IX. ON THE CHOLESTEROL CONTENT OF THE TISSUES OF GROWING RATS WHEN UNDER VARIOUS DIETS.

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It has for some time been known that growing animals are unable to thrive for any length of time on a diet consisting of a mixture of pure protein, fat, and carbohydrate. Stepp [1909, 1911] in a series of experiments on mice attempted to estimate the importance of lipoids in nutrition, and found that food mixtures after extraction with lipoid solvents could not maintain life, but that the extracted matter when added to the food made it once more efficient to do so; he could not obtain this result, however, by adding any known lipoid. A similar series of experiments has been carried out by Osborne and Mendel [1911] on rats. Their earlier work was directed to the question as to whether life could be maintained on a dietary containing a single individual protein, instead of mixtures of proteins such as ordinary normal dietaries comprise, and they were led to answer this question in the affirmative. Maintenance is possible so long as the protein stored is one not deficient in certain amino-acid groupings, but their earlier experiments led these authors to realise the importance of factors other than protein and energy supply and they found that synthetic dietaries which were able to maintain the life of fully grown rats were unable to do so with growing animals, but that a small addition of tissue extracts added to those synthetic diets induced normal growth.

In their later experiments these authors showed that the addition of milk products to the food promoted rapid growth in rats which had remained stationary in weight when on the original artificial diet. The material which was added they called "protein free milk"; it was prepared by removing

as far as possible by precipitation and coagulation, the proteins from fat free milk, the fluid being then evaporated at low temperatures and the residue ground to powder.

Hopkins [1912] in an extended series of experiments in which groups of young rats were fed upon artificial mixtures of isolated caseinogen, fat, carbohydrates and salts side by side with similar groups which were fed on the same basal dietary with the addition of a minute quantity of fresh milk, has found that in those experiments in which the basal diet contained fully purified material the rats without milk soon failed to grow. When the constituents were less completely purified then slow growth occurred. In all cases the milk addendum, although its total solids only amounted to 4 % or less of the whole food eaten, induced normal and continued growth.

The total energy consumption of these animals was determined and it was shown that the rats upon the purer dietary ceased to grow at a time when their intake was more than sufficient quantitatively to maintain normal growth. The absolute consumption of the animals growing rapidly with the milk addendum was greater than that of those growing slowly upon the basal dietary alone; but the consumption per unit of live weight was in comparable groups nearly the same. It was further shown in these experiments that if growth continued, as upon the less pure basal dietary, the small milk addendum reduced the food consumption necessary for a given weight increment to one half or less.

Cessation in increase of weight upon the pure dietary took place before any failure in appetite, although the consumption might, later, fall to a lower level. It was therefore suggested that any effect of the addendum upon appetite must have been secondary to a more direct effect upon growth processes.

Much discussion has taken place over the question as to what is meant by "growth." A living organism may increase its exterior dimensions without increasing its mass, and, on the other hand, the mass may be increased without any "growth processes" taking place.

When young rats are being fed on a normal diet they certainly grow, by which we mean that the amount of living material in their bodies increases and they increase in size permanently, but still we have to define our terms "grow" and "living material," and we find ourselves arguing in a circle.

The evidence now accumulated, however, points fairly to the conclusion that when "growing rats" are put on to a pure synthetic dietary the phenomena observed during their former growing period cease.

We may state what has happened either by saying that their "growth processes" have ceased, or by saying that they are being starved and hence cease to grow. It would seem that the latter more accurately states the case.

The synthetic diet seems to lack something which is necessary for the proper utilisation of the other food stuffs by the tissues of the animal.

We may compare the tissues of the rat and the food material to a heap of powder ready to unite on the application of a flame—the hormone present in a normal diet, but absent from a synthetic diet.

The fact then being fairly well established that "growth processes" cease when animals are fed on pure synthetic diets, or in other words, starved, it seemed to be of interest to carry out experiments to endeavour to determine how far variations of the cholesterol content of the bodies of growing rats occur, when fed on various dietaries, especially with regard to the question as to whether the growing organism can manufacture cholesterol when deprived of that substance in the food. It seemed that an answer might be obtained to this question, and to others bearing on the origin, fate and functions of cholesterol in the animal organism, by taking a number of sets of rats, from a common starting point—which was chosen as the period after birth at which the animals commenced to feed themselves—and estimating the total cholesterol content of the bodies of these rats at once, and then comparing the figures obtained with those from other sets of rats, which had been fed for definite periods on various dietaries of known composition. The original set of controls then gives us the normal quantity of cholesterol in the bodies of the rats when they start to feed themselves, whilst the numbers from the other sets give us an indication as to any increase or decrease in the quantity of cholesterol present.

