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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.

The other analytical methods used were as described for the neutral fraction (Riddell & Cook, 1955). Ester cholesterol was obtained by difference between total and free sterol.

Chromatography. In some experiments the methyl esters of the light petroleum-insoluble acids were separated by adsorption chromatography on alumina. The first experiments were made with untreated alumina (Savory and Moore) and poor recoveries of the initial material were obtained from the columns. In all later experiments acetic acid-washed and reactivated alumina was used (G. A. D. Haslewood, personal communication), when better recoveries were obtained, but the results were not consistent.

The adsorbent and material to be chromatographed were used in the ratios of 50 to 1 in a column of diameter 3.5 cm. fitted with a sintered glass base. The solvents used were light petroleum (b.p. 40–60°), benzene, ether and ethanol, all being of Analytical Reagent grade. The transition from one solvent to another was made by increasing the concentration of the more polar solvent by 20% (v/v) each time. Eluates were collected in fractions (50 ml.).

RESULTS AND EXPERIMENTAL

The values for total extracts, steam-volatile acids, ether-extractable acids and ester cholesterol are given in Table 1. Volatile acids were not determined in Expt. XIV, the main purpose of which was to investigate the light petroleum-insoluble fraction (see p. 673).

The steam-volatile acids were increased after sterol feeding in Expt. VII, where a low-fat diet was fed, but there was no increase on sterol feeding with the high-fat diet in Expt. XIII. The ether-extractable acids were increased on sterol feeding in all experiments and there was an increase in the amount of esterified sterol on all diets.

Fractionation of ether-extractable acids (Expts. VII and XIII)

The results obtained (calculated for the total amounts excreted) are given in Table 2. Some observations on the methods and results are

Table 1. *Weights of the ether-ethanol extracts and contents of steam-volatile and ether-extractable acids and ester sterol in the faeces of rats fed a low-sterol diet (C series) and a high-sterol diet (S series)*

	Expt. VII Low fat		Expt. XIII High fat		Expt. XIV High fat	
	<i>C</i> (g.)	<i>S</i> (g.)	<i>C</i> (g.)	<i>S</i> (g.)	<i>C</i> (g.)	<i>S</i> (g.)
Ether and ethanol extract	124	263	58	122	106	107
Steam-volatile acids	11.5	15.6	3.5	3.4	—*	—*
Ether-extractable acids	72.6	87.7	33.2	61.7	64.2	83.8
Ester sterol	4.6	12.4	0.8	4.0	3.2	12.5

* Not measured.

Table 2. *Values for the fractionation of ether-extractable acids calculated for the total amount excreted*

LP, light petroleum (b.p. 40–60°).

	Expt. VII		Expt. XIII	
	<i>C</i> (g.)	<i>S</i> (g.)	<i>C</i> (g.)	<i>S</i> (g.)
LP-soluble acids				
Solid acids				
Tetradecanoic	2.5	2.8	0	2.1
Hexadecanoic (Palmitic)	13.9	17.2	4.8	9.7
Octadecanoic	4.5	6.0	0.9	4.7
In distillation residue	4.3	3.4	2.7	1.8
Liquid acids				
Tetradecenoic	1.0	4.1	0	0
Hexadecenoic	8.4	9.8	5.4	5.4
Octadecenoic (Oleic)	18.2	14.5	12.0	25.3
'Linoleic acid'	4.4	0.5	1.6	0.9
In distillation residue	1.3	8.2	1.9	1.7
Total LP-soluble	58.5	66.5	29.3	51.6
LP-insoluble acids				
Initial fraction	7.8	15.1	2.4	3.2
Distillation residue; solid acids	2.2	2.6	0.3	3.0
Distillation residue; liquid acids	3.9	3.5	1.3	3.8
Total LP-insoluble acids	13.9	21.2	4.0	10.0

necessary. The initial treatment with light petroleum does not remove all insoluble acids, the presence of fatty acids increasing their solubility. When the bulk of the fatty acid esters are removed by distillation a further separation of a light petroleum-insoluble fraction from the distillation residues is possible. The total light petroleum-insoluble fraction is therefore composed of three fractions, viz. from the initial treatment and those obtained from the distillation residues of solid and liquid acids. The Twitchell separation into 'solid' or saturated acids and 'liquid' or unsaturated acids does make a broad separation, but some oleic acid is found in the solid acid fraction.

