

The Origin and Destiny of Cholesterol in the Animal Organism.
 Part V.—*On the Inhibitory Action of the Sera of Rabbits
 fed on Diets containing Varying Amounts of Cholesterol on
 the Hæmolysis of Blood by Saponin.*

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In an earlier paper* of this series it was shown that cholesterol is not excreted in the faeces of herbivorous animals, and that when rabbits are fed on a diet free from phytosterol but containing measured quantities of cholesterol, a portion of the latter substance is absorbed. The hypothesis was put forward that cholesterol is a substance which is strictly conserved in the animal economy; that when the destruction of the red blood corpuscles and possibly other cells takes place in the liver, their cholesterol is excreted in the bile, and that the cholesterol of the bile is reabsorbed in the intestine along with the bile salts, and finds its way into the blood stream to be used in cell-anabolism. It was also suggested that any waste of cholesterol might possibly be made up from that taken in with the food. In order to test this view, comparative estimations were made of the total cholesterol content of the blood of rabbits that had been respectively fed on bran which had previously been thoroughly extracted with ether, and on the same extracted bran with the addition of known amounts of cholesterol, care being taken that the animals were otherwise identically treated and were kept in good health. The results of the experiments showed that some, at any rate, of the cholesterol absorbed found its way into the blood stream. It seemed to us desirable to ascertain next whether the cholesterol was absorbed into the blood stream as such or in the form of esters or in both states, and also whether the phytosterol of vegetable food can be utilised for the formation of cholesterol in the organism. Owing to the small percentage of these substances in the blood, it did not seem probable that the chemical methods hitherto available for their estimation were sufficiently accurate to give reliable information unless in each experiment a larger number of animals was taken than we could conveniently attend to. It seemed likely, however, that a comparative study of the inhibitory effects of the sera of rabbits fed

* "Origin and Destiny of Cholesterol," Part III, 'Roy. Soc. Proc.,' B, vol. 81, 1909 p. 109.

on different diets on the hæmolytic action of saponin on blood might throw light on the points mentioned.

The experiments of Hausmann,* Abderhalden and Le Count† have proved that whereas cholesterol and phytosterol inhibit the action of saponin, their esters do not do so. More recently Windaus‡ has shown that the inhibitory action of cholesterol and phytosterol is due to the fact that they form pharmacologically inactive compounds with saponin. We therefore decided to make a comparative study of the inhibitory action of the sera of rabbits fed respectively on ether-extracted bran and extracted bran with cholesterol, extracted bran and extracted bran with cholesterol esters, and, finally, extracted bran and extracted bran with phytosterol.

In the present paper an account is given of these experiments.

Method of Feeding the Animals under Experiment.—In each experiment, two large healthy rabbits, A and B, were fed for nine or ten days on bran which had been thoroughly extracted with ether. Excess of food was placed in the cages so that the animals could eat as much as they wished. In the case of rabbit A, after the third day a weighed amount of cholesterol mixed with a small quantity of moist extracted bran was given daily in addition, care being taken that the animal ate the whole of it. The rabbit was killed three or four hours after the last cholesterol meal, and the blood collected in a sterile vessel. The blood was allowed to clot and was placed in the refrigerator until the serum separated. The rabbit B was killed at the same time and its blood collected and treated in a similar manner. Great care was taken to keep the animals in good health and as far as possible under the same conditions. It is also desirable not to extend the experiment over too long a period lest the continued sameness of the diet should have a deleterious effect. If the animals are in bad health, more especially if they waste away and become emaciated, the results are entirely vitiated. This is well illustrated in the following experiment, which was commenced for another purpose and only continued as a matter of curiosity. Six medium-sized rabbits, weighing respectively 1·3, 1·4, 1, 1·2, 1·5, 2·3 kilogrammes, which were, to begin with, in very poor condition, were fed for three weeks on extracted bran. They continued in poor condition and became very emaciated, and had the experiment been further prolonged would no doubt have died. They yielded altogether only 190 grammes of blood. The blood was analysed by the method described in an earlier paper.§ 0·1799 gramme

* 'Hofmeister's Beitr.,' vol. 6, p. 567.

† 'Ztschr. für exper. Path. und Ther.,' vol. 2, p. 199.

‡ 'Ber. d. deut. chem. Ges.,' 1909, vol. 42, No. 1, p. 238.

