On the Origin and Destiny of Cholesterol in the Animal Organism. Part XII.—*On the Excretion of Sterols in Man.*

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In Part X of this series [vol. 86, p. 13 (1912)]—"On the Excretion of Cholesterol by Man"—Ellis and Gardner, from analyses of the dried fæces collected during a series of experiments, carried out by R. H. A. Plimmer, M. Dick, and E. C. Lieb, at the Institute of Physiology, University College, and published under the title of "A Metabolism Experiment, with Special Reference to Uric Acid," came to the conclusion that in man the excretion of cholesterol in the fæces can be largely accounted for by that taken in with the food, provided that the body weight remains constant; if, however, a rapid loss in weight takes place, as in illness, the output of sterol exceeds the intake.

Further work has shown that this conclusion requires modification. In the above-mentioned investigation only one subject was experimented on and the cholesterol-content of the diet was not obtained by analysis of samples of the food actually consumed by the subject under experiment, since the examination of the fæces in question was not undertaken until long after the completion of Plimmer, Dick, and Lieb's investigation, and was of the nature of an afterthought.

The cholesterol ingested was estimated, partly from analyses of similar foods purchased long afterwards, and partly from published analyses of other observers. Further, in the examination of the fæces it was assumed that the only sterol present in the unsaponifiable matter was the crystalline coprosterol. This was separated from the accompanying oils, as far as possible, by crystallisation from alcohol or acetone. The last traces remaining in solution were obtained by conversion into the benzoate, which is only soluble with difficultly in cold alcohol. This method necessitates the use of large quantities of material to obtain accurate results.

Since the publication of this work, new and improved methods for the estimation of sterols in tissues, etc., have been introduced, and, further, our knowledge of the composition of the unsaponifiable matter of human fæces has been considerably extended.

It has been shown by one of us (1921) that though the crystalline sterols of human adult fæces consist mainly of coprosterol, there are also present smaller quantities of β-cholestanol, cholesterol, and possibly phytosterol, derived from the vegetable food. These substances can be quantitatively precipitated together from alcohol solution in the form of their digitonides, by means of an alcoholic solution of digitonin, and so estimated.

There is, however, no simple method by which the quantitative composition of the mixture of sterols recovered from the mixed digitonides can be determined. If a sufficient quantity of material is available, the bulk of the coprosterol may be separated by fractional crystallisation. The β-cholestanol may be separated from the coprosterol by conversion of the latter into ψ-coprosterol, which does not form a compound with digitonin, and the isolation of the unchanged β-cholestanol in the form of its digitonide. Cholesterol (with phytosterol) may be approximately estimated as dibromide.

These crystalline sterols, precipitable by digitonin, constitute only a proportion of the unsaponifiable matter of fæces, the rest may be washed away by means of ether or light petroleum from the precipitated digitonides and obtained as a reddish brown oil. This oil can be distilled, without any decomposition, in superheated steam and passes over into the condenser in the form of a solid emulsion, which is forced from the condenser, partly by the pressure of steam and partly by the action of the condensed water, in the form of solid white candles. These persist for a long time, but usually separate on long standing into oil and water. This oil can be distilled in a vacuum of 1 mm. without decomposition and passes over in a series of fractions ranging from 100° to 220°. The lower fractions contain aliphatic alcohols of high molecular weight, of which cetyl alcohol has been definitely identified. These fractions do not give any colour reaction with acetic anhydride and sulphuric acid. The higher fractions, boiling 200°—220° under 1 mm., constitute the main bulk of the oil. They are usually obtained in the form of transparent yellow glass, melting at 16°—18° C. and showing in bulk a greenish fluorescence. These glassy substances are marked by great stability, contain oxygen in a not very reactive form, and have a molecular composition of much the same order as cholesterol (Gardner, 1921).

They give the Burchardt-Liebermann reaction with acetic anhydride and sulphuric acid in a well-marked, though modified, manner, and though no crystalline esters have been prepared, they appear to be alcohols of a polycyclic type.

In view of the above facts, it seemed to us desirable to investigate more fully the intake and output of sterols in the case of normal human subjects on a rigidly known diet. For this purpose we made use of some of the

material obtained by a sub-committee of the Royal Society Food (War) Committee in experiments on the digestibility of breads made from different kinds of flour (1918), and by Gardner and Fox in their experiments on the digestibility of cocoa butter (1919).

The Diets consumed.

The daily diet of the subjects of the bread experiments consisted approximately of—

	grm.
Bread	800–1000
Minced meat	50
Butter	50
Jam	100
Milk	600
Cheese	50
Sugar	30
Tea	*ad. lib.*

The diet of the subjects of the cocoa butter experiments was the same, except that the butter was replaced by cocoa butter.

