LXIV. CHOLESTEROL SYNTHESIS IN THE ANIMAL BODY.

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Ellis and Gardner [1909] attempted to determine whether cholesterol synthesis occurred in the animal body by determining the amount of cholesterol in a number of unincubated eggs and in newly-born chicks from the same batch of eggs. They found that the amount in the newly-born chicks did not differ from that in the eggs, and they concluded that cholesterol synthesis did not occur. They laboured however under the difficulty that they were using a method of cholesterol estimation much less accurate than that available at the present time. Gardner and Lander [1914] approached the question from another point of view. They estimated the cholesterol in a number of newly-born chicks and fed the remainder of these chicks on various diets—chicken food, chicken food previously freed from sterols, and chicken food plus 2 % cholesterol, and, after 7, 14 and 21 days, further estimations were made on individuals from the various groups. Owing to irregular rate of growth and consequent irregular weights, the results were not very definite, but the authors conclude that cholesterol synthesis does not take place. Lander [1915], carrying out similar experiments on rats, found that the percentage cholesterol was, on the average, 161 mg. at 3 weeks of age. He fed other rats on mixed, and in some cases ether-extracted, diets, but none of his animals lived for any length of time owing to the fact that his diets were deficient in vitamin A. His inconclusive results are therefore open to objection, but he expresses the view that "there is no evidence that the living organism can manufacture cholesterol, but on the other hand, it is strictly conserved and readily picked out from the diet." His method of extracting the cholesterol was to mince the animals, grind them up with plaster of Paris, and extract the powdered material for 14 days with ether. Cholesterol was estimated in the extract by the digitonin method.

The work so far described has had as its basis the estimation of cholesterol in the whole body of the animals under experiment. Another method has been to measure the total sterol intake on mixed diets and the total excretion in the faeces. Thus Gardner and Fox [1921], working with eight adults on mixed diets, found that the faecal sterol output was about 2·5 times greater than the

intake. In all cases save one this large excess excretion over intake was maintained, and they conclude that there is some organ in the body capable of synthesising cholesterol. Similar results are reported by Gamble and Blackfan [1920] in which the sterol output was 1·3–3·4 times the intake. These concordant results lead the authors to conclude that cholesterol synthesis takes place, but there are certain facts which this type of experiment ignores. Thus, even though the sterol output is greater than the intake, no rigid conclusion as to cholesterol synthesis can be drawn unless knowledge exists as to the function of the cholesterol stored in the various organs of the body. Little is known on this subject and the excretion of cholesterol might possibly ensue as the result of some physiological function; in this case if the diet were poor in cholesterol, the reserve supplies in the liver, adrenals, etc., might be called upon to make up the deficiency, and hence, unless experiments of this type were carried out over a prolonged period, the possibility of the effect being due to cholesterol already stored in the body cannot be eliminated. A second objection is that bacterial synthesis of sterol in the large intestine may be the cause of the large excess of excretion over intake. It was with a view to investigating these possible sources of error that the work described in this paper was undertaken. The general plan was to estimate the amount of cholesterol in rats at various ages and weights when on the complete artificial diet suggested by Drummond and Coward [1920] and used in this laboratory, in others on a diet poor in cholesterol, and in others still on a diet completely free from cholesterol.

The difficulty with regard to the cholesterol-free diet has hitherto been that no diet has been devised which is free from cholesterol and yet sufficient for growth. The ether-extracted diets used by other workers have necessarily been free from fat and therefore from fat-soluble vitamins. Hence where such diets have been used the results have suffered from the fact that growth, if any, has been very slight, and in experiments of this type it would appear essential that the animals should exhibit normal growth throughout. This difficulty has been overcome in the present work by supplying the rats with a very small daily dose of the unsaponifiable matter from cod liver oil. This is prepared by extracting cod liver oil, after saponification with alcoholic potash, with ether. After washing the extract and removing the ether, the resulting product, which still contains about 5 % of oil, is again saponified and extracted with ether as before. The washed extract, after drying over anhydrous Na_2SO_4, is taken to dryness. The unsaponifiable matter so obtained contains about half its weight of cholesterol, the bulk of which can be removed by crystallisation from 95 % methyl alcohol. After filtration and removal of the solvent, the residue is dissolved in boiling 95 % ethyl alcohol and the remaining cholesterol precipitated by excess of a 1 % solution of digitonin in 90 % alcohol. After filtration and removal of the excess digitonin, the solvent is evaporated off. Suitable precautions are taken to prevent oxidation throughout the preparation.

