Lipid excretion

3.* Examination of faecal lipids of rats injected intravenously with serum lipoprotein containing ^{14}C-labelled cholesterol†

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The detailed fractionation achieved in our work on unsaponified human faecal lipids (Aylward & Wood, 1962; Wood & Aylward, 1962) encouraged us to combine refined chemical methods with tracer techniques in order to study the faecal excretion of serum cholesterol. In view of the possible hazards of using ^{14}C-labelled cholesterol in man, the rat was chosen for the experiments.

The chemistry of rat faecal lipids has been investigated by Riddell & Cook (1955), who saponified the total lipids and examined separately the unsaponifiable and acidic fractions. In our work we used unsaponified lipids in order to preserve ester structures (e.g. sterol esters) in the material, but have been able to confirm many of the findings of Riddell & Cook with regard to the unsaponifiable components.

Our radio-tracer methods were based on those of Lewis, Pilkington & Hodd (1961), who prepared rat serum lipoprotein containing labelled free and ester cholesterol derived from rats given a diet in which soya-bean oil or butter was the predominant fat; the main aim of the work to be described was to apply the chemical fractionation procedures to rat faeces as a means of following in detail the distribution of active lipids.

EXPERIMENTAL AND RESULTS

Methods

Silicic acid chromatography. Water-jacketed columns (25°) of 15 mm internal diameter containing 18 g 325-mesh silicic acid (Bio-Rad Laboratories, Richmond, California) were prepared as described by Hirsch & Ahrens (1958). Usually not more than 300 mg of lipid were applied to the columns, which were subjected to slight nitrogen pressure. Solvents were removed from fractions under a stream of oxygen-free nitrogen, and samples were stored at $-35°$ under nitrogen.

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Ion-exchange chromatography. Columns of the highly basic resin De-Acidite FF (SRA 71; Permutit Co. Ltd) were prepared in the hydroxyl form and washed with carbon dioxide-free water followed by diethyl ether saturated with water. Samples containing free acids were applied to the column in diethyl ether and eluted with diethyl ether saturated with water. Solvent blanks were always run, but solid dissolved from the resin by wet diethyl ether was usually insignificant in amount. Lipids emerging from the column always showed quantitative removal of acids as judged by titration and from experience with known mixtures. Elution of the column with 10% (v/v) acetic acid in diethyl ether resulted in recovery of 75–85% of the acids, and in our investigation this recovered material has been considered as representative of the acids as a whole. Recently Dole (1961) has described a procedure in which almost complete recovery of acids from a basic column was obtained.

Saponification of esters. A method similar to that of Benedict (1960) was used. Samples were refluxed for 3 h (12 h for sterol esters and long-chain waxes) with 2N-KOH in 95% (v/v) ethanol under nitrogen. The ethanol was evaporated under nitrogen, the residue dissolved in water and then acidified, and total fatty acids and unsaponifiable material were extracted with diethyl ether and weighed. Fatty acids and unsaponifiable material were then separated on a De-Acidite FF column and recovered.

Urea adduction. Straight-chain compounds were separated from branched or cyclic structures by dissolving the mixture in hot methanol (with addition of benzene, if necessary), saturating the solution with urea and cooling to 0° with stirring. The precipitate was filtered off and lipid isolated from adduct and filtrate by treatment with water and extraction with light petroleum (b.p. 40–60°).

Digitonin precipitation of sterols. The method of Cook (1958) was followed in order to obtain samples of sterols free from non-precipitable material. It was appreciated that the recovery of faecal sterols may be incomplete in this procedure (Wells & Mores, 1961).

Preparation of rat serum lipoprotein containing ^{14}C-*cholesterol.* Male Wistar rats (no. 1 strain; weight 150–200 g) were fed on Oxoid 41 diet (Oxo Ltd, London, E.C. 4) containing 25% (w/w) soya-bean oil or 25% (w/w) butter and after 10 days were injected intraperitoneally with 9·5 μc each of DL-2-^{14}C-mevalonate (Radiochemical Centre, Amersham, Bucks.). Serum lipoprotein containing free and esterified cholesterol labelled with ^{14}C was isolated by high-speed centrifugation and dialysis (Lewis *et al.* 1961).

Injection of rats with labelled lipoprotein and collection of faeces. Other groups of male Wistar rats (no. 1 strain; weight 150–200 g) were given similar soya-bean oil (group R1) or butter (group R2) diets; after 11 days they were transferred to unmodified Oxoid diet 41 (about 2·5 g fat and 0·12 g total sterol/100 g). One day later they were injected through the tail vein with 0·5 ml of either the labelled 'oil-lipoprotein' or 'butter–lipoprotein' preparation.

Faeces were collected twice each day for 10 days and stored under light petroleum at $-35°$; the results presented here refer to the composition and activity of only the first 2 days' collection from six rats in each group. Faeces were also collected from

rats maintained continuously on unmodified Oxoid 41 diet and not injected with labelled lipoprotein (low-fat diet, group R3).

Counting of ^{14}C. Samples of fractions (5 mg) were placed on aluminium planchets and the more active materials counted for 3000–5000 total counts on a windowless proportional gas-flow counter (Tracerlab Inc., Waltham, Massachusetts). The level of activity present was very low, but with careful allowance for background activity satisfactory results could be obtained, as judged by variation between successive counts of the same sample and recovery of activity during fractionation procedures. For the count rates observed (1·5–2·2 times background count; about 18–28 total counts/min) and counting for 3 or 4 h, the probable error of the determinations was about 5 % (Francis, Mulligan & Wormall, 1954).