§ *Ibid.*

of pure cholesterol benzoate was obtained, corresponding to 0.1423 gramme of cholesterol, or 0.0745 per cent. This is a higher percentage than we have ever found in healthy animals, even in the case of those fed on diets rich in cholesterol. We have not yet had an opportunity of making further experiments in this direction, but we do not think the result is in disagreement with the hypothesis referred to at the beginning of this paper.

Method of Carrying out the Hæmolytic Experiments.—The method employed is to mix together a suspension of blood corpuscles, a solution of saponin and the serum, making up to a constant volume with physiological salt.

For this purpose the following solutions are used :—

(1) Physiological salt—a 0.85-per-cent. solution of NaCl (specially purified) in distilled water.

(2) Saponin (Merck)—a 0.01-per-cent. solution in the physiological salt.

(3) Rabbit's blood corpuscles—a 5-per-cent. suspension in physiological salt.

In carrying out the experiments, the mixtures of blood, saponin, serum, and physiological salt are very carefully measured into glass tubes with accurately ground glass stoppers. The tubes are then placed in clamps fitted on to a circular plate of wood in such a manner that they radiate from the centre to the circumference. This disc, with the tubes attached, is slowly revolved in a vertical plane by means of a clockwork drum, the whole apparatus being kept at a constant temperature of 37° by placing it in an incubator.

As the tubes are completely inverted during the revolution of the disc, the corpuscles are kept equally distributed throughout the mixture without violently agitating the contents.

Elastic bands hold the stoppers securely in position to prevent their coming out of the tubes as they expand with the heat of incubation. The most favourable period of incubation was found to be three hours. After this the tubes are placed on ice and the corpuscles allowed to settle, or they may be at once centrifugalised. In either case the amount of hæmolysis is judged from the colour of the clear supernatant liquid.

By taking readings of a tintometer [Michael], on consecutive days, it was found that the tint of the tubes kept on ice did not vary, this showing that further hæmolysis than that taking place during incubation was prevented entirely by placing on ice. The ice method was adopted generally.

Experiments to ascertain whether Cholesterol administered with the Food and absorbed by the Animal appears in the Blood Stream as such.

Experiment I.—It was first necessary to ascertain the least amount of saponin solution which would produce complete hæmolysis in a given

volume of a 5-per-cent. suspension of rabbit's blood corpuscles. The results of the experiment are embodied in the following table:—

Table I.

Amount of blood, 5-per-cent. suspension.	Amount of saponin, 0·001 per cent.	Amount of NaCl, 0·85 per cent.	Result.
c.c.	c.c.	c.c.	
2	0·1	3·9	Incomplete hæmolysis
2	0·25	3·75	" "
2	0·5	3·5	" "
	0·01-per-cent. sol.		
2	0·1	3·9	" "
2	0·25	3·75	" "
2	0·5	3·5	" "
2	1	3	Complete hæmolysis
2	2	2	" "

Incubated for three hours at 37° C.

Thus 1 c.c. of a 0·01-per-cent. solution of saponin in a 0·85-per-cent. solution of NaCl completely hæmolyses 2 c.c. of a 5-per-cent. blood suspension in a total volume of 6 c.c.

It was found, however, in the course of the experiments, that the least amount of saponin needed to cause hæmolysis varied slightly with different specimens of rabbit's blood. For this reason it was necessary always to have the serum of a rabbit fed on extracted bran alone to compare with the serum of a rabbit fed on extracted bran and cholesterol, etc., so that the comparative experiments were carried out on the corpuscles of the same rabbit.

Experiment II.—Rabbit 1; fed on extracted bran for 10 days; weight of rabbit without blood = 1·7 kilogrammes.

Rabbit 2; fed for 10 days on extracted bran, during the last six days given $\frac{1}{4}$ gramme cholesterol per day. Weight of rabbit without blood = 2·5 kilogrammes.

Rabbit 3; fed for 10 days on extracted bran, during the last six days given $\frac{1}{4}$ gramme cholesterol per day for the first three days, 1 gramme per day for the last three days. Total, $3\frac{3}{4}$ grammes cholesterol.

Animals kept under the same conditions and killed at the same time. Sera collected 36 hours after death.

Results of experiment are given in the following tables:—

Table II.—Inhibitory Action of Serum of Extracted Bran-fed Rabbit (Rabbit 1).

Amount of blood, 5-per-cent. suspension.	Amount of serum.	Amount of NaCl, 0·85 per cent.	Amount of saponin, 0·01 per cent.	Result.
c.c. 2	c.c. 0·025	c.c. 2·975	c.c. 1	Almost complete hæmolysis
2	0·05	2·95	1	Slight hæmolysis
2	0·1	2·9	1	Trace hæmolysis
2	0·5	2·5	1	No hæmolysis
2	1	2	1	"

Table III.—Inhibitory Action of Serum of Rabbit fed on Extracted Bran + Cholesterol (Rabbit 2).

