indicates that if an animal is found by a reliable experimental method to contain 12 per cent. more or less blood than is deduced by calculation from the surface, the average constant of the species being used, it is *probable* that the blood volume of the animal is abnormal, whilst, if it is 20 per cent. smaller or larger, it is *almost certain* that the blood volume is abnormally small or large.

It may be pointed out, however, that if the blood volume were expressed as a percentage of the weight, it would only be possible to say with the same degree of certainty that the blood volume of an animal was abnormal when it differed by at least 40 per cent. from the calculated figure.

The Origin and Destiny of Cholesterol in the Animal Organism. Part VIII.—*On the Cholesterol Content of the Liver of Rabbits under Various Diets and during Inanition.*

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(Communicated by Dr. A. D. Waller, F.R.S. Received November 8,—Read December 7, 1911.)

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In Parts V* and VII† of this series of papers evidence was brought forward to show that when cholesterol, free and in the form of esters, is given with the food of rabbits, some is absorbed and finds its way into the blood stream, and that an increase of both free cholesterol and cholesterol esters takes place in the blood.

This result affords support to the working hypothesis with regard to the origin and destiny of cholesterol in the animal organism, which we were led to formulate in an earlier paper,‡ viz., that cholesterol is a constituent constantly present in all cells, and when these cells are broken down in the life process the cholesterol is not excreted as a waste product but is utilised in the formation of new cells. A function of the liver is to break down dead cells, *e.g.*, blood corpuscles, and eliminate their cholesterol in the bile. After the bile has been poured into the intestine in the processes of digestion, the

* 'Roy. Soc. Proc.,' 1909, B, vol. 81, pp. 230—247.

† 'Roy. Soc. Proc.,' 1910, B, vol. 82, pp. 559—568; see also Pribram, 'Biochem. Zeit.,' 1906, vol. 1, p. 413.

‡ 'Roy. Soc. Proc.,' 1908, B, vol. 81, pp. 110—128.

cholesterol is re-absorbed, possibly in the form of esters, along with the bile salts and is carried in the blood stream to the various centres and tissues for re-incorporation into the constitution of new cells.

It seemed to us that valuable data for the elucidation of the cholesterol problem might be obtained by a careful study of the cholesterol and cholesterol-ester content of the various organs and tissues of the body, in the case of rabbits fed on diets containing varying amounts of cholesterol, and also of rabbits kept in a state of inanition. In this paper we give an account of our experiments on the cholesterol content of the livers of such rabbits.

Method of Estimating the Cholesterol.

The animals after anæsthetisation were bled as completely as possible. The livers were then taken out and weighed. The material was then finely ground with sand and plaster of Paris. The ground mass was then allowed to set, after which it was finely powdered and extracted in Soxhlet's apparatus with ether for from two to three weeks. The ethereal extracts were made up to a known volume with ether and carefully divided into two equal parts. One part was evaporated to dryness, dissolved in alcohol, and the free cholesterol directly estimated. The other half of the ethereal solution was saponified with sodium ethylate. After separating the soaps, the total cholesterol in the ethereal solution was estimated. The ester cholesterol was determined by difference. The cholesterol was estimated by the digitonin method of Windaus,* using the modified procedure fully described in Part VII of this series of papers.†

Cholesterol Content of the Livers of Rabbits fed on Green Food only.

For this purpose a diet of cabbage leaf and cabbage stalk was selected, as these substances appear to form an efficient diet. Further, cabbage is rich in vegetable sterols which are largely passed unchanged in the fæces. This formed a convenient means of obtaining these sterols, which were required for another purpose, from the plant in quantity.

Experiment I.—A strong healthy rabbit (A) was fed on cabbage leaf from December 28, 1910, to February 1, 1911. It took 1·5 lbs. of leaf per day. Twice during the experiment the cabbage leaf was mixed with some extracted bran to prevent the fæces getting too moist, as they were required for another purpose. The rabbit maintained a practically constant weight

* Windaus, "Über die Entziftung der Saponin durch Cholesterin," 'Ber. d. Deutsch. Chem. Ges.,' 1909, vol. 42, pt. 1, p. 238, and 'Zeit. für physiol. Chem.,' 1910, vol. 65, p. 110.

