DESCRIPTION OF PLATE 3.

Coefficient of diffusion. Drawn by P. Nairn from a preparation of fresh blood cells which have been resting for 10 minutes at 37° C. on an agar film with an index of diffusion of 4. The nucleus of one polymorphonuclear leucocyte has just stained and the cell is showing three small red spots. The nuclei of two large lymphocytes have not yet stained, one cell is showing 1 centrosome and the other 3 centrosomes. The film also demonstrates an eosinophile leucocyte which is becoming achromatic, *i.e.*, its nucleus has lost its stain; and one granular red cell which contains two red spots. 2 mm. apochromatic objective, No. 4 eye-piece, 250 mm. draw-tube, 1 amp. Nernst lamp.

The Origin and Destiny of Cholesterol in the Animal Organism. Part III.—*The Absorption of Cholesterol from the Food and its Appearance in the Blood.*

By Charles Dorée, Lindley Student of the University of London, and J. A. Gardner, Lecturer in Physiological Chemistry, University of London.

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In his 'Text-Book of Physiology' Schäfer has suggested that the constant presence of lecithin and cholesterol in the bile may well be associated with the destruction of the red blood corpuscles which contain relatively large amounts of these substances, the latter, according to Hepner,* being present in the free state and not in the form of esters. This idea has recently received strong support from the investigations of Chasoburō Kosumoto† on the influence of toluylene diamine on the output of cholesterol in the bile. This reagent was found by Schmiedeberg to produce icterus, and Stadelmann, working in Schmiedeberg's laboratory,‡ observed that at the beginning of the action of the drug an increased production of bile took place. This, however, was only temporary, and soon the normal physical properties of the bile underwent an alteration; it became sticky, darker, and more concentrated.

Afanassiew§ showed that the effect of the drug is to destroy the red blood

* 'Pflüg. Archiv f. d. Ges. Physiol.,' 1898, vol. 73, p. 595.
† 'Bioch. Zeit.,' 1908, vol. 13, p. 354.
‡ 'Arch. f. experim. Pathol. u. Pharmak.,' 1881, vol. 14, pp. 231, 422.
§ 'Zeit. f. klin. Med.,' vol. 6.

corpuscles. The increased viscosity, which Afanassiew correlated with certain changes observed by him in the liver tissue, causes a hindrance to the flow of bile, and since the bile formation is, perhaps, increased beyond the normal, leads to icterus. When the action of the toluylene diamine ceases—according to Afanassiew owing to the removal of the hindrance which opposes the bile flow—the amount of bile increases again, possibly even above the normal. These statements made it appear probable that the cholesterol of the red corpuscles destroyed would appear in the bile, along with the bile colouring matter. By careful measurements of the quantities of bile produced in dogs in which permanent fistulæ had been established in a satisfactory manner, and by estimations of cholesterol in the bile, by methods which do not seem to us open to objection, before and after the administration of toluylene diamine, Kosumoto showed that this was the case. The conclusion seems to be justifiable that a part at any rate of the cholesterol of the bile arises from the *débris* of the normal destruction of red blood corpuscles in the liver.

On the other hand, the percentage of cholesterol in the fistula bile of dogs does not appear, according to the investigations of Goodman,* to depend upon the cholesterol content of the food taken by the animals, a result in accordance with the work of previous observers. Goodman made the interesting observation that with a diet of 725 grammes of coagulated white of egg, which contains little or no cholesterol, he was able, during five days, to collect 477 grammes of bile containing 0·208 gramme of cholesterol, whilst with a diet of 488 grammes of calves' brain, which is very rich in cholesterol, he obtained in four days 367 grammes of bile containing 0·145 gramme of cholesterol, so that the percentage content in cholesterol of the bile excreted in these two diets was 0·0436 in the case of the white of egg, and 0·0395 in that of brain. It seems probable, therefore, that the cholesterol of the bile is not derived directly from the food, and some of it at any rate is the result of the elimination of the cholesterol of dead blood corpuscles, and possibly also the *débris* of other tissues, by the liver. On the other hand, this cholesterol can in no sense be regarded as a waste product, as we have shown in previous papers† that herbivorous animals do not excrete cholesterol or any recognisable derivative of that body in their fæces, although their bile contains considerable quantities, in the case of the cow for example 0·07 per cent.‡ Further, in the case of the dog any cholesterol found in the fæces can be entirely accounted for by the cholesterol contained

* 'Hofmeister Beit.,' 1907, vol. 9, p. 91.

† 'Roy. Soc. Proc.,' B, vol. 80, p. 12.

‡ 'Journal of Physiol., Proc.,' 1907, vol. 36, p. ix.

in the food taken. In experiments in which cholesterol free food was given no cholesterol was found; thus a dog fed for 31 days on oatmeal and water passed no more than 0·1 gramme of impure cholesterol in the fæces.

From these observations we were led to the conclusion that the cholesterol of the bile must either be destroyed, or absorbed, along with the bile salts in the intestine, and taken into the blood stream.

The latter hypothesis is more in accordance with the great stability of the cholesterol molecule, and is supported by the observation of Pribram* that emulsions of cholesterol with olive oil, when injected into the stomach of the rabbit, cause an increase in the cholesterol content of the blood.

From a consideration of these facts we have been led to put forward with regard to the origin and destiny of cholesterol the following working hypothesis :—

1. Cholesterol is a constant constituent of 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.

2. A function of the liver is to break down dead cells, *e.g.*, blood corpuscles, and to eliminate their cholesterol in the bile.

3. After the bile has been poured into the intestine in the process of digestion the cholesterol is reabsorbed, probably in the form of esters, along with the bile salts, and carried by the blood to the various centres and tissues for re-incorporation into the constitution of new cells.

