XXXVI. CALCIFICATION OF THE BONES OF RATS ON A DIET LOW IN ERGOSTEROL.

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The statement is frequently made that ergosterol is a substance which cannot be made in the animal body but there does not seem to be any real evidence that this is the case.

Beumer [1927] attempted to trace the fate of ergosterol ingested by an infant; of 0·4 g. ingested, he only succeeded in recovering 0·147 g. from the stools, but the observation does not shed much light on the problem.

In contrast with earlier workers both Channon [1925] and Randles and Knudson [1925] concluded that the rat can synthesise cholesterol. One of the chief difficulties in such an experiment is to supply the fat-soluble vitamins to the experimental animals free from sterols. Channon met this difficulty by using the unsaponifiable matter from cod-liver oil, after having first freed it from cholesterol by precipitation with digitonin; such a procedure seems satisfactory and would presumably leave the rat supplied with vitamins A and D. Randles and Knudson used dried alfalfa leaves, which had been extracted with cold ether; the method was held to remove the sterols from the leaves, while not extracting the vitamin A; the extraction of the sterols by this method could however hardly be regarded as complete and the experiment would seem to lack conclusiveness on this ground.

Even if cholesterol can be synthesised in the rat's body, as Channon's work indicates, it cannot therefore be concluded that this is also true of ergosterol. It seemed possible that the problem might be attacked indirectly, not by an attempt to estimate the ergosterol balance but by ascertaining whether the administration of diets, rich in ergosterol on the one hand, and rendered as deficient as possible in it on the other, had any influence on calcification in rats irradiated with ultra-violet light and deprived of any source of vitamin D through the mouth.

It was obvious that the experiment must be a long one, in order to aim at a depletion of any reserves of ergosterol in the rat; the diet would therefore have to be as complete as possible, so that prolonged well-being might be assured, while it must at the same time be rendered as free as possible from vitamin D and from sterols. The chief problem lay in the preparation of sources of vitamins A and B which should fulfil this requirement.

The experiment lasted about 4 months and was therefore a prolonged observation on the behaviour of rats on a fat-free diet. Certain observations were made in the course of it, on the occurrence of a condition of scaliness of the tail, described in particular by Burr and Burr [1929], as occurring on diets free from fat. These observations are included in an ensuing note.

Experimental.

Preparation of the diet. The diet was composed as follows:

Caseinogen	...	20
Wheat starch	...	65
Salt mixture	...	5 (McCollum 185 [McCollum and Davis, 1914][1])

Daily supplements were given separately of a vitamin A concentrate equivalent to 1·0 g. of the original spinach, and of a vitamin B concentrate equivalent to 1·0 g. of dried yeast.

The caseinogen used was that of the British Drug Houses, "fat- and vitamin-free," which is extracted with alcohol and ether.

The wheat starch received one extraction with cold light petroleum, by which means a small amount of a yellow oil was removed.

The caseinogen, starch and salt mixture were mixed with freshly distilled water and steamed in a double saucepan. By this means the starch grains were burst and any risk of refection obviated [see Roscoe, 1927, 1]. The mechanism of refection is not understood, but if, as seems possible, it depends on the elaboration of the B vitamins by some organism within the intestinal tract and their subsequent absorption by the rat, it would also appear possible that such organisms, particularly if they were yeasts, might also elaborate ergosterol and provide it to the rat. It was advisable therefore to take special precautions against refection.

An extract from spinach leaves was prepared to be a source of vitamin A. It was hoped that this when rendered sterol-free would supply a source of vitamin A which would also be free from vitamin D. Willimott and Wokes [1927] found that an ether extract of spinach, when fed in the equivalent of 5·0 g. of fresh spinach daily, supplied adequate vitamin A and had no significant influence on calcification, but Chick and Roscoe [1926] and Roscoe [1927, 2] found that the fresh leaves of summer spinach did exercise a small but definite antirachitic effect. It is certainly possible to render spinach strongly antirachitic by artificial ultra-violet irradiation, but the amount of vitamin D appears to be negligible unless the spinach is exposed to special

[1] Composition of salt mixture:

Sodium chloride	51·9
Magnesium sulphate	164·0
Sodium dihydrogen phosphate	104·1
Dipotassium hydrogen phosphate	286·2
Calcium phosphate	162·0
Calcium lactate	390·0
Ferric citrate	35·4

conditions of irradiation or insolation. At any rate the ether extract of a green leaf appeared to offer the best hope of providing a source of vitamin A, devoid of vitamin D. In the present state of knowledge, it is easy to see that it would have been better to have used recrystallised carotene as the source of vitamin A, since Moore [1929] has shown that purified carotene has no antirachitic activity. At the time, however, when the present experiment was carried out, it seemed unwise to proceed further in fractionation than to prepare a light petroleum extract of spinach, to saponify it and to use what was left of the unsaponifiable fraction after the sterols had been precipitated with digitonin.