Amount of blood, 5-per-cent. suspension.	Amount of serum.	Amount of NaCl, 0·85 per cent.	Amount of saponin, 0·01 per cent.	Result.
c.c. 2	c.c. 0·025	c.c. 2·975	c.c. 1	No hæmolysis
2	0·05	2·95	1	"
2	0·1	2·9	1	"
2	0·5	2·5	1	"
2	1	2	1	"

Table IV.—Inhibitory Effect of Serum of Rabbit fed on Extracted Bran + Cholesterol (Rabbit 3).

Amount of blood, 5-per-cent. suspension.	Amount of serum.	Amount of NaCl, 0·85 per cent.	Amount of saponin, 0·01 per cent.	Result.
c.c. 2	c.c. 0·025	c.c. 2·975	c.c. 1	No hæmolysis
2	0·05	2·95	1	"
2	0·1	2·9	1	"
2	0·5	2·5	1	"
2	1	2	1	"

Incubated for three hours at 37° C.

In order to control these figures, the experiments were repeated the following day, with identical results. It was found, however, that when kept over a period of several days the sera lost their inhibitory effect to some extent, but even then the relative strengths remain in the same proportion.

Experiment III.—Rabbit 4; fed on extracted bran for 14 days, weight = 1·5 kilogrammes.

Rabbit 5; fed on extracted bran for 14 days, the last eight days with cholesterol in addition; $\frac{1}{4}$ gramme the two first, $\frac{1}{2}$ gramme the next four, and 1 gramme the last two days. Total cholesterol, $4\frac{1}{2}$ grammes; weight = 2 kilogrammes.

Animals kept under similar conditions and killed at the same time. Serum collected day after death.

The results of experiments are tabulated below:—

Table V.—Inhibitory Effect of Serum of Rabbit fed on Extracted Bran (Rabbit 4).

Amount of blood, 5-per-cent. suspension.	Amount of serum.	Amount of NaCl, 0·85 per cent.	Amount of saponin, 0·01 per cent.	Result.
c.c. 2	c.c. 0·025	c.c. 2·975	c.c. 1	Almost complete hæmo- lysis
2	0·05	2·95	1	Considerable hæmolysis
2	0·1	2·9	1	A trace hæmolysis
2	0·5	2·5	1	No hæmolysis

Table VI.—Inhibitory Effect of Serum of Rabbit fed on Extracted Bran + Cholesterol (Rabbit 5).

Amount of blood, 5-per-cent. suspension.	Amount of serum.	Amount of NaCl, 0·85 per cent.	Amount of saponin, 0·01 per cent.	Result.
c.c. 2	c.c. 0·025	c.c. 2·975	c.c. 1	Considerable hæmolysis
2	0·05	2·95	1	A trace hæmolysis
2	0·1	2·9	1	No hæmolysis
2	0·5	2·5	1	„

Exactly similar results were given by the sera heated for over an hour to 56° C., as the following table shows. The heating was performed with the object of showing that the inhibitory action of the sera on hæmolysis is not due to an organic enzyme.

Table VII.—Inhibitory Action of Heated Serum of Rabbit fed on Extracted Bran alone (Rabbit 4).

Amount of blood, 5-per-cent. suspension.	Amount of serum.	Amount of NaCl, 0·85 per cent.	Amount of saponin, 0·01 per cent.	Result.
c.c. 2	c.c. 0·025	c.c. 2·975	c.c. 1	Almost complete hæmo- lysis
2	0·05	2·95	1	Hæmolysis
2	0·1	2·9	1	Slight hæmolysis
2	0·5	2·5	1	No hæmolysis

Table VIII.—Inhibitory Action of Heated Serum of Rabbit fed on Extracted Bran + Cholesterol (Rabbit 5).

Amount of blood, 5-per-cent. suspension.	Amount of serum.	Amount of NaCl, 0·85 per cent.	Amount of saponin, 0·01 per cent.	Result.
c.c. 2	c.c. 0·025	c.c. 2·975	c.c. 1	Hæmolysed
2	0·05	2·95	1	A trace hæmolysis
2	0·1	2·9	1	No hæmolysis
2	0·5	2·5	1	„

A third series of observations was made the same day, with the sera in a 10-per-cent. solution of physiological salt. The results are given below :—

Table IX.—Inhibitory Action of 10-per-cent. Solution of Serum of Rabbit fed on Extracted Bran (Rabbit 4).