Diets used in the experiment.

I. Complete artificial diet	%	II. Cholesterol-free diet	%	III. Diet poor in cholesterol	%
Caseinogen	20	Caseinogen (ether extracted)	20	Caseinogen (ether extracted)	20
Rice starch	50	Rice starch	68	Rice starch	68
Butter	15	Butter	0	Butter	0
Yeast extract	5	Yeast extract	7	Yeast extract	7
Lemon juice	5	Lemon juice	0	Lemon juice	0
Salt mixture	5	Salt mixture	5	Salt mixture	5
		Unsap. matter (cholesterol-free)	0·0005 g. per rat per day	Unsap. matter	0·0005 g. per rat per day
				Cholesterol	0·004 g.

With a view to economising the unsaponifiable matter, it was dissolved in synthetic triolein and each animal of the group on the cholesterol-free diet received one drop of this solution per day, this being made up so that one drop contained approximately 0·0005 g. unsaponifiable matter. The cholesterol of diet III was given in a similar manner.

Fat and cholesterol content of the diets.

In the cholesterol-free diet (II), the fat (butter) was omitted and the starch increased to 68 %. The lemon juice was also omitted, as rats appear to be able to grow satisfactorily on a diet deficient in vitamin C. In order to render the diet more palatable, the percentage of yeast extract was increased to 7. As the caseinogen used had been thoroughly extracted, this diet was virtually fat-free. To obtain its fat content, 25 g., after grinding with anhydrous Na_2SO_4 were extracted with ether for 40 hours, and yielded 0·2 % ether-soluble material. This was saponified with sodium ethylate and the resulting unsaponifiable matter gave no precipitate with digitonin and none of the cholesterol colour reactions. As further confirmation that the diet was quite free from cholesterol, 25 g. were saponified first with aqueous and then with alcoholic potash. The unsaponifiable matter obtained by ether extraction also failed to give any of the cholesterol colour reactions; this diet was thus cholesterol-free, and constituted the basis of diets II and III. In diet III, each animal received three drops of a 4 % solution of cholesterol in triolein, thus receiving approximately 4 mg. cholesterol per day.

Diet	Basal fat g. per rat per day	Fat in supplement g. per rat per day	Total fat g. per rat per day	Total cholesterol g. per rat per day
I. Complete artificial	5·5	—	—	0·0015
II. Cholesterol-free	0·02	0·025	0·045	—
III. Poor in cholesterol	0·02	0·075	0·095	0·004

Animals used in the experiment.

All the rats used in this work were bred from a definite number of our own stock, with a view to eliminating abnormal results as far as possible. The original plan was to estimate the cholesterol content of rats at time of weaning

(age, 3–4 weeks, weight, 35–45 g.) and to take an average of the figures obtained as representing the normal cholesterol content of rats at this age and weight. Any animals put on to any diet at weaning were thus considered as starting with this average amount of cholesterol. Later it was decided to do estimations on numbers of rats on the complete artificial diet (diet I) at various ages and weights—in other words to obtain figures for a curve of cholesterol content of rats on this diet plotted against age or weight. This was to serve as a control of the results obtained from animals on the other diets. Every group of animals on any given diet was a mixture from the various litters, care being taken that the litters were well scattered among the different groups in order to obtain more average results.

Method of estimation.