It was determined at the outset to keep all the sets of rats on their particular dietaries until they had either doubled their weight or had been kept for a time equal to that in which a set of rats fed on ordinary bread and oatmeal had doubled their weight, which was found to be just over three weeks.

Owing, however, to the unpalatability of certain of the synthetic and alcohol-and-ether extracted diets, this was found to be difficult as the rats commenced to die after a certain time, so that in some cases I was unable to extend the observations beyond a period of about 14 days.

An endeavour was also made to find a synthetic diet which, by the addition of cholesterol and other substances such as cholesterol esters, lecithin, lemco, etc., would keep the rats in normal growth.

No synthetic diet was found satisfactory for this purpose and hence no reliable evidence is to hand as to how the cholesterol content of the rats would vary under such conditions.

Method. The animals employed in these experiments were ordinary tame white rats and were mostly bred in the laboratory. They were kept with the mothers until they were able to feed themselves and were then taken in sets of varying number and put into square wire cages of about eight cubic feet capacity. The bottoms of these cages were also of wire, the mesh being of sufficient size to allow the faeces to drop through into a trough placed beneath. Each cage had in it a large pad of cotton wool. It was not thought necessary for the purpose of the experiments to feed the animals separately; they flourish much better when not alone, and furthermore individual variations are eliminated. On the other hand, this plan is open to the disadvantage that if an animal dies the others commence to nibble it.

Diets employed. Altogether 13 sets of rats were analysed; the method of analysis being a modification of Windaus' digitonin process fully described by Fraser and Gardner [1910]. The details of the results are given in Tables I and II.

TABLE I.

Experi- mental set	Diet	No. of rats	Total weight in grams	Percentage of cholesterol			Weight of cholesterol per rat in grams			Average weight of rats
				Total	Free	Ester	Total	Free	Ester	
1	Starting feeding three weeks old	5	122.45	0.1467	0.0944	0.0523	0.0359	0.0229	0.0130	24.49
2	do. do.	6	133	0.1721	0.1373	0.0348	0.0380	0.0304	0.0076	22.20
3	Bread and oatmeal	4	142.4	0.200	0.144	0.056	0.071	0.051	0.020	35.60
4	Synthetic and milk salts	3	61.0	0.238	0.2122	0.0258	0.048	0.042	0.006	20.30
5	Synthetic and incinerated salts	1	26.8	0.136	0.136	nil	0.0366	0.0366	nil	26.8
6	Synthetic and mixed proteins	3	67.0	0.174	0.174	nil	0.0388	0.0388	nil	22.3
7	Synthetic and milk only	4	117.3	0.2162	0.1198	0.0964	0.0634	0.0351	0.0283	29.3
8	Synthetic and milk and cholesterol	3	76.6	0.5236	0.1608	0.3628	0.1340	0.0429	0.0911	25.50
9	Synthetic and cholesterol	2	34.6	0.428	0.4079	0.0201	0.074	0.0705	0.0045	17.3
10	do. do.	3	52.6	0.5046	0.4821	0.0225	0.0883	0.0812	0.0071	17.5
11	Synthetic and cholesterol esters	2	41.5	0.3606	0.3183	0.0423	0.075	0.066	0.009	20.75
12	do. do.	6	100.0	0.415	0.272	0.143	0.069	0.045	0.024	16.7
13	Ether extracted bread and oatmeal, and cholesterol	4	134.5	0.328	0.3178	0.0102	0.1104	0.1070	0.0034	33.60

TABLE II.

