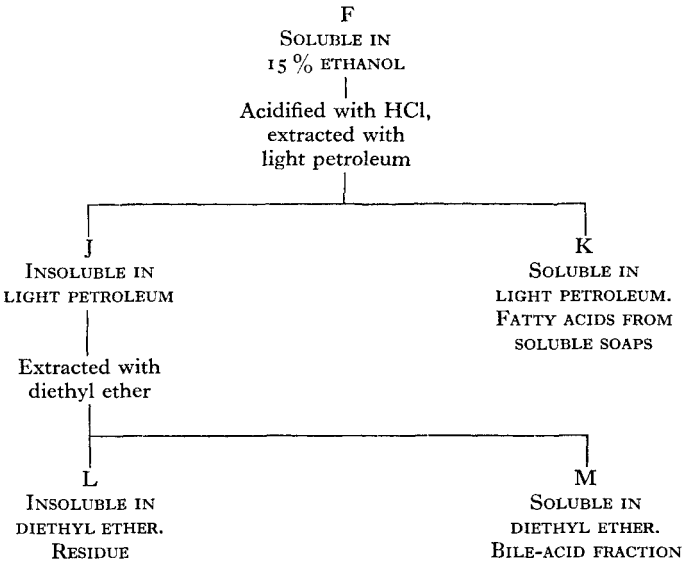


Fig. 2. Scheme of fractionation of aqueous ethanol extract (F) (see Fig. 1).

*Fractionation of lipid soluble in light petroleum*

Fraction (H) was treated as shown in Fig. 3.

*Removal of most-polar material.* Chromatography on silicic acid (Bio-Rad Laboratories) with 2% (v/v) methanol in chloroform removed most of the lipids (N), but about 4% were eluted with 50% (v/v) methanol in chloroform and then pure methanol as Fraction (O), a dark, sticky phospholipid mixture containing combined fatty acids, and with 1·56% nitrogen and 2·84% phosphorus. Separation of phospholipids by rubber dialysis (van Beers, de Iongh & Boldingh, 1958) gave results in close agreement, and both methods gave excellent recoveries on a weight basis.

*Removal of free acidic components.* The large proportion of acidic material present in Fraction (N) was removed at this stage by ion exchange on De-Acidite FF resin (strongly basic; Permutit Co. Ltd) in wet diethyl ether. This procedure was used because: (*a*) free fatty acids are difficult to separate from triglycerides by silicic acid chromatography (Hirsch & Ahrens, 1958); (*b*) the increased concentration of non-acidic lipids enabled more to be dealt with on a standard column; (*c*) faecal odours were removed, in agreement with observations that indole and skatole are retained on strongly basic columns (Martin & Alpert, 1950; Ikai, 1954); (*d*) as a means of obtaining acid-free neutral lipids, the ion-exchange method was much superior to alkali washing which gave extremely stubborn emulsions with unsaponified faecal lipids under a variety of conditions, probably due in part to the presence of monoglycerides and

small amounts of bile acids; and (*e*) no decomposition of lipids on the resin was observed. The non-acidic components were recovered as Fraction (P). Usually about 45% of the total lipid soluble in light petroleum (H) was retained on the column; of this amount about 80% could be recovered by elution with 10% (v/v) acetic acid in diethyl ether to give Fraction (Q).

