188. THE EFFECT OF RICKETS AND OF ERGOSTEROL ON THE BRAIN AND LIVER CALCIUM OF YOUNG RATS

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IN spite of the production of Ca deposits in the soft tissues by means of parathyroid extracts and irradiated ergosterol, the quantitative determination of Ca in the body tissues has not been the subject of many investigations. The concentration of Ca in normal tissues varies in a way which was not to be expected from the narrow range within which it fluctuates in the serum. On the other hand the changes in tissue Ca concentration produced by experiment and by disease are small, and the variability of its concentration in healthy tissues has made the significance of small changes difficult to assess except by statistical means.

The present contribution contains the results of Ca analyses of the brain and liver tissue of rats with low-phosphorus rickets and acute ergosterol poisoning.

EXPERIMENTAL

The rats were of Wistar strain and were killed when 9-12 weeks old and weighing 80-120 g. Normal controls were fed on dog biscuit, dried milk powder, poultry meal and fresh carrots. Cod liver oil was given to them twice a week and they were housed in a sunny room.

Rickets. Groups of rats received the high Ca low P diet RR401 [Jephcott & Bacharach, 1926] from the age of 4-6 weeks. They were shaded from light. When killed 28-56 days later the presence of advanced active rickets was shown by the very low level of the serum inorganic P and by the presence of marked rachitic changes in the epiphyses.

Ergosterol. These animals were placed in separate cages at the age of 9 weeks.

On the first evening of the experiment all food was removed from the cage except a small amount of ground dog biscuit containing 0.5 ml. of 10,000 X irradiated ergosterol solution. This was completely consumed by the next morning when poultry meal and carrot were offered and as a rule consumed in the course of the day. On the second evening surplus food was removed and a second dose of ergosterol on biscuit was given. By the second morning only part of the ergosterol had been taken and the rats were ill and refused other food. They passed small amounts of blood by the bowel. By the third morning all were very ill and some had died. The survivors were killed and these only were taken for the analyses.

Ergosterol controls. The ergosterol animals lost a large amount of weight during the experiment, part of this loss being due to refusal of food. To control this factor some similar animals were caged separately and allowed only the food corresponding to that actually eaten by the poisoned animals. These controls failed to gain weight or lost a few grams, but the weight loss was not as great as in most of the ergosterol animals.

The animals were killed by cutting the throat and blood was collected for Ca and inorganic P determinations. The brains were removed with care to avoid contamination with bone. Three brains were mashed together and the pooled material was passed through fine washed gauze. Sufficient material was obtained for duplicate ashing and analysis which were carried out as previously described [Linder, 1935]. The livers were treated in the same way.

McCance & Shipp [1933] state that if tissues to be analysed for Ca are not reduced to a white ash much Ca is lost by inclusion in, or adsorption on, particles of carbon. The ash obtained by the method used here contained a small amount of carbon. Duplicate determinations were made by this method and by the Stolte [1911] method, using ammonium nitrate to help the process of ashing and continuing until all the carbon was destroyed; the results were the same. The routine method was also compared with a modification in which heating was continued for 2 hr. in the furnace at a dull red heat; the carbon was destroyed but no more Ca was recovered. A satisfactory recovery of added Ca was also obtained.

Small portions of the ergosterol livers were taken for histological examination.

RESULTS

The results are given in Tables 1 and 2 and in Fig. 1. The tables show the number of determinations in each group and the mean Ca concentration in the fresh tissue with its standard deviation. The mean water content is given. The results in the different groups were compared by the statistical method described by Tippett [1931] for the comparison of the means of small samples. The value of t was calculated and the corresponding value of p was taken from Fisher's [1938] table. When p is less than 0.05 the means may be taken as significantly different. The results are grouped in pairs for comparison, and for each pair the right-hand columns of the tables give the difference between the means of the pair and the values of t and p derived from them. It will be seen that p in the cases in which differences are present is 0.01 or less, indicating a much greater degree of statistical probability.

Table 1. *Means of the series of brain Ca determinations and their statistical comparison*

Series	No. in series	Mean water content %	Mean Ca mg./100 g.	σ	Difference of mean Ca from normal	t	p
Normal	21	78.6	4.71	0.43	—	—	—
Rickets	12	78.8	5.30	0.62	0.59	3.22	0.01
Ergosterol	13	78.7	6.42	0.81	1.71	8.44	<0.01
Ergosterol control	4	78.7	4.63	0.45	0.08	0.337	0.7

Table 2. *Means of series of liver Ca determinations and their statistical comparison*

Series	No. in series	Mean water content %	Mean Ca mg./100 g.	σ	Difference of mean Ca from normal	t	p
Normal	14	71.7	3.28	0.51	—	—	—
Rickets	27	72.3	3.36	0.90	0.08	0.307	0.7
Ergosterol	24	72.7	4.97	0.95	1.69	6.06	<0.01
Ergosterol control	13	71.9	4.16	0.72	0.88	3.69	<0.01
Ergosterol control	—	—	—	—	0.81	2.67	0.01

Rickets. The mean serum Ca of the rachitic rats was 9.9 mg./100 ml.; this was practically the same as the normal mean, 9.7 mg. The mean inorganic P of the rachitic sera was 1.04 mg. and that of the normal sera was 5.5 mg.; the presence of rickets was therefore evident. Table 1 shows that the mean brain Ca in this type of rickets was greater than that of the normal brains by 0.59 mg./100 g.; p is less than 0.01, therefore this increase, although small, is unlikely to be due to chance. The mean water contents of the two groups were the same. From Table 2 it can be seen that the mean Ca content of the liver was unchanged in rickets, and there was no significant change in the water content.