Each rat was killed by chloroform; its alimentary canal was removed from the stomach downwards and washed through with water until quite empty. In this way only the cholesterol present in the tissues was estimated. The animal, together with the washed stomach, intestine, etc., was then placed in a flask with sufficient 10 % KOH and boiled for 3–5 hours, when complete solution, save of the bone fragments, had taken place. It was considered that in this way only could all the cholesterol be freed from the tissues. The results, which will be reported later, show a higher cholesterol content than those of Lander [1915] for animals of the same age and weight. This would suggest that either the cholesterol was not completely extracted by his method or else that his animals were considerably under the weight which those reared in this laboratory normally show at the same age. After saponification by aqueous KOH, the liquid was extracted with ether six times in a separating funnel after addition of sufficient alcohol to cause rapid separation of the ether and aqueous layers. The extract—usually about 500 cc.—was washed five times with water, and after removal of the ether the resulting fat was dried at 105° for half an hour. It was then boiled for 2 hours with sodium ethylate (2*N*) after solution in hot alcohol. Water was then added to dissolve the soap and the solution was extracted with ether and treated as already described for the previous saponification. The ether was then removed in a tared flask and the whole dried for 1 hour at 105° and re-weighed. In this way the weight of unsaponifiable matter in each rat was obtained. The cholesterol present in this unsaponifiable matter was then estimated by the method of Windaus [1910] in an aliquot part of a solution in light petroleum in order to economise digitonin. The fraction of the solution taken was always such that it contained, as far as possible, the same weight of unsaponifiable matter. After removal of the light petroleum the unsaponifiable matter was dissolved in 10 cc. 95 % ethyl alcohol and precipitated in boiling solution with 0·2 g. digitonin in 90 % alcohol. In this way the weight of cholesterol digitonide weighed was kept fairly constant. After standing over night the precipitate was collected in a Gooch crucible, washed first with ether and then with boiling water

until free from digitonin, dried for 1½ hours at 105° and weighed. From the weight of the digitonide, the total cholesterol in each animal can be readily calculated. The method is tedious, but ensures that all the cholesterol present in the animal is estimated. Owing to the large number of manipulative operations, great care is necessary; further, any error in the actual estimation of the cholesterol is greatly increased owing to the amount of unsaponifiable matter taken being only a fraction of the whole. The results obtained are taken as representing the amount of cholesterol present although the possible occurrence of other sterols precipitable by digitonin must be kept in sight.

Results.

1. *Complete artificial diet.*

	Wt. of rat (pairs) g.	Cholesterol mg.	Wt. of rat (pairs) g.	Cholesterol mg.	Wt. of rat g.	Cholesterol mg.
	11·0	16·4	11·8	20·6	11	25·6
	10·9	17·4	12·0	21·0	11	26·8
	11·3	17·2	12·1	20·3		
	9·6	14·8	13·2	22·9		
	10·4	17·8	13·0	20·4		
			12·9	21·6		
Mean	5·3	8·4	6·2	10·6	11	26·2

	Wt. of rat g.	Cholesterol mg.	Wt. of rat g.	Cholesterol mg.	Wt. of rat g.	Cholesterol mg.
	23·1	67·3	27·5	73	34·3	76
	20·8	57·5	24·0	69	34·1	71
	22·0	56·3	30·1	64	36·3	74
	21·5	58·3	27·0	86	34·8	73
	19·2	52·3	29·0	88	38·0	98
	21·2	50·3	30·3	78	38·0	103
	19·4	43·9			35·0	92
					35·0	87
					34·0	86
					33·2	98
					31·5	88
					32·5	92
					31·3	85
					38·0	71
					40·0	69
					37·6	71
Mean	21	55	28	76	35	83

	Wt. of rat g.	Cholesterol mg.	Wt. of rat g.	Cholesterol mg.	Wt. of rat g.	Cholesterol mg.	Wt. of rat g.	Cholesterol mg.
	68	134	83	141	109	238	137	262
	64	122	100	161	114	183	140	282
	65	110	100	167	110	179	139	262
	70	143	90	163	111	195	140	268
	59	142	90	194	110	197	155	288
	57	141	100	162	121	263	155	279
	63	131	94	165	122	210	152	299
					105	212	148	280
					113	187	146	278
					112	229		
					113	209		
Mean	64	132	94	165	113	208	146	278