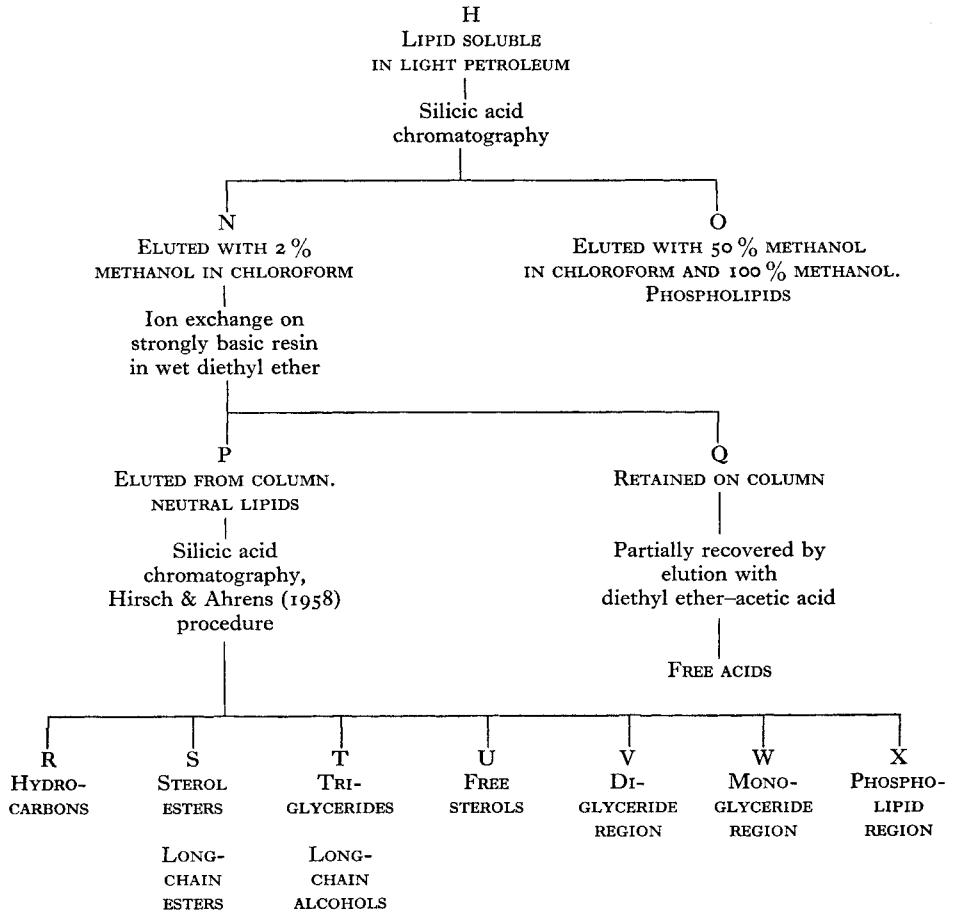


Fig. 3. Scheme of fractionation of lipids soluble in light petroleum (H) (see Fig. 2).

The ion-exchange procedure gave quantitative recovery of neutral lipids completely free from uncombined acids. We accepted the loss of 20% of the total acids, and considered the recovered 80% as representative of the whole. The clean separation of acidic and non-acidic lipids by alkali washing is very difficult when highly surface-active substances are present, and some form of ion-exchange procedure seems likely to provide a satisfactory method. Thus, very recently, Dole (1961) has been able to obtain almost complete recovery of fatty acids from a strongly basic ion-exchange resin by equilibrating the column overnight with eluting solvent, and McCarthy & Duthie (1962) have obtained quantitative separation of free fatty acids from other lipids using a silicic acid column treated with potassium hydroxide in isopropanol.

*Nature of acidic fraction* (*Q*). The bulk of the free acidic material soluble in light petroleum was contained in Fraction (Q). A portion of 300 mg was chromatographed on a column of 18 g silicic acid, prepared as described by Hirsch & Ahrens (1958). Four fractions were obtained:

(*a*) more saturated fatty acids, eluted with 50 ml 8% (v/v) diethyl ether in light petroleum (46% of applied sample, iodine value (Wijs's) 52, mean equivalent weight 273);

(*b*) less saturated fatty acids, eluted with a further 200 ml 8% (v/v) diethyl ether in light petroleum (37%, iodine value (Wijs's) 78, mean equivalent weight 267);

(*c*) a white solid, eluted with 100 ml 50% (v/v) diethyl ether in light petroleum (8%, mean equivalent weight 328, m.p. 68–72°, C 72·4%, H 11·6%);

