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Cholesterol Metabolism

4. THE NEUTRAL FRACTION OF THE FAECAL LIPID OF RATS FED DIETS WITH LOW AND HIGH CHOLESTEROL CONTENTS*

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In this communication an account is given of the neutral fraction or unsaponifiable matter (UM) of the extract (ether- and ethanol-soluble materials) of the faeces of rats fed diets with and without added fat (16·6%), containing either small amounts of sterols (*C* series) or with added cholesterol (1·6%; *S* series). Emphasis has been placed on the products of faecal excretion in the intact animal as an indication of the pathways of sterol degradation.

The excreted material represents the final result of a number of processes. There is the contribution by the unabsorbed lipid component of the diet—for example carotenoids and vegetable sterols—and in the case of the diets containing added cholesterol a considerable part is unabsorbed. The secretions of the intestine and the microbial population of the gut also contribute to the lipid component. A discussion of the factors is given by Cook (1952).

In calculating the effect due to the administered cholesterol it has been taken that the subtraction of the faecal material of the *C* series from that of the *S* series shows the gross changes undergone by the sterol in the entire animal. Here a number of processes again are concerned and the products include the compounds formed after absorption and excreted either via the bile or by the intestinal mucosa, those compounds metabolized by the intestine itself and those derived from microbial action. It is impossible to separate these processes, but the work is exploratory and is intended to devise methods suitable for studying metabolic degradation products of sterols. Apart from the major sterols (cholesterol and coprostanol) there is little information on the chemical nature of the neutral fraction of faeces. It has not been possible to isolate all the compounds present, but by the use of chemical and physical methods and by paper partition chromatography good evidence has been obtained for the presence in small amounts of a number of compounds. The main methods used have been adsorption chromatography on alumina and recrystallization, together with some chemical separations. The cholesterol used in these experiments, and in former work, contains small amounts of companion sterols such as cholestanol (see Fieser, 1953*b* and Discussion, p. 669).

The feeding experiments and preparation of initial UM and acidic fractions were done in collaboration with Dr D. C. Edwards and Dr R. O. Thomson. A preliminary account has been published (Cook & Edwards, 1952).

Nomenclature. The Ciba Foundation Rules (see *J. chem. Soc.* 1951, p. 3526) are followed generally, but some trivial names are used. Cholesterol is cholest-5-en-3β-ol, lathosterol cholest-7-en-3β-ol; 7-dehydrocholesterol is cholesta-5:7 dien-3β-ol and coprostanol coprostan-3β-ol (5β-cholestan-3β-ol). The name 'stenol' is used to designate unsaturated sterols, with the prefix Δ^5- or Δ^7- to indicate double bond position; 'stanol' is used for saturated sterols. The prefix 3α- or 3β- is used to indicate those sterols that are not and those that are precipitated as digitonides by an ethanolic digitonin solution. Some abbreviations are used to describe diols, e.g. '7β-diol' is cholest-5-ene-3β:7β-diol and '24- or 25-diol' is cholest-5-ene-3β:24- (or 25) diol. The term 'phytosterols' is used generically to indicate all sterols of vegetable origin.

METHODS AND MATERIALS

Animals. The same strain of rats, method of housing, feeding and collection of excreta as used previously were employed (Cook, Polgar & Thomson, 1950). Six male rats were used in each group in each experiment. The faeces were collected daily and dried *in vacuo* at 100°.

* Part 3: Cook & Thomson (1951*a*).

Diets. The basal diet consisted of powdered animal cake (North-Eastern Agriculture Co-operative Society Ltd., Aberdeen). The basic composition of this material is (as %) starch equivalent 66, provided by various cereals; protein equivalent 16, provided by dried milk and fish meal; fat (ether and ethanol extract) 4·6, mineral matter approx. 4·5 and fibre approx. 9·5. All necessary vitamins were present.

In the low-fat diet (Expt. VII) the cholesterol (20 g.) was mixed intimately with animal cake (1 kg.). In the high-fat diets the cholesterol (20 g.) was dissolved in olive oil (200 g.) by heating on a water bath and then mixed well with the animal cake (1 kg.).

Cholesterol. Recrystallized cholesterol (Glaxo Laboratories Ltd.) was used. The iodine value was 65·3.

Olive oil. This was obtained locally and had an iodine value 82·5. More detailed analyses of the lipid of the animal cake, the cholesterol used and of the unsaponifiable matter obtained from the olive oil are given later.

Extraction of animal cake and of faeces. The extractions were made on samples of 100–200 g. in a continuous extractor, first with ether and then with ethanol. The faeces were first extracted in the pellet form, powdered and then re-extracted. Analyses were made on both ether and ethanol extracts but the reported results are for combined extracts.

Saponification, and extraction of unsaponifiable matter (UM)

The procedure used was based on that described by Hilditch (1949). Care was taken to minimize contact with air and to avoid overheating during the procedures. The extracts (10 g.) were saponified with a solution of KOH (3 g.) in ethanol (50 ml.), the solution was boiled under a reflux condenser for 3 hr. and most of the ethanol was then removed by distillation. The solution was then diluted with a large volume of water, the UM extracted in a continuous extractor with ether, washed with water, dried with Na_2SO_4 and recovered. The acids in the combined soap solution and washings were recovered by acidification and extraction with ether. The acidic fraction contained about 5% of UM (mainly cholesterol) which was recovered and added to the initially extracted material (see Edwards & Cook, 1955).

Using the mild method of saponification with large amounts of extract, the process was not complete and small amounts of esters were found in the UM. The esters recovered during the chromatographic separations were resaponified (see p. 666).

Glass joints were lubricated, when necessary, with glycerol.

Detection and estimation of sterols

Liebermann–Burchard reaction (LB test). Use has been made of this reaction (addition of acetic anhydride and conc. H_2SO_4) in testing fractions. The test may be used to detect and estimate 'fast-acting' sterols such as 7-dehydrocholesterol and lathosterol (in general Δ^7- and $\Delta^{5,7}$-stenols) and 'slow-acting' sterols such as cholesterol (or Δ^5-stenols; see Moore & Baumann, 1952). A modification of this method by Cook, Kliman & Fieser (1954) has been adopted to estimate in a mixture 'fast-acting', 'slow-acting' and 'other' sterols. The last mentioned consist of stanols such as cholestanol and 3α-stanols which give no reaction, their value being obtained by difference. Coprostanol reacts as a slow-acting sterol and is estimated with cholesterol. The 7α- and 7β-diols give a 'fast' reaction, but because of their greater polarity they may be separated from the normal fast-acting companions of cholesterol.

A sample of cholesterol prepared from the dibromide was used as standard for slow-acting sterols, and a sample of lathosterol supplied by Dr L. F. Fieser as standard for fast-acting sterols.

The test is given by certain non-sterols, e.g. squalene, which gives a brown colour (Wheatley, 1953). Unidentified hydrocarbons (not precipitable by digitonin) give a reaction similar to that obtained with fast-acting sterols.

Free and total sterols. These were determined on the initial extract at first by the method of Schoenheimer & Sperry (1934) and later by the modification of Sperry & Webb (1950).

3β-Sterols. These were estimated gravimetrically as digitonides in some experiments.

Saturated sterols (3β-stanols). These were determined by the method of Schoenheimer (1930).

Zimmermann test (Z test). This was used to detect 3-keto and 17-keto steroids (cf. Fieser & Fieser, 1949; Broadbent & Klyne, 1954).

Lifschütz test (L test). This was used to show the presence of chromogens such as the 7α- and 7β-diols. (See Bergström & Wintersteiner, 1942.)

Sulphuric acid test. The colour and fluorescence developed on the addition of conc. H_2SO_4 have been used as a general test for steroids (cf. Bernstein & Lenhard, 1953).

Iodine value. On large amounts of fractions this was determined by the method of Rosenmund & Kuhnhenn (1923). On certain samples the micro-modification of Yasuda (1931) was employed.

Acetyl value. The method of West, Hoagland & Curtis (1934) was used. The value given is as mg. of acetyl taken up/g. of lipid.

