CLIX. CHOLESTEROL METABOLISM

I. ACIDS APPARENTLY CONCERNED IN THE METABOLISM OF CHOLESTEROL

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THE question of the origin of cholesterol in animals has been the subject of numerous researches but the problem of the metabolism of cholesterol has not commanded the same attention. Page & Menschick [1932] concluded that approximately 70 % of the cholesterol ingested by rabbits could not be accounted for by the sterol excreted in the faeces or by that stored in the animals. Schonheimer & Breusch [1933] found the cholesterol balance in mice to be negative or positive, depending on the supply of exogenous sterol. Beumer & Fasold [1933] showed that the rat also is apparently capable of metabolizing cholesterol. This conclusion was confirmed by Cook [1936, 1937], who found that rats fed a diet containing 2 % cholesterol were able to metabolize 20–30 % of the amount ingested.

This communication gives the results of further balance experiments. It is to be remarked that this work must be regarded as preliminary in nature and only indicative as to the possible fate of the cholesterol. Experiments on the acidic constituents of the faeces have yielded such consistent results that it is felt that, even if the direct relation of these acidic substances to cholesterol is not yet established, there is little doubt that certain fatty acids are concerned in, or associated with, cholesterol metabolism.

EXPERIMENTAL

The animals used were drawn from the laboratory piebald stock. As far as possible litter-mates were taken but if these were not available animals of approximately the same weights were used. The room in which the animals were kept was maintained at a constant temperature. The animals were kept in Hopkins metabolism cages, the urine and faeces being collected separately. The experiments can be divided into four dietary groups, three of which received different fats, and one no fat. The diets fed to the animals were made up as shown in Table I. In the diets of the control animals the cholesterol was omitted.

The cholesterol used was the specially recrystallized product of Glaxo, "Cholesterol G. L. Puriss. Recryst." The cholesterol was first dissolved in the fat portion of the diet by heating on a water bath, the molten mass being then well mixed with the other constituents.

The data with respect to the duration of the experiments, the weights of the animals and the amounts of food and cholesterol consumed are given in Table II. Exps. 1–4 belong to group A diet, exps. 5–7 to group B, Exps. 8 and 9 to group C and Exps. 10 and 11 to group D. Exps. 8 and 9 were done in collaboration with G. N. Jenkins. The results in Exps. 8 and 9, and Exps. 10 and 11 are the means of 3 animals in each case. In two instances, Exps. 1a and 6a, the animals died during the course of the experiment, and to counteract this effect as far as possible the

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Table I. *Rats' diets*

	All values in g.			
	Group and type of fat			
	<i>A</i> Arachis oil	<i>B</i> Triolein (B.D.H.)	<i>C</i> Beef dripping	<i>D</i> Fat-free
Caseinogen	23.0	23.0	23.0	23.0
Rice starch	40.0	40.0	40.0	50.0
Cane sugar	17.0	12.0	17.0	22.0
Fat	15.0	20.0	15.0	0.0
Salt mixture	5.0	5.0	5.0	5.0
Yeast	7.5	7.5	7.5	7.5
Cod liver oil	2.5	2.5	2.5	0.0
Radiostoleum	0.0	0.0	0.0	1 drop per diem
Cholesterol (Glaxo)	2.0	2.0	2.0	2.0
Calories per 100 g. food	426.0	448.0	426.0	347.0

Notes. The caseinogen used was "Glaxo ashless casein E"; the salt mixture No. 185 of McCollum & Davis [1915]. The yeast was a dried yeast preparation of trade name "Ceremealo". No calorogenic value is given to the cholesterol.