The calculations involved in determining the composition of each distillation fraction are a compromise, as three simultaneous equations have to be solved. Also the values for 'linoleic' acid are for a mixture of polyethenoid acids. Determinations of linoleic and linolenic acids by the method of Hilditch *et al.* (1945) on unsaponified extracts gave good agreement with the acids from the *C* series (4.1, cf. 4.4; and 1.8, cf. 1.6), but with the *S* series the results obtained were considerably higher than those given. Interfering compounds present in the *S* series extracts made the results of little value.

The results in Table 2 are given in absolute values for the various acid fractions and, as the same method was used throughout, the results are comparable. On both a low-fat and on a high-fat diet the absolute amounts of light petroleum-soluble and light petroleum-insoluble acids increased after the feeding of cholesterol. Of the light petroleum-soluble acids the solid acid fractions were increased in amount, this increase being greater when fat and cholesterol were fed. The palmitic acid fraction was more than doubled. The liquid acid fraction was

increased when cholesterol and fat were fed, the increase being mainly in oleic acid. The general picture is therefore of an increase in solid and liquid acid fractions which is most marked when fat and cholesterol are fed.

The light petroleum-soluble fractions from the distillation residues had acid equivalents ranging from 320 to 500, the higher values indicating the presence of residual UM. The iodine values ranged from 19 to 56. No further investigations were made.

The light petroleum-insoluble fractions were used for a preliminary exploration of methods for separating the components. By chromatography a number of non-crystalline optically active components were obtained. An experiment for the specific purpose of investigating the light petroleum-insoluble fraction was then undertaken.

Fractionation in Expt. XIV

The general procedure is shown in Fig. 1. The ether-extractable acids were converted into the methyl esters and distilled at 1 mm. to a final column temperature of 170°. After saponification the distillable esters (fraction 1) were separated by the Twitchell procedure into solid and liquid acid fractions. The distillation residues were treated with light petroleum and separated into soluble esters (fraction 2) and insoluble esters (fraction 3), both of which were dark coloured.

Fraction 2 was chromatographed on alumina. Fraction 22 was a dark tar which was not further investigated. Fraction 21 was saponified and extracted with ether to remove unsaponifiable matter which was added to that obtained in the initial separation. The acids were recovered, separated into solid and liquid fractions and analysed. The results for the acids obtained in fractions 1

Fig. 1. Fractionation in Expt. XIV. The solvent systems used are expressed as v/v mixtures. LP, light petroleum (b.p. 40–60°); B, benzene; Et₂O, ethyl ether; EtOH, ethanol.

and 2, and the total weight of the light petroleum-insoluble esters are given in Table 3.

The distillable esters contained a mixture of fatty acids presumably similar to those demonstrated in Expts. VII and XIII. The light petroleum-soluble fraction 21 contained acids with no demonstrable optical activity. They are probably mixtures of long-chain, C_{20} and higher, saturated and unsaturated acids. No further investigations were made. Fraction 22 was insoluble in light petroleum but was not further investigated.

masses. Samples of these were analysed for acetyl, but none was found. The process of acetylation would appear to be affecting other components.

It was decided to saponify each fraction separately, remove any remaining neutral matter, and recover and investigate the acids. The total amounts of neutral matter recovered were C 0.1 g.; S 0.2 g. The recovered acids solidified to amorphous solids within a short time, but they could not be crystallized. Melting points were indefinite. The weights of the recovered acids and analyses are

Table 3. *Weights and analyses of fractions 1 and 21 from the treatment of the methyl esters of the ether-extractable acids in Expt. XIV*

The weights of the light petroleum-insoluble fractions 22 and 3 are also given.

Light petroleum-soluble	Fraction 1		Fraction 21	
	<i>C</i>	<i>S</i>	<i>C</i>	<i>S</i>
Wt. (g.)	54.8	68.5	6.1	8.5
Residual UM (g.)	0	0	0.8	1.9
Total acids (g.)	54.8	68.5	5.3	6.6
Equivalent	284	326	340	360
Solid acids (%)	41	45	40	51
Liquid acids (%)	59	55	60	49
Light petroleum-insoluble	Fraction 22		Fraction 3	
	<i>C</i>	<i>S</i>	<i>C</i>	<i>S</i>
Wt. (g.)	0.4	0.7	2.8	6.1

Light petroleum-insoluble fractions 3. Samples of the methyl esters (C 2.2 g.; S 5.9 g.) were dissolved in benzene and chromatographed. There were obtained fractions 31 (C 0.41 g.; S 1.40 g.), fractions 32 (C 0.53 g.; S 2.80 g.) and final fractions 33 (C 0.16 g.; S 0.40 g.). Fractions 31 and 32 in both C and S series were yellow-brown gums, but fractions 33 were dark black tars. There was a loss of 50 % in the C series and of 20 % in the S series on chromatography of the initial material. Even after prolonged ethanol extraction the alumina was dark-coloured.