Amount of blood, 5-per-cent. suspension.	Amount of serum, 10 per cent.	Amount of NaCl, 0·85 per cent.	Amount of saponin, 0·01 per cent.	Result.
c.c. 2	c.c. 0·25	c.c. 2·75	c.c. 1	Almost complete hæmo- lysis
2	0·5	2·5	1	Hæmolysis
2	1·0	2·0	1	Slight hæmolysis
2	1·5	1·5	1	No hæmolysis

Table X.—Inhibitory Action of 10-per-cent. Solution of Serum of Rabbit fed on Extracted Bran + Cholesterol (Rabbit 5).

Amount of blood, 5-per-cent. suspension.	Amount of serum, 10 per cent.	Amount of NaCl, 0·85 per cent.	Amount of saponin, 0·01 per cent.	Result.
c.c.	c.c.	c.c.	c.c.	
2	0·25	2·75	1	Hæmolysed
2	0·5	2·5	1	A trace hæmolysis
2	1·0	2·0	1	No hæmolysis
2	1·5	1·5	1	„

We repeated these experiments with three other pairs of rabbits, and the results were so similar that we need not recapitulate them. It is obvious, therefore, that the cholesterol absorbed from the food appeared in the blood stream in the free state. Whether any entered in the form of esters it is impossible to say, but experiments are in progress which we hope will throw light on this subject.

It now seemed of interest to ascertain whether, the animals being fed on esters of cholesterol in addition to extracted bran, the esters were absorbed entirely as such, or whether they undergo hydrolysis in the process of digestion, and find their way into the blood stream as free cholesterol, in part, at any rate.

For this purpose we made use of pure cholesterol oleate and cholesterol stearate for feeding the animals.

Experiment IV.—Rabbit 6; fed on extracted bran for 18 days; weighed 2·2 kilogrammes at beginning, 2·3 kilogrammes at end of experiment.

Rabbit 7; fed on extracted bran for eight days, the last six days fed with cholesterol oleate in addition; $\frac{1}{4}$ gramme for four days, 1 gramme per day for two days; weight of animal = 2 kilogrammes at beginning and end of feeding.

Rabbit 8; fed on extracted bran for eight days, the last six days fed with cholesterol stearate in addition; $\frac{1}{4}$ gramme for four days, 1 gramme per day for two days; weight of animal = 2 kilogrammes at beginning and end of feeding.

Animals were kept under the same conditions and killed at the same time. Serum collected next day.

Results of experiment are tabulated below :—

Table XI.—Inhibitory Action of Serum of Rabbit fed on Extracted Bran (Rabbit 6).

Amount of blood, 5-per-cent. suspension.	Amount of serum.	Amount of NaCl, 0·85 per cent.	Amount of saponin, 0·01 per cent.	Result.
c.c. 2	c.c. 0·025	c.c. 2·725	c.c. 1·25	Almost complete hæmo- lysis
2	0·05	2·7	1·25	Considerable hæmolysis
2	0·1	2·65	1·25	"
2	0·25	2·5	1·25	Slight hæmolysis "

Table XII.—Inhibitory Action of Serum of Rabbit fed on Cholesterol Oleate in addition to Extracted Bran (Rabbit 7).

Amount of blood, 5-per-cent. suspension.	Amount of serum.	Amount of NaCl, 0·85 per cent.	Amount of saponin, 0·01 per cent.	Result.
c.c. 2	c.c. 0·025	c.c. 2·725	c.c. 1·25	Hæmolysis
2	0·05	2·7	1·25	"
2	0·1	2·65	1·25	Slight hæmolysis
2	0·25	2·5	1·25	No hæmolysis

Table XIII.—Inhibitory Action of Serum of Rabbit fed on Cholesterol Stearate in addition to Extracted Bran (Rabbit 8).

Amount of blood, 5-per-cent. suspension.	Amount of serum.	Amount of NaCl, 0·85 per cent.	Amount of saponin, 0·01 per cent.	Result.
c.c. 2	c.c. 0·025	c.c. 2·725	c.c. 1·25	Hæmolysis
2	0·05	2·7	1·25	"
2	0·1	2·65	1·25	Slight hæmolysis
2	0·25	2·5	1·25	No hæmolysis

The experiments were again performed with the sera after heating them to 56° C. with the same object as in the case of the previous experiments. The following tables give the results:—

Table XIV.—Inhibitory Action of Serum of Rabbit fed on Extracted Bran (Heated) (Rabbit 6).