Table II. *The duration of experiment, animals' weights, food and cholesterol consumptions of the experimental rats. In Exps. 8, 9, 10 and 11 three animals were used in each experiment. The weight figures are mean values.*

Exp. No.	Diet	Days on diet	Animals				Total amount of food g.	Total amount of cholesterol fed g.
			Sex	Wt. g.				
				Start	End	Gain		
1 <i>a</i>	15% arachis oil	40*	♂	32	65	33	1618	—
<i>b</i>	controls	123		30	222	192		
2 <i>a</i>	Plus cholesterol	98	♂	32	176	144	1574	28.1
<i>b</i>		100		34	192	158		
3 <i>a</i>	15% arachis oil	123	♀	32	160	128	2015	—
<i>b</i>	controls			29	137	108		
4 <i>a</i>	Plus cholesterol	109	♀	29	116	87	1611	28.7
<i>b</i>				30	121	91		
5 <i>a</i>	20% triolein	29	♂	82	145	63	550	—
<i>b</i>	control			93	144	51		
6 <i>a</i>	Plus cholesterol	22*	♂	86	76	-10	560	10.0
<i>b</i>		47		95	157	62		
7 <i>a</i>	Plus cholesterol	34	♂	44	97	53	503	9.0
<i>b</i>				39	92	53		
8	15% beef dripping controls	22	♀	193	187	- 6	715	—
9	Plus cholesterol	22	♀	212	231	19	818	14.6
10	Fat free diet	21	♀	104	117	13	645	—
11	Plus cholesterol	21	♀	97	133	36	657	12.0

* Animal died.

remaining animals were allowed to continue for a longer time so that the food intake was approximately the same as for the other members of the series. The results for growth and food intake are in good agreement with the previously published figures.

The animals were bled under anaesthesia, the blood being analysed with the carcasses. The livers were removed from the well-bled animal and weighed. The values given for liver as % body weight are the weights of the blood-free livers as % of the gross weights of the animals just before death. The animals were gutted, the gut and contents being analysed together. This material was kept free from mesenteric fat as far as possible. The remaining portions were analysed as carcass. In some cases the livers were analysed individually.

Methods of analysis. A revision has been made in the methods of analysis. It was found that the Leathes & Raper method tended to give low and inconsistent results. The method adopted is modelled on that of Kumagawa & Suto [1908]. A prolonged alkaline hydrolysis is necessary, and the acidified mass must be extracted at least thrice with ether. The crude lipoid was then further saponified and the unsaponifiable matter extracted. The alkaline portion was then acidified and extracted with light petroleum, the acids being weighed. The lipoid in the case of the livers is the sum of the values obtained for the unsaponifiable matter (U.M.) and the fatty acids; on the other hand the values given for "fat" with the carcasses and gut represent the values obtained for the crude ether extract.

The cholesterol is estimated as the unsaponifiable fraction of the lipoids. The criticism might be made that this fraction is too ill-defined and includes compounds other than sterols. Preliminary experiments show, however, that the digitonin-precipitable material represents a high proportion of the U.M. (90 % for the cholesterol-fed animals). It should be pointed out that the digitonin method shows a specificity for the normal series of sterols only; the *epi* series is not estimated by this method. Also as will be seen later coprosterol would be estimated by this method. The point at issue has been to decide whether cholesterol has been converted into a substance which would not be estimated in the unsaponifiable fraction and on this point the experiments yield suggestive results.

The question of the formation of coprosterol from cholesterol has not been studied but it will be realized that such a transformation would not affect the total sterols in the unsaponifiable matter isolated. Coprosterol can be readily isolated from the U.M. of the control faeces by recrystallization from acetone: water is added to the material dissolved in acetone until the solution becomes cloudy; it is then warmed and allowed to cool slowly; the first slight flocculent precipitate is filtered off and the solution left to stand at 4°; after some hours the coprosterol crystallizes out in needles; the crystals are filtered off rapidly on a chilled filter. After one recrystallization from acetone a material was obtained giving the following analytical figures (Weiler): C, 83.09; H, 12.40 %. $C_{27}H_{48}O$ requires C, 83.33; H, 12.48 %. The M.P. was 93.5°, and although this is lower than the value for synthetic material (102°) the M.P. was not depressed on admixture with an authentic sample of synthetic coprosterol obtained from Dr Rosenheim.

It has been found impossible to use the colorimetric method for cholesterol with any degree of accuracy. Samples of the various fractions have been kept and their analysis will be the subject of a further communication.