Urea adducts. Use has been made of the capacity of urea to form adducts with straight-chain organic compounds, in particular, paraffins and aliphatic alcohols (see Truter, 1951; Schlenk, 1954).

Separation of 3-ketosteroids. The reagent T (trimethylammoniumacetohydrazide chloride) of Girard & Sandulesco (1936) was used to separate 3-ketosteroids and other ketones. In general two treatments of the material were given.

Melting points are uncorrected. Combustion analyses were made by Weiler and Strauss, Oxford. Nitrogen was determined by the micro-Kjeldahl procedure.

Optical activity. This was measured on all fractions sufficiently light in colour, using 2% solutions in $CHCl_3$ with sodium light at 20°.

Spectrophotometry. A Unicam SP. 600 Spectrophotometer was used. Solvent: redistilled ethanol.

Polarography. The method of Robertson (1955) was used to detect and estimate 3-ketosteroids.

Chromatography

The major separations of the extracts were made by adsorption chromatography on alumina. The usual sequence of solvents was light petroleum (b.p. 40–60°), benzene, ether and ethanol. All solvents used were of Analytical Reagent quality and were not redistilled. Two lots of alumina were used: (*a*) Savory and Moore Ltd., London, (*b*) acid-washed alumina supplied by Merck and Co. Inc.,

Rahway, N.J. It was found that better recoveries were obtained from the Savory and Moore alumina if it was washed with acetic acid and reactivated. The activity was grade II (Brockmann & Schodder, 1941). The alumina was used in a ratio of 10 g./g. of lipid and the diameter of the column was generally 3·5 cm. The loss on the columns was 5–10%.

Paper partition chromatography of the sterols

These experiments were made in collaboration with Dr Margaret Thomson and Miss Anne Henderson.

The method used was adapted from that described by Neher & Wettstein (1952): stationary phase, phenylcellosolve (2-phenoxyethanol); mobile phase, heptane saturated with phenylcellosolve; Whatman no. 7 paper, temperature 24°. $SbCl_5$ (20%, w/v) in $CHCl_3$ is the best general reagent for detecting the sterols.

Table 1. *Paper partition chromatography of sterols*

Solvent system is described in text. I indicates immediate colour on spraying; S indicates colour develops slowly on heating.

	Double bond position	Colour with $SbCl_5$ in $CHCl_3$	Movement relative to cholesterol (5 hr. development)
	Monohydroxy compounds		
C_{27} sterols			
Cholesterol	5	Pink	1·0
Lathosterol	7	Reddish purple	0·9
7-Dehydrocholesterol	5, 7	Blue-green; I	0·7
Cholestanol	—	Pink; S	1·1
Coprostanol	—	Salmon pink; S	1·3
C_{28} sterols			
Brassicasterol	5, 22	Pink	1·0
Ergosterol	5, 7, 22	Blue-green; I	0·6
C_{29} sterols			
β-Sitosterol	5	Pink	1·0
α-Spinasterol	7, 22	Grey	0·9
Stigmasterol	5, 22	Pink	1·0
	Dihydroxy and trihydroxy compounds		Movement relative to cholest-5-ene-3β:7β-diol (20 hr. development)
Diols			
Cholest-5-ene-3β:7β-diol	5	Blue-green; I	1·0
Cholest-5-ene-3β:7α-diol	5	Blue-green; I	0·7
Cholest-4-ene-3β:6-diol	4	Faint brown	0·6
Cholest-5-ene-3β:24-diol	5	Maroon; I	0·4
Cholest-5-ene-3β:25-diol	5	Maroon; I	0·4
Triol			
Cholestane-3β:5α:6β-triol	—	Yellow-brown; S	0·5

Δ^7-Stenols and 7α- and 7β-diols gave brilliant colours immediately after spraying. Brief heating at 100° was necessary with the other sterols and prolonged heating was needed to demonstrate stanols. The development time for cholesterol and other sterols was 4–5 hr., but to separate the more polar diols and triols the time had to be increased to about 20 hr. In this time the mobile phase ran off the paper and R_F values could not be obtained. It was possible to obtain an index of the relative position of the various compounds by expressing ratios with respect to cholesterol, or after 20 hr. with respect to cholest-5-ene-3β:7β-diol.

Although pure samples of C_{27}, C_{28} and C_{29} Δ^5-stenols gave slightly different ratios, there was poor separation when they were mixed in equal amounts. Accompanying stanols may be separated from Δ^5-stenols. The components of mixtures of diols and triols are readily separated, but the method is unsatisfactory for ketones.

Test for artifacts

In view of the fact that under aerobic conditions cholesterol does undergo autoxidation, the behaviour of the Glaxo cholesterol to similar conditions as used in the extraction and saponification processes was studied.

Glaxo cholesterol (572 mg.) was refluxed for 18 hr. in ether, then for 4 hr. in ethanol; the solvents were then removed. The material was treated for 2 hr. with ethanolic KOH (10 ml.) of the same concentration and under the same conditions as used in the saponification process. The UM was recovered in the usual manner and gave a product (569 mg.) similar in appearance, m.p. and iodine value to the cholesterol initially taken. Paper chromatography of the original cholesterol and that obtained after treatment showed only one spot corresponding to cholesterol.

Additional evidence that cholesterol is virtually unaltered by the procedure used is shown by the fact that on a diet containing a minimal amount of fat and of known

composition and where there is no absorption, the sterol can be recovered from the faecal extract by using the procedures employed in this communication (Thomson, 1951). The reservation must be made that some changes may occur in the labile highly unsaturated compounds such as $\Delta^{5,7}$-stenols and hydrocarbons of the squalene type. A detailed study of their presence would require the use of special methods.

RESULTS

The diets fed, duration of experiments, the average of the initial and final total weights of the six rats in each group, total food, added fat and cholesterol ingested are shown in Table 2. The serial numbers used previously have been continued. All the animals ate the diets and increased in weight by approx. 60 g. per animal during the feeding period.

Table 2. *Duration of experiment, total weight of rats, total food, added fat and cholesterol ingested*

6 rats in each group; *C* indicates low sterol diet series; *S* indicates added sterol diet series.

Expt. no.	Duration of expt. (days)	Diet	Series	Average wt. during expt. g.	Total food (kg.)	Added fat (g.)	Added cholesterol (g.)
VII	53	Animal cake	*C*	1951	6·90	0	0
			S	1720	7·00	0	137·0
XIII	32	Animal cake + olive oil	*C*	1664	3·04	506	0
			S	1654	3·10	507	50·7
XIV	28	Animal cake + olive oil	*C*	1891	3·37	574	0
			S	2155	3·62	603	59·0

Table 3. *Analyses of the unsaponifiable matter of extracts of the animal cake, olive oil and Glaxo cholesterol*

Values as g./100 g. of original material.

	Animal cake	Olive oil	Cholesterol
UM	1·2	0·4	100
Digitonin-precipitable sterol	0·25	0·12	98
Sterols			
Fast-acting	0	~0·01	0·4
Slow-acting	0·12	—	96·6
Others	0·13	—	1·0
UM not precipitable by digitonin	0·95	0·3	2·0

The added cholesterol is the major source of sterol for the animals in the *S* series, but the animal cake and olive oil provide additional amounts of sterols. Analyses of the extract from the animal cake, the UM from the olive oil and the sterol distribution in the cholesterol fed are given in Table 3. The total animal cake and olive oil fed in each series of Expt. XIV over the experimental period may be presumed to contain approx. 2 g. of sterols of animal origin and approx. 2·5 g. of sterols of vegetable origin. The Glaxo cholesterol consists mainly of cholesterol, but some cholestanol and other sterols are present. A part (2%) is not precipitable with digitonin and may consist of 3α-sterols. Squalene is present in olive oil (Thorbjarnarson & Drummond, 1935) and presumably in small amounts in the animal cake. As it is present in both *C* and *S* series in small amounts and is probably absorbed, it may not be regarded as forming a large part of the unabsorbed UM. The nature of non-precipitable fraction is unknown but it probably consists of higher alcohols and carotenoids (see Discussion).