† 'Roy. Soc. Proc.,' 1910, B, vol. 82, pp. 559—568.

during the feeding period, as the following weighings show: December 28, 2·7 kgrm.; December 31, 2·5 kgrm.; January 1, 2·6 kgrm.; January 7, 2·45 kgrm.; January 11, 2·5 kgrm.; January 20, 2·5 kgrm.; January 23, 2·5 kgrm.; January 30, 2·5 kgrm.; February 1, 2·6 kgrm. When the animal was killed the stomach was full. The liver weighed 115·45 grm., and was unusually large for an animal of this weight, being 4·44 per cent. of the body weight. The total cholesterol, free and combined, was found to be 0·2425 grm., and the free cholesterol 0·2294 grm. The ester cholesterol was therefore 0·0131 grm.

Experiment II.—This rabbit (B) was fed on cabbage stalks from December 28, 1910, to February 16, 1911. It consumed 1·5 lbs. per day, and on two occasions during the feeding period it was also given some ether-extracted bran. The weight of the animal remained constant, the following being the weights during the feeding: December 28, 2·4 kgrm.; December 31, 2·35 kgrm.; January 3, 2·5 kgrm.; January 7, 2·45 kgrm.; January 11, 2·5 kgrm.; January 20, 2·5 kgrm.; January 23, 2·45 kgrm.; January 30, 2·3 kgrm.; February 6, 2·4 kgrm.; February 9, 2·4 kgrm.; February 11, 2·4 kgrm.; February 13, 2·34 kgrm.; February 16, 2·4 kgrm. The animal was killed on February 16 and the stomach was full. The liver weighed 82·91 grm., *i.e.*, 3·45 per cent. of the body weight. The total cholesterol, free and combined, was 0·1932 grm., and the free cholesterol 0·1228 grm. The ester cholesterol was then 0·0704 grm.

Cholesterol Content of the Livers of Rabbits fed on Bran which has been thoroughly extracted with Ether to remove all Fat and Phytosterols.

This diet was selected, as previous experiments had shown that rabbits can be kept at constant weight for many days together on this food. As the food was sterol-free, the influence of any absorption of vegetable sterols on the cholesterol content of the livers was eliminated.

Experiment III.—A healthy buck (C), weighing 2·3 kgrm., was fed on as much bran as it would consume from April 1 to April 8, 1910. The weights taken occasionally during the diet period were 2·3, 2·3, 2·2, 2·15, 2·2, 2·2, 2·2 kgrm. The liver weighed 62·2 grm., *i.e.*, 2·83 per cent. of the body weight. The total cholesterol, free and combined, was 0·1596 grm., and the free cholesterol 0·124 grm. The ester cholesterol was, therefore, 0·0356 grm.

Experiment IV.—This was a large doe rabbit (D), weighing 3·1 kgrm. It was fed from March 27 to April 11, 1911, and consumed during this period 1160 grm. of the bran. The weights of the animal taken occasionally were 3·1, 3·2, 3·1, 2·9, 2·9, 2·9, 3·1, 3·1 kgrm. The liver weighed 69·63 grm.,

or 2·25 per cent. of the body weight. The total cholesterol, free and combined, was 0·232 grm., free cholesterol 0·1527 grm., and ester cholesterol 0·0793 grm.

Cholesterol Content of the Liver of Rabbits fed on Extracted Bran to which Free Cholesterol had been added.

It has already been proved that when cholesterol is given with the food of rabbits, a portion only is excreted in the fæces, the remainder being absorbed in the intestine, giving rise to a well-marked increase in the cholesterol content of the blood. On the hypothesis mentioned at the beginning of the paper we should expect to find in such animals an increase in the cholesterol content of the liver.

Experiment V.—A healthy rabbit (E), weighing 2·8 kgrm., was fed from March 27 to April 14, 1911, on extracted bran to which cholesterol was added. It consumed during the period 1480 grm. of extracted bran, and 4·8 grm. of cholesterol. The cholesterol was given daily in 0·25 grm. portions mixed with a small quantity of the moistened bran, and care was taken that the animal ate the whole. The weights of the rabbit taken occasionally were 2·8, 2·8, 2·8, 2·7, 2·7, 2·7, 2·8, 2·8, 2·6, 2·6 kgrm. It thus lost during the whole period 0·2 kgrm.

The weight of the liver was 72·25 grm., *i.e.*, 2·77 per cent. of body weight. The liver contained 0·3315 grm. of cholesterol, and there was no ester present.

Experiment VI.—In this experiment a rabbit (F) was fed with as much extracted bran as it would eat. On the 1st, 2nd, 4th, and 5th days it received 0·25 grm. of cholesterol mixed with a little moist bran, and on the 6th, 7th, and 8th days 0·5 grm. It thus had 2·5 grm. during the period. The weights of the rabbit were as follows: 2·8, 2·7, 2·65, 2·6, 2·6, 2·65, and 2·7 kgrm. The liver weighed 74·7 grm., *i.e.*, 2·76 per cent. of body weight. It contained 0·341 grm. of free and combined cholesterol, 0·2144 of free cholesterol, the ester cholesterol thus being 0·1266.