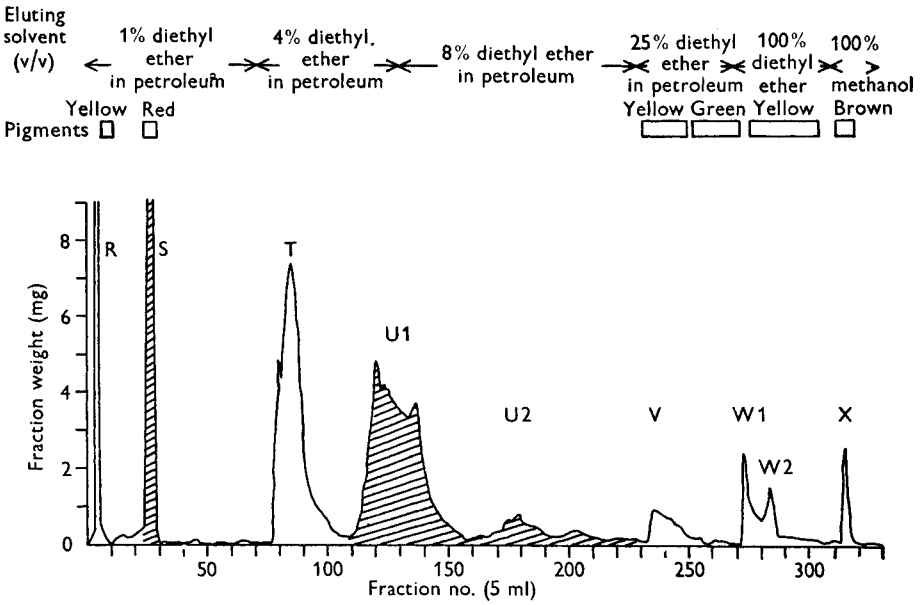


Fig. 4. Silicic acid fractionation of neutral lipids (P) from a sample of normal human faeces. Load, 281 mg; recovered, 279 mg. Silicic acid: 18 g, 325 mesh (Bio-Rad Laboratories) heated at 115° overnight. Fractions: 5 ml. Elution of pigments and composition of eluting solvents are shown, and fractions giving a strongly positive Liebermann–Burchard reaction are shaded.

(*d*) a more-polar dextrorotatory solid, eluted with a further 100 ml 50% (v/v) diethyl ether in light petroleum (3%, mean equivalent weight 396, m.p. above 100°, C 74·6%, H 10·3%).

The polarity and composition of (*c*) were consistent with the presence of hydroxy fatty acids. Fraction (*d*) apparently contained bile acids.

### *Silicic acid fractionation of neutral lipids* (*P*)

The lipids soluble in light petroleum, from which phospholipids and free acids had been removed, were chromatographed on silicic acid (Bio-Rad Laboratories) by a slight modification of the procedure of Hirsch & Ahrens (1958). The solvent sequence

and elution curve for a normal stool sample are show in Fig. 4. Material representing seven lipid regions was obtained.

*Peak R, hydrocarbon region.* Colourless hydrocarbons were followed closely by a small amount of carotene. On fractionation with urea, the former semi-solid material gave as a minor component (20%) a colourless, urea-adductable wax, m.p. 55°, mean molecular weight (cryoscopic) 330, and as a major component (80%) a colourless, viscous oil that would not adduct with urea. This oil had no detectable optical activity, gave a rather weak blue-green Liebermann–Burchard reaction, contained 86·3% C, 13·6% H, had a mean molecular weight (cryoscopic) of 340, a C-methyl content of 8·4% and iodine value (Wijs's) 16. The wax consisted of straight-chain, saturated hydrocarbons, but the liquid hydrocarbons appeared to be cyclic and branched. The infrared spectrums of the two fractions were consistent with these conclusions: relatively weak (wax) or strong (oil) absorption due to methyl groups at 3·38, 3·48 and 7·29 $\mu$, compared with the intensities of absorption due to methylene groups at 3·42, 3·51 and 6·82 $\mu$; absorption at 13·9 $\mu$ for wax, due to long methylene chains; and absence of absorption due to aromatic structures, for wax and oil. The spectrum of the oil was virtually identical with that of a liquid paraffin (BP) sample.

*Peak S, sterol ester region.* This fraction varied considerably in quantity and composition in different faeces samples, and straight-chain (urea-adductable) material was usually present. In one sample 52% of the fraction was urea-adductable, and this material was shown to consist of long-chain wax esters (infrared absorption at 5·74 and 8·5 $\mu$ due to ester groups and at 13·9 $\mu$ due to long methylene chains). Saponification of the esters gave fatty acids and unsaponifiable material with an infrared spectrum characteristic of long-chain alcohols (absorption at 2·75 and 9·5 $\mu$ due to hydroxyl groups; strong absorption at 6·8 $\mu$ due to methylene groups, weak at 7·3 $\mu$ due to methyl groups; absorption at 13·9 $\mu$ due to long methylene chains).

The lipid not able to form an adduct with urea was saponified and treated by digitonin precipitation; the wide melting range of the recovered sterols (for all samples examined) indicated their mixed nature.