The weight of faeces, the faecal excretion of ether- and ethanol-extractable material, unsaponifiable matter (UM), total, free and saturated sterols are given as total weights for the several experiments in Table 4. The value now given for UM and sterol in Expt. VII is higher than that reported previously (Cook *et al.* 1950). The new value is due to the better extraction method employed.

The amount of extract increased after sterol feeding, the difference between the *S* and *C* series group in each experiment corresponding approximately with the total amount of sterol fed. The amount of UM in the extracts from the *S* series was increased, the values for *S*–*C* (as % of sterol fed) are for Expt. VII 93, for Expt. XIII 60 and for Expt. XIV 71. Digitonin-precipitable sterols formed a large proportion of the UM and were proportionately greater in Expt. VII than in Expts. XIII and XIV, where additional fat was fed. The difference in UM and in the sterol in these experiments was due to better absorption (Cook & Thomson, 1951*b*). A portion of the sterol was esterified as shown by the difference between the total and free sterol; this aspect is dealt with subsequently. The saturated sterols were increased in amount after sterol feeding and even on the low fat diet there was an increase.

Table 4. *Weight of faeces, ether and ethanol extracts, and unsaponifiable matter (UM), total, free and saturated sterols in the extracts*

All weights as g.

Expt. no.	VII		XIII		XIV	
Series	*C*	*S*	*C*	*S*	*C*	*S*
Dry wt. faeces	1886	1900	738	841	944	978
Ether–ethanol extract	124	263	58	122	106	167
UM	18·0	134·8	11·1	41·7	15·7	58·7
Total sterol	11·4	122·4	5·2	26·1	5·3	34·1
Free sterol	6·8	110·0	4·4	22·1	2·1	21·6
Saturated sterols	9·2	40·4	4·0	16·7	4·1	11·7

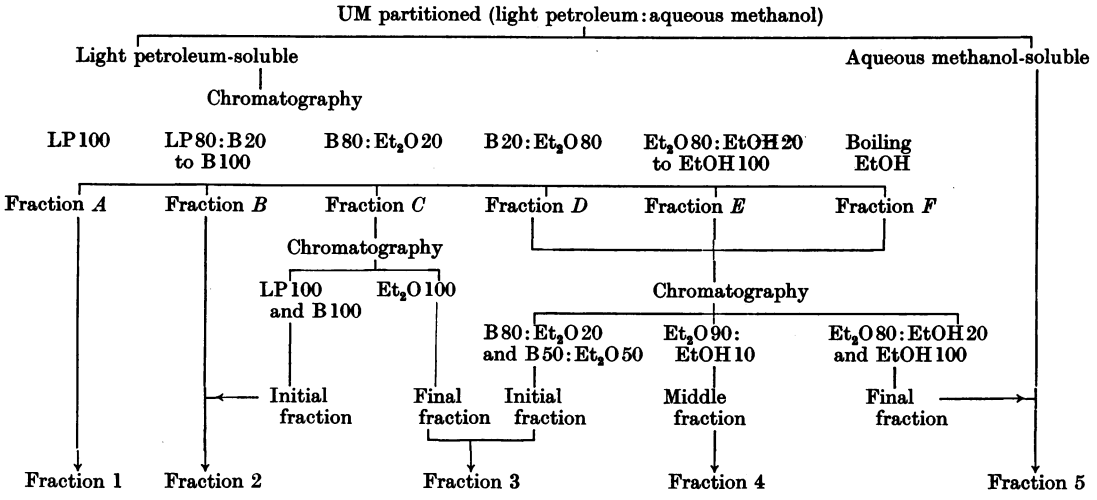

Fig. 1. Chromatographic separation used in method 1 to separate the unsaponifiable matter of the faecal lipids of rats fed diets containing small and large amounts of sterols respectively. Chromatography was on Al_2O_3; the solvent systems used are expressed as v/v mixtures. LP, light petroleum (b.p. 40–60°); B, benzene; Et_2O, ethyl ether; EtOH, ethanol.

EXPERIMENTAL

No further investigations on the UM from Expts. VII and XIII were carried out, but a more extensive study of the UM in Expt. XIV was made in an attempt to isolate the components. Two methods were used on three lots of material. In the first method approximately equal amounts of UM (approx. 10 g.) from both *C* and *S* series were studied so as to make a direct comparison of fractions. The investigations were mainly of an analytical nature and were exploratory, only a small number of components being isolated. In the second method a larger amount of UM (approx. 30 g.) from the *S* series was fractionated so as to yield material suitable as far as possible for specific identifications.

Method I

The procedure used for both *C* and *S* series is shown in Fig. 1. An initial separation was made into fraction soluble in light petroleum and fractions soluble in methanol: water (90:10 by vol.) (Haslewood, 1941). The light petroleum-soluble fractions were recovered and on chromatography gave five fractions. The weights and main properties of the fractions from the *C* and *S* series are shown in Table 5. Each fraction was investigated with the results given below.

Fraction 1. The fractions were treated with bromine in ether solution, the excess of bromine was removed by treatment with aqueous $NaHSO_3$ and the materials were recovered. Increases in weight were 5% for *C* and 23% for *S*. Light petroleum was then added and the soluble portion chromatographed on alumina. The eluates on removal of solvent gave white waxy semi-crystalline solids. The yields (as % of fraction) were 71 for *C* and 24 for *S*. The wax in both series had m.p. 38–41°. (Found: C, 86·4; H, 13·6%.) The wax is presumably a mixture of long-chain saturated hydrocarbons.

An unsuccessful attempt was made to recover the brominated compounds which were not identified. The unsaturated compounds increased after sterol feeding. Calculation of the unsaturated material by difference between the amount taken and the saturated hydrocarbon recovered showed that the *S* series contained 76% while the *C* series contained only 29%. The rapid LB test of the *S* series is noteworthy.

Fraction 2. This consisted of resinous material which in both series gave an LB test. Both fractions were treated with acetic anhydride in pyridine and gave acetyl values of 185 for *C* and 70 for *S*. The materials were recovered and chromatographed, but no crystalline fractions were obtained. The fraction is a mixture containing 3β-sterol (digitonin precipitation gave 9%), the remainder being made up presumably of higher alcohols which have a low optical activity.

Fraction 3. A positive Z test indicating the presence of ketones was obtained. The fractions were treated with Girard's reagent T and separated into ketonic and non-ketonic subfractions. The amount of ketones isolated were (as % of fraction 3) 5·4 for *C* and 5·3 for *S*. The individual ketones were not isolated, but polarography indicated the presence in both series of cholestanone with some coprostanone in the *S* series fraction. A spectrophotometric examination showed in the *S* series a compound with an absorption maximum at 234 mμ. This is possibly cholest-4-en-3-one. No similar absorption was found in the ketones from the *C* series.

The non-ketonic subfractions were yellow semi-crystalline waxes with ($[\alpha]_D$ approx. +15°). The materials were treated with benzoyl chloride in pyridine, the excess of reagents was removed, the materials were extracted with ether, recovered and chromatographed on alumina. No sharp differentiation of fractions was obtained. The materials from the benzene–ether eluates were crystalline but of indefinite melting point. The free sterols were recovered from the benzoates after saponification and crystallized from methanol. The white crystalline solids were freed from the mother liquors, which were added to fraction 5, as were the ethanol eluates from the column. The yields, properties of the crystals and sterol analyses are given in Table 6. Paper partition chromatography showed with both *C* and *S* series two spots, one corresponding to

Table 5. *Yields, and main properties of the chromatographic fractions separated from the unsaponifiable matter of the faecal lipids of rats fed a diet low in sterols (C series) and a diet high in sterol (S series)*

LB = quantitative Liebermann–Burchard reaction.

