XCIX. ON THE CHOLESTEROL CONTENT OF THE BILE, BLOOD AND FLESH OF THE HIPPOPOTAMUS.

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OUR knowledge of the origin and function of cholesterol in the animal organism, or of the processes involved in its storage and mode of transit to tissues requiring it, is still very scanty in spite of the very large amount of work expended on the subject in recent years.

In 1909 Dorée and Gardner from a consideration of the facts then available were led to formulate the following hypothesis: (1) cholesterol is a constant constituent of all cells, and when these cells are broken down in the life process their cholesterol is not excreted as a waste product, but is utilised in the formation of new cells; (2) a function of the liver is to deal with and break down products of dead cells, *e.g.* blood corpuscles, and to eliminate the cholesterol in the bile; (3) after the bile has been poured into the intestine in the process of digestion, the cholesterol is reabsorbed, possibly in the form of esters, along with the bile salts and carried by the blood to the various centres and tissues for reincorporation into the constitution of new cells.

This hypothesis received considerable support from the experiments of Dorée, Ellis, Fraser, Gardner and Lander [1908–1913], Wacker and Hueck [1913], who succeeded in demonstrating both in the case of herbivora—such as rabbit—and carnivora—such as dog and cat—a connection between the cholesterol ingested with the food and the amount in the blood, and from the careful investigation of Mueller [1915] who showed by analysis of chyle obtained from thoracic duct fistulae in dogs that cholesterol is readily absorbed from the intestinal tract through the chyle and may be recognised in the contents of the thoracic duct. The question whether the excretion and subsequent absorption of the cholesterol of the body forms a regular and exact cycle, or whether there is any wastage of cholesterol which would require to be made up by the animal either by actual synthesis in the body from simpler substances or by the utilisation of that taken in the food or by both processes is at present difficult to answer. The domestic herbivorous animals do not excrete appreciable quantities of cholesterol, or any recognisable derivative

of this substance in their faeces [Dorée and Gardner, 1908, 1], whilst their bile contains considerable quantities, in the case of cow for instance 0.07 %.

Loss however may occur in other ways. In man the sweat and sebum have been shown to contain cholesterol; in sheep the presence of relatively large quantities of cholesterol and cholesteryl esters has been demonstrated in wool fat: considerable quantities are also eliminated in milk.

The food of the herbivora contains no cholesterol, but instead the closely related sterols of the phytosterol group. Evidence has however been brought forward [Fraser and Gardner, 1910] which indicates that phytosterol can be absorbed and appear in the blood as cholesterol, but data are yet wanting to form any balance sheet of intake and output in various ways.

In the case of carnivora—dog and cat—cholesterol is a normal constituent of their food, and feeding experiments [Dorée and Gardner, 1908, 2; Kosumoto, 1908; Ellis and Gardner, 1909, 2] have shown that the total cholesterol found in the faeces can be approximately accounted for by that taken in with the food, provided the animal remains healthy and constant in weight. Nevertheless in experiments in which the intake of cholesterol was reduced to a minimum, *e.g.* on diets of bread, egg white and cream, etc., traces of sterols of the order of 0.03–0.05 g. per day were excreted.

On the other hand, in man the state of affairs is somewhat different. The adult human subject is marked off from other animals by the fact that the cholesterol passed into the intestines undergoes reduction at some stage to coprosterol and β -cholestanol, apparently by bacterial action, though a small quantity of cholesterol escapes this process [Gardner, 1921]. It would seem a probable assumption that this reduction limits the reabsorption of cholesterol, though this has not been definitely proved. Careful experiments on adults [Gardner and Fox, 1921] have shown that there is an average negative balance of 0.3 g. per day, so that there must be some synthesis going on in the body. Similar results have been published by Thannhauser [1923].

In recent years the chemical investigations of Wieland and his co-workers on the bile acids [1912], and of Windaus [1910] on the oxidation of the hydrocarbons, cholestane and coprostane, have definitely proved that cholic acid and coprosterol have the same carbon skeleton and that cholic acid only differs in this respect from the latter by the loss of a $\text{CH}(\text{CH}_3)_2$ -group. Windaus has, we believe, gone so far as to suggest that the bile acids may be derived from the cholesterol in the organism. If this be the case any bile acids lost in the faeces would have to be reckoned with in estimating the output of sterol derivatives, and the above-mentioned negative balance would be considerably increased. The hypothesis is however we think scarcely borne out by the results of Thannhauser. We think it more likely that the two classes are produced by collateral processes from a common origin.