In the Tables in this paper the diets are referred to as A, B, C, D. In A the bread was made from 80 per cent. milled flour, in B from 90 per cent. milled flour, and in C from 80 per cent. milled flour mixed with 20 per cent. maize. In D the bread used was the ordinary white bread of the shops (July, 1918). Some latitude was allowed for individual taste; for instance, subject F. J. in the bread experiments omitted the meat ration, and W. J. omitted the cheese, and subject C. took a certain amount of light beer.

Subjects used in the Experiments.

An account of the eight subjects, four in Cambridge and four in London who took part in the bread digestibility experiments is given in the above-mentioned report, and of the three subjects of the cocoa butter experiments in Messrs. Gardner and Fox's paper. The latter paper also contains an account of the fat utilisation in both series.

Experimental Methods.

The general procedure was fully described in the Royal Society report, but we may mention that the diets were consumed for periods of ten days, the six significant days being the fourth to ninth inclusive. The collection of fæces began one day later, and continued one day longer than the days of the diet. Accurate accounts of the weights of the food eaten were kept, and

analyses of samples of each food consumed by each set of subjects were made, no figures published in the literature being made use of. This was very necessary, as there was reason to suppose that war-time conditions of under-feeding might have an effect on the composition of various articles of the diet, particularly milk and meat.

Methods of Analysis.

The fæces were analysed in the following manner. A weighed portion of the dried fæces was subjected to a prolonged extraction in a Soxhlet apparatus with ether, and the ethereal solution was made up to known volume. Aliquot portions were then respectively titrated with standard alcoholic caustic soda, and evaporated to dryness and weighed. The rest of the ethereal solution was then mixed with a hot alcoholic solution of a very large excess of sodium, and allowed to stand forty-eight hours. The precipitated soaps were then filtered on the pump and thoroughly washed with ether. The ethereal filtrates and washings were then repeatedly shaken with alkaline water, and finally with distilled water, until quite free from soap. The ethereal solution of unsaponifiable matter thus obtained was made to known volume, and a suitable aliquot portion evaporated to dryness and weighed. The weighed unsaponifiable matter was then dissolved in alcohol, and the boiling solution mixed with a hot 1 per cent. alcoholic solution of digitonin, using at least a 10 per cent. excess, and allowed to stand overnight for the insoluble sterol-digitonides to separate. The alcohol was then evaporated at the lowest convenient temperature, and the residue washed by decantation with ether or light petroleum, to separate the portion of the unsaponifiable matter not precipitated by digitonin. The ether washings were passed through a weighed Gooch crucible, to guard against any loss of sterol-digitonide. The mixture of digitonide and excess of digitonin was then freed from the latter by washing by decantation with warm water, and finally the sterol-digitonide was brought into the Gooch crucible and the washing completed. The digitonide was then dried at 110° and weighed.

It was found advantageous to cover the asbestos mat of the crucible with a layer of pure sand. This prevented to some extent the sterol-digitonide from forming an impervious cake on the surface of the asbestos, and thus facilitated the filtration and washing. It was also very desirable to evaporate the alcohol before washing the digitonides, owing to the fact that the digitonides of both coprosterol and β-cholestanol are considerably more soluble in alcohol than cholesterol-digitonide.

The weight of sterol-digitonide, $\times 0{\cdot}234$, equals the weight of sterol in the

unsaponifiable matter taken. The sterol-digitonide, precipitated from fæces, consisted mainly of coprosterol digitonide with smaller portions of β-cholestanol digitonide, cholesterol digitonide, and perhaps also phytosterol digitonide.

The unsaponifiable matter not precipitated by digitonin was got by difference, but this was always checked by direct weighing of the oil washed away from the digitonide by ether. Fat in the foods was determined by extraction with ether in the usual way, and the unsaponifiable matter and sterols estimated as described in the case of fæces. In the case of bread and meat, however, the ether extraction was preceded by repeated extraction with boiling alcohol, as it is well known that in such substances the extraction of fat by ether alone is imperfect, even though the exhaustion may be prolonged. The alcohol extracts were then evaporated, taken up in ether, and the ether solution was added to the other ether extract.

Full details of the nitrogen balance in the subjects of diets A, B, C and D are given in the reports and papers mentioned.

Results.

The results of our experiments on the intake and output of unsaponifiable matter are summarised in the following Tables. Table I contains the daily intake and output of sterols precipitated by digitonin. The figures represent the daily average over a period of six days. The intake consists of the total cholesterol, in free and ester form, of the food consumed, together with traces of phytosterol. The sterol excreted consisted, as stated above, mainly of coprosterol, with smaller quantities of β-cholestanol and cholesterol, and perhaps phytosterol. The subjects are indicated by their initials. Eight of the subjects partook of diets A, B and C, except Mr. C., who was omitted from the experiment on diet B owing to an attack of diarrhœa, which came on during the experimental period.