Chemical separation

Chemical separation of the faecal lipids, based on our previous experience with human faeces (Aylward & Wood, 1962), was directed towards the quantitative separation of each class of lipid as far as possible free from contamination with other classes of compound. Further fractionation within one class (e.g. of fatty acids by gas–liquid chromatography) would then be possible. Fig. 1 shows in outline the steps involved in the fractionation of faeces from rats given soya-bean oil (R1), the fractions obtained, and their weights expressed in two ways: (*a*) as a percentage of the total faecal solids, and (*b*) as mg excreted/rat-day, averaged over the 12 rat-day sample. The total weight of fractions A1 and A2 was considered as the faecal total solids.

Extraction and fractionation of insoluble residue, A1. Acid hydrolysis and light-petroleum extraction of fraction A1 gave only a very small amount of material (B2). Further extraction with diethyl ether gave a fraction (C2) that had a mean equivalent weight (titration) of 440, and a final residue (C1).

Fractionation of lipid insoluble in light petroleum, B3. Solvents were removed from the primary lipid extract (A2), and the residue was treated with 15 % (v/v) ethanol in water (5 vol.) and shaken five times with light petroleum (5 × 1 vol.) in a separatory funnel to give petroleum-soluble lipids (B4) and a brown aqueous-ethanol suspension (B3). A small, insoluble fraction (C3) was obtained when the aqueous-ethanol suspension was made alkaline with sodium carbonate and centrifuged; this fraction would contain any calcium soaps present in the primary lipid extract, but in fact no ash was present. The material was soluble in diethyl ether and contained no acids, but tests for sterols were positive.

The clear alkaline solution was acidified, and a crude bile-acid fraction (D2) was extracted with diethyl ether. Further extraction of the aqueous solution with *n*-butanol yielded a clear, dark-brown solid (E2), probably containing conjugated bile acids and the pigment stercobilin. It was soluble in alkali and had a mean equivalent weight of 506 and a nitrogen content of 4·6 %. The final aqueous solution (E1) still contained a considerable proportion of the primary lipid extract (A2), and probably consisted largely of inorganic salts. It was not further investigated.

The crude bile-acid fraction (D2) was purified by removal on a basic ion-exchange column of non-acidic components (E4), which gave tests for sterols. Further fractiona-

tion of the acids (E3) on a silicic acid column indicated the presence of a small amount of fatty acids (F2). The bile acids were eluted in three fractions (collectively, F1) as shown in Fig. 2*A*, and materials from these peaks had carbon and hydrogen contents, mean equivalent weights and infrared (i.r.) spectrums consistent with their assignment as bile acids of increasing polarity. The i.r. spectrum of the material of intermediate polarity (as methyl esters) showed a split carbonyl peak, suggesting the presence of keto bile acids (absorption at 5·75 μ due to ester groups and at 5·8 μ due to ring keto groups).

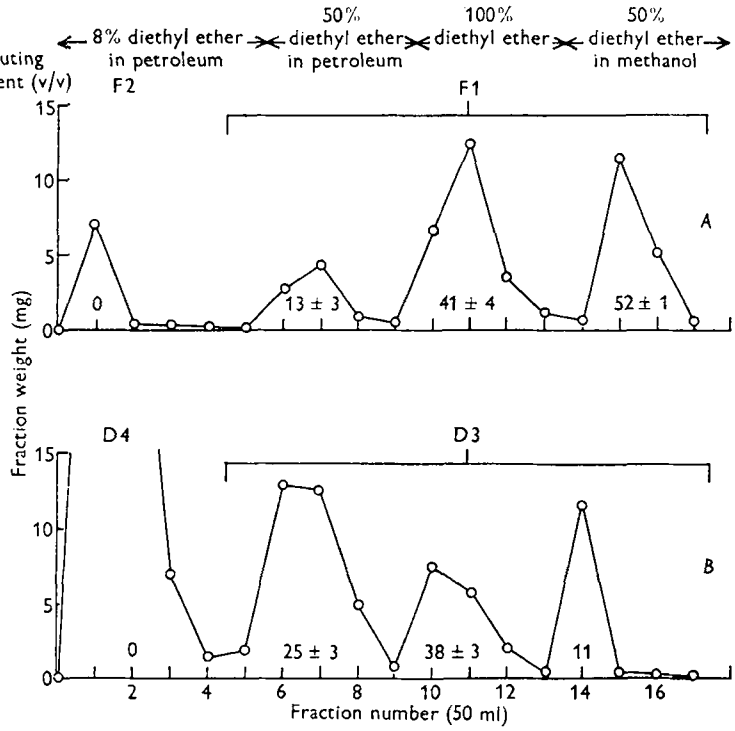

Fig. 2. Silicic acid fractionation of crude bile-acid fraction E3 (*A*) and of crude free fatty acid fraction C5 (*B*) from faeces of oil-fed rats, R1. Loads, 55 mg (*A*), 400 mg (*B*); recovered, 58 mg (*A*), 398 mg (*B*). Silicic acid: 18 g Bio-Rad 325 mesh, heated at 115° overnight. Fractions: 50 ml. Fraction symbols refer to Fig. 1. Figures within peaks show the mean ^{14}C specific activity of material from each region in counts/1000 sec mg ± SD.