Preparation of the extract of vitamin A. Two batches of extract were prepared. One was derived from 1500 g. of fresh prickly-seeded spinach (*Spinacia oleracea*) gathered in May. The product was used for a preliminary test to establish the vitamin A value of the extract. The second batch of 4200 g. of similar material was gathered at the end of September and beginning of October and the product was used for the main experiment.

The leaves were sorted and the larger pieces of stalk were removed. The leaves were dipped in boiling water and dried in front of a fan at a temperature of 37°. The dried leaves were powdered and sieved and extracted repeatedly with light petroleum (B.P. 40–60°), the solvent being subsequently distilled off at about 50°. From 1500 g. of fresh spinach about 2·6 g. of solid extract were obtained and from 4200 g. about 9·6 g. The extract was taken up with ether.

Saponification was carried out with sodium ethoxide, freshly prepared from metallic sodium and ethyl alcohol. Part of the alcohol was evaporated and water was added to the remaining solution, which was repeatedly shaken with fresh amounts of ether until the ether fraction ceased to show more than a little coloration. The ether was distilled off from the collected extracts, and after drying the yield of unsaponifiable matter was, from 1500 g. of spinach, 1·95 g., and from 4200 g., 3·8 g.

The unsaponifiable fraction was treated with digitonin in alcoholic solution. The solution was filtered to free it from the insoluble digitonide, which was washed with ether and alcohol. The alcohol was taken off and the residue was then taken up with ether and filtered to free it from the ether-insoluble excess of digitonin. When the ether was finally removed, a deep orange-coloured fraction was left, amounting to 0·92 g. from 1500 g. of spinach and 3·06 g. from 4200 g. of spinach.

The first batch of material, derived from 1500 g. of fresh spinach was taken up in hardened cottonseed oil and was fed to rats after a depletion period on a diet deficient in vitamin A. An amount corresponding with 1·0 g. of fresh spinach daily was found to supply a sufficient source of vitamin A over an extended experimental period of 70 days. The writers have found 0·02 and 0·03 g. of fresh spinach to supply adequate vitamin A for maintenance in the rat for an experimental period of 35 days [Hume and Smith, 1930].

The second batch of material, derived from 4200 g. of fresh spinach, was used for the sterol-free experiment. For this purpose the material was taken up with liquid paraffin (Internol of Messrs Allen and Hanbury); the preparation was stored in a brown bottle at about 0°; in these circumstances the yellow colour was well maintained and the preparation retained potency over a long period. It was so made up that one drop of the liquid paraffin contained the equivalent of 1·0 g. of fresh spinach; each rat received one drop daily of the preparation throughout the whole period of the experiment.

Preparation of the extract of the B vitamins. The concentrate of B vitamins was prepared from brewer's yeast by the method described by Chick and Roscoe [1929].

The yeast was washed four or five times with ice-cold water and pressed. About 15 kg. of such moist yeast were thrown into about 30 litres of boiling distilled water containing 0·01 % of acetic acid. The whole was again brought to the boil and filtered through Büchner funnels while hot. The clear filtrate was concentrated to a convenient small bulk, acidulated with sulphuric acid to about p_H 3·0 and stored at 0°. It was diluted and filtered again just before use.

The preparation was tested on rats, kept on open wire screens and fed on a diet deficient in the B vitamins, *i.e.* caseinogen (British Drug Houses, "fat- and vitamin-free") 300, wheat starch 750, hardened cottonseed oil 225, salt mixture (McCollum 185) 75, cod-liver oil 3–5 drops per rat daily. The diet was mixed with water and steamed. On an equivalent of about 1·0 g. of dried yeast daily, young rats grew well for an experimental period of 35 days. A similar dose was used throughout the main experiment. No source of vitamin C was given.

For a part of the animals ergosterol was added to the diet and for that purpose ergosterol supplied by the British Drug Houses was used. It was dissolved in "Internol" so that one drop contained $\frac{1}{10}$ mg. In this concentration it showed a tendency to crystallise out so that the bottle had always to be shaken before use.

Method of experiment.