Fraction no.	Wt. (g.)	Appearance	Iodine value	$[\alpha]_D$	Sterol as % of fraction by: Digitonin	Sterol as % of fraction by: LB
		C series				
1	0·69	Pale-yellow oil	45	+2°	0	2
2	0·35	Brown-green transparent wax	65	0	9	7
3	2·96	Mustard-coloured wax	36	+22°	10	20
4	2·47	Brown crystalline solid	56	−20°	51	66
5	1·79	Brown gum	—	+8°	51	—
Loss	0·94					
Total	9·20					
		S Series				
1	0·74	Pale-yellow oil	42	+14°	1	29
2	0·91	Brown opaque wax	62	+9°	9	7
3	1·89	Semi-crystalline yellow solid	11	+27°	21	31
4	4·99	Brown crystalline solid	61	−30°	96	93
5	0·92	Brown gum	—	+8°	47	—
Loss	1·09					
Total	10·54					

Table 6. *Yields, properties and results of paper partition chromatography of crystals from fractions* 3 *and* 4

	Fraction 3 (non-ketonic)		Fraction 4	
	C	*S*	*C*	*S*
Yield (g.)	1·69	0·97	0·45	4·83
M.p. (°)	75–77	88–90	120	136
$[\alpha]_D$	+25°	+27°	−28°	−39°
Iodine value	10	22	44	65·5
Sterols (%)				
Fast-acting	0	0	13	4
Cholesterol	15*	34*	87	96
Coprostanol	75	54	0	0
Others	10	12	0	0
Paper partition chromatography				
Cholesterol (or Δ^5-stenol)	+	+	+	+ (one spot)
Other stenols	0	0	+	0
Stanols	+	+	0	0

* Calculated from iodine value.

cholesterol, the other to a saturated sterol. The fraction consisted mainly of stanols with an admixture of cholesterol, the iodine values corresponding to a concentration (as %) of 15 for *C* and of 34 for *S*. By difference the remainder was calculated to be made up of cholestanol or other non-LB-reacting stanols, e.g. 3α-stanols.

Fraction 4. The chemical and physical properties suggested that the major components present were unsaturated sterols. The fractions were found to contain 13% of 'fast-acting' sterols for the *C* series and 4% for *S* series. The presence of 7-dehydrocholesterol (*C* series approx. 1%, *S* series 0·2%) was indicated spectrophotometrically. The fractions from both series were crystallized from methanol, the crystals were recovered and the materials from the mother liquors were added to fraction 5. The yields, properties and analyses are given in Table 6. The crystals from the *C* series consisted mainly of a stenol with properties different from those of cholesterol. On crystallization from ethanol solid needles, grouped in tufts, were obtained. The acetate had m.p. 120° (cholesteryl acetate 114°). It is presumed that phytosterol derived from the animal rat cake formed part of the fraction. The crystals from the *S* series consisted of nearly pure cholesterol.

Fraction 5. This consisted in both series of a brown gum which gave an LB test, a strongly positive L test which was more marked in the *S* series and a red colour with a red fluorescence with conc. H_2SO_4. The fractions were treated with ethanolic digitonin solution and the digitonides separated from the non-precipitable fractions. The 3β-compounds were recovered from the digitonides by cleavage with pyridine, the yields being 59% for the *C* series, and 75% for the *S* series. Separation of this fraction by the method of Mosbach, Nierenberg & Kendall (1953) on celite columns was unsuccessful, although trial mixtures of the diols and triol could be separated. Paper partition chromatography on the fraction was carried out with the results shown in Table 7. The non-precipitable fraction

Table 7. *Components of the digitonin-precipitable fraction* 5 (*method I*) *as demonstrated by paper partition chromatography*

0, no test; ±, trace; +, small amount; + +, medium amount; + + +, large amount.

	C series	*S* series
Cholesterol or Δ^5-stenol	+ +	+ +
Cholest-5-ene-3β:7α-diol	+	+ +
Cholest-5-ene-3β:7β-diol	+	+ +
Cholest-4-ene-3β:6-diol	±	+
Cholest-5-ene-3β:25-diol and/or :24-diol	+ +	+
Cholestane-3β:5α:6β-triol	+	±

formed 41% for the *C* series, and 25% for the *S* series and was a yellow gum which gave a yellow colour with a yellow-green fluorescence with conc. H_2SO_4. On chromatography four non-crystalline fractions were obtained. The $[\alpha]_D$ of the only determinable fraction was +8°. This is possibly due to the presence of 3α-sterols.

Pigments. The fractions obtained were light yellow to dark brown and obviously contained pigment. Some studies were made on fraction 4 in which the pigment was eluted from the column. The mother liquor from the crystallization was examined spectrophotometrically, and from the absorptions it was presumed that xanthophyll and zeaxanthin were present (cf. Karrer & Jucker, 1950). Other carotenoids are also present (see Discussion).

Method II

The procedure used for the remainder of the matter from the *S* series of Expt. XIV is shown in Fig. 2. Use has been made of the method of Hardegger, Ruzicka & Tagmann (1943) to prepare a 'cholesterol-poor unsaponifiable fraction' (CPUM). The total UM (30·0 g.) was refluxed in methanol (400 ml.) for 1 hr. The solution was decanted from the tar and allowed to stand at room temperature for 16 hr. The crude crystalline cholesterol (9·74 g.) which separated was filtered and washed with small amounts of cold methanol. The material in the mother liquor and washings was recovered and pooled with the tar remaining in the flask to form CPUM.

Separation. The CPUM (19·79 g.) was dissolved in a small amount of light petroleum and chromatographed on alumina to give three primary fractions *A*, *B* and *C*. Fraction *C* contained residual cholesterol, much of which was removed by crystallization and was pooled with the crude cholesterol. Fraction *B* was rechromatographed, the crystals of coprostanol (0·92 g.) being designated fraction 2. The final fractions containing the most polar components were termed fraction 3.

Fraction 1. This contains fraction *A* and the least polar components of fraction *B*. In the chromatography of this fraction heptane was used as the initial eluent, six fractions being obtained. Fraction 11 was a white semi-solid (1·07 g.). It was dissolved in acetone (5 ml.) and allowed to stand at −15° for 3 hr. The waxy semi-crystalline solid (0·90 g.) was removed by filtration and a viscid oil (0·16 g.) was recovered from the mother liquor. The oil reacted with bromine, gave an immediate LB test, but was not precipitable by digitonin. A sample of the oil dissolved in ether and treated with gaseous HCl gave a small amount of a semi-crystalline solid insufficient for characterization. A sample of fraction 12 (0·27 g.) was dissolved in light petroleum and treated with urea (2 g.) moistened with methanol (0·2 ml.). The urea adduct was removed by filtration, washed with light petroleum and decomposed with water. A semi-crystalline wax (0·02 g.) was recovered similar in appearance to the material obtained from the acetone treatment of fraction 11 to which it was added. The combined waxes were crystallized from cold acetone and gave a material of m.p. 38–40° which on analysis gave C, 83·8%; H, 12·8%. The material not forming an adduct on recovery from the light petroleum solution gave a yellow wax (0·25 g.) which was fluorescent in ultraviolet light and gave an immediate LB test but was not precipitable by digitonin. No further investigations were made. Fraction 13 was a brown gum (0·94 g.) which gave a positive Z test. Polarography indicated the presence of coprostanone. Separation was made with Girard T reagent to yield ketones and non-ketones. The ketonic fraction is described later. Fraction 13 NK was combined with material from the rechromatography of fraction 3 and is described with that fraction.