In some of the modern text-books of physiological chemistry it is stated that the bile of the hippopotamus differs from that of other animals in containing no cholesterol. For instance, A. P. Mathews in his text-book of

Physiological Chemistry (1921, p. 432) states that "cholesterol has been found in the bile of all animals in which it has been looked for, with the exception of the hippopotamus," and on p. 434 he again draws attention to this and goes on to suggest "it would be interesting to know whether the blood of the hippopotamus contains cholesterol. Perhaps some African physiologist may make some interesting discoveries by studying the bile and blood of this animal." The statement is based on some work of Hammarsten [1911]. On looking up the original paper we found that the statement in such definite form was perhaps scarcely warranted. Hammarsten found that the bile of the hippopotamus was relatively poor in ether-soluble matter. From the bile of two animals he only obtained 0.756 g. of ether extract. 0.2 g. he used in testing for the presence of phosphorus compounds and the rest he hydrolysed with alcoholic potash in order to examine the unsaponifiable matter. He states "Der Nachweis von Cholesterin gelang jedoch nicht, als Endprodukt wurde nur eine äussert kleine Menge einer durchsichtigen firnisähnlichen Masse erhalten." He does not explain how he treated this unsaponifiable matter, or how he tested for cholesterol. In his general conclusions at the end he remarks "Cholesterin konnte nicht nachgewiesen werden, und die in reinem Äther löslichen Stoffe, deren Menge nur sehr klein war, bestanden hauptsächlich aus Olein mit ein wenig festem Fett." This statement of Hammarsten with regard to the bile of the hippopotamus stands in the way of the acceptance of the above-mentioned hypothesis of a cholesterol cycle, or indeed of any other hypothesis on cholesterol and bile in relation to digestion. It seemed therefore well worth while to subject the bile of this animal to closer investigation. For this purpose it was deemed necessary to obtain the materials from a healthy animal in a natural state.

For the specimens we are indebted to the kindly interest of the Under-Secretary of State for the Colonies and his staff and the Governor of Kenya. The specimens were taken from an adult male shot on the lower Tana river by Mr Keith Caldwell in March, 1923.

BILE OF THE HIPPOPOTAMUS.

Mr Caldwell reported that the animal killed had a very small gall bladder and that he doubted whether it contained much more than half a pint of bile. The bile was poured into excess of alcohol in a Winchester quart bottle for transit to England. The alcoholic solution was green in colour and contained a moderately bulky precipitate. The precipitate was filtered, well washed with alcohol and dried *in vacuo*. It dried up to a tough scale dark green in colour and weighing 1.1588 g. The alcoholic filtrate and washings were made up to 2 litres, and an aliquot portion (25 cc.) on evaporation gave a residue which after drying *in vacuo* weighed 0.1522 g. The total solid matter in solution was therefore 12.176 g. and the total solid matter of the whole bile 13.3348 g. If Mr Caldwell's estimate of the volume of the original bile is approximately correct the solid matter per 100 cc. would be 4.7 g. The residue obtained on evaporating the sample was soluble in warm water giving a clear yellowish

solution, with a slight green fluorescence, which was neutral to litmus. On cooling it became slightly turbid. This was added to the rest of the alcoholic solution (1975 cc.) and the bulk of the alcohol distilled off. The concentrated solution was then diluted with water and thoroughly extracted first with ether, then light petroleum and finally with ether. The combined extracts were made up to 500 cc. An aliquot portion (50 cc.) was evaporated and the residue after drying *in vacuo* to constant weight weighed 0.0836 g. This residue was dissolved in alcohol and the free cholesterol estimated by precipitation with digitonin, using the procedure of Fraser and Gardner [1910]. 0.0225 g. of cholesterol digitonide was obtained, equivalent to 0.00547 g. of cholesterol. The ether washings from the digitonide were evaporated to dryness, taken up in chloroform and the solution tested with acetic anhydride and sulphuric acid. A brown colour developed, which on standing turned a dusky green, so that possibly traces of cholesterol esters were present. The remaining ether extract (450 cc.) was evaporated, taken up in alcohol and hydrolysed by boiling with excess of an alcoholic solution of sodium ethoxide. After evaporating the greater portion of the alcohol, the product was diluted with water and extracted with ether in the usual manner. 0.1227 g. of an unsaponifiable matter was obtained. This was dissolved in alcohol and the cholesterol estimated, using in this case the procedure of Windaus [1910]. The cholesterol digitonide separated in typical crystalline flakes and weighed 0.2003 g. Thinking that owing to the volume of alcohol used a small portion of cholesterol digitonide might have been retained in solution, the filtrate was carefully evaporated to dryness, the residue washed well with ether and freed from excess of digitonin by washing with warm water. A further quantity of cholesterol digitonide weighing 0.0061 g. was obtained. The total cholesterol in the whole 500 cc. of ether extract was therefore 0.05575 g. and the free cholesterol was 0.0547 g. so that by difference the ester cholesterol was 0.001 g. This difference was probably real and not due to error of experiment, since the filtrate from the digitonide of the free cholesterol gave the sterol colour reaction with acetic anhydride and sulphuric acid. The cholesterol digitonide was dissociated in the usual manner in the vapour of boiling xylene, and after distilling off the xylene in steam the residue was crystallised from alcohol. It crystallised in the typical cholesterol plates and after twice recrystallising melted at 146–147°. The benzoate, prepared by the pyridine method, crystallised in typical form and, on heating, melted at 146° to a turbid liquid, which cleared at 170–180°, and on cooling showed the colour changes characteristic of cholesterol benzoate. The bile of the hippopotamus contains, according to Hammarsten, ethereal sulphates. This we were able to confirm, and thinking it possible that some cholesterol might be present as an ethereal sulphate, the aqueous mother liquors after extraction with ether were evaporated to dryness, dissolved in 100 cc. glacial acetic acid and boiled under a reflux for 4 hours. The solution was then largely diluted with water and thoroughly extracted with ether. The ethereal solution was freed from acid by thoroughly