The question arises whether the excretion and subsequent absorption of the cholesterol of the body form 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. With reference to the wastage of cholesterol it must be pointed out that not inconsiderable quantities are excreted through the skin; the sweat and sebum of men have been shown to contain cholesterol, and in the case of the sheep the sweat, being absorbed by the wool, enables us to demonstrate the presence of large quantities of cholesterol and isocholesterol. How is the wastage made up? Considering the remarkable chemical nature of cholesterol it would appear less probable that it is synthesised by the animal than that the loss is made up by the absorption of cholesterol obtained from the food. In the case of carnivora the cholesterol is contained in their food as such, and might easily be utilised. On the other hand, the food of the herbivora contains no cholesterol, but instead the closely related phytosterols, and the question arises whether this closely allied substance can be utilised by the animal.

* 'Bioch. Zeit.,' 1906, vol. 1, p. 413.

If the wastage is made up in this way we should expect to find variations in the cholesterol content of the blood according as the food was free from, or rich in, cholesterol. If, further, it were able to take in from its richer diets more cholesterol than was at the moment required, storing it up in the intervals of feeding to replace loss we should expect considerable variations in the cholesterol content of the plasma with the variety of the food. If, however, the animal only takes up what is required to supply immediate waste we should not expect more than a slight variation on different diets, and this variation might easily be entirely masked owing to the different quantities of bile caused to flow into the intestine under the influence of foodstuffs of different kinds. With regard to the mechanism of the absorption of cholesterol in the intestine it would seem probable that it is first esterified, being converted into the oleic and palmitic esters. These compounds, which are stated to possess the property of forming with aqueous fluids lanoline-like emulsions,* were found by Hürthle to be constantly present in the blood plasma of various animals.†

With a view to testing the validity of these considerations we planned a series of experiments, the first instalment of which is described in the present paper.

Experiments to Ascertain whether Cholesterol is Absorbed by Herbivorous Animals when given with their Food.

The animal selected for this investigation was the rabbit. Preliminary experiments showed that the bulk of the phytosterols of bran can be extracted along with the fat by means of ether, without altering the appearance of the bran and without impairing its feeding value, except for the elimination of the fat. It was found that rabbits could be kept for long periods on this diet without apparent injury to health, and often without much loss in weight, though individuals varied considerably in this respect.

The general method adopted in these experiments was as follows:—A rabbit was fed for several days previous to the commencement of an experiment on extracted bran. It was then given each morning 0·25 gramme of cholesterol mixed with a few grammes of extracted bran, care being taken to see that the animal ate the whole. After eating the cholesterol-bran mixture the animal was allowed during the rest of the day as much extracted bran as it would eat. This procedure was followed until the animal had eaten 2 grammes of cholesterol, after which it was fed on extracted bran only for three days in order to sweep all cholesterol from its gut. The fæces during

* Ivor Bang, 'Ergebnisse d. Physiologie,' vol. 2, p. 180.

† 'Zeit. Physiol. Chemie,' vol. 21, p. 331.

the experiment were carefully collected, dried in the oven at 80° to 90° and weighed. The fæces were then extracted with ether in a Soxhlet's apparatus for a week or ten days. The ethereal solution was saponified according to the method described in our former paper* by means of sodium ethylate. The precipitated soap was filtered off and washed thoroughly with ether. The ethereal filtrate and washings were freed from excess of alkali and alcohol by repeatedly washing with water, dried, and the ether distilled off. The dry residue was weighed and then fractionally crystallised from alcohol until no further crystalline matter could be obtained. The oily residues were then dried, dissolved in pyridine, and treated with excess of benzoyl chloride and after standing over night poured into water. The precipitated matter was filtered, taken up with ether, dried, and the ether again evaporated. The residue was boiled with a little alcohol and any cholesterol that had remained in the oily residue was thus obtained in the form of the highly insoluble benzoate.

Experiment I.—In order to ascertain whether it was possible to extract the whole of the cholesterol from fæces by the method used, 2 grammes of cholesterol were ground up with moistened fæces that had already been extracted, in the proportions usually found. The mixture was dried and subjected to the whole process detailed above, when 2·098 grammes of slightly brown-coloured cholesterol were obtained—a quantitative recovery. As the cholesterol recovered from natural fæces is often highly coloured and can only be readily purified by treatment with animal charcoal, it was also desirable to ascertain what loss occurred under the conditions usually followed. The two grammes of recovered cholesterol were therefore dissolved in about 50 c.c. of alcohol, boiled with about half the weight of animal charcoal, and filtered by means of a hot funnel. The charcoal was then washed with hot alcohol. On evaporating the alcohol and crystallising the cholesterol 1·8 grammes were recovered. The loss due to boiling with charcoal was therefore about 10 per cent.

Experiment II.—In order to ascertain (1) how far it was possible to extract the phytosterol from bran by simply extracting with ether for several days; (2) whether any cholesterol could be detected in the fæces, after phytosterol had been eliminated as far as possible from the diet; and (3) how far the quantity of the oily unsaponifiable matter was affected by the use of ether extracted food; and (4) whether an animal could be kept in a healthy state on a prolonged, ether extracted diet, a rabbit weighing 2·4 kilogrammes was fed from December 30, 1907, to January 15, 1908, inclusive on extracted bran, moistened with a little water. The fæces were

* 'Roy. Soc. Proc.,' B, vol. 80, p. 212.

collected from January 2nd to the 15th inclusive and weighed, after drying, 462 grammes. During this period the animal was given 1·12 kilogrammes of extracted bran, most of which it ate with apparent satisfaction. On January 15th its weight was 2·4 kilogrammes and during the period of the experiment varied from 2·4 to 2·5 kilogrammes.