Fraction 14 was a brown gum (2·01 g.) ($[\alpha]_D$ +9°). It was treated with urea and the adduct-forming material—fraction 141 (0·26 g.)—was separated. To this were added other similar fractions (see later) giving a total of 0·48 g. The combined material had an acetyl value of 104 and had

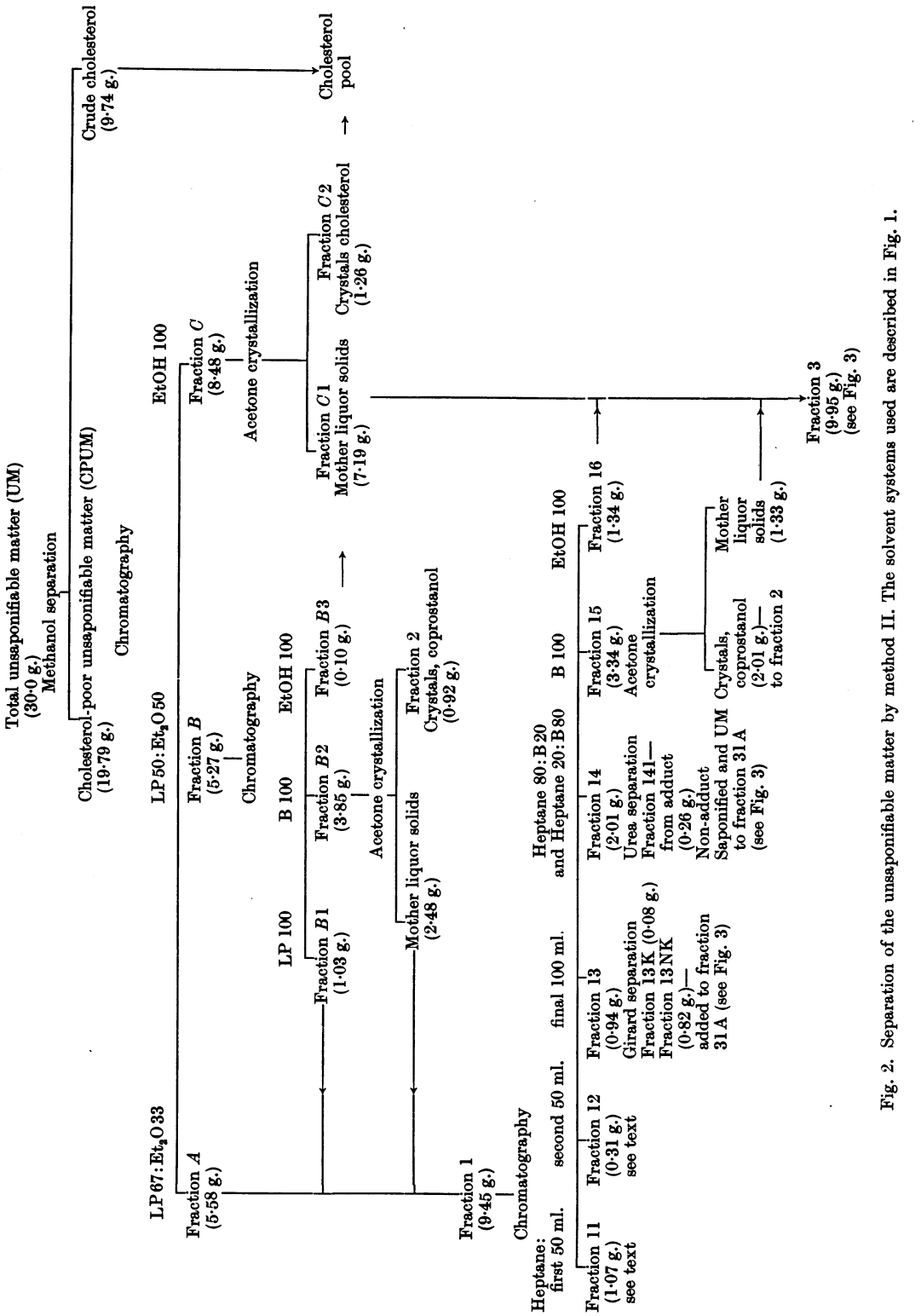

Fig. 2. Separation of the unsaponifiable matter by method II. The solvent systems used are described in Fig. 1.

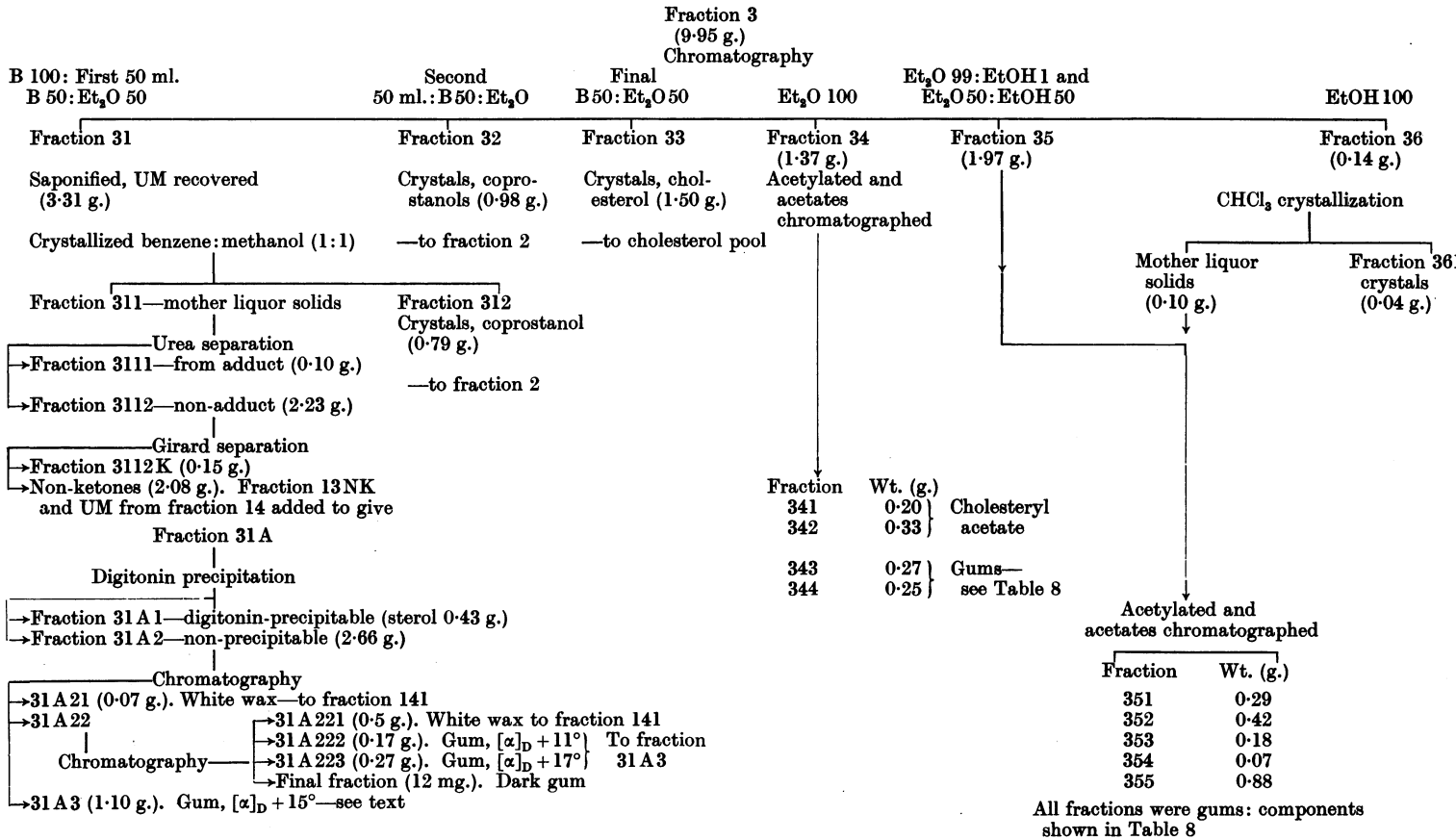

Fig. 3. The separation of fraction 3. The solvent systems are described in Fig. 1.

no optical activity. It is presumed to consist of higher aliphatic alcohols. The material not forming an adduct consisted of esters which were saponified, the UM was recovered and combined with similar material from fraction 3 (see later).

Fraction 15 consisted mainly of crystalline coprostanol (3·34 g.). (For identification, see p. 667.) The fraction was crystallized from acetone and the crystals (2·01 g.) were pooled with fraction 2. The material from the mother liquor was added to fraction *C*1 as was the ethanol eluate fraction 16 to form fraction 3.