Expt set	Diet	No. of rats analysed	Average wt. of rat at beginning of experiment	Average weight of rat at end of experiment	Average increase in weight	Length of expts in days
3	Bread and oatmeal	4	18	36.7	18.7	25
4	Synthetic diet and milk salts	3	22.2	20.3	-1.9	16
5	" " " incinerated salts	1	25.7	27	1.3	16
6	" " " mixed proteins	3	20.7	22.8	2.1	15
7	" " " milk only	4	18.2	30.2	12	24
8	" " " milk and cholesterol	3	19.5	26.7	7.2	24
9	" " " cholesterol	2	19.3	18.25	-1.05	13
10	" " " "	3	20.0	18.3	-1.7	15
11	" " " cholesterol esters	2	19.3	20.7	1.4	16
12	" " " "	6	16.6	17.0	0.4	15
13	Ether extracted bread and oatmeal, and cholesterol	4	18.8	34.7	16	31

Set (1). This set consisted of 5 rats which were killed and analysed at the period when they were just commencing to feed themselves. They were then 22 days old, and weighed altogether 126.45 g., the average weight per rat being 25.29 g. The actual minced up material used was 122.45 g. This mass was pounded up with sand and plaster of Paris and allowed to dry. It was then extracted with ether in a Soxhlet apparatus for about 14 days. The ethereal extract was made up to one litre, one half of which was analysed at once, and gave by direct precipitation with digitonin in hot absolute alcohol 0.236 g. of the digitonin compound corresponding to 0.0573 g. of cholesterol. The other half was saponified and gave 0.3698 g. of the compound corresponding to 0.0898 g. of cholesterol. This represents a total cholesterol content of 0.1798 g. corresponding to 0.0359 g. of cholesterol per rat which may be taken as our control standard.

Set (2). This set, consisting of six rats, was treated in a similar way to the last. They were 24 days old and weighed 134.5 g., the average weight per rat being 22.4 g. The actual minced up material used was 133 g.

Total cholesterol content 0.2289 g., or an average of 0.038 g. of cholesterol per rat.

Set (3). Ordinary diet. This set consisted of four rats which commenced to feed themselves about 28 days after birth, when their total weight was 72 g. They were then put on a diet of bread and oatmeal and doubled their weight in 25 days. During this period they were given 377 g. of dry bread and 155 g. of oatmeal, the total being 532 g., 447 of which were eaten. The dry bread was soaked in water after being weighed and mixed with the oatmeal.

The total weight of the four rats when killed was 146.8 g., their average weight being 36.7 g., whilst the actual weight of material minced up was 142.4 g. Total cholesterol content 0.2846 g.; corresponding to 0.071 g. cholesterol per rat.

This diet of bread and oatmeal was analysed for phytosterol, 0.0235 % of total phytosterol being found. The method employed was to heat the food up with a 10 % solution of alkali on a water bath for some hours, and then after making acid with strong hydrochloric acid, to continue the heating for a similar period. The mass was then treated with ether and estimated

Curve 1. Four rats, ordinary diet (Set 3). Total weight curve.

in the ordinary way. We see now that the increase in the cholesterol per rat over set (1) is $0.071 - 0.0359 = 0.035$. Therefore the total increase in cholesterol in the bodies of the four rats is 0.14 g.

Altogether 447 g. of food, containing 0.0233 % of phytosterol were eaten, which means that 0.105 g. phytosterol were ingested. Hence taking into consideration individual variations in the rats and possible errors of experiment there is here no evidence of any synthesis of cholesterol; all the phytosterol contained in the food seems to have been laid hold upon and stored. Curve 1 represents the growth of these rats.

Set (4). Basal synthetic diet + milk salts. This set consisted of six rats which were not started on the dieting until they were 28 days old, when they weighed 133.5 g., their average weight being 22.2 g. These animals were fed on the synthetic diet used by Hopkins [1912] in his extended series of observations on the growth of rats when fed on various diets, which consisted of pure protein, fat, and carbohydrate, with salts added of the same nature and percentage as those found in milk.

Composition of food.

Protein (Hammarsten's pure casein)	..	..	22	%
Potato starch	..	..	42	%
Cane sugar	..	..	21	%
*Lard	..	..	12.4	%
Calcium phosphate	..	..	1.95	%
Magnesium phosphate	..	..	0.35	%
Potassium chloride	..	..	0.15	%
Sodium chloride	..	..	0.15	%

* The lard was carefully purified and was quite free from cholesterol.