The fæces were extracted with ether and the ethereal solution saponified in the manner described; 1·215 grammes of dry unsaponifiable matter was obtained as a viscid stiff oil. This dissolved in a small quantity (20 c.c.) of absolute alcohol, with the exception of 0·05 gramme of insoluble tar. On adding enough water to make the alcohol about 85 to 90 per cent. strength and allowing to stand, some crystalline matter separated and, after filtration and drying, was obtained in the form of dark brown greasy crystals weighing 0·4 gramme.

This brown crystalline matter was decolorised by animal charcoal and carefully recrystallised fractionally from alcohol. The first and main crop melted at 135° to 136°, and under the microscope had the form of transparent long hexagonal plates. It was identical with the "phytosterol" which we had isolated from the bran. The mother liquors yielded a small crop of crystals which under the microscope were more indeterminate, being grouped in masses and stars. On recrystallisation, however, long hexagonal crystals were again obtained melting at 133° to 135°. *No trace of cholesterol could be discovered.*

Experiment III.—Immediately on the close of the above experiment the same rabbit was fed from January 16 to January 25 inclusive, on 80 grammes of extracted bran with 0·25 gramme of cholesterol per day, except on two days on which no cholesterol was given. It received therefore in this period 2 grammes of cholesterol. It was then fed for three days on extracted bran alone, and the fæces were collected during the whole period. After drying, the fæces weighed 497 grammes. The weight of the animal remained constant all through the experiment.

The ethereal extract was reddish in colour, with a green fluorescence, which was just as marked after saponification; 2·56 grammes of dry unsaponifiable matter were obtained. This dissolved in absolute alcohol leaving 0·06 gramme of insoluble tar. Water was then added to reduce the alcohol to 90 per cent., and on standing, brown crystalline matter was deposited weighing 1·14 grammes. On further standing another crop weighing 0·28 gramme was obtained. The mother liquors were then evaporated to dryness, dissolved in pyridine and treated with benzoyl chloride; 0·1035 gramme of crude benzoate of cholesterol was obtained. The total weight of crude cholesterol was thus 1·5 grammes. The two crops of

cholesterol were decolorised by animal charcoal and recrystallised from alcohol. The larger crop melted at 139° and was practically pure cholesterol, but the smaller crop softened at about 125° and melted at 135°. Under the microscope the latter appeared to consist mainly of cholesterol, but was contaminated with phytosterol. The benzoate after recrystallisation melted at 144° to 145° to a turbid liquid which cleared at 180° and on cooling showed the characteristic play of colour in a well-marked manner. About 1 gramme of purified cholesterol was in this way obtained. Evidently therefore the rabbit had absorbed between 0·5 and 1 gramme of cholesterol during the time of the experiment. The animal remained in good health for some weeks afterwards, its weight remaining constant, when it was killed for another purpose.

Experiment IV.—The rabbit used in this experiment weighed 1·7 kilogrammes, and was very thin. It was fed for the three days prior to the commencement of the experiment on extracted bran; it was then given 0·25 gramme of cholesterol and 60 to 70 grammes of extracted bran daily for eight days, and extracted bran alone for three more days. Care was taken that the animal took the whole of the cholesterol, but it wasted a good deal of the bran. The animal lost weight during the whole experiment, and died the day after, when its weight was 1·3 kilogrammes. A *post-mortem* examination showed that the animal was very thin, and in poor condition. The intestine was filled with a watery fluid, the liver very dark in colour, and the stomach dilated with gas. It may be noted that during this experiment the weather was very cold, and for a few days the heating apparatus was out of order, so that possibly this may have had something to do with the death of the animal, which was not in the best of condition at the start.

Three hundred and twelve grammes of dry fæces were obtained. The ethereal extract was pale yellow in colour, and on evaporation gave 1·46 grammes of unsaponifiable matter as a greasy brown solid. This dissolved in 85 to 90 per cent. alcohol, with the exception of a small amount of tar which was insoluble in absolute alcohol, though soluble in ether. On standing, the solution deposited 0·79 gramme of brown crystalline matter. A further crop, weighing 0·32 gramme, was obtained on long standing. This was more granular in appearance, and rather sticky. The mother liquors were evaporated to dryness and benzoylated in pyridine solution, but no matter difficultly soluble in alcohol could be isolated.

The two crops were decolorised by animal charcoal and recrystallised. The first deposit melted at 142° to 143°, and under the microscope was seen to consist of practically pure cholesterol. The second crop of crystals

melted at 141° to 142°, but a microscopic examination showed that it contained some phytosterol, as the shape of the crystals deviated from those of cholesterol in the well-known manner occasioned by traces of phytosterol. A final crop melted at 138° to 139°, and contained a larger amount of phytosterol. In this experiment it is evident that at least 1 gramme of cholesterol was absorbed.

Experiment V.—A rabbit, weighing 1·7 kilogrammes, was treated exactly as in Experiment IV. At the end it weighed 1·6 kilogrammes, and appeared to be in good health; 277 grammes of dry fæces were obtained, which were extracted for 14 days in a Soxhlet's apparatus with ether. The ethereal solution was deep red, with a strong green fluorescence. The dry unsaponifiable matter weighed 2·275 grammes, and was dissolved in 70 c.c. of hot alcohol, leaving 0·05 gramme of insoluble tar. After adding 6 c.c. of water to the hot alcohol solution, 1·39 grammes of red crystalline matter were deposited, from which 1·125 grammes of fairly pure cholesterol were obtained. The mother liquors, on standing, deposited a small quantity of crystalline matter mixed with oil, from which, after boiling in alcoholic solution with animal charcoal, 0·11 gramme of white crystals was isolated. All the mother liquors were evaporated and benzoylated, but only a small trace of difficultly soluble matter resulted. The weight of cholesterol recovered was therefore 1·23 to 1·4 grammes, which, however, would contain any phytosterol present.