The complex nature of this chromatographic region is to be expected in view of the similar behaviour on silicic acid columns of a number of substances, including sterol esters and long-chain wax esters (Hirsch & Ahrens, 1958; Wren, 1960).

*Peak T, triglyceride region.* The fraction separated quite well from the less-polar sterols and was Liebermann–Burchard negative. An urea-adductable component was again usually present, and could not be separated from triglycerides on silicic acid under the conditions used (Fig. 4). This material was a white, non-saponifiable wax, and constituted 27% of the fraction in one sample. Infrared examination (absorption at 2·75 and 9·5 $\mu$ due to hydroxyl groups; absorption at 13·9 $\mu$ due to long methylene chains; absence of ester absorption) and satisfactory analysis for elements showed it to consist of long-chain alcohols.

The part of this region not able to adduct with urea was liquid and very probably consisted of triglycerides, since saponification gave 95% fatty acids and some water-soluble, non-saponifiable component. Glycerol: fatty acid ratios were not determined.

The fatty acids (iodine value (Wijs's) 153; mean equivalent weight 288) were quite highly unsaturated compared with the faecal free and soap fatty acids.

*Peaks U1 and U2, free sterol region.* Peak U1 contained saturated sterols and the separated peak U2 contained unsaturated sterols (rapid Lieberman–Burchard reaction at 0°). U1 was predominantly coprostanol (m.p. 100–101°, $[\alpha]_D + 26°$ in chloroform, after one crystallization; Cook (1958) gives m.p. 101°, $[\alpha]_D + 28°$ in chloroform, for coprostanol). Esters were absent from both peaks, as judged by infrared absorption in the carbonyl region. The contribution of unsaturated sterols was clearly small relative to that of the saturated sterols (Fig. 4).

*Peak V, diglyceride region.* The material was a green grease without urea-adductable components; it gave on saponification about 60% of fatty acids and some unsaponifiable matter soluble in diethyl ether. The green pigment present was probably chlorophyll or a degradation product and constituted less than 4% of the total region, as judged by nitrogen content. According to Zill & Harmon (1959) chlorophylls are partially degraded on silicic acid. Infrared absorption of the total region indicated the presence of long hydrocarbon chains (13·9 $\mu$), hydroxyl groups (2·78 and 9·5 $\mu$) and ester groups (5·72 and 8·5 $\mu$). The elution behaviour of this material strongly suggested that the combined fatty acids were present as diglycerides, but glycerol:fatty acid ratios were not determined. However, triglycerides containing one hydroxystearic acid radical (James, Webb & Kellock, 1961) might be eluted in this region.

*Peaks W1 and W2, monoglyceride region.* The total region was a green grease without urea-adductable components. The nitrogen content was 0·6%, which may indicate a chlorophyll content of 10%, and a yellow pigment was also present. Saponification gave about 60% fatty acids and some unsaponifiable matter soluble in diethyl ether. The method of Pohle & Mehlenbacher (1950) indicated a 1-monoglyceride content of 48% in the fraction (an equivalent weight of 284 being assumed for the fatty acids). The infrared spectrum showed absorption due to long hydrocarbon chains, hydroxyl groups and esters.

Although the elution curve (Fig. 4) suggests partial separation of components, both esters and unsaponifiable substances were found in each peak, W1 and W2. Further fractionation of the total region on a silicic acid column, with 80% (v/v) diethyl ether in light petroleum as eluent, gave some fractions containing only crystalline, non-saponifiable material, the infrared spectrum of which was not inconsistent with the presence of steroid diols or triols (strong absorption due to hydroxyl groups at 2·78 and 9·6 $\mu$; absorption at 6·8 $\mu$ due to methylene groups of similar strength to that at 7·3 $\mu$ due to methyl groups; absence of absorption due to esters or long hydrocarbon chains). Compounds of this type have been found in unsaponifiable fractions of human faeces by Cook, Edwards & Riddell (1956).

*Peak X, phospholipid region.* A yellow-brown wax was eluted, with 1% nitrogen, but only 0·1% phosphorus; it gave a positive Molisch test. The major part of the faecal phospholipids had been removed earlier as Fraction (O).

*Pigments.* In previous separations of faecal lipids, pigments and odours have often been associated with most of the fractions obtained. In this work odours were absent from the fractions prepared by silicic acid separation, and pigments did not present

a major problem. Colourless hydrocarbons were obtained by careful collection of fractions, closely followed by carotene; and the triglycerides, free sterols and uncombined fatty acids were free of pigments. A red pigment, probably lycopene, was often present in the sterol ester region, and green and yellow pigments were present in the di- and mono-glyceride regions.