2. *Cholesterol content of* 100 *g. rats receiving the cholesterol-free diet.*

	Weight when weaned and put on to diet g.	Final weight when used for estimation g.	Cholesterol mg.	Cholesterol mg. per 100 g. rat
	42	103	225	219
	42	102	237	232
	46	98	219	223
	47	103	225	218
	42	102	227	224
	47	107	230	214
	41	107	226	210
	41	102	230	225
	37	100	224	224
	37	97	215	222
	50	94	214	229
	37	98	213	217
Mean	42	101	224	221

3. *Cholesterol content of* 100 *g. rats receiving cholesterol-free diet plus* 4 *mg. cholesterol per rat per day.*

	Weight when weaned and put on to diet g.	Final weight when used for estimation g.	Cholesterol mg.	Cholesterol mg. per 100 g. rat
	47	113	227	214
	37	106	243	225
	54	108	241	213
	55	110	241	213
	48	108	246	228
	56	110	238	216
Mean	49	109	240	218

It is recognised that there are objections to taking averages of figures such as these but the variations are not as great as might be expected considering the biological nature of the experiment, that the animals used were from many litters and that the ages of animals of similar weight often varied. It would have been an advantage if the number of animals used for estimation had been greater, but this was limited by the difficulty of raising sufficient litters from the limited number of stock animals selected for breeding for this experiment, and also by the tedious nature of each estimation. However, from the average figures it is possible to construct the curve in Fig. 1 in which the abscissae give the weights of the rat in g. and the ordinates the cholesterol content in mg. From this curve it will be seen that the cholesterol content of the young rat at weaning time is about 100 mg. and this figure is taken as the approximate content of all the other animals put into the other groups.

It would appear from this curve that a linear relationship exists between cholesterol content and body weight; further, the curve may be divided into two parts, one from birth to a weight of about 35 g., and a second part which is less steep, and it would appear that the increase of cholesterol with increase of body weight is definitely more marked in the early stages. This is shown more clearly by the curve in Fig. 2 in which are plotted as abscissae the ages of the

animals and as ordinates the amount of cholesterol expressed as mg. per 100 g. body weight.

It will be observed that there is a rapid increase in the percentage cholesterol to an age of 3 weeks followed by a fall to a value slightly above that at birth. The figures from which this curve has been constructed have been obtained by the conversion of some of the figures in the previous table to cholesterol percentages and their rearrangement into groups of animals of the same age. This was not possible with all, as records of all the ages wer

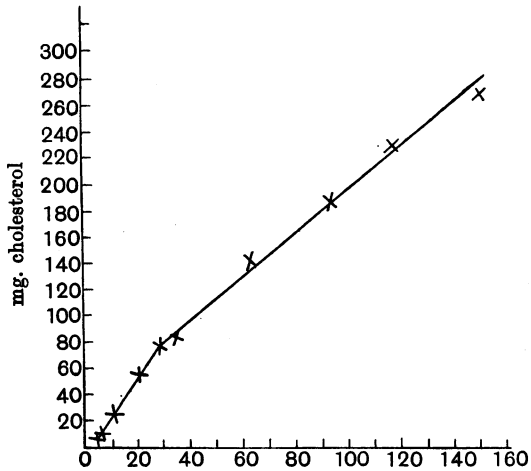

Fig. 1.

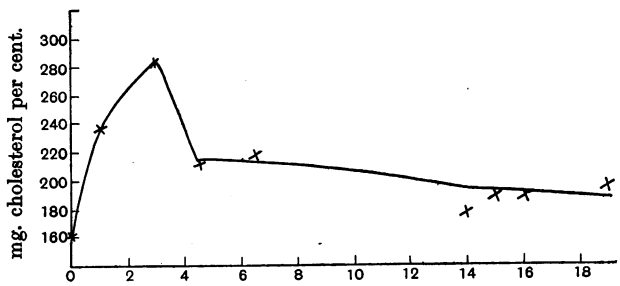

Fig. 2.

not kept. This curve is much less regular than the preceding one—a not unexpected result—but it would seem to indicate that the maximum cholesterol percentage corresponds approximately to the time of weaning at the age of 3 weeks, and this would suggest that it was due to completion of construction of nervous tissue. One other possibility which may explain this rise to a maximum is that the digestive tracts of the animals which were killed before weaning were not removed before estimation of the cholesterol content, and hence they may have retained in it some sterol from their mother's milk, but it is difficult to explain the results on this fact alone.