Experiment VI.—A rabbit, weighing 1·7 kilogrammes, was fed on extracted bran and cholesterol as in the previous experiments. The weight remained practically constant, but after the experiment, on being put on ordinary diet, it began to lose weight, and died on the eighth day. The weight of the dead animal was 1·4 kilogrammes, but a *post-mortem* examination revealed nothing abnormal.

The weight of dry fæces collected was 283 grammes, and they were extracted for 14 days. The ethereal extract was pale yellow in colour, but without any fluorescence. The unsaponifiable matter weighed 3·13 grammes, but was not free from calcium chloride. It was boiled with absolute alcohol, when 0·145 gramme of insoluble matter was left. The alcoholic solution after dilution with water until the strength was 85 to 90 per cent., gave 1·47 grammes of not very coloured crystalline matter. The mother liquors yielded between 0·1 and 0·2 gramme of impure crystalline matter. After recrystallisation of the whole from the least amount of 90 per cent. alcohol, the melting point was 135°.

The results of these experiments are summarised in Table I.

It is clear from these experiments that (1) cholesterol is not excreted by rabbits unless they are fed on it, which is in agreement with our previously

Table I.

Exp.	Duration of experiment, in days.	Weight of rabbit at beginning, in kilogrammes.	Weight at end.	Weight of cholesterol given.	Weight of bran, in kilogrammes.	Weight of dry fæces.	Total unsaponifiable matter dry, in grammes.	Weight of crude cholesterol recovered, including phytosterol.
II.	14	2·4	2·4	—	1·12	462	1·215	0·4 of phytosterol
III.	11	2·4	2·4	2	1·01	497	2·56	1·5
IV.	11	1·7	1·3	2	0·67	312	1·46	1·11
V.	11	1·7	1·6	2	0·73	277	2·27	1·23—1·4
VI.	11	1·7	1·7	2	0·73	283	3·13	1·4—1·6

published results; (2) that when cholesterol is administered with the food a portion of it is absorbed, in our experiments about 50 per cent. It is also clear that vegetable food such as bran or grass can be freed from fat and phytosterols by extraction with ether without impairing its feeding value.

Is Cholesterol Absorbed from the Food by Carnivorous Animals?

In our former paper on the excretion of cholesterol by the dog* we described experiments which, although they were carried out primarily for the purpose of showing that the cholesterol content of the fæces was a function of the cholesterol in the food taken, may yet be considered as evidence for the absorption of some cholesterol from the food in this animal. But such evidence cannot be regarded as of the same conclusive nature as that afforded by the experiments on the rabbit, because the cholesterol content of the various foodstuffs given is not known with any degree of certainty. The estimations of Dormeyer†, for the cholesterol content of dry muscle (0·23 per cent.), are perhaps as satisfactory as any, though they probably err, if anything, in being too high. However, if we take such a value and apply it to our own data we arrive at such results as the following. In one experiment a dog in 20 days ate 7470 grammes of cooked beef and mutton, the percentage of solids in which we found to be about forty. Allowing for the fact that the meat did not consist entirely of muscle and for variations of other kinds, we may perhaps halve Dormeyer's value in this case. On this assumption, then, the animal consumed 3·4 grammes of cholesterol, whereas 0·8 gramme only was found in the fæces, so that a disappearance of about 2·5 grammes of cholesterol is indicated.

* 'Roy. Soc. Proc.,' B, vol. 80, p. 227.

† 'Pflüg. Archiv,' vol. 61, p. 341.

In another experiment the animal ate 6758 grammes of horseflesh in 17 days. Making the calculation as before, we find that the dog may have eaten 4 grammes of cholesterol, whereas only 1 gramme was discovered, pointing again to an absorption of possibly 3 grammes.

We did not institute any further experiments with the dog on these lines on account of the numerous uncertainties involved. We have, however, lately been successful in discovering a cholesterol-free diet comparable with extracted bran on which cats can be fed, and have begun an elaborate series of experiments on the question which we hope to communicate in the near future. Preliminary experiments on cats showed us that, as in the case of dogs, the cholesterol of animal food is passed in the fæces as such, but that on a brain diet these animals also convert the cholesterol into coprosterol. The two following experiments may be quoted here as bearing on this interesting point.

Experiment VII.—A cat weighing 2·8 kilogrammes was fed on raw sheep's brain for 14 days. The fæces were somewhat liquid, and dried at 100° to a soft, sticky, glue-like mass, which was ground up with excess of sand before extracting with ether. Weight of dry fæces, 245 grammes. During the period of diet the animal lost 0·5 kilogramme in weight, which loss, however, it subsequently regained on ordinary diet; 28 grammes of unsaponifiable matter, in the form of a dark red viscid oil were obtained. By fractional crystallisation from acetone between 18 and 19 grammes of brown crystalline matter were separated. This consisted of coprosterol, and after further purification from dilute alcohol 12 grammes of perfectly pure coprosterol were obtained. This melted at 99° to 100° C., and had a specific rotatory power (in chloroform) $[\alpha]_D^{16} = +20°{\cdot}4$.