Fractions 31 and 32 in each series were treated with acetic anhydride in pyridine and the materials recovered. The yields (as g.) were fraction 32 (C 0.45; S 1.28) and fraction 33 (C 0.57; S 2.96). After this treatment the materials were soluble in a mixture of light petroleum and benzene. The treated fractions 31 and 32 from the C and S series were chromatographed separately, but in such a way that eluates of comparable nature were obtained. Nine fractions were obtained—fractions 311 to 313 and fractions 321 to 324 were light-coloured gums, but the final fractions 314 (C 0.09 g.; S 0.21 g.) and 325 (C 0.19 g.; S 1.05 g.) were black tars. None of the fractions 311–313 and 321–324 could be crystallized from the usual solvents, but on standing some of these solidified to amorphous

given in Table 4, where values for cholic and tri-ketocholanic acid are also shown. Fraction 322 from the S series was increased after sterol feeding and was further investigated. It gave a yellow Hammarsten test (Haslewood, 1943) and a yellow colour with conc. H_2SO_4 . It gave a precipitate with an ethanolic 2:4-dinitrophenylhydrazine solution. Polarographic investigation by Mr D. M. Robertson showed a complex polarogram which was difficult to interpret but indicated the presence of a reactive carbonyl group. Infrared spectrophotometry by Dr R. Stevenson at the Converse Memorial Laboratory (Harvard University) showed a typical bile acid 'finger print' with a marked carbonyl inflexion. The evidence suggests a ketonic bile acid. The nature of the other acids is unknown, but from the properties they could consist of bile acids with and without keto groups (see Discussion).

Final fractions. The acids recovered from the black tars had equivalents in the C series ranging from 246 to 376 and in the S series from 296 to 456. The acids from each series were pooled. The solutions were fluorescent in indirect light. On analysis the acids from both series were found to contain 3.3 % N. These fractions probably contain acids of a pyrrolic nature, derived from the bile pigments, but there is evidence for the presence of other acids.

Table 4. *Yields, properties and analyses for C and H of acids obtained by chromatography of the methyl esters of the light petroleum-insoluble fraction of Expt. XIV*Solvents used were light petroleum (b.p. 40–60°)-LP; benzene-B, ether-Et₂O as v/v mixtures.

Fraction (no.)	Solvents	Series	Wt. (mg.)	Acid equivalent	[α] _D	Analyses	
						C (%)	H (%)
311	LP70:B30	C	121	323	+27°	72.8	10.8
		S	252	349	+35°	71.0	10.2
312	LP30:B70	C	85	335	+41°	67.1	9.5
		S	299	340	+40°	72.9	10.0
313	B100	C	139	358	+9°	70.7	9.5
		S	308	296	+12°	71.6	9.6
321	LP70:B30	C	88	341	+42°	—	—
		S	86	356	+46°	—	—
322	LP30:B70	C	99	403	+23°	61.2	9.2
		S	609	356	+43°	69.9	9.8
323	B100	C	50	482	+28°	58.4	9.0
		S	345	388	+48°	71.2	9.9
324	B95:Et ₂ O5	C	26	379	+22°	—	—
		S	287	360	+41°	69.7	9.5
Cholic acid (C ₂₄ H ₄₀ O ₅)			—	408	+37°	70.6	9.8
Triketocholanic acid (C ₂₄ H ₃₄ O ₅)			—	402	—	71.6	8.5

Livers

The ether-ethanol extracts of the livers in Expt. XIV were saponified, the unsaponifiable matter was removed, the acids were recovered and converted into the methyl esters (*C* 8.7 g.; *S* 2.6 g.). The esters were chromatographed on alumina and from the light petroleum 50: benzene 50 and benzene 100 eluates semi-crystalline fractions, soluble in light petroleum, were obtained. They had no demonstrable optical activity and consisted presumably of fatty acid esters (yields *C* 2.0 g.; *S* 6.3 g.). From the ether 50:ethanol 50 eluates light-coloured gummy dextro-rotatory fractions were obtained (yields *C* 0.30 g.; *S* 0.54 g.). The gums were insoluble in light petroleum, but no further investigations were made. Black tars were obtained from the extruded columns with boiling ethanol.