Cholesterol content of rats on the cholesterol-free diet at approximate weight of 100 *g.*

From an initial average weight of 42 g. to a final average weight of 101 g., the cholesterol has increased from 100 mg. (obtained from the curve in Fig. 1) to 224 mg. Using the figures reduced to 100 g. body weight, it is seen that the ratios of the final to initial body weights, and of the final to initial cholesterol contents are 100/42 and 221/100, *i.e.* 2·4 and 2·2 respectively; in other words, the rate of increase in cholesterol content has been very nearly as rapid as that of body weight. As the animals received no cholesterol over this period of 6 weeks during which their average weight rose from 42 to 100 g. and as their cholesterol content rose from 100 to 221 mg., an increase of 0·121 g. per animal, it would seem clear that cholesterol has been synthesised.

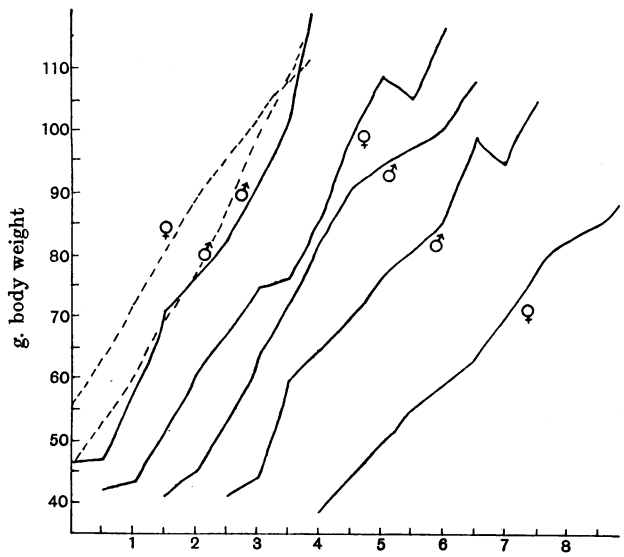

Fig. 3.

That the growth of the animals on the cholesterol-free diet was quite normal is shown in Fig. 3 in which are given the growth curves of six animals receiving this diet and taken at random. For comparison, there are given as dotted lines what are considered in this laboratory to be normal curves for animals receiving a diet complete in every respect.

Each animal received during the 6 weeks of diet III approximately 170 mg. of cholesterol, and had it all been stored, the total content of the animals which already had 100 mg. at the beginning, would have been 270 mg. even if synthesis had not occurred. It is curious that the average figure per 100 g. body weight in this group is 219 mg., whereas of those receiving no cholesterol (group 2) the average figure is 221 mg., the ingestion of 170 mg. having made no difference to the final cholesterol content of the animals of

group 3. It is to be observed that in group 1 (complete artificial diet) the cholesterol content of 100 g. rat is about 190 mg. whereas in both groups 2 and 3 the figure is about 220. No explanation of this difference has been obtained.

Sterol excretion.

Cholesterol was estimated in the ether extract of the dried faeces after 72 hours' extraction. The average figure per animal over a weekly period on the cholesterol-free diet was 0·0198 g. which would give for the 6 weeks of the experiment a total of 0·119 g. On diet III (poor in cholesterol) the excretion for the whole period was 0·126 g. per animal.

Thus the total sterol synthesised (that in the tissues was 0·121 g.) was 0·240 g. by each animal in 6 weeks.

SUMMARY.

1. A curve showing the relation between the body weight of rats from birth to 150 g. weight and the cholesterol content has been constructed for animals reared on the complete artificial diet used in this laboratory.

2. Rats fed from weaning time until they had reached 100 g. weight on a cholesterol-free diet showed an increase in cholesterol content from 100–220 mg.; the animal body would thus seem capable of synthesising cholesterol.

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