By feeding the rats on this diet I wished to determine firstly whether any growth occurred or not, and secondly whether any increase of cholesterol occurred in the body, none being present in the food. Before administering the food, the protein, starch and sugar were mixed dry, and the lard was then carefully rubbed in by hand and the whole mixed together till of a uniform composition. Thus prepared the mixture represented the dry weight of the food. Each day's ration for the set of rats was weighed out dry, then water was added, a little at a time, the whole being thoroughly mixed so as to make the mass into a light paste, of such consistency that the rats would eat it freely. More food was always given than the rats would eat, and was placed in a shallow vessel so as to be easily accessible; water was placed in a separate vessel. It was found possible to determine the food eaten with great accuracy; the rats spilled out very little, and practically none of the faeces became mixed up with it. The food left over from the previous day was taken out each morning, before feeding, carefully dried and weighed.

During the period of dietary the rats were given altogether 211 g. of food, 175 g. of which were eaten, but in considering these figures one must remember that the rats died progressively, during the experiment, apparently owing to the unpalatability of the food, and by the end of the 16th day only one remained, two having died earlier in the day.

The average weight of these three rats was 20.3 g., and as the average weight of the rats at the commencement of the period of dieting was 22.2 g., we see that no growth has taken place. Curve 2 represents the growth curve of these rats, their average weight being plotted against days of dieting, and the small crosses represent the periods at which successive rats died.

Curve 2. Synthetic diet + milk salts (Set 4). Average weight curve.

Some difficulty was experienced in keeping the rats from nibbling those that died. No nibbling of any importance occurred in this and the next sets, but some did occur in those sets which were fed on an ether and alcohol extracted diet.

Total cholesterol content 0.1454 g., corresponding to 0.048 g. cholesterol per rat. We thus see that this figure is very near that found for set (1) and that no apparent increase of cholesterol has taken place during the period of growth.

Curve 3. Synthetic diet + incinerated salts (Set 5). Average weight curve.

Set (5). This set also consisted of six rats taken from the same litter as the above set, and the rats were put on the special diet 28 days after birth. Their total weight at this time was 154 g., or an average weight of 25.7 g. They were fed on exactly the same diet as set (4) except that the salts used

were obtained from incinerating bread and oatmeal. During the period of dieting the rats were given 165.5 g. of food, 125 g. of which were eaten. As in the case of set (4) the rats did not thrive well on this diet, dying at intervals during the course of the experiment, until at the end only one rat was left weighing 27 g., so again we see that practically no increase in weight has taken place.

Curve 3 represents the growth curve of these rats. The last rat was killed on the 16th day and analysed, the actual weight of material used being 26.8 g. Total cholesterol found 0.0366 g. per rat. This figure again very closely approximates to that found for set (1), no increase in the cholesterol content of the organism having taken place.

Set (6). This set consisted of five rats which were fed on a pure synthetic diet as in the case of set (4), but half the protein was replaced by protein obtained from bulls' testicles.

Curve 4. Synthetic (mixed proteins) + lecithin (Set 6). Average weight curve.

This was obtained as follows:

The tissue was carefully ground up and boiled with alcohol, added in the ratio of one litre of alcohol to 300 g. of tissue. The alcohol was filtered off and the boiling repeated. The tissue was then extracted with ether in a Soxhlet apparatus for a fortnight, after which it was again extracted by petroleum ether for a considerable time. In addition 0.5 % of lecithin prepared from bulls' testicles was added. The diet was entirely cholesterol free.

At the beginning of the experiment the five rats weighed 103.5 g., being an average of 20.7 g. One rat died on the 7th day and one on the 8th. On the 16th day, the remaining three rats weighed 68.5 g., being an average of 22.8 g. During this period they were given 150 g. of food, 130 of which were eaten. The actual amount of material analysed was 67 g.

Total cholesterol content per rat 0.0388 g.

Curve 4 shows average growth during the course of the experiment.