The rats used were of the Lister Institute black and white strain; they were about 33–39 g. in weight and about 20–23 days old. They were kept singly in cages on open wire grids of mesh 3 squares to the inch to prevent consumption of faeces. The cages were washed several times a week, if necessary; it was feared that even a small contamination with faeces might supply ergosterol to the rats, through micro-organisms in the faeces.

Four litters of rats were used and were distributed evenly as regards sex and litter amongst four experimental groups; each of the four groups included five individuals, which were identical in sex and litter for each group. For the 1st week of experiment all four groups were treated alike; after that two of the groups received a daily addition of one drop of liquid paraffin, containing 0·01 mg. of ergosterol, while the other two groups received the same amount of liquid paraffin only. The experiment lasted 120 days, except in the case of one animal in each group (one litter), where it lasted 112 days.

About the 80th day of experiment it was hoped that the rats' ergosterol reserve, in those groups not receiving ergosterol, might be exhausted. Irradiation with a mercury vapour quartz lamp (Hewittic Electric Co.) was therefore instituted for one of the two groups receiving ergosterol and for one of the two not receiving ergosterol. The four groups therefore were receiving treatment as follows:

Group 1.	No irradiation	No ergosterol
Group 2.	No irradiation	Ergosterol
Group 3.	Irradiation	No ergosterol
Group 4.	Irradiation	Ergosterol

Irradiation was for 10 minutes every weekday at a distance of about 60 cm. It was found necessary to shield the eyes of the rats from time to time,

whenever they became sore from the irradiation, otherwise the animals ceased to thrive.

The animals were weighed at regular intervals. At the end of the experiment, all were killed and the femur and tibia of both legs were removed. These were dried, extracted with ether and alcohol and the ash content was determined.

Results.

The rats did well throughout the experiment and all survived the full period. The eyes of those which had not been irradiated were all normal or nearly normal at the end of the experiment; the condition of the eyes in those which had been irradiated could not be judged as regards xerosis, since, in spite of being frequently shielded, the eyes of these rats were rendered abnormal by the action of the mercury vapour lamp. Only three out of the twenty animals developed "snuffles" and only two showed small patches of congestion in the lungs at autopsy. The vitamin A supply throughout the experiment would therefore appear to have been quite adequate.

All the four rats belonging to one litter (No. 1228) developed small sore patches on the skin about the face and shoulders, towards the close of the experiment. This condition has often been observed before by the writers, in rats on other diets; it appears to be caused by an abnormal multiplication of lice, which cause irritation to the rat; the writers are inclined to correlate it with an unknown dietary deficiency.

All the rats developed some signs of the "scaly tail" condition, described by Burr and Burr [1929]. The tails were excoriated, shiny, annulated and scaly in parts and in some instances there were signs of the same condition on the skin of the feet. It would be natural to conclude with Burr and Burr that the condition was due to the absence of fat from the dietary, were it not that the same condition developed in the rats which were used for testing the material employed as a source of vitamin B. These latter rats received about 16 % of hardened cottonseed oil in their diet, together with 3–5 drops of cod-liver oil per head daily. The presence or absence of fat could not therefore have been the factor governing the development of scaly tail. The factors differentiating these experiments from others which the writers have carried out, and in which the condition has not been seen, would appear to be the type of caseinogen, the source of the B vitamins and the type of cage, with open wire screens to prevent coprophagy. A further investigation and discussion of the problem is included in the following paper.

Growth was strong at the start, being at first fully normal but after about 50 days, in most cases, it tended to slacken off; females had then reached a weight of about 150 g. and males of 200 g. An increase in the supply of B vitamins towards the close of the experiment did little to restore the growth rate and it is probable that the slackening was due to the lack of an unknown dietary factor, either that one, the lack of which caused the scaly tail condition, or another. A little acceleration of the growth rate took place after

the institution of irradiation about the 80th day of experiment in Groups 3 and 4, but if growth was being limited in any case by the lack of some other factor, any benefit to growth from the administration of vitamin D would be thereby obscured. The total growth response in the four groups, in the period after the institution of irradiation in Groups 3 and 4, was as follows:

Group 1.	No irradiation, no ergosterol	106 g.
Group 2.	No irradiation, ergosterol	112 g.
Group 3.	Irradiation, no ergosterol	129 g.
Group 4.	Irradiation, ergosterol	147 g.

The figures appear to show a small but distinct superiority in growth in the two groups which were irradiated.