Amount of blood, 5-per-cent. suspension.	Amount of serum.	Amount of NaCl, 0·85 per cent.	Amount of saponin, 0·01 per cent.	Result.
c.c. 2	c.c. 0·025	c.c. 2·725	c.c. 1·25	Almost complete hæmo- lysis
2	0·05	2·7	1·25	Considerable hæmolysis
2	0·1	2·65	1·25	"
2	0·25	2·5	1·25	Slight hæmolysis "

Table XV.—Inhibitory Action of Heated Serum of Rabbit fed on Extracted Bran + Cholesterol Oleate (Rabbit 7).

Amount of blood, 5-per-cent. suspension.	Amount of serum.	Amount of NaCl, 0·85 per cent.	Amount of saponin, 0·01 per cent.	Result.
c.c. 2	c.c. 0·025	c.c. 2·725	c.c. 1·25	Hæmolysis
2	0·05	2·7	1·25	"
2	0·1	2·65	1·25	Slight hæmolysis
2	0·25	2·5	1·25	No hæmolysis

Table XVI.—Inhibitory Action of Heated Serum of Rabbit fed on Extracted Bran + Cholesterol Stearate (Rabbit 8).

Amount of blood, 5-per-cent. suspension.	Amount of serum.	Amount of NaCl, 0·85 per cent.	Amount of saponin, 0·01 per cent.	Result.
c.c. 2	c.c. 0·025	c.c. 2·725	c.c. 1·25	Hæmolysis
2	0·05	2·7	1·25	"
2	0·1	2·65	1·25	Slight hæmolysis
2	0·25	2·5	1·25	No hæmolysis

They were also carried out with 10-per-cent. solution of the sera in physiological salt.

Table XVII.—Inhibitory Action of Serum in 10-per-cent. Solution.
Extracted Bran-fed Rabbit (Rabbit 6).

Amount of blood, 5-per-cent. suspension.	Amount of serum, 10 per cent.	Amount of NaCl, 0·85 per cent.	Amount of saponin, 0·01 per cent.	Result.
c.c. 2	c.c. 0·25	c.c. 2·5	c.c. 1·25	Almost complete hæmo- lysis
2	0·5	2·25	1·25	Considerable hæmolysis
2	1·0	1·75	1·25	"
2	2·5	0·25	1·25	Slight hæmolysis "

Table XVIII.—Inhibitory Action of Serum of Rabbit fed on Extracted Bran
+ Cholesterol Oleate in 10-per-cent. Solution (Rabbit 7).

Amount of blood, 5-per-cent. suspension.	Amount of serum.	Amount of NaCl, 0·85 per cent.	Amount of saponin, 0·01 per cent.	Result.
c.c. 2	c.c. 0·25	c.c. 2·5	c.c. 1·25	Hæmolysis
2	0·5	2·25	1·25	"
2	1·0	1·75	1·25	Slight hæmolysis
2	2·5	0·25	1·25	No hæmolysis

Table XIX.—Inhibitory Action of Serum of Rabbit fed on Extracted Bran
+ Cholesterol Stearate in 10-per-cent. Solution (Rabbit 8).

Amount of blood, 5-per-cent. suspension.	Amount of serum, 10 per cent.	Amount of NaCl, 0·85 per cent.	Amount of saponin, 0·01 per cent.	Result.
c.c. 2	c.c. 0·25	c.c. 2·5	c.c. 1·25	Hæmolysis
2	0·5	2·25	1·25	"
2	1·0	1·75	1·25	Slight hæmolysis
2	2·5	0·25	1·25	No hæmolysis

These experiments clearly show that the esters of cholesterol undergo hydrolysis during the digestive process and appear, partially at any rate, in the blood stream as free cholesterol.

As cholesterol is not a normal constituent of the food of rabbits, it appeared interesting to find out (1) whether vegetable phytosterol is absorbed by the animal if given in the food; (2) whether this can be utilised by the animal.

Experiment V.—To ascertain whether the phytosterol of the food in the

case of rabbits is excreted unchanged or whether any is absorbed or destroyed during digestion.