Fractionation of lipid soluble in light petroleum, B4. Acidic and neutral components were separated on a De-Acidite FF column. The acids (C5) were fractionated on silicic acid (Fig. 2*B*) to yield a major region consisting of saturated and unsaturated fatty acids (D4) and three minor regions (collectively, D3). Of the minor regions, the first two eluted apparently contained bile acids as judged by mean equivalent weights, analysis for elements, and i.r. absorption; material from the third region contained phosphorus and nitrogen, probably owing to the retention on the basic resin of a small proportion of the total phospholipids in B4 (cf. Lea, Rhodes & Stoll, 1955). However, 90% of the phosphorus present in B4 was eluted from the resin but

was retained on silicic acid chromatography of the non-acidic fraction (C6) with 2% (v/v) methanol in chloroform, giving phospholipid-free neutral lipids (D6).

The phospholipid fraction (D5) was eluted from silicic acid with 50% (v/v) chloroform in methanol, and contained 1·1% P. On saponification it gave fatty acids and an unsaponifiable fraction soluble in diethyl ether and containing 5·1% N, and 0·04% P, suggesting the presence of sphingosine or allied bases (sphingosine, 4·7% N).

Fractionation of neutral lipids, D6. The neutral lipids contained only traces of N, and no P, ash or uncombined acids. Silicic acid chromatography yielded eight fractions, five of which were further subdivided. Fig. 3 shows a typical elution curve, obtained with inactive faecal lipids from normal rats maintained on Oxoid diet 41 (R3). The

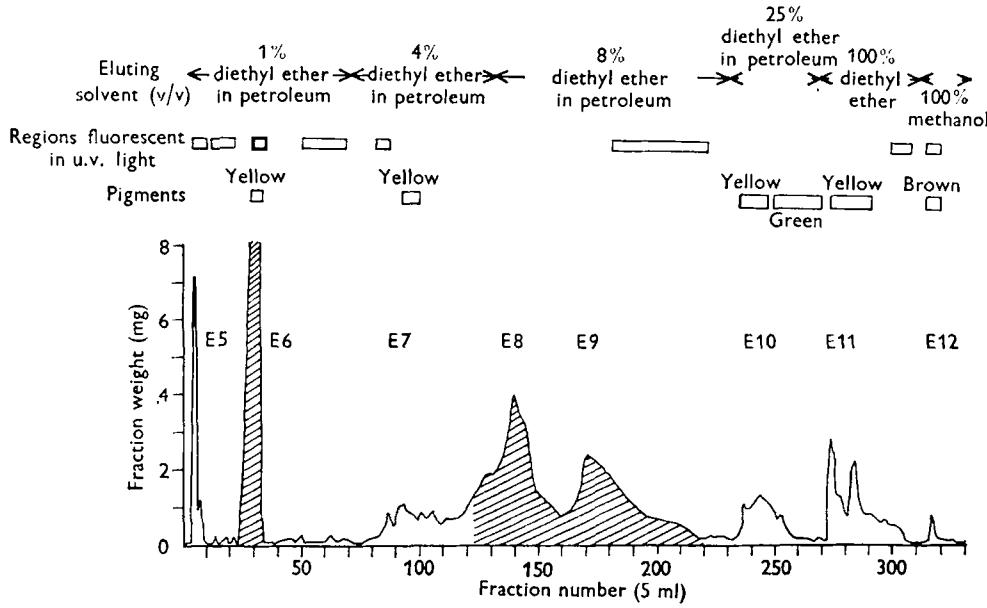

Fig. 3. Silicic acid fractionation of neutral lipids (D6) from faeces of normal rats maintained on Oxoid diet 41 (rat group R3; low-fat diet). Load, 291 mg; recovered, 295 mg. Silicic acid: 18 g Bio-Rad 325 mesh, heated at 115° overnight. Fractions: 5 ml. Elution of pigments and regions fluorescent in u.v. light is shown, together with the composition of eluting solvents. Fractions giving a strongly positive Liebermann–Burchard reaction are shaded. Fraction symbols refer to Fig. 1.

same sequence of eluting solvents was employed to obtain the results shown in Fig. 1, and a similar distribution of coloured bands, regions fluorescent in u.v. light and fractions showing a strong positive Liebermann–Burchard reaction was observed. As noted in work on human faecal lipids (Aylward & Wood, 1962), the movement of coloured and fluorescent bands provided a good indication of the progress of elution of the lipid regions.

Fractionation of hydrocarbon region, E5. Colourless material was obtained that yielded on urea fractionation a white wax (F4, straight-chain hydrocarbons) and a colourless oil (F3, cyclic and branched paraffins). Carotene was absent.

Fractionation of sterol ester region, E6. Urea fractionation removed a white solid (F6), identified by i.r. absorption and saponification as long-chain esters. The non-

adductable portion (F5) was saponified, the fatty acids were recovered (G2) and sterols were precipitated with digitonin and recovered (G1). The sterol fraction was a mixture of substances, as judged by m.p. determinations.