washing first with water, then with alkali and finally with water. The ether was then distilled off, and the slight residue taken up in alcohol and boiled for 2 hours with sodium ethoxide. On diluting and extracting with ether in the usual way a residue weighing 0.076 g. was obtained. This yielded 0.0257 g. of cholesterol digitonide corresponding to 0.0062 g. of cholesterol. The ether washings from this digitonide were evaporated and taken up in chloroform. The solution gave with acetic anhydride and sulphuric acid a deep brown colour, which showed no further change on standing. We had not sufficient material to attempt to decide the interesting question whether this small quantity of cholesterol was really present in the bile as an ethereal sulphate or in chemical combination with some bile acid [Wieland and Weil, 1912]. The dark green solid from the alcoholic bile was rather difficult to powder. It was insoluble in alcohol but yielded to ether a trace of pigment which coloured the solution pink. Thinking that possibly some sterol matter, probably in ester form, might have been occluded in this material, it was largely dissolved by boiling under a reflux with an alcoholic solution of sodium ethoxide for several hours. The solution was then diluted with water and thoroughly extracted with ether. The washed ethereal extract on evaporation left a slight residue, which was taken up in alcohol and precipitated by digitonin, using the method of Fraser and Gardner. 0.0044 g. of cholesterol digitonide was obtained, corresponding to 0.001 g. of cholesterol.

We can generally confirm Hammarsten's description of the properties of the hippopotamus bile, with the exception of his statement about cholesterol, but the quantity at our disposal was insufficient for any attempt to extend his interesting account of the bile acids present.

BLOOD.

The fresh blood taken from the animal was carefully poured into excess of alcohol for transit. The proteins were precipitated in a fairly finely divided condition without any large clots, and beyond this precipitation the blood appeared to have undergone no autolytic change during the journey. It was filtered and the precipitate was well washed first with alcohol and then with ether and allowed to dry in the air on a porous tile. The air-dried material was bright red in colour and weighed 84.08 g. A small sample was dried to constant weight at 100°, and the result showed that the 84.08 g. of air-dried material contained 15.02 g. moisture.

The filtrate and washings were made up to 2200 cc. An aliquot portion (100 cc.) was evaporated on the water-bath and dried to constant weight *in vacuo*. The dry residue weighed 0.245 g. so that the total solid matter in the whole solution was 5.39 g. Assuming that the fresh blood contained 80 % of water, the weight of blood corresponding to the total dry matter would be about 372 g. 8.1887 g. of the air-dried red solid were dissolved in 2 % caustic soda solution, and the fluid repeatedly extracted with ether according to the method recommended by Fex [1921]. In this way 0.0318 g. of ether extract

was obtained. This was dissolved in alcohol and precipitated with excess of digitonin to obtain the amount of free cholesterol. 0.0074 g. of cholesterol digitonide was obtained, corresponding to 0.001799 g. of cholesterol.

To estimate the total cholesterol 9.1426 g. were similarly treated and the ether extract hydrolysed by means of sodium ethoxide in the usual way. The alcoholic solution of the unsaponifiable matter on precipitation with digitonin gave 0.0095 g. of compound corresponding to 0.002309 g. cholesterol.

84.08 g. of the air-dried red matter contained therefore 0.02124 g. total cholesterol, 0.01847 g. of free cholesterol and by difference 0.00277 g. of cholesterol in ester form.