After removal of the final Fraction (X) the silicic acid column usually showed no residual colour. However, if the red pigment present in the sterol ester region was allowed to remain on the column for more than a few hours, it tended to change into a yellow compound that was not eluted by any of the solvents used subsequently. There was no evidence of any other modification of lipids during the fractionation procedure.

It was found after some experience with silicic acid fractionation of human neutral faecal lipids that the movement of coloured bands (and bands fluorescent in u.v. light) provided a reliable indication of the elution of some of the major lipid fractions. Total recovery of material added to the column (P) was usually 98–99%, as judged by the combined weight of all fractions.

## DISCUSSION

### *The pattern of lipid excretion*

In Tables 1 and 2 we have given for the purpose of illustration the quantitative results obtained from the fractionation of the faecal lipids of one volunteer living on a mixed diet. Table 2 shows that excreted lipids and associated compounds include the following: free fatty acids, soaps, glycerides, phospholipids, long-chain alcohols

Table 1. *Composition of a sample of normal human faeces*

| Basis | Total solids* (%) | Primary lipid extract (Fraction C) (%) | Lipid soluble in light petroleum (Fraction H)† (%) |
|---|---|---|---|
| Wet faeces | 29·4 | 9·7 | 3·8 |
| Dry faeces* | 100 | 32·8 | 12·8 |
| Primary lipid extract (Fraction C) | — | 100 | 39·2 |

Total solids, primary lipid extract and lipid soluble in light petroleum are expressed as a percentage of wet weight, of total solids and of primary lipid extract respectively for a single faeces sample. Fractions (C) and (H) are explained in Fig. 1.

* Determined by steam-bath drying. There was some loss of volatile fatty acids.

† For fractionation see Table 2.

and esters, sterols and their esters, bile acids and hydrocarbons. Metallic salts and amino acid conjugates of bile acids may also be present, together with a variety of pigments. Within these classes of compounds there is a range of individual components (such as different fatty acids, different sterols).

The extraction and fractionation methods used have been tested on several batches of material from various volunteers and the procedures outlined represent those finally chosen after preliminary experimental work at each stage. As would be expected, there were quantitative differences from one individual to another but the same type

of compound and the same pattern of excretion were always found, although the pattern would no doubt be modified with great variation in dietary conditions and in pathological conditions.

Our work suggests that faecal lipids form a much more complex mixture than that likely to be found under normal conditions in any body tissue such as plasma or liver, and that this complexity arises from the multiple sources of the lipids: (*a*) unabsorbed food residues; (*b*) material derived from bile, intestinal secretions and from the intestinal mucosa; and (*c*) modifications of (*a*) and (*b*) by bacteria as well as products synthesized by bacteria. The action of bacteria can result not only in the formation of new lipids from simple systems, but also in changes (through processes such as hydrogenation and oxidation) of lipids entering the large intestine.

Table 2. *Lipid components of a sample of normal human faeces*

| Lipid class | Contained in fractions | Percentage of faecal total solids | Percentage of lipids soluble in light petroleum |
|---|---|---|---|
| Total soaps (as fatty acids) | E, G, K | 7·7 | |
| Bile acids* | M, Q | 1·1 | |
| Petroleum-soluble (Fraction H) | | | |
| Free fatty acids | Q | 5·5 | 42·9 |
| Free sterols | U | 2·7 | 21·1 |
| Triglycerides | T | 1·2 | 9·4 |
| Hydrocarbons | R | 0·96 | 7·5 |
| Phospholipids* | O, X | 0·61 | 4·8 |
| Long-chain esters | S | 0·47 | 3·7 |
| Long-chain alcohols | T | 0·45 | 3·5 |
| Sterol esters | S | 0·44 | 3·4 |
| Mono- and di-glycerides* | W, V | 0·3 | 2·3 |
| Unsaponifiable matter from mono- and di-glyceride regions | W, V | 0·2 | 1·6 |
| Total | | 21·63 | 100·2 |

The major lipid classes isolated are expressed as a percentage of the faecal total solids, and the individual lipids soluble in light petroleum as a percentage of the total lipid soluble in light petroleum. The derivation of the fractions is shown in Figs. 1, 2 and 3.