A large healthy rabbit was fed for eight days on a mixture of equal parts of extracted bran and extracted wheat germ, and the faeces were collected during the last seven days. The faeces, which when dried weighed 60 grammes, were extracted with ether and the ethereal solution saponified with sodium ethylate. The unsaponifiable matter was obtained in the form of a clear stiff oil, weighing 0.4115 gramme. This was dissolved in 5 c.c. absolute alcohol and left to crystallise. A small quantity of greasy crystalline matter separated, too small to be readily purified. It was not cholesterol, but consisted of the crystalline "phytosterol" of bran, from which it is difficult to free completely the original bran by simple extraction.

The animal was then fed for one day on the same diet, the faeces being discarded, and for the following eight days on a daily ration of $\frac{3}{4}$ gramme of phytosterol (from wheat-germ), 30 to 40 grammes of extracted bran and an equal quantity of extracted germs of wheat, care being taken that the animal ate the whole of the phytosterol—2 grammes in all. It was then fed for four more days on the same diet, but without phytosterol. The faeces collected during the 12 days weighed, after drying, 189 grammes. The animal remained in good health and its weight was constant all through the experiment. The faeces were treated as before and yielded 2.3965 grammes of greasy unsaponifiable matter. This was repeatedly recrystallised from alcohol and two crops of pure phytosterol were obtained, the weights and melting points of which were: crop 1, 0.6465 gramme, M.P. = 132° C.; crop 2, 0.603 gramme, M.P. = 132° C. The mother liquors and residues were then evaporated to dryness and heated for a few minutes at a temperature of 180° — 200° C. with benzoyl chloride. The product was then poured into a suitable quantity of alcohol and allowed to stand. The difficultly soluble crystalline matter which separated was recrystallised from hot alcohol. In this manner 0.149 of white phytosterol benzoate was obtained which on recrystallisation from ethyl acetate was obtained in characteristic crystalline form and melted at 141° C. 1.3675 grammes of pure phytosterol were therefore recovered out of the 2 grammes administered.

A consideration of the quantity of phytosterol recovered and also of the relative quantities of unsaponifiable matter obtained in the two parts of the experiment makes it clear that some of the phytosterol of the food was either destroyed or absorbed, but most probably absorbed.

Experiment VI.—In order to ascertain whether phytosterol given in the food has the same effect as cholesterol.

For this purpose animals were fed with actual bran and wheat-germ, which is rich in phytosterol, and other animals were fed on extracted bran with the addition of measured quantities of pure phytosterol.

Rabbit 9; fed on extracted bran for 10 days; weight of animal = 1·4 kilogrammes.

Rabbit 10; fed on extracted bran for three days, then ordinary bran and wheat-germ for six days; weight of animal = 1·7 kilogrammes. The following table gives the result of experiment:—

Table XX.—Inhibitory Effect of Serum of Rabbit fed on Extracted Bran (Rabbit 9).

Amount of blood, 5-per-cent. suspension.	Amount of serum.	Amount of NaCl, 0·85 per cent.	Amount of saponin, 0·01 per cent.	Result.
c.c. 2	c.c. 0·025	c.c. 2·975	c.c. 1	Almost complete hæmo- lysis
2	0·05	2·95	1	Slight hæmolysis
2	0·1	2·9	1	A trace hæmolysis
2	0·5	2·5	1	No hæmolysis

Table XXI.—Inhibitory Effect of Serum of Rabbit fed on Ordinary Bran and Wheat-germ (Rabbit 10).

Amount of blood, 5-per-cent. suspension.	Amount of serum.	Amount of NaCl, 0·85 per cent.	Amount of saponin, 0·01 per cent.	Result.
c.c. 2	c.c. 0·025	c.c. 2·975	c.c. 1	Hæmolysis
2	0·05	2·95	1	Slight hæmolysis
2	0·1	2·9	1	No hæmolysis
2	0·5	2·5	1	"

This experiment was repeated the following day with identical results.

Rabbit 11; fed on extracted bran for 11 days; weight of animal = 2 kilogrammes.

Rabbit 12; fed on ordinary bran and wheat-germ for nine days, having been fed with extracted bran for two days previously; weight of animal = 2 kilogrammes.

The animals were kept under exactly similar conditions and killed at the same time. Serum collected day after death. The results are tabulated below:—

Table XXII.—Inhibitory Action of Serum of Rabbit fed on Extracted Bran (Rabbit 11).

Amount of blood, 5-per-cent. suspension.	Amount of serum.	Amount of NaCl, 0·85 per cent.	Amount of saponin, 0·01 per cent.	Result.
c.c. 2	c.c. 0·025	c.c. 2·975	c.c. 1	Almost complete hæmo- lysis
2	0·05	2·95	1	Hæmolysis
2	0·1	2·9	1	Slight hæmolysis
2	0·5	2·5	1	No hæmolysis

Table XXIII.—Inhibitory Action of Serum of Rabbit fed on Ordinary Bran and Wheat-germ (Rabbit 12).