Fractionation of triglyceride region, E7. Under the conditions used this fraction was not well separated from the less-polar free sterols. Saponification gave fatty acids (F8) and unsaponifiable material (F7). Part of the unsaponifiable material was urea-adductable (G4); it was shown by i.r. examination (absorption at 2·78 and 9·52 μ due to hydroxyl groups, and at 13·9 μ due to long methylene chains; no absorption due to esters) to consist of long-chain alcohols. The fraction of F7 not able to precipitate with urea (G3) gave coprostanol (H1) on digitonin treatment (m.p. 101°, $[\alpha]_D$ +26° in chloroform) and also a soluble fraction (H2). H2 probably consisted of coprostanol not removed by digitonin treatment (Wells & Mores, 1961) together with ketonic material such as coprostanone, which would be concentrated in this fraction (Rosenfeld, Fukushima, Hellman & Gallagher, 1954), and perhaps additional unidentified components.

With other samples of faeces the composition of this fraction was further investigated to ascertain whether the long-chain alcohols were present initially in the free state, or combined as waxes. Urea treatment of E7 (without saponification) gave adductable material with an i.r. spectrum indicative of a mixture of long-chain esters and free long-chain alcohols (absorption at 2·78 and 9·53 μ due to hydroxyl groups; at 5·75 and 8·58 μ due to ester groups, and at 13·9 μ due to long methylene chains). The fatty acids (F8) were thus derived at least in part from long-chain esters, so that the triglyceride content of the faecal lipids must have been very low.

Less-polar free sterols, E8. The i.r. spectrum of this fraction was virtually identical with that of pure coprostanol (Rosenkrantz, Milhorat & Farber, 1952), and indicated the absence of esters. After one crystallization from methanol, the optical rotation and melting point of this material were also correct for coprostanol ($[\alpha]_D$ +28° in chloroform, m.p. 100–101°).

More-polar free sterols, E9. This region was incompletely separated from the previous one, and contained unsaturated, laevorotatory sterols giving an immediate positive Liebermann–Burchard reaction at 0°. The i.r. spectrum was similar to that of cholesterol, and indicated the presence of traces only of carbonyl compounds or esters.

Fractionation of diglyceride region, E10. Yellow and green pigments were eluted here in small amounts. Unsaponifiable components (F9) constituted about 39% of the region; a rapid Liebermann–Burchard reaction was given, and about 30% of the material could be precipitated by digitonin. The fatty acid components of the region were obtained as fraction F10.

Fractionation of monoglyceride region, E11. Yellow pigments were present. Unsaponifiable matter (F11) constituted 29% of the fraction, fatty acids (F12) 52%, and water-soluble material, presumably glycerol, made up the remainder. Fraction F11 gave an immediate purple Liebermann–Burchard test, changing to green, and about 25% of the material could be precipitated with digitonin. The material not precipitable with digitonin gave a yellow-brown Liebermann–Burchard reaction.

Phospholipid region, E12. Only a small quantity of lipid was eluted here, as would be expected after the removal of the major part of the phospholipids as fraction D5. It contained no phosphorus.

^{14}C *activity of fractions*

Fig. 1 shows for rats given oil (R1) the results of counting the separated fractions for ^{14}C activity, expressed in two ways: (*a*) the specific activity of the fraction in counts/1000 sec mg, and (*b*) the total activity excreted in the fraction per rat-day, in counts/1000 sec. Fig. 2 shows the distribution of activity during further fractionation of the crude bile-acid fraction (E3) and the acidic fraction (C5). The ^{14}C activity found in various fractions from faeces of the rats given butter (R2) is recorded in Tables 3 and 4.

The total activity injected into six rats in the labelled lipoprotein was about 274000 counts/1000 sec for the rats given oil and about 282000 counts/1000 sec for the rats given butter.

Table 1. *Amounts and relationships of total solids, primary lipid extract and lipid soluble in light petroleum in faeces of rats on different diets*

	Weight of fraction excreted								
	g/100 g total solids			g/100 g primary lipid extract			mg/rat-day		
Fraction*	R1	R2	R3	R1	R2	R3	R1	R2	R3
Total solids (A1 + A2)	100	100	100	—	—	—	1847	1565	1720
Primary lipid extract (A2)	15·3	19·1	13·0	100	100	100	282	298	223
Lipid soluble in light petroleum (B4)	4·5	4·3	5·2	29·3	22·3	40·2	82	67	90

R1: six rats maintained on 25% soya-bean oil diet (11 days) followed by low-fat diet (1 day); faeces then collected for 2 days.

R2: six rats maintained on 25% butter diet (11 days) followed by low-fat diet (1 day); faeces then collected for 2 days.

R3: six rats maintained on low-fat diet only; faeces collected for 4 days.

* See Fig. 1.

DISCUSSION

Pattern of lipid distribution

The amounts excreted per rat-day of faecal total solids, primary lipid and lipid soluble in light petroleum, and the relationships between these values for three groups of rats are shown in Table 1. Results for rats maintained on a low-fat diet of unmodified Oxoid 41 (R3) are compared with those for rats fed on a 25% butter diet (R2) (see p. 90) and others fed on a 25% soya-bean oil diet (R1) (see Figs. 1 and 2 for detailed fractionation). The excretion of total solids by the three groups suggests that roughly similar amounts of the diets were ingested by each group. The amount of petroleum-soluble lipid excreted was less in the rats on diets with 25% fat than in animals on the low-fat diet, suggesting that large amounts of unabsorbed petroleum-soluble lipids from the diet (e.g. plant sterols from soya-bean oil) did not contribute to the faecal lipids of animals on the high-fat diets. Differences in the three groups

due to unabsorbed dietary lipids have probably been eliminated, as was intended, by the 1-day period on the Oxoid 41 diet alone, immediately before collection of faeces.