Only two of these subjects, E and B, were available for the experiments with diet D. Another subject, P, joined in this experiment.

In Table II, the daily intake and output of the portion of the unsaponifiable matter of the fat which is not precipitated by digitonin is given. These figures are only approximate, and are no doubt slightly too high owing to the presence of traces of resinous matter produced by the action of the alkali on the alcohol during hydrolysis of the fat (Gardner and Fox, 1921).

Discussion of Results.

It will be noticed from the figures in Table I that in every case there is an excess of output over intake, except subject B on diet C, who shows a

Table I.—Daily Intake and Output of Sterols precipitable by Digitonin in grammes.

	E.	C.	B.	A.C.	H.W.H.	F.J.	W.J.	A.L.	
Diet A—									
Intake of sterol in food	0·28	0·28	0·30	0·22	0·26	0·15	0·19	0·23	
Output of sterol in fæces ...	0·67	0·54	0·56	0·45	1·07	0·28	0·65	0·31	
Balance	−0·39	−0·26	−0·26	−0·23	−0·81	−0·13	−0·46	−0·08	Average loss of sterol −0·34.
Diet B—									
Intake of sterol in food	0·25	—	0·26	0·23	0·27	0·18	0·25	0·25	
Output of sterol in fæces ...	0·59	—	0·29	0·70	0·51	0·84	0·71	1·20	
Balance	−0·34	—	−0·03	−0·47	−0·24	−0·66	−0·46	−0·95	Average loss of sterol −0·46.
Diet C—									
Intake of sterol in food	0·28	0·27	0·33	0·28	0·40	0·22	0·34	0·30	
Output of sterol in fæces ...	0·79	0·43	0·28	0·55	0·60	0·44	0·60	0·39	
Balance	−0·51	−0·16	+0·05	−0·27	−0·20	−0·22	−0·26	−0·09	Average loss of sterol −0·21.
Diet D—		P.							
Intake of sterol in food	0·19	0·19	0·19						
Output of sterol in fæces ...	0·35	0·27	0·50						
Balance	−0·16	−0·08	−0·31	—	—	—	—	—	Average loss of sterol −0·18.

Average of all subjects :—

Intake	0·2534
Output	0·5604
Balance	−0·3070

Table II.—Daily Intake and Output of "Unsaponifiable Matter" not precipitated by Digitonin in grammes.

	E.	C.	B.	A.C.	H.W.H.	F.J.	W.J.	A.L.	
Diet A—									
Intake in food	2·03	2·04	2·10	1·84	3·02	2·23	2·31	2·83	
Output in fæces	0·40	0·36	0·40	0·32	0·53	0·21	0·24	0·15	
Difference	1·63	1·68	1·70	1·52	2·49	2·02	2·07	2·68	
Percentage utilisation	80·30	82·34	80·95	82·69	82·45	90·58	89·61	94·70	Average utilisation 85·45.
Diet B—									
Intake in food	2·42	—	2·67	2·47	3·22	2·63	2·41	3·06	
Output in fæces	0·36	—	0·25	0·17	0·41	0·65	0·39	0·40	
Difference	2·06	—	2·42	2·30	2·81	1·98	2·02	2·66	
Percentage utilisation	85·12	—	90·63	93·11	87·27	75·28	83·81	86·92	Average utilisation 86·02.
Diet C—									
Intake in food	2·08	2·04	2·22	1·99	2·95	2·34	2·12	2·82	
Output in fæces	0·21	0·46	0·65	0·43	0·44	0·47	0·70	0·45	
Difference	1·87	1·58	1·57	1·56	2·51	1·87	1·42	2·37	
Percentage utilisation	89·90	77·45	70·72	78·39	85·08	79·91	66·98	84·04	Average utilisation 79·06.
Diet D—		P.							
Intake in food	1·04	1·09	1·14						
Output in fæces	0·35	0·16	0·27						
Difference	0·69	0·93	0·87						
Percentage utillsation	66·34	85·32	76·31	—	—	—	—	—	Average utilisation 75·99.

Average intake by food	2·197
Average output in fæces	0·378
Balance	+1·819
Percentage utilisation	82·79

small positive balance. The negative balances are very variable, ranging from 0·03 to 0·95 grm. per day. The average negative balance on diet A was 0·34, on diet B 0·46, on diet C 0·21, and on diet D 0·18. The high output on diet B, with coarse bread, may possibly be accounted for by the somewhat laxative effect of the brown bread on some of the subjects—subject C, for instance, was so relaxed that he was obliged to retire from the experiment. He stated that brown bread always had this effect on him. The average negative balance in the twenty-six experiments was 0·307. On comparing the same individuals on different diets, a similar great variation will be noticed.