Experiments in which Rabbits were fed on Extracted Bran, but the Cholesterol, instead of being given by the Mouth, was injected in Olive Oil Solution into the Peritoneal Cavity.

In order to ascertain whether cholesterin absorbed from other parts of the body than the intestine would be carried to the liver and cause an increase in the cholesterol content of that organ, two rabbits were fed on extracted bran. In one, the control, pure olive oil was injected into the peritoneal cavity, and in the other a solution of cholesterol in olive oil.

After recovering from the operations and feeding for some days the animals were killed and their livers analysed.

Experiment VII.—As a control a rabbit (G), weighing 3·4 kgrm., was anæsthetised with ether on July 13, and 10 c.c. of sterilised olive oil injected into the peritoneal cavity. The animal did not eat well until July 18, when its weight was 2·8 kgrm. After this date it took its food (extracted bran) readily, and its weight remained quite constant until July 28. On the following day another 10 c.c. of olive oil was injected as before. It was killed on August 6, when its weight was 2·4 kgrm. The liver, which was normal in appearance, weighed 51·27 grm., *i.e.*, 2·05 per cent. of body weight. Some oil still remained unabsorbed in the cavity. The weight of free and combined cholesterol in the liver was found to be 0·1698 grm., the free cholesterol 0·1418 grm., and the ester cholesterol, by difference, 0·028 grm.

The fæces of the animal were collected during the whole experiment, and after drying weighed 364 grm. They were extracted with ether, and the fats in the ethereal solution saponified with sodium ethylate. After separating the soaps and washing, the ethereal solution was evaporated to dryness. The oily residue obtained was taken up in alcohol and precipitated with digitonin. The precipitate was thoroughly washed with ether and then with water, and after drying weighed 0·254 grm. This precipitate was decomposed by heating in xylene vapour, according to the method of Windaus for recovering the cholesterol from its digitonin compound.

After evaporating the xylene and crystallising from alcohol, crystals were obtained which under the microscope had the form of typical cholesterol crystals. The fæces therefore contained 0·0617 grm. of cholesterol, an output of 0·0028 grm. per day.

Experiment VIII.—A vigorous rabbit (H), weighing 3·7 kgrm., was anæsthetised with ether, and 10 c.c. of olive oil, containing 0·5 grm. of cholesterol in solution, injected into the peritoneal cavity on July 13. As in case of rabbit (G) it took very little food (extracted bran) until July 16, when its weight was 3 kgrm. The weight remained fairly constant until July 29, when it weighed 2·8 kgrm. Another 10 c.c. of oil containing 0·5 grm. of cholesterol was again injected. The animal was killed on August 6, when its weight was 2·8 kgrm. Some of the oil was still unabsorbed. The liver, normal in appearance, weighed 59·01 grm., or 2·1 per cent. of the body weight. It contained 0·3485 grm. of free cholesterol and no ester cholesterol. During the experiment it passed 669 grm. of fæces (dry). This was treated as in the control experiment, and 0·695 grm. of digitonin compound was obtained, corresponding to 0·1689 grm. cholesterol, an output of 0·0073 grm. per day.

Cholesterol Content of the Livers of Rabbits during Inanition.

If the hypothesis put forward at the beginning of this paper is correct, we should expect that, during inanition, when an animal is living on its own tissues and the ordinary processes of digestion are in abeyance, an accumulation of cholesterol would take place in the liver. In order to test this, two rabbits, which had been long in stock and well fed, were selected. One was a fat animal, and the other a thin one, which, though well fed, showed little tendency to lay on fat.

Experiment IX.—This animal (I), at the beginning of the experiment, was fat, and weighed 3 kgrm. It was fed for three days on extracted bran, after which it was kept without food from October 28 to November 3, 1910, but was allowed water *ad lib.* It steadily decreased in weight: 2·9, 2·8, 2·65, 2·6, 2·5, 2·45 kgrm., and at the end of the period was apparently in good health. It appeared to suffer no inconvenience. It passed no fæces during the inanition period. After it had been killed, it was found that there was still some fat round the kidney and in other parts. The stomach and intestines contained a dark semi-fluid material, and the stomach was full of wind. Some fæces were found in the rectum. The gall bladder was distended. The loss in weight was 18 per cent. The liver was normal in appearance and weighed 43·01 grm., *i.e.*, 1·75 per cent. of the body weight. The total cholesterol, free and combined, was 0·3406 grm., and the free cholesterol 0·1831 grm. The ester cholesterol, by difference, was thus 0·1575 grm.