Rat faeces usually had a much higher total solids content than samples of normal human faeces. Thus a typical human sample contained 29·4% total solids (Aylward & Wood, 1962), whereas faeces from rat group R3 contained 78·2% total solids. This difference may be due in part to some drying out of rat faeces between collections. In the same human sample petroleum-soluble lipid constituted 12·8% of total solids (for rats, the value is 4·3–5·2%) and 39·2% of primary lipid extract (for rats, the value is 22·3–40·2%).

Only a very small quantity of combined lipid remained in the residue after the initial extraction of rat faeces. Thus, for group R1 (Fig. 1), 15·2% of faecal total solids was removed by ethanol–diethyl ether extraction; after acid hydrolysis of the extracted residue only 0·02% total solids became soluble in light petroleum, while a further 0·27% became soluble in diethyl ether.

Table 2 gives details of daily excretion of some lipid fractions for rats on three different diets. The different lipid classes will be considered individually, first from a chemical standpoint, and then with regard to the distribution of activity.

Table 2. *Amounts of lipid fractions in daily faecal excretion of rats on high-fat (soya-bean oil or butter) and low-fat diets*

Lipid class*	Mean weight of fractions excreted (mg/rat-day) R1, oil diet	R2, butter diet	R3, low-fat diet
Free fatty acids (D4+F2)	43·9	41·4	52
Combined fatty acids (G2+F8+F10+F12+acids in phospholipid fractions D5 and E12)	9	6	ND
Free bile acids (F1+D3)	24·9	21·3	13
Coprostanol, cholesterol and sterols of similar polarity (H1+E8+E9)	11·6	9·0	13·8
Esterified sterols (G1)	1·8	1·2	1·9
Phospholipids (D5+E12)	4·5	1·9	ND
Hydrocarbons (E5)	1·4	1·1	1·2

R1, R2 and R3, see note to Table 1.
ND, not determined.
* See Fig. 1.

Soaps and fatty acids

Soaps. Only very small amounts of calcium or magnesium soaps of fatty acids were present in any of the samples of rat faeces examined. The appearance of soaps as a suspension in the light-petroleum layer during fractionation of the primary lipid extract (A2), noted during examination of human material (Aylward & Wood, 1962), was not observed, and very little lipid soluble in light petroleum resulted from acid hydrolysis of the extracted residue (A1).

Free fatty acids. This fraction amounted to 40–50 mg/rat-day, and was the major lipid class present. Fig. 1 shows that 7% of the total free fatty acid was not removed from the primary lipid extract by light petroleum, and was separated from bile acids

later (F 2). The total free fatty acids from the faeces of the rats given oil had an equivalent weight of 288, and there was i.r. spectral evidence of the presence of *trans* unsaturation (10·3 μ). The values given are low, since some loss of fatty acids of shorter chain length occurred with the methods used. Ratios of free to combined fatty acids were approximately 4·9:1 (R 1) and 6·9:1 (R 2).

Glycerides and phospholipids

Mono- and di-glycerides. Fatty acid esters were eluted from silicic acid (together with unsaponifiable material) in the mono- and di-glyceride regions of Hirsch & Ahrens (1958). Although the partial glycerides were not characterized by determination of fatty acid: glycerol ratios, it seems very probable that mono- and di-glycerides were present in fractions E 10 and E 11.

Triglycerides. It is clear from Fig. 1 that with the rats given oil the major part of the 'triglyceride' region (E 7) consisted of lipids other than triglycerides, which was also true with the group given butter. Further investigation indicated that part of the total fatty acid (F 8) of the region was combined as wax esters, so that the triglyceride content of the sample corresponded to less than 1 mg/rat-day (cf. free fatty acid excretion of 43·9 mg/rat-day).

Phospholipids. Phosphorus-containing lipids (D 5) were present in all samples examined. A small amount of material also appeared in the 'phospholipid' region E 12, but contained no P. There was some evidence for the occurrence of sphingolipids in the total phospholipid fraction, as suggested by Riddell & Cook (1955).

Sterols and sterol esters

Free sterols. Fraction E 8 consisted of coprostanol, and there was some overlapping with the triglyceride region (E 7). Glycerides, however, did not contaminate the free sterol regions. The incompletely separated sterol region E 9 (cholesterol and other unsaturated sterols) was of greater significance in the samples of rat faeces than in some human faeces we have examined (Aylward & Wood, 1962). However, for rats given diets containing different free fatty acids Wilson (1961) has found great variation in total sterol excreted, and in coprostanol: cholesterol ratio. The daily excretion of cholesterol, coprostanol and sterols of similar chromatographic behaviour, shown in Table 2, may be compared with a mean daily excretion of such sterols of 4·6 mg found by Coleman, Wells & Baumann (1956) for adult male rats on a fat-free diet, and of up to 10·5 mg found by Wilson (1961) for rats on a sterol-free diet containing free fatty acids. Our results almost certainly include unabsorbed dietary sterols, since the total dietary intake of sterols per rat-day may be estimated as 10–12 mg for the low-fat Oxoid 41 diet.