The sterol excreted in the fæces is derived partly from the food and partly from the bile. The determination of the amount of bile excreted per day by a human being is a very difficult problem, and as yet reliable data are wanting. The amount is given in the text-books as from 500 to 1000 c.c. per day. The figures are derived mainly from the study of patients with biliary fistulæ. We may quote a case of a woman with a biliary fistula studied in some detail by Pfaff and Balch at the Massachusetts General Hospital (1897). They found that the total bile excreted per day was 525 grm., but the amounts excreted at different periods of the day were very variable. Such figures must, however, be taken with great reserve as an index of the amount of bile produced in the normal subject. It is known that, if bile is passed into the intestine, the secretion is increased both in concentration and in amount, and that fistula bile differs from bladder bile both in concentration and even in composition. Thus, Pfaff and Balch's bile contained only some 3 per cent. of total solid matter, while various observers have given the solid content of bladder bile as 10 to 20 per cent. Further, the percentages of inorganic constituents in fistula and bladder bile are of quite different order. However, there appears to be a general consensus of physiological opinion that in health, and when bile finds its way into the intestine, the excretion is probably larger (rather than smaller) than in the fistula cases.

Very variable values have been recorded in the literature for the cholesterol-content of bladder bile, and many of the figures must be accepted with considerable reserve, particularly those obtained before the development of modern methods of estimation of sterols. We know that cholesterol is very soluble in bile, and Moore and Roaf have shown that ordinary bladder bile is able to dissolve a good deal more than is ordinarily found. The cholesterol of the bile is mainly found in the non-ester condition. In more recent years, Peirce (1912) has examined the cholesterol-content of the gall-bladder bile in a variety of conditions by the digitonin method. The results are variable, but he regards the normal figure as about

0·15 to 0·16 per cent. If we take 550 c.c. as the amount of bile secreted per day and the cholesterol-content as 0·16, each of the subjects of our experiment should have passed into the intestine about 0·9 grm. per day, more or less, in bile solution. It is evident, therefore, that the subjects have reabsorbed a considerable amount of the cholesterol along with the bile salts in the intestine.

It has been shown in earlier papers of this series that in the case of herbivora (1912, 2), and also carnivora such as dog and cat (1913), that cholesterol given with the food appears in the blood, the cholesterol having been absorbed in the intestine with the bile salts. This is more difficult to demonstrate in the human subject, but Widal, Weill and Laudat (1912) have shown that heavy fat meals produce hypercholesterinemia, though this state is transitory. The adult human subject is marked off from other animals by the fact that the cholesterol passed into the intestine undergoes reduction at some stage to coprosterol and β-cholestanol, probably by bacterial action, though a small quantity of cholesterol escapes this process. It would seem a probable assumption that this reduction limits the reabsorption of the cholesterol. This has not been definitely proved, though feeding experiments are in progress with herbivorous animals to gain evidence on this point.

We think, however, that the considerations detailed above fully explain the very variable negative balances recorded in Table I.

It also follows that since cholesterol is an integral constituent of all cells of the body, and there is an excess of output over intake, *there must be some organ in the body capable of synthesising cholesterol.* This question we are at present investigating.

It will be seen from Table II that the intake of unsaponifiable matter not precipitated by digitonin is very much larger than the output, and that, as an average of the twenty-six experiments, there is a percentage utilisation of 82–83 per cent. The ratio of the average amount of fæcal sterols precipitable by digitonin to unsaponifiable matter not so precipitated was 1 : 0·25 to 1 : 1·18, but in the majority of cases the individual ratios are not far from the mean value.

As already mentioned, the fæcal unsaponifiable matter not precipitated was volatile in superheated steam without appreciable decomposition; thus 9 grm. only left in the distilling flask 0·14 grm. of carbonaceous matter.

On fractionation of the oil from the whole of the experiments under a pressure of 1 mm., the ratio of the lower boiling portions which did not give the Burchardt-Liebermann reaction to the high boiling "sterol" portion which did was about 1 : 4.

In the paper already referred to ("On the Composition of the Unsaponifiable Matter of the Ether Extract of Human Fæces") Gardner suggested two sources for these non-precipitable oils: (1) the substances which accompany cholesterol in the unsaponifiable matter of tissue fat; (2) the bile acids or their derivatives. With regard to the first source, it is clear from the figures in Table II that the quantity taken in with the food would fully account for that in the fæces. This, however, cannot be decided until the unsaponifiable matter of the tissue fats has been thoroughly investigated and compared with that of the fæces. With regard to the second source, probably oxidisation experiments will throw light on this. Preliminary experiments, however, proved inconclusive.

Work is being continued in both these directions, and we hope to have the honour of communicating the results at some future time.

We take this opportunity of thanking the Grant Committee of the Royal Society for help in defraying the expenses of this work.

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