Set (7). This set consisted of four rats born on May 30, 1913, and the animals were put on their special diet on June 30, 1913. This diet was the same as the synthetic diet employed in sets (4) and (5); in addition to which 20 cc. of milk were given each day. We wished to see how the addition of milk alone to the synthetic diet would influence growth and whether the small quantity of cholesterol in the milk would be picked out by the organism and utilised for growth. The weight of the four rats at the commencement of the experiment was 75 g., corresponding to an average of 18.2 g. per rat. At the end of the experiment their total weight was 120.9 g., representing an average of 30.2 g. Each rat had therefore put on during the course of the experiment 12 g., which is a greater increase than in the following case in which cholesterol was also added to the diet, but not so great as in set (13), which had ether extracted bread and oatmeal and cholesterol. During the

Curve 5. Synthetic diet + milk only. No cholesterol (Set 7). Average weight curve.

period of dieting of 24 days, the rats were given 366 g. of food, and 480 cc. of milk, 265 g. of the food being eaten.

Milk contains about 4 % of fat, and milk fat about 0.5 % of cholesterol. The milk given to the rats would thus contain altogether about 0.096 g. of cholesterol.

All the rats remained perfectly healthy during the course of the experiment, none having died. The rats were killed on July 22 and analysed, the actual weight of material used being 117.3 g. Total cholesterol found 0.252 g.; corresponding to 0.063 g. of cholesterol per rat. Now assuming that each rat had in its body the normal quantity (set 1) of cholesterol, viz. 0.0359 g., the total quantity would be 0.144 g. for the four rats. Therefore the total increase for the four rats is $0.252 - 0.144 = 0.108$.

The milk given contained as we have seen about 0.096 g. of cholesterol altogether. These figures would therefore indicate that the cholesterol in the milk was greedily picked out and stored in the organism. All the cholesterol

found can thus be accounted for by that contained in the milk, and, taking into account the errors of experiment, there is no evidence of any synthesis having taken place. Curve 5 represents the average growth curve for this set.

Set (8). This set, consisting of four rats from the litter born on May 30, 1913, were put on their special diet on June 30. The diet was exactly the same as the last except that 2 % of cholesterol was added to the food and only 10 cc. milk given each day instead of 20. The dry food after being weighed was carefully mixed with water as in the case of set (4). The object of feeding the rats on this diet was to see how the addition of the milk affected growth, and whether, should normal growth occur, the cholesterol content of the tissues would approach or exceed the normal figure.

The weight of the four rats at the commencement of the period of feeding was 78 g., corresponding to an average of 19.5 g. During the period of dieting the rats were given 338 g. of the food, 230 cc. of milk and 6.8 g.

Curve 6. Synthetic diet (milk salts) + milk + cholesterol 2 % (Set 8). Average weight curve.

cholesterol. As in the previous cases the food left over was carefully dried and weighed and it was found that the rats had eaten approximately 200.7 g. food and 4 g. of cholesterol. The total cholesterol in the 230 cc. of milk added was about 0.048 g. since milk contains 4 % of fat, and the fat 0.5 % of cholesterol.

One rat which met with an accident on the 15th day was killed at once. At the end of the period of dieting the remaining three rats weighed 80.3 g., corresponding to an average weight of 26.7 g. The increase in weight per rat was therefore 7.2 g. during the period of 23 days, which is less than half the average increase of the previous set.

The rats were killed on July 22. Total cholesterol found 0.4012 g., corresponding to 0.134 g. per rat, a figure approximating to that found for set (13), viz. 0.1104 g. per rat. However, growth was much slower on this synthetic diet with milk and cholesterol than on the partly extracted food with cholesterol alone added (set 13). As the total cholesterol found was

only 0.4012 g. for the three rats and 4 g. had been eaten in the food, it is clear that only about one-tenth of that ingested is stored up.

Curve 6 represents the average weights of these rats plotted against days.

Set (9). This set consisted of six rats which were fed with the synthetic diet used in set (4) to which 2 % of cholesterol was added. It has been seen that in the case of sets (4) and (5) no increase either of growth or of cholesterol content took place on a pure synthetic diet alone, and one wished to see what effect the addition of cholesterol would have on each of these factors. The weight of the six rats at the commencement of the period of dieting was 115.5 g., being an average of 19.3 g. Two rats died on the 4th day, one on the 5th and one on the 6th, but no nibbling whatever occurred as the bodies were removed in time to prevent it. The remaining two rats were killed on the 13th day and analysed. Their weight was then 36 g., being an average of 18 g. No increase in weight had therefore taken place. During the period of dieting the rats were given 116 g. of food, 76 of which were eaten including 1.52 g. of cholesterol. The two rats were killed on the 13th day and analysed. Total cholesterol content for the two rats 0.148 g.; or 0.074 g. per rat.