* Approximate values. There is some doubt about the freedom of the fraction from other lipids.

### *Extraction methods*

We chose an initial (ethanol–diethyl ether) procedure which would give complete extraction of lipids and associated compounds, although we recognized that it would lead to the presence of various non-lipid substances in the primary extract and to the presence of some soaps in the extract rather than in the residue.

The methods described worked smoothly with all the samples examined and we were able in our subsequent fractionation techniques to avoid difficulties arising from pigments which had led in earlier work to the use of a preliminary saline extraction (Aylward & Wills, 1961, 1962).

Thin-layer chromatography on silicic acid of unsaponified faecal lipids provides a rapid method for partial separation of the major classes, and results have been reported by Williams, Sharma, Morris & Holman (1960). Some of the unidentified lipids separated by these workers have been identified by us.

### *Soaps and fatty acids*

*Soaps.* As already noted, the calcium and magnesium salts of fatty acids occurred in the initial ethanol–diethyl ether extract (C) as well as in the residue (B). In the separation described, soaps formed about 10% of the extract (C) corresponding to about 20% of the total soaps in the faeces. This distribution of soaps is to be expected in view of the known differences in solubility of soaps of different fatty acids in the mixtures of solvents (cf. the old-established Twitchell procedure for the separation of fatty acids) but has not always been noted in excretion studies.

*Free fatty acids.* In the separation described, the free acids comprised over 5·5% of dry faecal weight and soaps 7·7% (as free acids). Thus these two fractions together (13·2% dry faecal weight) comprised the greater part of the lipids. It is known that great variations take place in the amounts of these components depending on the fat and mineral content of the diet and on other factors (cf. Fowweather (1926), who reported in eighty-four subjects a range of from 1·05 to 10% for free fatty acids and from 0·54 to 11·4% for soaps).

Our experiments provide evidence for fatty acids containing hydroxyl groups. Infrared investigations on several of the fatty acid samples have indicated the presence of *trans* unsaturation (absorption at 10·3 $\mu$), in agreement with other recent work. James *et al.* (1961) have examined the free fatty acids in normal faecal lipids and in samples obtained from patients with steatorrhoea, and have found various unusual isomeric octadecenoic acids, 50% of them having the *trans* configuration. They have reported also the presence of 10-hydroxystearic acid, in small amounts in normal material and in large amounts in the free fatty acids derived from the faeces of patients with steatorrhoea.

Losses of lower fatty acids certainly occurred during the separation procedures used, because of volatility under reduced pressure and appreciable solubility in water. Special techniques must be employed to ensure complete recovery of these acids.

### *Glycerides and phospholipids*

*Mono- and di-glycerides.* The procedure of silicic acid fractionation provided evidence of mono- and di-glycerides, as well as triglycerides. The presence of partial esters in faeces may be expected because of their occurrence in human intestinal contents, as shown by the studies of Frazer and his colleagues (Frazer, 1952) and of other workers (Kuhrt, Welch, Blum, Perry, Weber & Nasset, 1952). The partial esters may be derived in part from the lipolytic action of bacteria in the large intestine.

*Triglycerides.* Triglycerides containing one or more hydroxy-acid radicals are likely to have polar properties similar to those of mono- and di-glycerides, and may therefore be present in small amounts in fractions (W) and (V) (cf. James *et al.* 1961).

The fatty acids of our triglyceride and other fractions have not as yet been submitted to a complete analysis by gas–liquid chromatography, but preliminary investigations indicate that the triglycerides are relatively unsaturated in contrast to the free and soap fatty acids. It is generally accepted that unsaturated fats are absorbed more efficiently in man than are saturated fats. Our finding that faecal triglycerides are

relatively unsaturated seems to suggest that they are predominantly endogenous in origin, products of synthesis in the body or in the large intestine. It has also been observed (Wood, 1961, unpublished) that, in a single subject consuming a normal diet supplemented with 90 g/day of maize oil, the faecal triglycerides were not increased in amount or degree of unsaturation compared with those excreted on the normal diet alone.

*Phospholipids.* The bulk of the phospholipid present in the lipid soluble in light petroleum (H) was removed at an early stage; although separation of phospholipids could probably be deferred until the main silicic acid separation of lipids, when they would appear in Fraction (X). However, retention of part of the total phospholipids might then occur during removal of free acids by ion-exchange chromatography.