Fraction 3. This contained unhydrolysed material (waxes), residual coprostanol, cholesterol and ketones, and the bulk of the most polar components, e.g. diols and triols. The fraction was treated as shown in Fig. 3, six main fractions being obtained.

Fraction 31 was waxy in appearance and consisted in part of unhydrolysed esters. It was saponified and a semi-crystalline UM obtained; the acids (0·18 g.) were recovered and had an equivalent weight of 228. They were not further investigated. The UM was crystallized and the coprostanol was added to fraction 2. The solid from the mother liquor was treated with urea and the fraction forming an adduct was added to fraction 141. The material not forming an adduct gave a positive Z test and was treated with Girard T reagent. The ketonic fraction will be described later. The non-ketones were combined with fraction 13K and the UM from the non-adduct forming portion of fraction 14 to give fraction 31 A (3·76 g.).

Fraction 31*A* was treated with a solution of digitonin and the insoluble digitonides (1·82 g.) were removed. They gave a LB test and consisted mainly of coprostanol (0·43 g.), which after recovery was added to fraction 2. The non-precipitable fraction 31 A2 (2·66 g.) was chromatographed to give three primary fractions, the first of which was added to fraction 141. The second fraction was re-chromatographed to give four fractions, the first being added to fraction 141. The second and third fractions were dextro-rotatory gums which gave a yellow-brown LB test and the H_2SO_4 test was yellow with a yellow-green fluorescence. They were combined with fraction 31 A3 which had similar properties. The combined material was treated with urea but no complex was formed. The material was then treated with acetic anhydride and pyridine and gave an acetyl value of 59. It was not possible to obtain crystals from this fraction, but it is suggested that it is a mixture of oxidized unsaturated hydrocarbons and alcohols which are responsible for the LB test and of 3α-stanols probably coprostan-3α-ol (*epi*-coprostanol). An approximate value of the amount of this compound was obtained from the $[\alpha]_D$ and acetyl value and this corresponded to approx. 50%. A mixture of 3α-stanols was probably present.

Fraction 32 consisted mainly of coprostanol (0·98 g.) and was pooled with fraction 2. Fraction 33 consisted of nearly pure cholesterol (1·50 g.) which was added to the crude cholesterol pool. Fraction 34 was a brown gum (1·37 g.), which on paper partition chromatography behaved as a complex mixture of sterols. The acetylated material (acetyl value 76) on chromatography gave four fractions. The first two, 341 and 342, consisted mainly of cholesteryl acetate, giving m.p. 116°, $[\alpha]_D$ −48°. It is considered probable that phytosteryl acetates were also present. Fractions 343 and 344 were brown gums of complex nature, the constituents of which after saponification are shown in Table 8.

Table 8. *Results of the Lifschütz test and nature of compounds demonstrated by paper partition chromatography on some chromatographic fractions from fraction* 3 *(method II)*

Markings as for Table 7.

Fraction no.	343	344	351	352	353	354	355	361
Lifschütz test	±	±	+ + +	+ + +	+	±	+	0
Paper partition chromatography								
No. of compounds demonstrated	5	6	6	6	5	4	3	1
Cholesterol or Δ^5-stenol	+	+	+	+	0	0	0	0
Cholest-5-en-3β:7α-diol	+	+	+ +	+ +	+	±	+	0
Cholest-5-en-3β:7β-diol	+	±	+ +	+ +	+	±	+	0
Cholest-5-en-3β:24- and/or 25-diol	±	+	+	+	+	0	0	0
Cholestane-3β:5α:6β-triol	+ +	±	±	0	0	0	0	0
Brown-black spot at origin	0	+	+ +	+ +	+ +	+ +	+ + +	0
Green spot	0	0	0	+	+	+	0	+ +

Fraction 35 was a brown gum (1·97 g.). To it was added the mother liquor from the $CHCl_3$ crystallization of fraction 36. The combined material was acetylated (acetyl value 107) and chromatographed to give five fractions consisting of gums which would not crystallize from the usual solvents. The several fractions were saponified, the neutral material was recovered and chromatographed on paper. The components present are shown in Table 8. The demonstrable components present could not be separated on alumina.

Fraction 36 was a gum (0·16 g.), but on crystallization from $CHCl_3$ a small crop of crystals was obtained. The amount was too small for the usual methods of characterization, but paper partition chromatography and spraying with $SbCl_5$ in $CHCl_3$ showed a material giving a green spot. The complexity of the final fractions with regard to demonstrable sterol components is shown in Table 8. The 7α- and 7β-diols are responsible for the L test. These compounds are further considered in the Discussion.

In method II separation of the final fractions by digitonin was not done, but it may be deduced from the results of method I that 75% of the combined fractions 343, 344, and of 351–355 consisted of non-precipitable compounds (approx. 1·8 g.). The large brown-black spot at the origin of the chromatogram suggested the presence of more polar compounds. An unsuccessful attempt was made to isolate

batyl alcohol (or similar glyceryl ethers), but its presence cannot be excluded. Fraction 355 contained 8% N. This may be due to the presence of sphingosine or a similar N-containing base.

Ketones. The yield of ketones was taken as the material separable by the Girard reagent. Investigation of the nature of this fraction was undertaken by Mr D. M. Robertson and some preliminary results are shown in Table 9. Apart from fraction 13K it is evident that a large amount of ketonic material is unaccounted for. The 2:4-dinitrophenylhydrazones were prepared and chromatographed no alumina and with all fractions five components were present but none as yet has been isolated.

Fraction CC/ML from the mother liquor was a semi-crystalline solid. On analysis it gave (as %) sterols fast-acting 2, slow-acting 72, others 26; digitonin-precipitable sterol was 86. Paper partition chromatography showed the presence of large amounts of cholesterol or Δ^5-stenols and small amounts of diols and stanols.

Attempts to separate the components were unsuccessful, but from the analysis it is presumed that in this fraction the total crude cholesterol contains (as g.) fast-acting sterols 0·07 and cholesterol with phytosterols 2·63. The difference between total digitonin-precipitable sterols and the sum of fast-acting sterols and cholesterol gives 12% of the fraction as 3β-stanols (0·44 g.). From the known

Table 9. *Ketones present in various fractions*

		S series			
	C series	Method 1	Ketone-rich		
Fraction	3K	3K	13	13K	312K
Separated by Girard reagent (mg.)	160	40	—	83	150
Coprostanone (mg.)	None	Present	69	67	25
(%)	—	—	8	80	17
Cholestanone (mg.)	12	6	—	15	7
(%)	8	15	—	18	5
Cholest-4-en-3-one (mg.)	None	0·2	—	*ca.* 0·1 mg.	—
(%)	—	0·5	—	*ca.* 0·1	—
Cholesta-3:5-dien-7-one (mg.)	None	None	1·4	None	None
(%)	—	—	0·15	—	—

Coprostanol. The crystalline materials from fractions 15, 2, 31 and 32 were pooled (5·13 g.). A sample (4·7 g.) was recrystallized from acetone. The white crystalline solid did not form a complex with urea or react with bromine. The $[\alpha]_D$ was +23°. The sterol had m.p. 99–101° and the acetate had m.p. 88°. These were unchanged by admixture with authentic samples kindly supplied by Dr L. F. Fieser. On analysis it gave (as %) fast acting sterols 0, coprostanol 96, other sterols 4. The digitonin-precipitable sterol was 100. It is suggested that the difference is due to the presence of a 3β-stanol, presumably cholestanol. Paper partition chromatography showed the presence of saturated sterols only.

Treatment of crude cholesterol. The fractions which consisted mainly of cholesterol were from the initial separation and fractions C2, 33, 341 and 342 (13·03 g.). Use has been made of the observation of Fieser (1953*a*) that cholesterol may be freed from its companions by crystallization from acetic acid.