Analyses of the animals

The results obtained for the analyses of the livers, carcasses and guts of the animals on the various diets are shown in Table III. It will be observed that when fat is included in the cholesterol-containing diet "fatty" livers result. On a fat-free diet there is no increase of fat in the liver. It will also be seen that the results obtained with the triolein-containing diet are not consistent. In Exp. 6a the animal died and the amount of fat in the liver had not increased. This animal

had not eaten for some days before its death. In Exp. 7 the amount of fat in the liver has increased slightly but the amount is not comparable with that observed in Exp. 6. Exp. 7 was actually carried out first but it was uncontrolled. The animals showed a severe diarrhoea which fact probably accounts for the relatively small absorption. In Exp. 6 and with the control animals there was an initial period of diarrhoea which lasted for about a week but thereafter the animals, with the exception of no. 6a, adapted themselves to the diet.

It will be seen from the table, and with the reservations concerning the triolein-fed animals, that cholesterol is absorbed on a fat-containing diet irrespective of the type of fat fed. Also from the results of Exps. 2 and 4 there is apparently no difference in behaviour of the two sexes.

Analysis of the faeces

The faeces were collected over the whole period and finally dried at 70° to approximately constant weight. They were then weighed and powdered. An aliquot was extracted in a Soxhlet apparatus with ether for at least 72 hr. It has been found that more complete if somewhat more highly coloured extracts are obtained by using this solvent in place of light petroleum. The results for the analysis of the faeces are given in Table IV. They are in agreement with the

Table IV. *Details of the analyses of the lipoid and unsaponifiable matter (U.M.) in the faeces of the rats fed on the various diets*

Exp. no.	Diet	Dry wt. faeces g.	Aliquot estimation			Total in faeces		Lipoid as % faeces	U.M. as % lipoid
			Amount g.	Lipoid g.	U.M. g.	Lipoid g.	U.M. g.		
1	♂ controls arachis oil	59.7	20.0	2.62	0.93	7.8	2.97	13.0	38.1
			20.0		1.06				
2	Plus cholesterol	97.8	20.0	7.59	4.89	37.20	23.80	38.0	64.0
3	♀ controls arachis oil	79.4	20.0	2.16	0.84	8.60	3.23	10.8	37.6
4	Plus cholesterol	94.2	40.0	17.05	10.39	40.10	24.50	43.5	61.0
5	Controls triolein	21.9	20.0	2.71	1.26	2.96	1.38	13.6	46.5
6	Plus cholesterol	34.6	20.0	7.72	5.31	13.35	9.20	38.6	68.7
7	Plus cholesterol	29.3	29.3	9.92	6.63	9.92	6.63	33.9	66.7
8	Controls beef dripping	37.0	20.0	3.40	0.545	6.28	1.01	17.0	16.0
9	Plus cholesterol	52.1	20.0	7.96	3.82	20.74	9.96	39.8	48.0
10	Controls fat-free diet	22.2	10.0	0.79	0.45	1.75	1.00	7.9	57.0
11	Plus cholesterol	36.0	10.0	3.71	3.41	13.36	12.30	37.1	92.0

previously published results. The lipoid is the ether-soluble material. This was saponified for at least 5 hr. with 10% alcoholic KOH. The material was then diluted with water and extracted with ether. The ether extracts were washed 5 times with water. No soaps have been found in tested portions of the U.M. The remaining alkaline material was acidified with conc. HCl and the acids extracted with light petroleum. These results will be dealt with later.

Analysis of the urine

Qualitative experiments had indicated that the amount of cholesterol excreted in the urine of cholesterol-fed rats was not greatly augmented but no quantitative experiments had been made. The urines of the animals in Exps. 1, 2, 3 and 4 (Table II) were collected over the period of experiment and the results shown in Table V were obtained.

The urinary lipoids were estimated by first heating the urine on a water bath for 1 hr. with HCl and extracting with ether. The ethereal extracts were saponified and the U.M. extracted. The U.M. gave a precipitate with digitonin solution.

Table V. *Analyses of rats' urine*

Exp. no.		Vol. ml.	U.M. in aliquot	U.M. calculated in total vol. mg.
1	Normal	774	—	—
2	Cholesterol	596	—	—
3	Normal	1706	270 ml. gave 8.8 mg.	55.5
4	Cholesterol	630	200 ml. gave 17.4 mg.	54.8

A point of interest is the decrease in the urine volume of the cholesterol-fed animals. It was also noticed that the urine was much darker in colour. The results indicate that excretion of U.M. in the urine is a negligible factor in the metabolism of cholesterol although the reservation must be made that other breakdown products may be present.