Although the components of Fraction (O) have not been identified, their phospholipid nature has been indicated by several tests, including determination of their content of phosphorus, nitrogen and fatty acids, and their retention within a rubber membrane during dialysis in light petroleum solution.

It may be noted that the amount of faecal phospholipid isolated in the various experiments was quite appreciable (0·61% of the faecal total solids) in comparison with, for example, that of triglycerides (1·2%).

### *Sterols and sterol esters*

*Free sterols.* The heterogeneity of the free sterol fraction has already been noted. Separation of coprostanol from unsaturated sterols was achieved by chromatography on silicic acid, which is in agreement with the results of Coleman, Wells & Baumann (1956) who used this adsorbent to separate coprostanol from a zone containing cholesterol, $\Delta^7$-cholestenol and 7-dehydrocholesterol.

The sterols were not completely recovered by precipitation as digitonides and washing, which provides further evidence of the difficulty of measuring accurately faecal sterols by the Sperry & Webb (1950) or allied procedures. Recent work has drawn attention to these difficulties (Wells & Mores, 1961; Aylward & Wills, 1962) and it appears that high-temperature gas–liquid chromatography may prove the best method for resolution and determination of these mixtures (cf. Beerthuis & Recourt, 1960; Wilson, 1961).

In the experiment reported (Table 2) the amount of free sterol (2·7% of the faecal solids) was exceeded only by those of free fatty acids (5·5%) and soap fatty acids (7·7%), and represented over 20% of the fraction soluble in light petroleum.

It seems probable that part of the unsaponifiable component of the mono- and di-glyceride regions may consist of more-polar sterols than those eluted as Fraction (U) (e.g. steroid diols and triols). Such compounds have been found by Cook *et al.* (1956) in the unsaponifiable matter of human faeces.

*Sterol esters.* The true sterol ester content of the region (S) varied in different samples from 26 to 56%. Clearly, with faecal lipids, it is not possible to assume that the material eluted in this region from silicic acid columns is entirely sterol ester, although it may be so with simpler mixtures, such as serum lipids. This point has

been made by Lovern (1956). Long-chain wax esters have been found in this region, and may be conveniently removed by precipitation with urea.

In the sample described in Table 2 the sterol present as esters accounted for only 0·26% of the faecal solids. Thus 9% of the total sterol in this sample (2·96% of the faecal solids) was present in the esterified form (cf. Aylward & Wills, 1962).

In most experimental work the presence of sterol esters has been deduced by subtracting the amount of free sterol from that of total sterol, but recently the esters have been isolated (Schön, 1959; Aylward & Wills, 1961).

The origin of faecal sterol esters is not entirely clear; they may come partly from the diet, and recently cholesterol esters have been found in human bile (Phillips, 1960).

### *Long-chain alcohols and hydrocarbons*

*Long-chain alcohols.* We have found long-chain alcohols in the combined state as esters in the sterol-ester region (S), and as the free alcohols in the triglyceride region (T). Cook *et al.* (1956) have found higher aliphatic alcohols in the unsaponifiable matter of human faeces; there was no evidence to show whether they were present originally in the free or combined state. The long-chain esters and the free alcohols may be derived from dietary or bacterial sources or both.

*Hydrocarbons.* Fraction (R) (0·96% of the total faecal solids) consisted of about 20% waxy, solid, straight-chain hydrocarbons and about 80% of liquid, cyclic and branched-chain hydrocarbons showing many properties, such as infrared absorption, corresponding to those of liquid paraffin (BP), but differing from it in giving a weak, positive Liebermann–Burchard reaction. Similar fractions were found in all faecal samples examined (range 0·20–0·96% of the faecal solids). Riddell & Cook (1955) and Cook *et al.* (1956) have reported the presence in rat and human faeces of hydrocarbons with high carbon : hydrogen ratios, for which a positive Liebermann–Burchard reaction was recorded. In view of the considerable number of steroidal substances known to occur in human faeces, the presence of small amounts of steroidal hydrocarbons would not be altogether surprising. For example, $\Delta^{3,5}$ cholestadiene gives a positive Liebermann–Burchard reaction (Brew, Dore, Benedict, Potter & Sipos, 1959) and would provide an explanation for the iodine absorption of the cyclic fraction.