DISCUSSION

The results obtained confirm and extend the observations of Cook (1938) that the feeding of cholesterol is associated with the increased faecal excretion of acids. The experiments have been made using the balance method with unlabelled sterol, but there is evidence from experiments using ¹⁴C-labelled cholesterol that acids are indeed formed. Siperstein & Chaikoff (1952), using cholesterol labelled in positions C₄ and C₂₆, have shown that 90% of the sterol labelled in the nucleus (C₄) was recovered in the saponifiable fraction in the faeces and reached the intestinal tract via the bile.

A more specific fate of the cholesterol is its conversion into bile acids, a transformation first sug-

gested by Bloch, Berg & Rittenberg (1943). This observation has now been confirmed (see Byers & Biggs, 1952; Bergström, 1952) and may be regarded as a major pathway of cholesterol metabolism. Our experiments showing the large increase in light petroleum-insoluble acids and what are probably ketonic bile acids is indicative of this pathway. The analyses given in Table 4 show the diverse nature of these acids and the preliminary experiments on the liver suggest that similar acids arise in part at least in that organ. The subject is being further investigated. The increase in the excretion of volatile acids is discussed in more detail in the succeeding communication (Cook, Edwards, Riddell & Thomson, 1955).

The large increase in the light petroleum-soluble acids is still to be explained. Part of this is due to an increase in the excretion of sterol esters after feeding cholesterol. If the amount of acid (calculated as oleic acid) so esterified is deducted from the total light petroleum-soluble acid excreted a considerable portion is still unaccounted for. The conversion of cholesterol into other acidic components is thus indicated. This subject is discussed further in the succeeding communication.

SUMMARY

1. The faecal excretion of acids in rats fed low-fat and added fat (16.6%) diets containing small amounts or large amounts (1.6%) of cholesterol has been investigated.

2. The amounts of ether-extractable acids and of ester cholesterol are increased after sterol feeding (Table 1).

3. The ether-extractable acids were separated into solid and liquid acid fractions, converted into methyl esters and fractionally distilled. The light petroleum-insoluble compounds were separated from the fractions. Analyses of the acids are given (Table 2): these show that the increases after cholesterol feeding occur in the solid, liquid and light petroleum-insoluble fractions.

4. The methyl esters of the light petroleum-insoluble esters were chromatographed and gave a number of fractions, some of which are probably ketonic bile acids (Table 4).

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Cholesterol Metabolism

6. THE FATE OF INGESTED CHOLESTEROL IN THE INTACT RAT

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Some of the neutral and acidic products of the faecal excretion after the ingestion of cholesterol have been described (Riddell & Cook, 1955; Edwards & Cook, 1955). The object of this communication is to put the results of the several experiments on a comparable basis and to give a picture of the metabolism of cholesterol in the entire animal. The method used has been to feed large amounts of cholesterol to one group of animals and to keep a control group on a diet low in sterols. The difference between the two groups has been taken to indicate the changes undergone by the ingested cholesterol. Schoenheimer & Breusch (1933) demonstrated that in mice there was a balance between cholesterol synthesis and destruction, and that on diets rich in cholesterol destruction exceeded synthesis. Using labelled cholesterol, this conclusion has been confirmed for other animal species (Gould, Taylor, Hagermann, Warner & Campbell, 1953; Langdon & Bloch, 1953). It can thus be considered that differences between the total sterol fed and that excreted represents the cholesterol metabolized, this term being used to include all processes occurring within the animal. The method lacks the finesse of experiments using isotopically labelled sterols but has the advantage that large amounts of material are

available for study. In work of this nature the isolation of intermediates in the biological degradation of cholesterol is of importance as pointer to further experiments.

The greater part of the sterol metabolic products appears in the gut and is excreted in the faeces. Some slight excretion occurs in the urine and sebum, but for practical purposes these pathways may be neglected. In addition to the experiments in which animals were fed animal cake which has a high fibre content, some experiments were carried out on synthetic diets low in fibre to determine the amount and nature of the steam-volatile acids. A short account of this aspect of the study has been given (Thomson, 1951). Balance experiments on the rat after cholesterol ingestion show that the absorbed cholesterol is localized in the liver, and this organ has been made the subject of preliminary study. The rat has a well-developed tolerance for ingested cholesterol and is a good subject for the study of its metabolism.

METHODS AND MATERIALS

The diets having animal cake as basis have been described (Riddell & Cook, 1955). In addition, two experiments were made with synthetic diets low in fibre content, with and without added fat, to determine the nature of the faecal