A sample of crude sterol (9·25 g.) was dissolved in hot acetic acid (100 ml.), the solution cooled rapidly and filtered from the crystals. The cholesterol crystals were dissolved in ether, washed with alkali, then with water and the material was recovered (6·52 g.). The crystals had m.p. 146° and $[\alpha]_D$ −39°. Paper partition chromatography showed one component. The material consisted of pure cholesterol. The mother liquor was diluted with water, the aqueous solution was extracted with ether and the greater part of the acetic acid was removed. The ethereal extract was recovered, saponified with ethanolic KOH solution, diluted with water and extracted to give fraction CC/ML 2·60 g.).

behaviour of cholestanol it is considered that this is the major component. The remaining material (0·51 g.) consisted of 3α-stanols and/or other compounds.

Composition of unsaponifiable matter

From the yields and analyses of fractions obtained it is possible to assess the probable composition of the main components of the UM in the *C* and *S* series animals. The values given in Table 10 have been calculated for the total UM excreted: *C* series, 15·7 g. and *S* series, 58·7 g. These values are for fractions isolated and analysed, no corrections being made for losses or for the acids obtained from unhydrolysed esters. The working loss of about 15% has been regarded as being distributed evenly among the different fractions.

The values for the *C* series were obtained by method I and those for the *S* series mainly by method II, but some values have been calculated from the results of method I, in particular the amounts of saturated and unsaturated hydrocarbons in fraction 1 were taken from percentage values, as have also the values for digitonin-precipitable and non-precipitable material in fraction 3. The value for 3α-stanols (as 3α-coprostanol) was taken from the $[\alpha]_D$ value for fraction 31A2 and is an approximation.

As the basal diet of animal cake and olive oil and the amounts consumed are of the same order in both series, the values of *S*–*C* may be regarded as indicating the changes which cholesterol undergoes in the animal. It is difficult to give an accurate value to the amount of unabsorbed cholesterol, but the amount of crystalline cholesterol recovered (17·7 g.) may be used as an approximation. If this amount is deducted from the total UM and the percentage

Table 10. *Estimated composition of the total unsaponifiable matter (UM) of the faecal lipid in Expt. XIV*

In the *S* series (high-sterol diet) the total UM was 58·7 g. and in the *C* series (low-sterol diet) the UM was 15·7 g.

		S series			*C* series		
		Fraction	*%	Wt. (g.)	Fraction	%	Wt. (g.)
Hydrocarbons	Saturated	11/12	24	0·6	1	71	0·8
	Unsaturated	11/12	76	2·1		29	0·3
Alcohols	Urea-adduct forming	141, 311, 31 A 21/221	—	0·9			
	Others	—	35	4·0	2	—	0·6
Stanols	3α-Stanols (polarimetry)	31 A 2 + crude cholesterol	35	2·2			
	Cholestanol	Crude cholesterol and coprostanol	—	1·3	3NK	—	0·5
	Coprostanol	Crude coprostanol	—	9·6			4·3
Stenols	Crystalline cholesterol	Crude cholesterol	—	17·7	—	—	—
	Cholesterol and phytosterols	CC/ML	—	5·1	4	—	4·1
	Fast-acting sterols	CC/ML	—	0·1			0·1
	Ketones	13K, 3112K	—	0·5	3K	—	0·3
Final fractions	Digitonin-precipitable	343/4, 35/6	75	3·8	5	59	1·8
	Not precipitable	343/4, 35/6	25	1·2		41	1·3

* Values as % of fraction (see text).

values are calculated for a 'cholesterol-poor' UM, it is found that these are similar to those obtained in the *C* series except that the proportion of unsaturated hydrocarbons is three times greater in the *S* series. The proportion of 'other alcohols' is also increased, these increases being mainly at the expense of the cholesterol and phytosterol fraction. It would thus seem that the feeding of large amounts of cholesterol to rats causes an absolute increase in metabolic products of sterols but their relative proportions are similar to those found in an animal kept on a low-sterol diet.

DISCUSSION

The results show the value and the limitations of adsorption chromatography on alumina as a method of separating the components of a complex mixture. The separation of a major component, e.g. cholesterol, from the initial material by crystallization is to be recommended, although the sterol recovered is not homogeneous. The fact that cholesterol forms mixed crystals with a number of other sterols ('companions') and that recrystallization from the usual solvents or separation by chromatography is of little value in obtaining pure cholesterol has been emphasized by Fieser (1953*a*). If the crude cholesterol is crystallized from acetic acid, the companion sterols may be recovered from the mother liquor. The crystallized material is of a high degree of purity.

For a complete analysis the interposition of chemical methods, e.g. formation of urea adduct, digitonin precipitation, etc., would appear to be necessary. In the fraction containing diols and triols the use of adsorption-elution chromatography on alumina appears to be limited. The success of paper partition chromatography with these fractions is a pointer to a more satisfactory method. A consideration of the components of the UM and their probable origin is of interest.

Hydrocarbons. The initial non-polar chromatographic fractions seem to be mixtures of saturated and unsaturated hydrocarbons. Similar mixtures have been isolated from the UM of liver by Schwenk, Todd & Fish (1954). The semicrystalline wax fraction 11 isolated is probably a mixture of higher paraffins. Mackenna, Wheatley & Wormall (1952) have isolated *n*-pentacosane from sebum. The origin of the saturated hydrocarbons is probably in part from intestinal secretion, but part may be derived from the vegetable constituents of the animal cake (see Deuel, 1951). The amount present is not increased after feeding excess of sterol.

The unsaturated hydrocarbons are increased in amount after sterol feeding. There is insufficient evidence to decide whether squalene is a component. This interesting fraction, particularly that giving a rapid LB test, is being further investigated.

Higher alcohols. The material (fraction 141), which forms an adduct with urea, has an acetyl value of 104 and does not give sterol reactions, was presumed to be a mixture of higher alcohols. Such mixtures have been found in sebum-like materials (Wheatley, 1954). The origin is probably similar to the paraffins, as the amount is not increased on feeding excess of sterol.

3α-Stanols. The evidence for the presence of these substances is indirect. Fraction 31 A 2 in method II is considered from its chromatographic behaviour and optical rotation to consist of approximately half 3α-stanols. The remainder of

the fraction may consist of oxidized or changed unsaturated compounds which are optically inactive, but there is no evidence as to their nature.

Coprostan-3α-ol (*epi*-coprostanol) has been found in the UM of the dog's faeces by Marker, Wittbecker, Wagner & Turner (1942) and Lederer, Marx, Mercier & Perot (1946) have demonstrated the presence of this compound in ambergris, the intestinal concretion of certain species of whales. It is probable that 3α-stanols derived from the other sterols are also present.

3β-Stanols. The 3β-stanols form 34% of total UM in the *C* series, and in the *S* series 35% after correction for unabsorbed cholesterol. The major stanol in both series is coprostanol which forms 31% in both *C* and *S* series. The difference between these values (approx. 4%) is presumably due to cholestanol which is precipitable by digitonin but does not give the LB test. It has not been possible to isolate this compound either as the free sterol or as a derivative but evidence for its presence is indirect. The crude coprostanol contained other sterols precipitable by digitonin and the material from the mother liquor of the recrystallization of the crude cholesterol contained similar compounds. These have been estimated as cholestanol. Cholestanol is present in the cholesterol fed and part may be derived from this, but the greater amount is probably produced in the animal. There is evidence that cholestanol is absorbed to some extent in the rat (Dam & Brun, 1935).

Other 3β-stanols derived from the plant sterols are probably present. Rosenheim & Webster (1941) showed that β-sitosterol (24b-ethylcholest-5-en-3β-ol) is converted into β-sitocoprostanol (24b-ethyl-5β-cholestan-3β-ol) in a manner similar to the conversion of cholesterol into coprostanol when the dietary factor (possibly phrenosin) is fed. We have not been able to demonstrate the presence of related stanols, as the large amounts of coprostanol mask the presence of other stanols.

Unsaturated sterols (stenols). The major stenol in the faecal UM of the animals of the *S* series is unabsorbed cholesterol which forms at least 40% of the material. The properties of the stenols in the *C* series suggest that they are plant sterols derived from the cereals and olive oil in the diet fed. The total intake of these is approx. 2·5 g. It is generally considered that the absorption of these compounds is small, but they may be changed chemically in the intestine (see above).