Errors in faecal lipid analysis (and in particular an abnormally high percentage of unsaponifiable material) have sometimes been traced (*a*) to contamination of solvents or extraction of grease from apparatus, or (*b*) to the presence in faeces of ingested medicinal paraffin or the contamination of the samples by externally applied liquid paraffin or vaseline (see, for example, Holt, Tidwell, Kirk, Cross & Neale, 1935). Exhaustive checks were carried out to show that technical errors arising from (*a*) did not occur in our work, and (*b*) was also ruled out.

It would appear therefore that the hydrocarbons must arise (i) from metabolic (and bacterial) synthesis or degradation—which could account for the unsaturation of the liquid fraction and the weak positive Liebermann–Burchard reaction, or (ii) from material present in the diet.

Small quantities of straight-chain hydrocarbons are known to be present in vegetable

foods (Waldron, Gowers, Chibnall & Piper, 1961) and it has been demonstrated that processed foods may contain trace amounts of paraffins (Cookson, Coppock & Schnurmann, 1953). The amounts of hydrocarbons excreted were significant in all our samples; nevertheless, calculations based on the assumptions that all the hydrocarbon was derived from food, and that none was absorbed, suggested a mean concentration in the diet of the subjects below that of the legal limit for mineral oil in food (O'Keefe, 1956).

### *Bile acids*

Various experiments were carried out on extraction procedures for bile acids, and we are satisfied that the techniques described concentrate the bulk of the bile acids into Fraction (M). However, 17% of the free bile acids dissolved in the light petroleum extract (H), and were recovered by fractionation of the free acids (Q). In addition, bile-acid soaps may be present in Fractions (B) and (G), and the small proportion of conjugated bile acids found in faeces (Bergström & Norman, 1953) may concentrate in Fraction (L). Further investigations of these bile-acid fractions are being made in view of the comment by Bergström (1961) that in much previous work analysis for total bile acids has apparently accounted for only part of this fraction.

Bile acids reported as present in human faeces are deoxycholic acid (estimated as two-thirds of the total by Frantz & Carey, 1957), cholic acid (Jenke & Bandow, 1937) and lithocholic, isolithocholic and 12-ketolithocholic acids (Heftmann, Weiss, Miller & Mosettig, 1959).

The assessment of bile-acid excretion has recently assumed importance in relation to sterol balance studies, as these acids are thought by some workers (Gordon, Lewis, Eales & Brock, 1957; Bergström, 1959) to be the major product of cholesterol degradation for subsequent elimination. From the results obtained, the amount of bile acids (1·1% of the faecal solids) corresponds to a daily excretion of 270–480 mg, if a daily excretion of 25–45 g solids be assumed. This range is in fair agreement with results from several other laboratories (Lewis, 1957; Haust & Beveridge, 1958; Curran, Azarnoff & Bolinger, 1959), although Bergström (1959) suggests a figure of 1 g per day.


## SUMMARY

1. A technique is described for the extraction and fractionation of the lipids and associated compounds in human faeces, the three main stages being: (*a*) extraction with ethanol–diethyl ether and partition of the extract between 15% (v/v) ethanol in water and light petroleum, (*b*) fractionation of the 15% ethanol extract, and (*c*) fractionation of the light-petroleum extract by silicic acid and ion-exchange chromatography.

2. The main lipid classes isolated and characterized were soaps and fatty acids, sterols and sterol esters, long-chain waxes and alcohols, hydrocarbons and bile acids. There was evidence for the presence of mono-, di- and tri-glycerides, and several pigments were noted.

3. The qualitative and quantitative results revealed the complexity of the pattern

of faecal lipids; the distribution was similar in the different samples from normal subjects examined.

4. The complexity of the pattern is attributable to the fact that faecal lipids can be derived from several sources. These include unabsorbed food residues, material derived from absorbed dietary lipids subsequently eliminated through bile or intestinal secretions, compounds produced by biosynthesis and then excreted, products of bacterial biosynthesis and the results of bacterial modification of intestinal lipids.

5. The possible origins of the different lipid fractions are discussed. Further evidence was obtained that sterol esters as well as free sterols occur in human faeces; attention is drawn to the relatively high excretion of hydrocarbons derived in whole or part from the diet; the highly unsaturated nature of faecal triglycerides was noted.

6. The results indicate the deficiencies of classical methods used for routine work in nutritional and metabolic studies on faecal lipids and the desirability of new, relatively simple techniques suitable for routine use.

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