Experiment X.—This animal (J) was vigorous but thin, and at the commencement of the experiment weighed 1·9 kgrm. It was fed for three days on extracted bran, after which it was kept without food from November 11 to 17, 1910, but allowed plenty of water. It lost weight steadily, the weights being 1·8, 1·7, 1·6, 1·55, 1·45, 1·4, a percentage loss of 26·2. The animal suffered no obvious inconvenience during the fast. It was killed on November 17. The stomach contained a dark semi-fluid matter, and there were some fæces in the rectum. No fat was noticed round the organs. The animal passed no fæces during inanition. Unfortunately, the dark matter in the stomach and intestines was not analysed. The liver was normal in appearance and weighed 32·87 grm., *i.e.*, 2·35 per cent. of the body weight. The total cholesterol, free and combined, was 0·1234 grm., the free cholesterol 0·1123, and the ester cholesterol, by difference, 0·0111 grm.

Cholesterol Content of the Livers of Newly-born Rabbits.

Experiment XI.—Five newly-born animals were taken, the mother having been fed on an ordinary mixed diet of bran, oats, and green stuff.

The livers of the five animals weighed 17·12 grm. They were found to contain 0·0369 grm. of cholesterol, free and combined, 0·0317 grm. of free cholesterol. The ester cholesterol, by difference, was 0·0052 grm.

The results of the above 11 experiments are gathered together in the following table (p. 468).

Discussion of Results.

On comparing the figures in the following table it will be seen that in Experiments III, IV, and VII, on animals fed on extracted bran alone, the total free and combined cholesterol per kilogramme of body weight is remarkably constant. This figure may be taken as representing the normal cholesterol content of the liver under conditions in which the body weight is kept constant, but no cholesterol or phytosterol is absorbed with the food. On comparing these figures with those in Experiments I and II, in which the animals had been fed for a very long period on green food containing phytosterol, a small increase is noticed, indicating that some phytosterol was absorbed from the food and appeared in the liver in the form of cholesterol. It would of course require a much larger number of experimental data to be certain on the point, but the result is in agreement with the observations on blood published in Part VII of the series, in which a similar increase in the cholesterol content of the blood of rabbits fed on extracted bran *plus* phytosterol compared with that of similar animals fed on extracted bran alone was observed. If we consider the percentage contents of the livers themselves the increase is not observed. It will be noticed, however, that the livers of the two animals fed on green cabbage are extraordinarily large compared with those of the other animals of about the same weight. Whether this is accidental or brought about by the nature of the food we are unable to say, though the animals, as far as general and *post-mortem* appearances were concerned, seemed to have been in good health.

In the case of the animals E and F, fed on extracted bran to which an excess of cholesterol had been added, or H, in which the cholesterol was injected into the peritoneal cavity, a marked increase in the total cholesterol of the liver is noticeable, no matter whether the actual cholesterol found, or the percentage in the liver, or the weight per kilogramme of body weight is considered. This increase is much too large, we consider, to be due to chance.

In Experiments IX and X, on animals kept in a state of inanition and

Number of experiment and letter of rabbit.	Food of rabbit.	Weight of animal when killed, in kgrm.	Weight of liver, in grammes.	Weight of total cholesterol free and combined, in grammes.	Weight of free cholesterol, in grammes.	Weight of ester cholesterol, in grammes.	Weight of cholesterol per cent. of liver.			Weight of liver cholesterol per kgrm. of body weight.		
							Total, free and combined.	Free cholesterol.	Ester cholesterol.	Total, free and combined.	Free.	Ester.
I, A	Cabbage leaf.....	2·60	115·45	0·2425	0·2294	0·0131	0·211	0·199	0·012	**0·093**	0·088	0·005
II, B	Cabbage stalk	2·40	82·91	0·1932	0·1228	0·0704	0·233	0·148	0·085	**0·081**	0·051	0·030
III, C	Extracted bran.....	2·20	62·20	0·1596	0·1240	0·0356	0·257	0·199	0·057	**0·073**	0·056	0·017
IV, D	Extracted bran.....	3·10	69·63	0·2320	0·1527	0·0793	0·333	0·219	0·114	**0·075**	0·045	0·030
VII, G	Extracted bran (oil injected into peritoneal cavity)	2·40	51·27	0·1698	0·1418	0·0280	0·331	0·277	0·054	**0·071**	0·059	0·012
V, E	Extracted bran and cholesterol	2·60	72·25	0·3315	0·3315	Nil	0·459	0·459	Nil	**0·128**	0·128	Nil
VI, F	Extracted bran and cholesterol	2·70	74·70	0·3410	0·2144	0·1266	0·457	0·287	0·170	**0·126**	0·079	0·047
VIII, H	Extracted bran (cholesterol injected into peritoneal cavity)	2·80	59·01	0·3485	0·3485	Nil	0·591	0·591	Nil	**0·125**	0·125	Nil
IX, I	No food (water allowed)	2·45	43·01	0·3406	0·1831	0·1575	0·792	0·426	0·366	**0·139**	0·075	0·064
X, J	No food (water allowed)	1·40	32·87	0·1234	0·1123	0·0111	0·376	0·286	0·090	**0·088**	0·080	0·008
XI	Five newly-born rabbits (unfed)	—	17·12	0·0369	0·0317	0·0052	0·216	0·185	0·031	—	—	—