On a diet of raw sheep's brain, therefore, the cat changes cholesterol into coprosterol in the same way as we showed was the case with the dog.* If we assume that sheep's brain contains 2 per cent. of cholesterol, our cat should have consumed in the 14 days some 34 grammes of cholesterol, which would correspond with an absorption of about 15 grammes, or 1 gramme per day. Owing to the difficulty of crystallising coprosterol completely from the oily matter with which it is mixed in the unsaponifiable residue, one cannot claim for this estimation any high degree of certainty. It is, however, significant that the total weight of unsaponifiable matter obtained was less than the total cholesterol that should have been consumed, and there can be no doubt that the extraction of the fæces by ether was a very thorough one, as it was allowed to go on for 14 days, the ether distilling over during the day and being allowed to soak on the material during the night.

* 'Roy. Soc. Proc.,' B, vol. 80, p. 227.

Experiment VIII.—In this experiment the sheep's brain was lightly fried in its own oil before being given, in order to ascertain whether the cooking process had any influence on the conversion of the cholesterol to coprosterol. The cat selected weighed 2·9 kilogrammes, and during the feeding period of 14 days lost 0·4 kilogramme in weight; 1913 grammes of brain (weighed uncooked) were consumed, and 365 grammes of dried fæces obtained. These were of very much the same constituency as before, and were treated in a similar manner; 23 grammes of unsaponifiable matter in the form of a red oil were obtained. This proved very difficult to purify, owing to the tarry, sticky oils present, and we were only able to isolate 7·4 grammes of white crystalline matter, which proved to be a mixture of cholesterol and coprosterol. The cholesterol taken (on the above assumption) should therefore be 38 grammes, the total unsaponifiable matter being only 23 grammes. The cooking and consequent partial sterilisation of the brain seem, therefore, to have interfered with the conversion of the cholesterol into coprosterol.

Experiments to ascertain whether any of the Cholesterol which disappears from the Food can be found in the Blood.

Herbivorous Animals.

Pribram* has stated that on administering cholesterol to rabbits *per os*, an increased percentage of this substance could be found in the blood. His method consisted in injecting into the stomach of the animal an emulsion of cholesterol, cholesterol oleate, or cholesterol palmitate, made up with olive oil. After some hours the animal was killed, and the cholesterol in the blood determined in the usual way. But from the point of view of proving that cholesterol taken by the mouth can be absorbed into the blood stream these experiments seem to us for several reasons by no means conclusive. In the first place, the rabbits studied were far from being under normal conditions of diet. A comparatively large dose of oil was put into the stomachs of animals who are not accustomed to take or assimilate fats in this form. Pribram mentions that the oil passed into the blood as the serum became opalescent, and it seems to us not improbable that, with a quantity of oil in the stomach which cannot be assimilated in the ordinary way, some of the oil might percolate, if one may use the term, into the blood, carrying the cholesterol with it. The supposed increase, therefore, found in the blood might not unreasonably be due to a mechanical rather than to a metabolic process. But a further consideration of the data given by Pribram led us to doubt whether they could be considered as showing that there was an increased percentage of cholesterol.

* 'Bioch. Zeit.,' vol. 1, p. 414.

The standard of comparison employed by him was the cholesterol content of the blood of a starving rabbit, which again is an abnormal case, since conceivably some cholesterol might disappear from the blood during starvation, and if this is so it is probably a variable standard, as different animals would vary in the rate at which the cholesterol was removed from their blood. Again, the quantities of cholesterol isolated and weighed were extremely small, and were admittedly not pure. No melting points or other constants were given, and we are consequently left uncertain as to whether the matter weighed was cholesterol in a more or less pure state, or whether it was largely composed of crude unsaponifiable matter, or whether it contained any cholesterol at all. As the percentages found were so small, a very slight variation in the amount of impurity present would invalidate and even entirely reverse the conclusions deduced by the author. In a second series of experiments, however, which bring out the increased inhibitory power of the serum of the cholesterol-fed rabbit towards the hæmolytic effect of saponin, the results are more satisfactory, and certainly speak for an assumption of cholesterol by the blood. But our objection to the method of dosage adopted still holds, and the number of hæmolytic experiments carried out was too few. Our own experiments in comparing the action of sera in this respect have shown us that there is a very considerable variation in the action of the sera of individual animals of the same species, when treated under precisely similar conditions. The discussion of this point, however, we leave for the present, as we hope to make it the subject of a communication in the near future.

In the experiments we have carried out to ascertain the fate of the cholesterol which disappears from the food we have endeavoured to avoid the difficulties pointed out in the preceding paragraph in the following way:—

1. We adopted as a standard of comparison a rabbit which was fed for a long period on a cholesterol-free diet, viz., bran thoroughly extracted with ether. The blood of such an animal was compared with that from others which had been fed in an exactly similar way as to times and quantities of extracted bran, but whose food contained in addition a measured daily quantity of cholesterol. In this way we had two rabbits feeding on practically the same diet under the same conditions, and accordingly the chance of variations, especially in the bile flow, due to differences in the food taken was reduced to a minimum, and the only variation likely to interfere was that due to the individual peculiarities of different rabbits which are inevitable in such experiments.

2. With regard to the estimation of cholesterol in the tissues and the

blood, we must again emphasise the fact that the weight of crude unsaponifiable matter obtained from them gives little or no idea of their cholesterol content. In our experience, the ether extract of animal tissues always contains relatively large quantities of low melting oily or resinous bodies, which may prove to be of very considerable importance in biochemistry, but they are non-crystalline and cannot be considered as cholesterol. Furthermore, their amount is very variable, so that even for purposes of comparison the weights of crude unsaponifiable matter are useless. Our own procedure was as follows: The blood, if dried in the ordinary way, becomes a very hard horny mass which even if powdered is difficult to extract. We therefore mixed the blood after whipping to prevent coagulation with sand and plaster of Paris in sufficient quantity to form a friable mass. This was ground up and extracted for 14 to 30 days, the heating being stopped during the night so that the ether might thoroughly soak into the material. The extract was saponified in the manner we have previously described, and the non-saponifiable residue converted directly to benzoate in pyridine solution, the cholesterol being thus weighed in the form of cholesterol benzoate.