Unidentified free sterols were present in fractions C 3 and E 4. The unsaponifiable component of the diglyceride (F 9) and monoglyceride (F 11) fractions probably consisted partly of more-polar sterols, for instance diols or triols (found in rat faeces by Riddell & Cook, 1955). Consideration was given to the possibility that steroidal material eluted from silicic acid after cholesterol might consist of esters of sterols with unsaturated fatty acids, the acid components of which had become more polar by

oxidation before or during fractionation. Böttcher, Woodford, Boelsma-van Houte & van Gent (1959) have shown how readily such changes may occur in serum lipids; and sterol esters were certainly present in the rat faeces. However, examination of the unsaponifiable fractions F 9 and F 11 showed that the increased polarity of the steroidal material was a property of the nucleus, and not of an acid side-chain. Boyd & Mawer (1961) have reported the presence in rat serum and other tissues of derivatives of 7-hydroxycholesterol in which the hydroxyl group at C 3 is esterified, but these compounds were eluted from silicic acid with free cholesterol.

Sterol esters. Esterified sterols were present in all samples examined. The ratio of free sterols (excluding more-polar sterols) to esterified sterols (i.e. ratio H 1 + E 8 + E 9 : G 1) was 6·5 : 1 (R 1), 7·5 : 1 (R 2) and 7·3 : 1 (R 3). Riddell & Cook (1955) deduced the presence of esterified sterols in all rat faeces examined by them from determinations of free and total sterols, but recently Gerson, Shorland & Adams (1961) were unable to find sterol esters in rat faeces.

Long-chain esters, alcohols and hydrocarbons

Long-chain esters and alcohols. Urea-adductable esters were found in the sterol ester region of the faeces of rats given oil (R 1), but not in the faeces of rats given butter (R 2). There was evidence for the presence of both long-chain alcohols and esters in the triglyceride region (E 7). Long-chain alcohols, if present, would be expected in this region (Wren, 1960) but long-chain esters should be eluted with sterol esters unless some more-polar group is present. The origin of faecal long-chain waxes may be dietary or bacterial, and vegetable and bacterial waxes often contain hydroxy fatty acids; the elution of long-chain esters partly with sterol esters and partly with triglycerides and free long-chain alcohols may result from the presence of hydroxyl groups in some, but not all, of the long-chain ester molecules.

Hydrocarbons. All samples examined contained hydrocarbons that could be separated with urea into straight-chain (F 4) and cyclic (F 3) fractions, as has been found with human faeces (Aylward & Wood, 1962). The greater part of this material probably came from the diet, and the (unmodified) Oxoid diet 41 was found to contain 15·3 mg hydrocarbons/100 g.

Bile acids

Solvent partition of the total lipid extract (A 2) resulted in the extraction of 29 % of the total free bile acids into the light-petroleum solution. Fig. 2 indicates that the bile acids soluble in light petroleum (D 3) were predominantly compounds less polar than the major fraction of acids soluble in aqueous ethanol (F 1). Solvent fractionation of this sort may be expected in view of the considerable complexity of the total bile-acid mixture in rat faeces (Bergström, 1959).

Small amounts of conjugated bile acids that have escaped bacterial splitting of the peptide link have been found in rat faeces (Bergström & Norman, 1953) and there was evidence of the presence of such compounds in fraction E 2. The complexity of the major bile-acid fraction (E 3) was apparent from the chromatographic behaviour of the mixture (Fig. 2*A*) and from the carbon and hydrogen contents, i.r. spectrums and equivalent weights of the components. The ethyl esters of acids eluted from silicic

acid with pure diethyl ether usually showed a split carbonyl peak in their i.r. spectrums, suggesting the presence of keto bile acids. The complete recovery of this complex assembly of bile acids, free from other lipid classes, is a difficult matter (Gustafsson, Norman & Sjövall, 1960) and some previous work has been criticized by Bergström (1961) on the grounds of incomplete recovery of bile acids. In our work the major bile-acid fractions obtained (Fig. 2) were: (*a*) entirely composed of acidic material; (*b*) free from fatty acids; (*c*) largely free from acidic pigments; and (*d*) composed predominantly of bile acids as judged by equivalent weights, carbon, hydrogen and nitrogen contents and i.r. spectrums. Table 2 shows the mean daily excretion of free bile acids for the three groups of rats; further amounts of bile acid were present as conjugates in fraction E2. There was a small difference in the mean daily bile-acid excretion of the groups given oil and butter, but a large difference between rats on the 25% fat diets and rats on the low-fat diet, which suggests that 25% oil or butter in the diet stimulates the rate of bile-acid excretion compared with a low-fat diet. However, all three values were very much higher than the 5 mg bile acids suggested as the daily excretion for a 200 g rat by Bergström (1959).

The quantity of cholic acid and its metabolites excreted per day by the rat is influenced by factors other than the type of dietary fat. With a complete bile fistula, rats lost about 50 mg/day of bile acids (Bergström, 1959). In germ-free rats excretion was about 2 mg/day of conjugated bile acids, compared with about 5 mg of largely free acids in conventional rats; *Escherichia coli* alone was not responsible for the changed excretion of cholic acid (Gustafsson *et al.* 1960). However, quantity and type of dietary fat may influence bile-acid excretion as a result of changes induced in the intestinal flora.