Now up to the end of the 6th day 35 g. of food had been eaten containing 0.71 g. of cholesterol. Thus the remaining 1.52 — 0.71 or 0.81 g. of cholesterol was eaten by the two rats which were analysed. The amount of cholesterol to be accounted for in their bodies is obviously $2(0.074 - 0.0359) = 0.0762$ g. taking 0.0359 as standard, and this is quite covered by the quantity of cholesterol ingested. It will be noted that only about one-tenth of the cholesterol ingested is stored, the remainder being excreted in the faeces.

Curve 7 shows the average weights of the rats during the experiment.

Curve 7. Synthetic diet + cholesterol (*Set 9*). Average weight curve.

Set (10). This set consisted of four rats whose diet was the same as in the last set but with the following modifications:

(1) The fat employed was manufactured by dissolving glycerol in dry pyridine and adding palmitic chloride. This was allowed to stand over

night and then poured into water and filtered through a filter pump. Any excess of acid chloride was dissolved out by a dilute solution of sodium carbonate and the residual fat carefully dried.

(2) 0.5 % of pure lecithin prepared from bulls' testicles was added to the food, being carefully rubbed in so as to be uniformly distributed.

(3) Half of the pure protein ordinarily employed was replaced by protein obtained from bulls' testicles which was quite cholesterol free (*vide* set 6).

The weight of the four rats at the beginning of the experiment was 81 g., being an average of 20.25 g., and at the end three rats remained whose total weight was 55 g., being an average of 18.3 g., one rat dying on the last day. It was considered that the unpalatability of the synthetic food might be mitigated by flavouring it with meat extract; this was done all through this particular experiment which lasted 15 days. The average weight nevertheless went down, neither the lemco added nor the lecithin seeming to have any pronounced effect on growth or even sufficing to sustain the original weight. During the period of dieting of 15 days the rats were given 144 g. of food and 2.88 g. of cholesterol; 101.3 g. of food were eaten; the cholesterol intake thus being 2 g.

The actual weight of material analysed was 52.6 g. Total cholesterol content 0.2654 g.; or 0.0883 g. per rat. The quantity of cholesterol which the three rats have put on is $3(0.0883 - 0.0359) = 0.572$ g. These rats ingested in their food about 2 g. of cholesterol. All found is therefore accounted for by that contained in the food.

Curve 8 represents the average growth during the course of the experiment.

Curve 8. Synthetic + cholesterol (artificial fat + lecithin and lemco) (Set 10). Average weight curve.

The results so far obtained do not indicate that cholesterol itself has any appreciable influence on the growth of an animal; all that is shown is that when an animal is fed on an artificial diet which does not promote growth

and to which cholesterol is added, no increase of growth occurs, but that part of the added cholesterol is stored bringing the total cholesterol content of an individual rat up to or above that found in the body of a rat fed on an ordinary diet [*vide set (3)*].

Whether the esters of cholesterol have any influence in this direction is a question which has been investigated by adding them to the synthetic diet instead of cholesterol.

Set (11). This set consisted of six rats fed on an ordinary synthetic diet with the addition of 2 % of cholesterol esters. The ester employed was palmitic ester which contains 0.6 of its weight of cholesterol; this is equivalent then to adding 1.2 % of cholesterol.

The weight of the six rats, at the beginning of the experiment, was 116 g., being an average of 19.3 g. per rat. On the 6th day three rats died and one on the 7th day; the two remaining rats remained alive for 16 days in good health when they weighed 43.5 g., being an average of 21.75 g. No lemco was added to the food, and no growth took place. After the four rats had died the remaining two were given 70 g. of food of which 50 were eaten, and the amount which they ate during the previous six days was calculated at 16 g. This represents 66 g. of food containing 1.2 % of cholesterol or 0.792 g. of cholesterol. These two rats were killed on the 16th day when they weighed 43.5 g., or an average of 21.75 g. Total cholesterol content 0.1496 g., or 0.075 g. per rat, representing an increase for the two rats of $2(0.075 - 0.0359) = 0.08$ g.