Experiment IX.—Rabbit A. A rabbit, weighing 2·8 kilogrammes, was fed for 21 days with 70 to 80 grammes of extracted bran per day, and then killed 24 hours after the last supply of food had been placed in the cage. A *post-mortem* examination showed that the stomach still contained some food. The animal during this period lost 0·3 kilogramme in weight. The weight of blood obtained was 73 grammes, from which 0·14 gramme of unsaponifiable matter in the form of a stiff oil was obtained. The quantity of cholesterol contained in this was so small that it was not found possible to isolate any in a pure state.

Experiment X.—Rabbit B. This rabbit, weighing 2·2 kilogrammes, was fed for three days on extracted bran, then during 10 days on 540 grammes of extracted bran, mixed with $2\frac{1}{4}$ grammes of cholesterol, care being taken that the whole of the cholesterol was eaten. The weight of the animal remained unaltered during this period, and it was killed 24 hours after the last meal had been placed in the cage. The blood obtained weighed 71 grammes and yielded 0·29 gramme of crude unsaponifiable matter, from which 0·0375 gramme of pure cholesterol benzoate was obtained. The specimen, which was actually weighed without further crystallisation, melted at 142° to 143° to a turbid liquid which became clear at 170°, and on cooling showed the characteristic play of colours. This quantity corresponds to a yield of 0·0295 gramme of cholesterol or 0·0415 per cent.

Experiment XI.—Rabbit C. In order to compare the cholesterol content

of an animal fed on a normal diet which contained phytosterol but not cholesterol, a rabbit weighing 2·8 kilogrammes was fed on a liberal mixed diet of cabbage, oats, and bran for a month, and killed after 24 hours as in previous experiments. The blood obtained weighed 75 grammes, which yielded 0·117 gramme of unsaponifiable matter as a brown oil mixed with crystalline material. After treating with benzoyl chloride in the usual way 0·028 gramme of greasy crystals was obtained, which were obviously not pure. Under the microscope these appeared as star-shaped aggregates of needles mixed with indeterminate matter, but no typical crystals of cholesterol benzoate were observed, and the substance could not be further purified. It is obvious that there is not sufficient cholesterol in the blood of a single rabbit, when fed on a non-cholesterol, or on a normal diet, for an accurate quantitative estimation. We therefore fed six rabbits, weighing 1·5, 1·7, 1·4, 1·9, 1·5, 1·9 kilogrammes respectively, on a liberal diet of oats, bran, and greens for a week. They were then killed and the total blood taken. This weighed 500 grammes. On treatment in the usual way 0·464 gramme of unsaponifiable matter as a brown, slightly greasy solid was obtained. This was crystallised from alcohol; the first crops, weighing respectively 0·091 and 0·049 gramme, consisted, as a microscopic examination showed, mainly of cholesterol, plate-like crystals of which were mixed with minute spherules of some other substance. These crystals were dried and treated in pyridine solution with benzoyl chloride. All the mother liquors remaining were evaporated to dryness and treated with pyridine and benzoyl chloride. The benzoate found was recrystallised from a measured quantity of absolute alcohol; 0·1523 gramme of pure cholesterol benzoate in all was thus obtained, which, without further purification, melted correctly, and gave the colour play of cholesterol benzoate; 0·1199 gramme of cholesterol was thus obtained from the blood of six rabbits or 0·024 per cent.

It is clear from these experiments that Pribram was correct in his conclusions, and that cholesterol can be absorbed from the intestines into the blood of the animal, since in the case of the rabbits which had been fed on cholesterol we were easily able to prepare and weigh pure cholesterol benzoate, whereas in the case of a rabbit fed on extracted diet, or on normal diet, the quantity was so small that we were unable to obtain any cholesterol from it in a pure state. In order to get a precise figure for the cholesterol content of the blood of the rabbit under normal conditions we were obliged to deal with the blood of six rabbits.

In the case of rabbits A, B, and C we made estimations of the cholesterol contained in the brain and spinal cord, and in the rest of the animal respectively. We can, however, draw no conclusions from the results of the

experiments, but we think the actual determinations are of sufficient interest and accuracy to be placed on record. The method we adopted for the brain and cord was to mix with plaster of Paris in a mortar, and, after the mass has hardened, to powder it thoroughly. This was then thoroughly extracted with ether (21 days) and the extract treated in the usual way. The rest of the rabbit carcase (including the fur) was finely minced in a machine, mixed with plaster of Paris, and again passed through the machine. After it had set to a dry mass it was ground up in a mortar with coarse sand and plaster of Paris, and extracted for three weeks with ether. The results are collected together in the following table :—

Table II.

	Weight in grammes.	Unsaponifiable matter.	Pure cholesterol found.	Cholesterol per cent.
Rabbit A, weight 2·5 kilos., fed on extracted bran for 20 days.				
Blood	73	0·14	(trace)	—
Brain and spinal cord	14·3	0·68	0·427	3·0
Rest of rabbit	2413	3·75	2·168	0·09
Rabbit B, weight 2·2 kilos., fed on extracted bran + $2\frac{1}{4}$ grammes of cholesterol for 10 days.				
Blood	71	0·29	0·0295	0·0415
Brain and spinal cord	13·31	1·31	(lost)	—
Rest of rabbit	2116	3·50	1·908	0·09
Rabbit C, weight 2·8 kilos., fed on a mixed diet of cabbage, oats, and bran for 1 month.				
Blood	75	0·117	(trace)	—
Brain and spinal cord	17·5	0·776	0·5225	3·0
Rest of rabbit	2708	5·11	2·716	0·10
Six rabbits, fed on above mixed diet.				
Blood	500	0·464	0·1199	0·024

EXPERIMENTS TO ASCERTAIN WHETHER THE CHOLESTEROL CONTENT OF THE BLOOD CAN BE CORRELATED WITH VARIATION IN THE CHOLESTEROL CONTENT OF THE FOOD IN CARNIVOROUS ANIMALS.