Distribution of ^{14}C activity

Activity correlations for the fractions of very low specific activity were sometimes poor, but in spite of the generally low level of activity it was considered that a useful indication of the distribution of ^{14}C derived from the injected lipoprotein cholesterol could be obtained with the more active fractions. Sixteen terminal fractions were derived from fraction D6 (Fig. 1) and, of these, eight or nine contained activity. The similar distribution of activity in the fractions from faeces of animals given oil or butter, and the overall recovery of activity are shown in Table 3. Fraction E8 consisted of essentially pure coprostanol, whereas H1 contained sterol overlapping from the free sterol region into the triglyceride region, and also consisted of coprostanol. The good agreement between the specific activities of these two fractions in both groups of rats is apparent.

Fatty acids, long-chain esters, long-chain alcohols, hydrocarbons and phospholipids

Activity was either not detected or was of a very low order in all these fractions, as would be expected since they are unlikely to be derived from lipoprotein cholesterol.

Fatty acids. No activity was detected in the purified free fatty acid fractions, in agreement with earlier work (e.g. Siperstein, Jayko, Chaikoff & Dauben, 1952). Cook, Edwards, Riddell & Thomson (1955) have suggested that cholesterol may be converted in the rat into fatty acids and excreted in the faeces. In our experiments

faecal free fatty acids were not derived to any significant extent from serum cholesterol.

Hydrocarbons. It seems possible that the low level of activity found in the cyclic hydrocarbons (F3) may indicate the presence of some hydrocarbon material derived from cholesterol. The major part of this fraction was almost certainly derived from the Oxoid diet, which contains traces of liquid paraffin; however, a weak positive Liebermann–Burchard reaction was given by the faecal material, as has been observed

Table 3. *Distribution of ^{14}C activity in fractions derived from D6 (neutral lipids) in faeces of rats given oil (R1) or butter (R2), and overall recovery of activity*

Fraction*		Specific activity of fraction (counts/1000 sec mg)		Activity excreted in fraction (counts/1000 sec rat-day)	
		R1, oil diet	R2, butter diet	R1, oil diet	R2, butter diet
D6		18	19	531	406
E8	Free sterol fractions	36	40	198	172
E9		28	31	126	105
H1		34	40	54	52
F11	Unsaponifiable from monoglyceride region	11	11	20	18
G1	Sterols from sterol esters	10	14	18	17
H2	Component of triglyceride region	14	10	11	16
F9	Unsaponifiable from diglyceride region	6	11	4	7
F3	Cyclic hydrocarbons	4	4	4	3
F6	Long-chain esters	3	Absent	1	Absent
Total activity in fractions derived from D6		—	—	436	390

R1, R2, see note to Table 1.
* See Fig. 1.

for hydrocarbons from rat faeces (Riddell & Cook, 1955) and from human faeces (Cook, Edwards & Riddell, 1956; Aylward & Wood, 1962). Liquid paraffin (BP) does not give a Liebermann–Burchard reaction. Squalene, if present, would be found in F3 and, in view of the biosynthetic relationship of this hydrocarbon to mevalonate and cholesterol, its presence might account for the weak activity of this fraction. The hydrocarbon fraction of the injected lipoprotein contained only traces of activity.

Free sterols and sterol esters

Free sterols. Sterol activity was largely concentrated in the coprostanol and cholesterol regions (E8, E9 and H1). Unequal dietary dilution of activity by inactive sterols probably explains the higher specific activity of E8 compared with E9. Sterol activity was also present in H2 (which may have contained ketonic material), C3 and E4. The nature of C3 and E4 was not determined, but in the absence of other criteria activity present in these non-acidic fractions was assumed to be due to sterols.

The specific activity of the unsaponifiable fractions F9 and F11 was low relative to that of the major sterol fractions. Components derived from lipoprotein cholesterol were apparently present in both fractions, probably diluted with inactive material of similar polarity. The precipitation of only 25–30% of these fractions with digitonin

suggests the presence of active, polar sterols considerably diluted with inactive, non-precipitable substances of bacterial or dietary origin. Unfortunately the activity of the digitonides was not determined.

Sterol esters. Activity was present in the sterols isolated from sterol esters (G1). Of the total activity excreted as sterols (excluding more-polar sterols), 4·6% (R1) and 4·9% (R2) was present in sterol esters. The active esters may have originated from cholesterol esters present in bile, but clearly their excretion represented only a minor pathway for elimination of serum cholesterol.

The specific activity of the sterols present as esters was only 28% (oil diet) and 35% (butter diet) of that of the free coprostanol (E8). These results are interpreted as indicating a large dilution of active cholesterol esters with sterol esters from the diet, and this conclusion seems valid even if the active sterol entered the intestine in the free state and became esterified by bacterial action. Other evidence supports the concept of dietary dilution of endogenous cholesterol esters: (*a*) the sterol region G1 was found to be a mixture; (*b*) dietary components, for example vegetable oils, contain sterol esters; and (*c*) sterol esters are relatively difficult to hydrolyse in vitro, and hence may tend to escape hydrolysis during passage through the alimentary tract.