Amount of blood, 5-per-cent. suspension.	Amount of serum.	Amount of NaCl, 0·85 per cent.	Amount of saponin, 0·01 per cent.	Result.
c.c. 2	c.c. 0·025	c.c. 2·975	c.c. 1	Hæmolysis
2	0·05	2·95	1	Slight hæmolysis
2	0·1	2·9	1	A trace hæmolysis
2	0·5	2·5	1	No hæmolysis

Experiments were also carried out with the sera after heating for over an hour to 55° C., and in 10-per-cent. solutions made up with physiological salt. The results agreed entirely with the above tables.

The serum of the rabbits fed on ordinary bran and wheat-germ shows a slightly greater inhibitory power than the serum of the rabbit fed on extracted bran. This seems to indicate (1) that some of the phytosterol of the wheat-germ found its way into the blood stream, or (2) possibly caused an increase of cholesterol in the blood. Rabbits experimented on disliked the wheat-germ so that it was often neglected, the extracted bran being always given the preference. We therefore resolved to give, in subsequent experiments, weighed quantities of pure phytosterol.

Rabbit 13 ; fed on extracted bran for seven days.

Rabbit 14 ; fed on extracted bran for 12 days with phytosterol in addition, eight days with $\frac{3}{4}$ gramme per day, then four days with $\frac{1}{2}$ gramme per day.

Animals kept under similar conditions.

Table XXIV.—Inhibitory Action of Serum of Rabbit fed with Extracted Bran (Rabbit 13).

Amount of blood, 5-per-cent. suspension.	Amount of serum.	Amount of NaCl, 0·85 per cent.	Amount of saponin, 0·01 per cent.	Result.
c.c. 2 2 2 2	c.c. 0·025 0·05 0·1 0·5	c.c. 2·975 2·95 2·9 2·5	c.c. 1 1 1 1	Complete hæmolysis Considerable hæmolysis Slight hæmolysis No hæmolysis

Table XXV.—Inhibitory Action of Serum of Rabbit fed with Extracted Bran + Phytosterol (Rabbit 14).

Amount of blood, 5-per-cent. suspension.	Amount of serum.	Amount of NaCl, 0·85 per cent.	Amount of saponin, 0·01 per cent.	Result.
c.c. 2 2 2 2	c.c. 0·025 0·05 0·1 0·5	c.c. 2·975 2·95 2·9 2·5	c.c. 1 1 1 1	Hæmolysis not complete A trace hæmolysis No hæmolysis „

These experiments were repeated with the same sera, on consecutive days, three times altogether, and in every case the result was identical.

Further, it was found that on addition of greater quantities of saponin to the same volume of blood, if the serum were added in proportional quantities, hæmolysis was in every case prevented, if the ratio between saponin and serum were the same as that which prevented hæmolysis in the above experiment.

The table is given below :—

Table XXVI.—Inhibitory Effect of Serum of Rabbit fed on Extracted Bran with Phytosterol in addition (Rabbit 14).

Amount of blood, 5-per-cent. suspension.	Amount of serum.	Amount of NaCl, 0·85 per cent.	Amount of saponin, 0·01 per cent.	Result.
c.c. 2 2 2 2 2 2 2	c.c. 0·025 0·05 0·1 0·15 0·2 0·25	c.c. 2·975 2·95 2·9 2·35 1·8 1·25	c.c. 1 1 1 1·5 2 2·5	Hæmolysis A trace hæmolysis No hæmolysis „ „ „

This is interesting as giving an indication of the quantitative reaction between the serum and saponin, and therefore of its chemical nature.

In order to ascertain whether the result would be affected by heating or dilution, however, another pair of rabbits was used.

Rabbit 15; fed for 14 days on extracted bran. Weight of animal = 1·5 kilogrammes.

Rabbit 16; fed on ordinary bran and wheat-germ for 13 days, the last eight days with phytosterol in addition; for six days $\frac{1}{4}$ gramme per day, then $\frac{3}{4}$ gramme the next day, and 1 gramme the last day.

Animals kept under similar conditions and killed at the same time. The results of experiments are given below:—

Table XXVII.—Inhibitory Effect of Serum of Rabbit fed on Extracted Bran (Rabbit 15).