A. *Experiments in which the Animals were killed two to four hours after a Meal.*

Experiment XII.—A dog, weighing 7·36 kilogrammes, was fed for 10 days on a daily ration of 200 to 300 grammes of bread, the whites of two eggs,

and a teaspoonful of cream, the whole being moistened with a solution of Liebig's extract of beef, and lightly fried. The animal was killed three and a half hours after the last meal. The blood weighed 480 grammes, and from this 0·687 gramme of unsaponifiable matter was obtained. This was at once benzoylated in pyridine solution, and a total crop of cholesterol benzoate weighing 0·4865 gramme was separated. This corresponds to 0·3892 gramme, or 0·0811 per cent. of cholesterol.

A *post-mortem* examination showed that the stomach was practically empty. The gall-bladder contained 5·2525 grammes of bile which on evaporation yielded 0·892 gramme of solid matter. From this 0·002 gramme of cholesterol benzoate was obtained, or 0·18 per cent. of cholesterol (calculated on the dry solids).

Experiment XIII.—A dog, weighing 8·13 kilogrammes, was fed for nine days on a daily ration of 250 grammes of raw brain. At first it did not take kindly to this food, but in the last few days it consumed the whole of the brain given. The animal was killed two hours after a meal. The weight of blood obtained was 430 grammes, which yielded 0·74 gramme of unsaponifiable residue. This was benzoylated directly in pyridine solution, and 0·484 gramme of pure cholesterol benzoate was obtained, melting correctly. This corresponds to 0·3872 gramme, or 0·09 per cent. of cholesterol.

A *post-mortem* examination showed that the stomach contained some undigested food. The gall-bladder contained 2·06 grammes of bile of a pale yellow colour, and left 0·2245 gramme of solid matter. From this 0·004 gramme of unsaponifiable matter was obtained, but only a trace of benzoate could be separated from it.

Experiment XIV.—A dog, weighing 9·77 kilogrammes, was fed for nine days on a daily ration of 200 grammes of dry oatmeal made into porridge with water. The animal ate about half the last meal only, and was killed four hours afterwards. The blood weighed 680 grammes and yielded 0·91 gramme of unsaponifiable matter. This, on benzoylation in pyridine solution, gave 0·6775 gramme of pure cholesterol benzoate, corresponding to 0·542 gramme, or 0·0797 per cent. of cholesterol in the blood.

A *post-mortem* examination showed that the stomach contained a quantity of undigested food. The gall-bladder contained 6·36 grammes of bile which, on evaporation, yielded 1·1065 grammes of solid residue. From this only 0·001 gramme of cholesterol benzoate could be obtained, corresponding to 0·07 per cent. of cholesterol.

Experiment XV.—A dog weighing 8·5 kilogrammes was fed for six days on a daily ration of 200 to 300 grammes of brain, mixed with some bread. The

animal was killed four hours after the last meal. The blood was unfortunately lost. The gall-bladder contained 702 grammes of bile which, on evaporation, yielded 1·3947 grammes of solid residue. From this, 0·0028 gramme of cholesterol benzoate was obtained, or 0·16 per cent. of cholesterol.

B. *Experiments in which the Animals were killed 24 hours after a Meal.*

Experiment XVI.—A dog weighing 7·8 kilogrammes was fed for 7 days on porridge made by boiling about 100 grammes of oatmeal with water, daily. It was killed 24 hours after the last meal. The quantity of blood obtained was 524 grammes, from which 0·9845 gramme of greasy unsaponifiable matter was obtained. The first crop of crystals from alcohol weighed 0·2915 gramme, and a microscopic examination showed that these consisted of practically pure cholesterol. The residues were benzoylated in pyridine solution and yielded 0·1795 gramme of crystals, which melted correctly and gave the characteristic colour play of cholesterol benzoate. The total cholesterol obtained was therefore 0·435 gramme or 0·083 per cent.

A *post-mortem* examination showed that the stomach was quite empty. The gall-bladder was distended and contained 4·55 grammes of bile, which, after drying at 100° C., left 0·8257 gramme of solid matter. We attempted to estimate the cholesterol in this quantitatively, but the amount was too small for an accurate determination. However, 0·0025 gramme of cholesterol benzoate was isolated, or 0·24 per cent. of the total solids.

Experiment XVII.—A dog weighing 9·1 kilogrammes was fed for 14 days on raw brain. It was given 300 to 500 grammes of fresh brain per day, but the animal did not take the food very well and often left a portion uneaten. On the last day of the period the dog developed feverish symptoms and was killed 24 hours after the last meal. The quantity of blood obtained was 507 grammes and yielded 1·43 grammes of unsaponifiable matter. This was not completely soluble in alcohol. The first crop of crystals weighed 0·5075 gramme and melted at 145° C. A second crop weighed 0·0785 gramme and melted at 144° C. From the residues on benzoylation 0·2115 gramme of cholesterol benzoate, melting at 145°, was isolated. The total cholesterol obtained was therefore 0·7526 gramme, or 0·1486 per cent.

A *post-mortem* examination showed that the stomach was empty and distended with gas, and the lungs appeared to be congested. The gall-bladder contained 0·844 gramme of bile, which was very thick and stringy, and contained 0·1617 gramme of solid matter. From this 0·0025 gramme of cholesterol benzoate was obtained, or 1·2 per cent. of the total solids.