living on their own tissues, we find, as was expected, a similar storing up of the cholesterol in the liver. This is very marked in the case of the fat rabbit in Experiment IX, which probably used up the fat directly, and less marked in the case of the lean rabbit in Experiment X, which made a greater demand on its tissues. This result, we think, was also to be expected. In Experiment XI the percentage total cholesterol content of the liver of newly-born rabbits is of the same order of magnitude as that of adult rabbits. What factors govern the relative proportions of free cholesterol and cholesterol esters the experiments do not indicate.

These results we submit afford striking evidence in support of the hypothesis advanced at the beginning of this paper.

Addendum on the Examination of the Unsaponifiable Matter in the Fæces of Rabbits fed on Ether-extracted Bran.

In former investigations on the subject we never succeeded in crystallising cholesterol from the fæces of rabbits fed on extracted bran, but, as in Experiment VIII we succeeded in isolating cholesterol by the digitonin method from the fæces of rabbit G, which had a diet of extracted bran, but into the peritoneal cavity of which olive oil had been injected, it became necessary to examine more clearly the fæces of a normal animal fed in the same way. For this purpose a rabbit was fed for 15 days on extracted bran and the fæces collected. They weighed, after drying, 295 grm., and yielded, after treatment in the manner described, 0·6445 grm. of unsaponifiable oily matter, soluble in alcohol. This residue was dissolved in alcohol and mixed with as much of an alcoholic solution of digitonin as would have completely precipitated the residue had it consisted of pure cholesterol. This was allowed to evaporate to dryness spontaneously, and was obtained free from unchanged oil by means of ether. This oil weighed 0·3076 grm., and did not give any sterol colour reaction in chloroform solution with acetic anhydride and sulphuric acid. The digitonin precipitate, after washing repeatedly with hot water, weighed 1·2175 grm., but it was not free from digitonin, and was yellowish in colour. It was then finely powdered and washed with about 200 c.c. of ether until colourless. The ether dissolved about 0·25 grm. of solid matter. This solid was insoluble in petroleum ether, but soluble in benzene, and on testing with acetic anhydride and sulphuric acid only gave the sterol colours in a slight and indefinite manner. The 0·99 grm. of digitonin compound was then heated in xylene vapour until completely decomposed, and the clear xylene solution on evaporation gave a yellow, oily solid. This was only partly soluble in ether, leaving a white, insoluble powder, which did not give any sterol reaction. The ethereal

solution was evaporated and the solid recrystallised from 95-per-cent. alcohol. The crystalline matter which separated was impure, and, even after recrystallisation, we could recognise under the microscope no crystals which could be definitely described as cholesterol. The whole was then converted into benzoate by the action of benzoyl chloride in pyridin solution; 0·068 grm. of a benzoate was obtained, which, after repeated recrystallisation from alcohol, was still slightly yellow in colour. It melted at 142° C. to a clear brown liquid, which, on cooling, gave a brilliant *green* play of colours at the moment of solidification, gradually changing to brown. This behaviour was quite different from cholesterol benzoate, which melts at 145° to a turbid liquid, only becoming clear at about 180°, and, on cooling, gives a play of purple and blue colours of quite characteristic appearance. Under the microscope the crystalline matter was indefinite in appearance and one could find none of the characteristic square envelopes of cholesterol benzoate.

Had the 0·97 grm. of insoluble digitonin compound consisted entirely of cholesterol digitonide, it would have corresponded to 0·24 grm. of cholesterol, an output of only 0·016 grm. per day.

In order to determine satisfactorily the nature of the unsaponifiable residue, it will be necessary to prepare it in large quantity, and this we must reserve for a further investigation.

We take this opportunity of thanking the Government Grant Committee of the Royal Society for help in carrying out this work.