Amount of blood, 5-per-cent. suspension.	Amount of serum.	Amount of NaCl, 0·85 per cent.	Amount of saponin, 0·01 per cent.	Result.
c.c. 2	c.c. 0·025	c.c. 2·975	c.c. 1	Hæmolysis not quite complete
2	0·05	2·95	1	Considerable hæmolysis
2	0·1	2·9	1	Slight hæmolysis
2	0·5	2·5	1	No hæmolysis

Table XXVIII.—Inhibitory Effect of Serum of Rabbit fed with Phytosterol in addition to Ordinary Bran and Wheat-germ (Rabbit 16).

Amount of blood, 5-per-cent. suspension.	Amount of serum.	Amount of NaCl, 0·85 per cent.	Amount of saponin, 0·01 per cent.	Result.
c.c. 2	c.c. 0·025	c.c. 2·975	c.c. 1	Hæmolysed
2	0·05	2·95	1	A trace of hæmolysis
2	0·1	2·9	1	No hæmolysis
2	0·5	2·5	1	„

Table XXIX.—Inhibitory Effect of Rabbit fed on Extracted Bran heated to 56° C. for an hour (Rabbit 15).

Amount of blood, 5-per-cent. suspension.	Amount of serum.	Amount of NaCl, 0·85 per cent.	Amount of saponin, 0·01 per cent.	Result.
c.c. 2	c.c. 0·025	c.c. 2·975	c.c. 1	Hæmolysed (not quite complete)
2	0·05	2·95	1	Considerable hæmolysis
2	0·1	2·9	1	Slight hæmolysis
2	0·5	2·5	1	No hæmolysis

Table XXX.—Inhibitory Effect of Serum of Rabbit fed on Phytosterol in addition to Ordinary Bran and Wheat-germ. Heated to 56° C. for an hour (Rabbit 16).

Amount of blood, 5-per-cent. suspension.	Amount of serum.	Amount of NaCl, 0·85 per cent.	Amount of saponin, 0·01 per cent.	Result.
c.c. 2	c.c. 0·025	c.c. 2·975	c.c. 1	Hæmolysed
2	0·05	2·95	1	A trace hæmolysis
2	0·1	2·9	1	No hæmolysis
2	0·5	2·5	1	„

Table XXXI.—Inhibitory Effect of Serum of Rabbit fed on Extracted Bran in 10-per-cent. Solution made up with Physiological Salt (Rabbit 15).

Amount of blood, 5-per-cent. suspension.	Amount of serum, 10 percent.	Amount of NaCl, 0·85 per cent.	Amount of saponin, 0·01 per cent.	Result.
c.c. 2	c.c. 0·25	c.c. 2·75	c.c. 1	Hæmolysis almost com- plete
2	0·5	2·5	1	Considerable hæmolysis
2	1·0	2	1	Slight hæmolysis
2	1·5	1·5	1	No hæmolysis

Table XXXII.—Inhibitory Effect of Serum of Rabbit fed on Phytosterol in addition to Ordinary Bran and Wheat-germ in 10-per-cent. Solution of Physiological Salt (Rabbit 16).

Amount of blood, 5-per-cent. suspension.	Amount of serum.	Amount of NaCl, 0·85 per cent.	Amount of saponin, 0·01 per cent.	Result.
c.c.	c.c.	c.c.	c.c.	
2	0·25	2·75	1	Hæmolysed
2	0·5	2·5	1	A trace hæmolysis
2	1	2	1	No hæmolysis
2	1·5	1·5	1	„

These experiments fully confirm the conclusion arrived at from a comparison of the sera fed on extracted bran and on ordinary bran + wheat-germ.

Conclusions.

1. When cholesterol is given with the food of rabbits, some is absorbed and finds its way into the blood stream as free cholesterol, only a portion of the total cholesterol given in the food is absorbed, the rest being excreted unchanged. The amount of cholesterol which finds its way into the blood stream was not increased in our experiments by increasing the amount given in the food. It would appear probable, therefore, that the animals only take up such an amount of cholesterol as they can utilise.

2. Cholesterol when in the form of esters undergoes hydrolysis in part, at any rate, during digestion, and appears in the blood stream as free cholesterol.

3. When animals are fed on phytosterol, this substance is in part absorbed, just as in the case of cholesterol, and appears in the blood stream either itself or in the form of cholesterol. The latter point can, however, only be decided by the examination of very large quantities of the blood of animals fed on phytosterol.

Experiments are now in progress which we hope will decide this question.

We take this opportunity of expressing our thanks to the Government Grant Committee of the Royal Society for assistance in carrying out this work.