The crude cholesterol obtained is associated with a number of companions and, after crystallization from acetic acid, the presence in the mother liquor of some companions, such as fast-acting sterols, cholestanol and unidentified compounds, was inferred. A large amount of cholesterol and probably phytosterols is present also in this material. The amount of endogenous cholesterol produced is not known but it is considered that the feeding of cholesterol diminishes, if not eliminates, cholesterol biosynthesis. This aspect is dealt with in a subsequent communication.

The amount of fast-acting sterols found in the faecal UM was small and was not significantly increased after sterol feeding. It has been shown that the amount of 7-dehydrocholesterol is increased in the intestinal mucosa of guinea pigs when cholesterol is fed (Glover, Glover & Morton, 1952; Glover & Green, 1954). As this process occurs only in the mucosa it is presumed that none of the products is excreted.

Ketones. Ketones separable by the Girard reagent were found in both *C* and *S* series and the amounts present were approximately doubled after sterol feeding. The detectable ketone present in greatest amount was coprostanone but cholestanone was also present, particularly in the *C* series. A number of other ketones were present as shown by the number of 2:4-dinitrophenylhydrazones separable by chromatography. There is evidence (polarographic and spectrophotometric) for the presence of small amounts of cholest-4-en-3-one and traces of cholest-3:5-dien-7-one in the UM of the *S* series.

Rosenheim & Webster (1943) isolated cholestenone as the *o*-tolylsemicarbazone from the faecal UM of rats fed cholesterol in the form of brain. They obtained from the Girard derivative a total yield of 3·3% of ketones (as % UM). They estimated the cholestenone as being 1% but they did not account for the remaining two-thirds of ketones present. Coprostan-3-one has been shown to be present in ambergris (Lederer *et al.* 1946) and its presence might be expected in the faecal UM of terrestrial animals. Our yield of ketones is less than that obtained by Rosenheim & Webster (1943) and the subject is being further investigated.

Final fractions. These fractions contained the most polar compounds. Separation by digitonin gave a precipitable fraction consisting presumably of 3β-hydroxy steroids and a non-precipitable fraction. A number of sterols were demonstrated by paper partition chromatography in the precipitable fraction (see Tables 7 and 8). The 24- and/or 25-diols have been demonstrated in samples of cholesterol. The 24-diol has been isolated from brain cholesterol by Ercoli & Ruggieri (1953), a finding confirmed by Fieser (1954). Fieser has also found that the 25-diol is present in old samples of cholesterol and is a product of autoxidation. Our results do not permit us to state which diol is present or whether the amount is increased after sterol feeding. A triol is present in fair amount. There is a possibility that the diols and triol are artifacts, in part at least, and are produced during

the manipulations. Cholesterol does undergo autoxidation (cf. Mosbach *et al.* 1953), but the conditions used were somewhat more drastic than those used here. It is worth observing that the autoxidations undergone by cholesterol may prove a guide as to the possible biochemical transformations. Paper partition chromatography (Tables 7 and 8) indicated a number of unidentified compounds which may be derived from cholesterol or be the product of metabolism of steroid hormones which are excreted in the bile and appear in the faeces (e.g. see Hyde, Elliott, Doisy & Doisy, 1954). This aspect has not been investigated, but some of the products formed seem to be water-soluble (Leblond, 1951).

The fraction not precipitable by digitonin was not studied in any detail. An unsuccessful attempt was made to isolate batyl alcohol (or a similar glyceryl ether). These compounds have been found in the UM of organ extracts (cf. Hardegger *et al.* 1943). Our observations do not, however, preclude its presence. The large amount of nitrogen in the final samples is of interest and one possibility is that sphingosine or a similar nitrogen-containing base is present (Hardegger *et al.* 1943). The fluorescence also suggests that traces of bile pigments or pyrrolic compounds are present.

The carotenoids form part of the UM, as they are poorly absorbed, but no attempt was made to estimate the amounts present. Vitamin E is also probably present and it is suggested that it forms part of fraction 31A2. This compound is poorly absorbed and is altered in its passage through the gut (Harris, 1950).

The general picture of the faecal UM is that it is dominated by the sterols and their metabolic products. These form at least 80% of the total material. Whether the intestine is acting solely as an excretory pathway for sterols or whether it possesses special metabolic functions is impossible to state. Further consideration to the sterol metabolism of rats is given in the succeeding communications.

SUMMARY

1. Rats were fed diets having as a basis animal cake with and without olive oil (16·6%) containing either no added cholesterol or added cholesterol (1·6%). The neutral fraction or unsaponifiable matter of the faecal extracts was analysed (Tables 2–4).

2. Separation of the unsaponifiable matter by adsorption chromatography on alumina (Fig. 1) gave fractions consisting of saturated and unsaturated hydrocarbons, stanols, stenols, ketones and more polar compounds (Tables 5 and 6).

3. Using paper partition chromatography (Table 1) and more elaborate methods of fractionation (Figs. 2 and 3), the neutral fraction was shown to be complex in nature (Tables 7 and 8). Preliminary results are reported on the ketones using polarographic methods (Table 9).

4. A balance is given of the changes which the neutral fraction derived from cholesterol undergoes in the intact rat (Table 10).

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Cholesterol Metabolism

5. THE EXCRETION OF ACIDS AFTER CHOLESTEROL INGESTION IN RATS

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When rats were fed a diet rich in cholesterol, an increase in the excretion of faecal acids was observed which bore a relation to the amount of sterol missing when a balance, based on unsaponifiable matter as a measure of sterol, was made (Cook, 1938). The subject was investigated further by Cook, Polgar & Thomson (1950), and the presence of an optically active acid was demonstrated in the light petroleum-insoluble fraction. In the present work a more detailed fractionation has been made of the faecal acids of rats fed diets with and without added fat (16·6 %), containing either small amounts of sterol (*C* series) or with added cholesterol (1·6 %; *S* series). The total steam-volatile acids, the components of the non-volatile fraction and the constituents of the light petroleum-insoluble fraction have been investigated. Some preliminary experiments made on the livers of the animals indicate that the light petroleum-insoluble acids originate in part at least in that organ.

Preliminary accounts of this work have been given (Edwards & Cook, 1951; Cook & Edwards, 1952).

METHODS AND MATERIALS

The animals, diets used, method of extraction of faeces and separation of unsaponifiable matter (UM) are described in the preceding paper (Riddell & Cook, 1955). The compounds present in the remaining soap solution were investigated thus:

Volatile acids. Total steam-volatile acids were determined on a sample of the extracted soap solution by the method of Friedmann (1938).

Ether-extractable acids. The remainder of the soap solution was acidified with 10% (v/v) H_2SO_4, extracted with ether which was washed with water; the ether extract was dried with Na_2SO_4 and the acids were recovered. In Expts. VII and XIII the acids (1 g.) were treated with light petroleum (100 ml.) and allowed to stand for 24 hr. at room temperature. The light petroleum-soluble portion was decanted from the insoluble gum, recovered and weighed. The light petroleum-insoluble fraction was dissolved in ethanol, recovered and weighed.

The light petroleum-soluble fractions were separated into 'solid' and 'liquid' acid fractions by the Twitchell separation of lead salts. Each of these fractions was then converted into methyl esters and fractionally distilled *in vacuo*. The fractionation column was heated and was essentially that described by Longenecker (cf. Hilditch, 1949). Saponification equivalents and iodine values of the distillation fractions were determined. The procedures employed and the calculations used in assessing the composition of the distillation fractions were based on those described by Hilditch (1949).

Polyethenoid acids were estimated as linoleic acid. Determinations of linoleic and linolenic acids on samples of unsaponified extract were made by the method of Hilditch, Morton & Riley (1945).

Acid equivalents. These were measured on dark-coloured fractions from the light petroleum-insoluble material by potentiometric titration, using a Mullard conductivity controller and bright platinum electrodes.