We have seen that 0.792 g. of cholesterol was ingested so that about one-tenth of this quantity has been stored in the bodies of these two rats.

Curve 9. Synthetic + cholesterol esters (*Set 11*). Average weight curve.

Curve 9 gives the average weight of these rats during the course of the experiment.

Set (12). This set, consisting of six rats, was fed on exactly the same diet

as the last with the addition of 0.5 % lecithin; the fat used was synthesised as in set (10) and half the protein was replaced by the pure protein obtained from bulls' testicles [see set (6)]. On June 24, when the dieting was commenced the six rats weighed 99.5 g., being an average of 16.6 g. For the first five days lemco was used to flavour the diet, at the end of which time the total weight had gone up to 116 g. On the 6th, 7th, 8th and 9th days the lemco was omitted, by which time the total weight was 120.5 g. Lemco was now again added to the diet, but the total weight steadily declined till the 15th day, when it was 105 g. and two rats died. All through the experiment the rats had been quite well, but now began to show signs of diminished vitality; it was therefore decided to analyse them at this point when their average weight, 17 g., was practically what it was at the beginning of the experiment, viz. 16.6 g. The total amount of material analysed was 100 g. Total cholesterol content, 0.416 g., or 0.069 g. per rat. During the 15 days the six rats were given 180 g. of food of which 157 were eaten. This means that about 1.8 g. of cholesterol were also ingested. The total cholesterol put on by the six rats is $6(0.069 - 0.0359) = 0.1986$ g., that is to say about one-tenth of that ingested, as was the case in the last set.

Curve 10 gives the average weight during the course of the experiment.

Curve 10. Synthetic + cholesterol esters (artificial fat, mixed proteins, 5 % lecithin, and lemco (Set 12). Average weight curve.

We notice that this set of six rats were kept alive and quite well for a period of 15 days when two died. The rats increased in weight about the middle of the experiment when it was thought interesting to omit the lemco from the diet; after four days from the omission they began to decline in weight, and the re-addition of lemco to the food failed to check the decline which progressed steadily to the end.

It has been an endeavour in these experiments to keep the rats in health for a long period on synthetic diet either with or without cholesterol and to

note the cholesterol content at the end, but so far complete success has not been obtained.

The results obtained from those sets of rats which were fed on a synthetic diet to which cholesterol and cholesterol esters were added do not go to show that these substances have any effect on the growth of the rats. The inability of a synthetic diet to enable growth processes to go on is evidently not due to the absence of cholesterol.

It seemed interesting, however, to see what would be the result of feeding rats on an ordinary diet of bread and oatmeal which had been extracted with ether, or to which cholesterol was added. Should we find the same results as were obtained in the last four sets or not? Unfortunately, the food material used was not thoroughly extracted as was proved by subsequent examination. 50 g. of the food were heated on a water bath for several hours with a 10 % alkali solution, after which the mixture was made acid and again heated for several hours. The mass was then analysed for phytosterol by the modification of Windaus' method. In the 50 g. taken was found 0.0076 g. of phytosterol, that is 0.0152 %.

From 50 g. of similar food, viz. bread and oatmeal, which had been extracted by alcohol, 0.0074 g. of phytosterol was obtained, i.e. 0.0148 %.

Set (13). This set was fed on bread and oatmeal which had been extracted with ether together with the addition of 2 % cholesterol in the daily ration. We have seen in set (5) how the rats behaved on a fat free diet, and I wished to see what effect the addition of cholesterol would have on the growth and condition of the rats. A litter of 12 rats was born on May 30, 1913. Four of these were put on the special diet on June 30 and during the period of dieting of 31 days, were given 278 g. of bread and 276 g. of oatmeal and 11 g. of cholesterol; 428 g. of bread and oatmeal were eaten and 8.5 g. of cholesterol by the four rats, none of which died. The bread and oatmeal were weighed dry, and, with the cholesterol added, were made into a paste with water of such consistency that the rats would eat it freely. The weight of the four rats at the beginning of the experiment was 75.2 g., representing an average of 18.8 g. per rat. The rats readily ate the mixture and were quite healthy throughout the period of 31 days. At the end of this period their total weight was 138.8 g., corresponding to an average of 34.7 g. per rat. Each rat had therefore gained in weight practically 16 g. Curve 11 represents the average growth during the experiment. There were no deaths and all the rats remained quite healthy. The rats were killed on July 30; the weight of the actual tissue analysed being 134.5 g. Total cholesterol 0.4544 g.,