The alcoholic filtrate, remaining after estimating total solids (2100 cc.) was evaporated to get rid of most of the alcohol, diluted with water and thoroughly extracted with ether. The ethereal solution was concentrated to 100 cc. 50 cc. on evaporation yielded 0.2889 g. of ether extract, so that the "fat" in 5.39 g. of solids was 0.6053 g. This was dissolved in alcohol and the free cholesterol precipitated in the usual manner with excess of digitonin. 0.2359 g. of compound corresponding to 0.05737 g. of cholesterol was obtained.

To estimate the total cholesterol the remaining 50 cc. were evaporated and hydrolysed with sodium ethoxide. On extraction with ether 0.1185 g. unsaponifiable matter yielded with digitonin 0.2345 g. of compound, equivalent to 0.057 g. cholesterol. Evidently this portion of the blood contained no cholesterol in ester form.

The total cholesterol in the 5.39 g. of solid matter dissolved in the alcohol was 0.119 g., all in the free state. In the whole blood the cholesterol in ester form was only 2.0 % of the total. This of course is on the assumption that no change had taken place in the ratio of free to ester cholesterol on keeping in alcohol for 6 months.

FLESH.

Pieces of muscle were cut off from the animal and placed in a stoppered jar with a little alcohol for transit. The material was cut into thin slices and minced. The meat was free from obvious fat and seemed quite sweet. As far as one could judge by the eye it did not appear to have undergone any autolytic change. The alcohol (about 40 cc.) was evaporated and the small residue added to the mince.

50.35 g. were taken for analysis. This was covered with 2 % solution of caustic soda, allowed to stand overnight, and then heated on the water-bath for half an hour, when practically the whole of the solid matter went into solution (method of Fex [1921]). This was thoroughly shaken with ether and allowed to stand 12 hours for the ether to separate. This process was repeated three times when it was found that nothing more was extracted. The combined ethereal solutions were evaporated and the residue dried to a constant weight. 0.1032 g. of ether extract, *i.e.* 0.205 % was obtained.

This ether extract was dissolved in alcohol and the free cholesterol pre-

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cipitated with excess of digitonin. 0.114 g. compound, corresponding to 0.0277 cholesterol, was obtained. The ether washings of this compound were evaporated and the residue, hydrolysed by means of sodium ethoxide in the usual manner, yielded 0.0201 g. of unsaponifiable matter. This on precipitation with digitonin gave 0.0084 g. of compound, equivalent to 0.00204 g. cholesterol in ester form.

The alkaline liquors left after extraction of the original stuff with ether were acidified and again extracted with ether. A minute trace of crystalline matter was obtained, but it contained no cholesterol.

The percentage of cholesterol in ester form was 6.8 of the total.

The results are summarised in the following table:

	Bile			Blood		Flesh
	Wt. in g. for whole bile	As % of the total solids	Per 100 cc. of bile*	% of total dry matter	Per 100 g. of blood assuming 80 % water	% of material (as received)
Ether extract	0.836	6.27	0.298	—	—	0.205
Total cholesterol	0.063	0.472	0.0224	0.1892	0.0379	0.059
Free cholesterol	0.0547	0.410	0.0195	0.1854	0.0372	0.055
Ester cholesterol	0.0083	0.062	0.0029	0.0037	0.0007	0.004

* Assuming Mr Caldwell's estimate is correct.

CONCLUSIONS.

(1) The bile of the hippopotamus, as Hammarsten found, is poor in fat. It contains, however, cholesterol both in the free and ester forms. In the specimen examined the total cholesterol was 0.47 % of the total solid matter.

The portion of the bile solids soluble in ether contained 6.54 % of free and 0.126 % of cholesterol in ester form. The portion insoluble in alcohol contained 0.001 g. of cholesterol, most probably in ester form. The part soluble in water and alcohol contained 0.0062 g. of cholesterol most probably in the form of an ethereal sulphate, since it could not be extracted by ether, and the cholesterol was only liberated by acid hydrolysis. This constituted about 74 % of the total ester cholesterol. The percentage of total cholesterol in this bile is much lower than the values given for the bladder bile of domestic animals such as the cow; data are, however, wanting for comparison with animals at grass.

(2) The quantities of free and ester cholesterol found in the blood and in the flesh, though on the low side, are we believe within the limits found for grass-fed animals, though available data are as yet scanty.

I desire to express my sincere thanks to the Under-Secretary of State for the Colonies and his Staff at the Colonial Office, and to the Governor of Kenya for their kindness in arranging to procure the specimens: also particularly to Mr Keith Caldwell, Big Game Warden at Nairobi for his trouble in finding and shooting a suitable animal and for the great care he took in cutting out, preserving and packing the specimens for transit. I also have to thank the Government Grant Committee of the Royal Society for help in defraying the laboratory expense of the investigation.

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