The cholesterol (as U.M.) balance sheet

From the values that have been obtained it is now possible to construct a balance sheet (Table VI).

Table VI. *The U.M. balance sheet*

The values for U.M. in the table have been obtained by subtracting from the U.M. of the cholesterol-fed animals the amount of U.M. present in the control animals.

U.M. in	Arachis oil		Triolein	Beef	Fat-free
	♂ g.	♀ g.	Exp. 6 g.	dripping g.	
Faeces	20.83	21.27	7.82	8.95	11.30
Livers	0.43	0.41	0.24	1.12	0.00
Carcasses	0.04	0.03	0.03	—	0.44
Gut	0.02	0.08	0.09	—	0.22
Total	21.32	21.79	8.18	10.07	11.96
Cholesterol fed	28.10	28.70	10.00	14.60	12.00
% accounted for	76.00	76.00	82.00	69.00	99.70

These results show that when fat is included in the diets of cholesterol-fed rats there is a loss of cholesterol irrespective of the type of fat fed or the amount of cholesterol. The loss of cholesterol varies between 20 and 30 %. The higher value was obtained in Exp. 8 and here the results are not complete as the U.M. was not determined in the carcasses or gut. It is extremely probable that here a higher percentage of cholesterol would have been recovered had these values been determined. The results for the fat-free diet are of interest. It will be seen that here there is virtually complete recovery of the cholesterol fed. The value for the carcass U.M. is rather high and might possibly be correlated with the fact that the cholesterol-fed animals grew better than the control animals. The results make it exceedingly probable that in the rat cholesterol is only metabolized in the presence of fat. It would also appear that the type of fat fed has but slight effect on the metabolism of cholesterol.

The fate of the cholesterol

Some indications of a possible fate of the cholesterol or some association with acidic substances may now be given. Table VII is what might be called the lipid balance sheet of cholesterol metabolism as reflected in the faeces. It would appear from the results that have been given that the lipid constituents of the carcasses do not give any clue as to the fate of the cholesterol.

In column A are the values for the extra faecal lipoids due to or caused by cholesterol feeding. In column B are the values for U.M. These figures appear in

Table VII. *The faecal lipid balance sheet showing the difference in the distribution of the faecal lipoids of the control and cholesterol-fed animals on the various diets*

All values in g.													
Exp. nos. and diet	Lipoid			U.M.			Diff. between A and B	Acids			Cholesterol		
	Controls	Plus cholesterol	Diff. A	Controls	Plus cholesterol	Diff. B		Controls	Plus cholesterol	Diff. D	Fed	Accounted for as U.M.	% Metabolized E
1 and 2 Arachis oil ♂	7.80	37.20	29.40	2.97	23.80	20.83	8.60	4.45	10.10	5.65	28.10	21.32	6.78
3 and 4 Arachis oil ♀	8.60	40.10	31.50	3.23	24.50	21.27	10.20	5.23	10.38	5.15	28.70	21.79	6.91
5 and 6 Triolein	2.96	13.35	10.40	1.38	9.20	7.82	2.58	1.41	3.44	2.03	10.00	8.18	1.82
8 and 9 Beef dripping	6.28	20.74	14.46	1.01	9.96	8.95	5.51	4.80	8.25	3.45	14.60	10.10	4.50
10 and 11 fat-free	1.75	13.36	11.61	1.00	12.30	11.30	0.31	0.73	0.97	0.24	12.00	11.96	0.04

the U.M. balance sheet as faecal U.M. The difference between A and B shown in column C would therefore represent material other than U.M. caused to be excreted in the faeces by cholesterol feeding. This may be regarded as unaccounted lipid. It will be obvious that this material should be present in the alkaline fraction as the potassium salt of an acid. If the alkaline fraction is acidified and extracted with light petroleum it is found that "fatty" acids are indeed present. In column D are given the differences between the amount of acids found in the faeces of the cholesterol-fed rats and those of the control animals. This can be regarded as the increase in the amount of acids caused by cholesterol feeding. In column E are given the values for the amount of cholesterol metabolized. This was obtained by subtracting the amounts of cholesterol recovered as shown in the balance sheet (Table VI) from the total amount of cholesterol fed in each separate series.