Experiment XVIII.—A large dog weighing 19·2 kilogrammes was fed for 24 days on raw brain together with a little bread. This animal took the

food readily and consumed daily from 500 to 800 grammes of brain, and at the end of the period was in good health. It was killed 24 hours after a full meal. The weight of blood obtained was 1140 grammes, which yielded 1·5 grammes of unsaponifiable matter. On crystallisation from alcohol a first crop, weighing 0·304 gramme, melting at 144° to 145°, was obtained, and a second weighing 0·184 gramme and melting at the same temperature. The mother liquors and residues, on benzoylation, gave 0·41 gramme of cholesterol benzoate. The total cholesterol obtained was therefore 0·816 gramme, or 0·072 per cent.

A *post-mortem* examination showed that the stomach was empty. The gall-bladder contained 15·82 grammes of bile, which gave 3·947 grammes of solid matter, and from this 0·017 gramme of cholesterol benzoate was obtained, or 0·34 per cent. of cholesterol.

Experiment XIX.—A dog weighing 13·4 kilogrammes was fed for 10 days on a diet practically free from cholesterol. The daily ration consisted of 250 to 300 grammes of bread, the whites of two eggs, two teaspoonfuls of cream lightly fried together after moistening with a solution of Liebig's extract of beef. The animal continued in good health and was killed 24 hours after the last meal. The blood weighed 760 grammes and yielded 1·326 grammes of unsaponifiable residue. On crystallising from alcohol the first crop weighed 0·4615 gramme and melted at 145°, the second weighed 0·2135 gramme and the same melting point. After benzoylation, the residues yielded 0·1728 gramme of cholesterol benzoate, melting at 145°, and showing the colour changes. The total cholesterol obtained was therefore 0·8132 gramme, or 0·107 per cent. A *post-mortem* examination showed that the stomach was empty. The gall-bladder contained 10·29 grammes of bile which, on evaporation, gave 2·632 grammes of solid matter. From this, 0·003 gramme of cholesterol benzoate was obtained, or 0·09 per cent. of cholesterol.

These results are collected together in Table III.

The experiments differ fundamentally from those carried out on the rabbit described in the earlier part of this paper. In the case of these animals a standard diet free from cholesterol and similar bodies, to which measured portions of cholesterol could be added, was available; and as they are practically continuous feeders the bile flow and consequently the cholesterol content of the blood, due to this source, would remain practically constant. In the case of the dogs we had no such cholesterol-free standard diet and were obliged to make use of a number of entirely different foods, differing as far as possible in cholesterol content, though even the non-cholesterol ones contained phytosterol. Little is known concerning the

Table III.

Experiment.	Weight of dog, in kilogrammes.	Diet.	Diet period, in days.	Weight of blood, in grammes.	Weight of cholesterol in the blood.	Percentage of cholesterol in the blood.
A.—Animals killed *two* to *four* hours after a meal. (Terriers.)						
XII.	7·36	Bread, egg-white, cream	10	480	0·3892	0·0811
XIII.	8·13	Raw brain	9	430	0·3872	0·0900
XIV.	9·77	Meal	9	680	0·5420	0·0797
B.—Animals killed *twenty-four* hours after a meal.						
XVI.*	7·8	Meal	7	524	0·4350	0·083
XVII.†	9·1	Raw brain	14	507	0·7526	0·148
XVIII.‡	19·2	Raw brain + bread	24	1140	0·8160	0·072
XIX.§	12·67	Bread, egg-white, cream	10	760	0·8132	0·107

* Terrier. † Terrier; dog jaundiced.
‡ Retriever dog. § Collie dog.

influence of food on the excretion of bile, but from experiments that have been made by various observers there is good reason to suppose that the nature of the diet would not be without influence. Furthermore, the dog is a discontinuous feeder and the flow of bile into its intestine would be intermittent. Under these circumstances the portion of the floating (as distinguished from the constitutional) cholesterol in the blood due to the reabsorption of the cholesterol of the bile, would not necessarily be strictly comparable in these different cases. As to what would be the limits of such variation, if any, we have no data at present from which to form an opinion, but a variation of the kind suggested might wholly or partially mask any variation due to the cholesterol absorbed from the food, which, at best, would not be great in absolute magnitude. In the first series of experiments (A) in which the animals are fairly comparable in weight and variety, the blood of the dog fed on brain shows a small increase in the percentage of cholesterol in its blood, though it would not be unreasonable to ascribe this to an extra bile flow due to the fatty nature of the diet. In the second series of experiments (B) in which the blood was taken after the digestive process was completed, the percentages found were of much the same order of magnitude as in the first series, with the exception of Experiment XVII. It will be noticed, however, that the percentage in the blood of the retriever dog fed on brain was slightly less than that in the blood of the dogs fed on meal, and bread and egg-white respectively. These animals were, however, very different in weight and were of different varieties.

Little value can be given to the high figure obtained in Experiment XVII, as the animal was ill at the time its blood was taken.

It seems to us very doubtful whether the chemical methods of estimating cholesterol, which we have endeavoured to make as perfect as possible, are sufficiently accurate to enable us to draw definite conclusions without making an enormous number of experiments of this type. We have, however, recently found a material suitable for the food of cats, which can be rendered cholesterol-free, and a series of experiments are in progress to compare the effect on the blood of the addition of cholesterol to such a diet both by chemical analysis and by comparisons of the anti-hæmolytic effect of the sera. The results of these experiments, which we expect to give more definite information on this subject, we hope to make the subject of a communication in the near future.

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