corresponding to 0.1104 g. of cholesterol per rat. It will thus be seen that each rat has put on roughly about 0.0748 g. of cholesterol, again taking 0.0359 from set (1) as our standard. As each rat ate during the course of the experiment about 2 g. of the pure cholesterol given, it will be seen that only a small proportion of that ingested is stored. It is as well to note that the 428 g. of food eaten would contain in all 0.064 g. of sterol which had not been extracted, but this will in no way alter our results.

Curve 11. Ether extracted bread and oatmeal + 2 % cholesterol (Set 13). Average weight curve.

It would seem, in view of the results obtained from sets (9), (10), (11) and (12) which were fed on synthetic diets to which cholesterol and cholesterol esters were given and in which no growth occurred, that the growth in this case is due rather to the fact that the food was imperfectly extracted than to the fact that cholesterol was added.

The results of the experiments detailed above are gathered together in Tables I and II (pp. 81, 82).

SUMMARY OF RESULTS.

1. The rats which were just^{*}beginning to feed themselves contained in their bodies a normal standard quantity of cholesterol, viz. 0.0359 and 0.038 g. for sets (1) and (2), respectively. The total percentage is 0.1467.
2. After being fed on an ordinary diet of bread and oatmeal for three weeks, the total quantity of cholesterol per rat goes up to 0.071 g., the percentage being 0.2.
3. When the rats are fed on a pure synthetic diet containing no cholesterol, no growth occurs, nor is there any increase in the cholesterol content of the organism over a period of 16 days, and correspondingly there is no loss (sets 4 and 5).
4. The replacement of half of the pure caseinogen used in the synthetic diet by cholesterol-free protein obtained from bulls' testicles, together with

the addition of lecithin, also causes no increase either in the growth or the cholesterol content of the rats (set 6).

5. When rats were fed on the ordinary synthetic diet, with the addition of considerable quantities of milk, viz. 20 cc. *per diem*, they increased their weight by 66 % in 23 days. The cholesterol in the milk was eagerly picked out by the organism and stored, and the quantity found could be accounted for by that contained in the milk. There was no evidence of any synthesis (set 7).

6. When half the above quantity of milk was given and in addition 2 % of cholesterol, growth was considerably less during the same period of 23 days. About one-tenth of the cholesterol ingested was stored, bringing the total quantity per rat up to 0.1340 g. and the total percentage to 0.5236 (set 8).

7. The addition of cholesterol and cholesterol esters to a synthetic diet, together with lecithin and lemco, did not in any way enable normal growth to proceed. At the end of 15 or 16 days the average weight of the rats fed on a synthetic diet with cholesterol added had slightly decreased, and in the sets which had cholesterol esters added the increase per rat was less than 1 g. during 16 days. In all of these cases the rats stored in their bodies about one-tenth of the total cholesterol eaten with the food (sets 9, 10, 11 and 12).

8. When a diet of bread and oatmeal which had been imperfectly extracted with ether was given together with 2 % of cholesterol, slight growth occurred and the total quantity of cholesterol in the rat was found to be about three times that of set (1). (Set 13.)

GENERAL CONCLUSIONS.

A pure synthetic dietary is insufficient to enable "growth processes" to go on in young rats, in other words the rats are starved on such a diet and neither cholesterol, cholesterol esters nor lecithin appears to supply the deficiency. Lemco, however, appears to have a beneficial effect in some cases, rats fed on a diet with this addition showing no eagerness to nibble their dead comrades.

There is no evidence that the living organism can manufacture cholesterol, but on the other hand it is a substance which is strictly conserved and readily picked out from a diet in which it is present in only small quantities. When, however, it is present in large quantities in the food then the percentage in the body goes up considerably but only a small quantity of that ingested is actually stored up.

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