It thus seems probable that a considerable proportion of the esterified sterol isolated recently from human faecal lipids (Schön, 1959; Aylward & Wills, 1961) may be regarded as an unabsorbed dietary component.

Bile acids

The highest specific activities found in the fractionation of faecal lipids were those of the bile-acid fractions. The question of dietary dilution does not arise here, but it may be noted that cholesterol biosynthesized from [2-^{14}C]mevalonate contains five labelled carbon atoms per molecule, of which one is lost (as carbon dioxide) during formation of a bile-acid molecule (Cornforth, Cornforth, Popják & Gore, 1958).

The finding of 16% (rats given oil) and 24% (rats given butter) of the total bile-acid activity in fraction E2 supported the chemical evidence that this fraction contained conjugated bile acids. Fractionation of the major free bile-acid region (E3; specific activity 38 counts/1000 sec mg) on silicic acid yielded a small amount of inactive fatty acid, and three bile-acid fractions of specific activity 13, 41 and 52 counts/1000 sec mg (Fig. 2*A*). The fraction with the highest specific activity probably contained predominantly cholic acid; the other fractions probably contained less-highly hydroxylated acids and various keto derivatives formed by bacterial action in the intestine and partially reabsorbed through the enterohepatic circulation (Bergström, 1959). Since this circulation is a relatively slow process, and the samples examined by us were collected during the first 2 days after intravenous injection of labelled lipoprotein, it seems reasonable that the newly formed cholic acid should have the highest specific activity, whereas the less-polar, bacterially modified acids might at this stage have received a relatively smaller contribution of labelled material, and thus have lower specific activities.

The ratio of bile acid ^{14}C activity to sterol activity

Addition of all ^{14}C activity present in bile-acid fractions and in sterol fractions for the two groups of rats (R 1 and R 2) gave the results shown in Table 4.

Table 4. *^{14}C activity excreted in bile acids and sterols by six rats given oil (R 1) or butter (R 2), and total recovery of injected lipoprotein activity in first 2 days' collection of faeces*

	R 1, oil diet	R 2, butter diet
Total lipoprotein activity injected (counts/1000 sec)	274000	282000
Activity recovered in faecal bile acids, days 1 and 2 (counts/1000 sec)	14340	13400
Activity recovered in faecal sterols, days 1 and 2 (counts/1000 sec)	6640	6330
Ratio, activity recovered in faeces as bile acids:activity recovered as sterols	2·16:1	2·12:1
Total activity recovered in faeces, days 1 and 2 (counts/1000 sec)	20980	19730
Total activity recovered in faeces in first 2 days after injection (%)	7·66	7·00

In both groups rather more than twice as much activity was excreted in the form of bile acids as was excreted in the form of sterols, and about 7 % of injected activity was recovered in each group in 2 days. The question of the relative importance of faecal elimination of bile acids and sterols in the rat as pathways of cholesterol excretion has been subject to considerable discussion. Our results with labelled lipoprotein indicate that bile-acid excretion is the major pathway under the experimental conditions used, and chemical separation of faecal lipids also shows the excretion of larger quantities of bile acids than sterols (Table 2); further, part of the sterol excretion is almost certainly due to unabsorbed dietary sterols. It must be emphasized, however, that we have dealt only with the faeces obtained during the 2 days after intravenous injection of labelled lipoprotein and that other trends may become apparent (e.g. in the relative excretion of activity in rats given oil compared with rats given butter) on examination of faecal lipids excreted over longer periods.

SUMMARY

1. Detailed fractionation procedures have been applied to faeces from rats and have been combined with ^{14}C tracer studies.

2. Faeces from three groups of rats have been examined: one group was maintained on a diet with 2·5 % fat ('low-fat' diet), and the faeces were not active; two groups were maintained on diets with 25 % fat ('high-fat' diets) containing either soya-bean oil or butter, and the faeces were active.

3. The diet of the rats given oil was changed to a low-fat diet and 1 day later the animals were injected intravenously with serum lipoprotein derived from other rats given the 25 % oil diet and containing ^{14}C-labelled free and ester cholesterol; the

faeces examined were those collected during the 2 days after injection. A parallel procedure was employed with the rats given butter.

4. The chemical fractionation techniques used for human faeces proved successful and indicated a pattern of lipid distribution in rat faeces similar in many respects to that in human faeces. Lipids present included free fatty acids, sterols and sterol esters, long-chain waxes and alcohols, phospholipids, hydrocarbons and free and conjugated bile acids. There was evidence of the presence of mono- and di-glycerides. The rat faeces examined contained much smaller proportions of fatty acid soaps, pigments and triglycerides than found in typical human faeces.

5. The daily excretion of bile acids by rats on the high-fat diets was considerably greater than that of the rats on the low-fat diet.

6. The distribution of ^{14}C in the faecal lipid fractions from the injected rats could be satisfactorily studied in spite of the low level of activity. Activity was negligible in free or combined fatty acids, phospholipids, long-chain waxes and long-chain alcohols, but was present in all bile-acid and sterol fractions. For rats given oil and also for those given butter the ratio, activity excreted as bile acids:activity excreted as sterols was rather more than 2:1.

7. Activity was present in sterol esters and in unsaponifiable substances more polar than cholesterol, probably including steroid diols and triols. The specific activity of these fractions suggested that in each substance active material derived from serum cholesterol had been considerably diluted with substances of dietary or bacterial origin.

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