Table I. *Percentage ash in the dried extracted bones of rats fed on a diet made as deficient as possible in ergosterol (Group 1), to which ergosterol was added (Group 2), which were irradiated with ultra-violet light for the latter part of the experiment (Group 3) and which were both irradiated and received added ergosterol (Group 4).*

Litter No.	Sex	No irradiation and no ergosterol	No irradiation and ergosterol	Irradiation and no ergosterol	Irradiation and ergosterol
1228	♀	60·6	62·4	61·9	62·4
1227	♀	59·0	59·2	60·7	60·4
1255	♂	60·3	61·0	61·9	61·8
1255	♂	60·8	61·7	62·3	61·7
1272	♂	59·8	61·2	61·5	61·0
	Average	60·1	61·1	61·7	61·5

The percentage ash in the dried extracted bones is set out in Table I. It is seen at once that all bones, even those in the negative control, Group 1, without ergosterol or irradiation, show a very high measure of calcification. In comparison with these, however, the two irradiated groups show a small but definite superiority in calcification; the range in each group is so small and the superiority of each individual irradiated rat over its corresponding litter mate in the negative control group is so regular that it would appear permissible to accept the result, in spite of the relatively high calcification in all groups. There is no difference between the calcifications of the two irradiated groups, indicating that abundance or deficiency of ergosterol in the diet made no difference to the calcification when the rats were exposed to ultra-violet light from a powerful artificial source. The second group, those rats which received ergosterol without irradiation, show a curious result. The individual values are more scattered and, while on the whole, barely inferior to the values in the irradiated groups, are definitely superior to those in the negative control group. The result seems to suggest that a rich supply of ergosterol in the diet promotes calcification when the only source of ultra-violet light is diffuse daylight in a North room. It must not be forgotten that ergosterol feeding in Groups 2 and 4 took place over the whole period of the experiment.

The whole experiment is marred by the high calcification of the negative controls, which is not easy of explanation. The salt mixture used is certainly not one on which a low calcification would be expected, but past experience suggests that some other explanation is needed. The reserves of the rats in vitamin D might have been high, but this has not been found to be the case when similar rats from the Lister Institute stock have been fed on rickets-producing diets. The only other explanation would seem to be that the vitamin A concentrate, prepared from spinach, also contained vitamin D; that possibility has already been discussed and dismissed as improbable, particularly in view of the smallness of the dose used, which was the equivalent of 1·0 g. of fresh spinach.

The result suggests that limitation of ergosterol in the diet did not act as a limiting factor in calcification when the rats were powerfully irradiated with ultra-violet light. It is not however possible to deduce from this result whether ergosterol can be synthesised in the rat's body or not; that some measure of synthesis takes place is one possible interpretation, but ignorance as to the rat's possible reserve of ergosterol and uncertainty as to whether the diet was completely devoid of, or only relatively deficient in, ergosterol, make it impossible to draw a definite conclusion, the results being such as they are.

If synthesis did take place, addition of ergosterol to the diet was still able to raise the calcification to a slightly higher level, when the supply of ultra-violet light was only very small.

Summary.

1. Experiments are described in which rats were fed on a diet made as free as possible from ergosterol, with the object of ascertaining to what extent this sterol can be manufactured in the animal body. Other rats were fed on the same diet with an abundant addition of ergosterol. After about 80 days half the rats in each series were irradiated with a mercury vapour quartz lamp daily, for about 40 days. At the end of the experiment the percentage ash in the dried fat-free bones of all the rats was determined.

2. The bones of all the rats showed a high percentage of ash. That in the bones of the irradiated rats receiving ergosterol was the same as in those of the irradiated rats not receiving ergosterol. The ash of the group receiving neither irradiation nor ergosterol was significantly lower than that of the other groups. The group which received ergosterol but not irradiation showed an ash content of the bones which did not differ significantly from that of the two irradiated groups.

3. No answer therefore was obtained to the main question of the enquiry, *i.e.* the possibility of ergosterol synthesis in the rat body. Since however, when the supply of ultra-violet light was abundant, a variation in the amount of ergosterol in the diet made no difference to the calcification, it must be concluded that the rat was, in those circumstances, sufficiently supplied with ergosterol. Such ergosterol may have been supplied by some measure of

synthesis or it may have been derived from a residual amount in the diet or from the faeces, for in spite of all precautions it was found impossible to prevent the rat from consuming traces of the latter.

4. When, however, the supply of ultra-violet light was very small, the supply of ergosterol from any of the possible sources just mentioned was not sufficient to promote the maximum calcification, which was only then attained when abundant ergosterol was added to the diet.

5. In the course of these experiments, which represent a prolonged maintenance of rats on a fat-free diet, the animals developed the "scaly tail" condition of Burr and Burr, who attributed it to lack of fat in the diet. The observation is discussed in the next paper.

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