It will be seen from the table that the values shown in columns C, D and E in each experiment are of the same order. In Table VIII are given the results of previously published experiments [Cook, 1937] to which the amounts of acid have been added. These experiments are not complete because the acid was not estimated in the first series of faeces collected. A calculated value has therefore been added. The extraction of the faeces in these experiments was made with light petroleum.

Table VIII. *The lipid balance in the faeces on varying fat concentrations plus cholesterol*

Diet fat %	Cholesterol			Lipoid unaccounted for g.	Acid found g.	% cholesterol metabolized
	Fed g.	Found in faeces and animals g.	Difference g.			
15	30.19	18.90	11.29	7.62	6.62	37.4
20	29.77	20.17	9.60	9.75	7.13	32.2
30	26.99	18.95	8.04	9.17	8.19	29.8
Totals			28.93	26.54	21.94	
Add calculated acid for first series					4.25	
					26.19	

It will be seen that here, as in the experiments described above, the missing cholesterol bears some relation to the production of acids which appear in the

faeces. It will be realized that if these acids are derived from cholesterol this might be reflected in an increase in the mean mol. wt. or equiv. wt. of the acids. The equiv. wt. has been determined by titrating with standard alkali in alcoholic solution at 60°. The results obtained are shown in Table IX.

Table IX. *The equiv. wt. of the mixed faecal fatty acids*

	Arachis oil		Triolein			Beef dripping		Fat-free	
Controls	320	316	354	359	350	285	303	323	332
	333	320							
	Mean 322		Mean 354			Mean 294		Mean 327	
Plus cholesterol	378	375	320	328	323	329	333		
						329	320	340	
	Mean 375		Mean 323			Mean 328			

It must be emphasized that the values given are those of the mixed fatty acids and the results are comparative only. It will be seen that with the exception of the triolein and fat-free diets there is a tendency for the equiv. wt. of the acids to rise on cholesterol feeding. On the triolein and fat-free diets the equiv. wts. of faecal acids from the control animals are high in value. It is possible that with the arachis oil and beef dripping diets there is a selective excretion of higher fatty acids in association with the cholesterol. The appearance of these fatty acids was first observed on the arachis oil diet. The mixed acids were crystallized out of alcohol and on recrystallization an acid of m.p. 73.5° was obtained. On analysis this compound gave (Weiler): C, 78.86, 78.69 and 78.53, 78.58; H, 13.01, 12.97 and 13.03, 12.97%. The mol. wt. (Rast) was 404 and the equiv. wt. 386 and 390. The compound did not take up bromine in alcoholic solution. The formula $C_{25}H_{50}O_2$ corresponds with the analytical figures but the substance is probably a mixture of high fatty acids. It is possible that this acid is derived from the "lignoceric acid"¹ which is present in relatively large amount in arachis oil. No systematic fractionation of the faecal acids has yet been made. A preliminary Twitchell separation indicates that the saturated acids form a high percentage of the material.

The results with the other fats cannot be so easily explained. The concentration of lignoceric acid in beef dripping is very low and triolein naturally does not contain any. The position is far from clear but it would appear that the excretion of cholesterol in the faeces, and possibly its metabolism, are associated with the appearance of acids of high mol. wt.

SUMMARY

1. Rats have been fed on synthetic diets containing cholesterol in the presence of arachis oil, beef dripping and triolein. The cholesterol was also fed with a fat-free diet.

2. Cholesterol is absorbed irrespective of the type of fat fed. It is not absorbed on a fat-free diet.

3. Balance sheets of the cholesterol recovery on the various diets have been prepared. There is complete recovery on the fat-free diet but on the fat-containing diets only 70–80% of the cholesterol can be recovered.

4. A lipid balance sheet of the faecal excretion has been constructed. This indicates apparently that cholesterol metabolism is bound up with an increase of fatty acids in the faeces. The amount of cholesterol missing is approximately

¹ The position with regard to this acid in naturally occurring substances is well described by Chibnall *et al.* [1936].

equal in amount to the acids recovered. These acids are of high mol. wt. but it cannot be stated as yet that they are derived from cholesterol.

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