Ergosterol. In the ergosterol group the mean serum Ca concentration was 13.2 mg./100 ml. This was 3.5 mg. higher than the mean of the normal group

Fig. 1. Individual observations on the Ca contents of the brain and liver of young rats. Effect of rickets and of ergosterol.

and is an indication of the degree of ergosterol toxæmia which had been established. The mean brain Ca was 1.71 mg. higher than the normal; the value of t shows that this difference is highly significant. That this was not due to partial starvation is shown by the last comparison in Table 1, which demonstrates that the mean brain Ca of the partially starved controls did not differ appreciably from that of the normal animals.

The concentration of Ca in the livers was also increased, the mean increase being the same as in the case of the brains. In the case of the liver, however, the mean Ca concentration of the partially starved controls showed a significant increase over that of the normal group, though the difference between the means of the ergosterol and partially starved groups was still sufficiently large to remain of significance. Of the increase in liver Ca it seems that half was directly due to

the ergosterol and that half may have been due to the lowered intake of food, the result of the ergosterol.

The water content of the tissues is included in the tables. The results have been examined on the basis of dry tissue, but the validity of the comparisons was not affected.

Microscopic examination of the livers showed diffuse areas in which the cytoplasm of all the cells and the nuclei of some of them stained very poorly; in two livers small areas of calcification were seen, and in a third there was a comparatively large area in which the cells were necrotic and there was extensive deposition of Ca.

DISCUSSION

In the following remarks the Ca figures quoted from the literature have been reduced to mg./100 g. of fresh tissue. When the authors gave figures for dried tissue the values for fresh tissue were calculated by dividing the figures by 4.

Hess *et al.* [1932] made an extensive study of the Ca concentration in rat brain. The brains of their normal rats contained 25–65 mg./100 g. When rickets was produced by high Ca low P diets the brain Ca was reduced to between 9 and 19 mg., and a similar reduction followed a low Ca high P diet. An abrupt change from a high Ca low P diet to a normal diet caused tetany, the serum Ca falling and the brain Ca remaining low. They found that Ca. could be rapidly removed from the brain by rachitogenic diets, but could only be restored slowly by normal diets and vitamin D.

Beumer [1936] found that the mean Ca content of 27 normal rat brains was 3.55 mg. and that of 27 rachitic rat brains was 2.08 mg. He agreed with Hess in regard to the lowered concentration in rickets, but the amounts of Ca which he found were very much smaller. Farago [1936] reported that the different parts of rat brain varied considerably in their Ca contents; the cortex contained 6 mg. and the cerebellum 0.4 mg. He does not give figures for the whole organ but they must have been about the same as those of Beumer [1936] and those reported here. Watchorn & McCance [1937] gave the brain Ca of black and white piebald rats as 56 mg. and Patwardhan & Chitre [1938] reported 13–20 mg.

The following amounts have been found in the brains of other animals: cat 8–17 mg. [Heubner & Rona, 1923] and 4–7 mg. [Katzenelbogen, 1932]; dog 2–8 mg. [Underhill & Jaleski, 1933], 14 mg. [Reed *et al.* 1933] and 26 mg. [Hetenyi, 1935]; rabbit 5.9 mg. [Denis & Corley, 1925]; guinea-pig 4–11 mg. [Linder, 1935]; calf 16 mg. and sheep 10.8 mg. [McCance & Shipp, 1933]; man 10.6 mg. [Magnus-Levy, 1910] and 4–6 mg. [Eaves, 1931].

Hess *et al.* [1932] pointed out that there was no reason to anticipate a fall in tissue Ca on a high Ca low P diet, and the results obtained in the present experiments do not show such a decrease either in brain or liver. On the contrary the brain Ca was slightly increased.

Investigations into the Ca content of rat liver tissue are even fewer. Favarger & Rutishauser [1937] found 4.5 mg. in normal rat liver and Patwardhan & Chitre [1938] 7 mg. The mean of the present determinations was 3.3 mg. and no change was found in low P rickets.

In other animals the following results for liver Ca have been recorded: dog 8.6 mg. [Voit, 1880], 22 mg. [Hetenyi, 1935] and 4.5 mg. [Reed *et al.* 1933]; rabbit 7.5 mg. [Denis & Corley, 1925] and 5 mg. [Smith & Elvove, 1929]; guinea-pig 4.2 mg. [Linder, 1935]; ox 8.1 mg. [Corley & Denis, 1925] and 8.7 mg.

[McCance & Shipp, 1933]; and man 7.1 mg. [Stoeltzner, 1899] and 7.2 mg. [Magnus-Levy, 1910].

The effect of large doses of ergosterol on the tissues has been extensively studied by histological methods [cf. Reed *et al.* 1933]. The extent of the calcification depends to a large extent on the nature of the diet, for the most extensive calcification has been found when the P intake was high and this occurred even if the intake of Ca was minimal [Shelling, 1930]; on a low P diet even with excess of Ca there may be no deposits [Smith & Elvove, 1929]. Similarly the height of the serum Ca was no indicator of the amount of tissue deposit. Harris & Innes [1930] added increasing amounts of Ca phosphate to a low Ca high P basal ration and found that ergosterol calcification increased with the Ca intake, but as phosphate was added as well as Ca these results are not in complete disagreement with those of Shelling [1930] and of Smith & Elvove [1929]. It seems likely that greater calcification occurs in prolonged, moderately severe overdosage, although deposits appear very quickly when large and rapidly fatal doses are given.

Localized histological deposits of Ca may not involve a large increase of Ca in the whole organ, but few analyses of tissues after dosing with ergosterol have been made. Reed *et al.* [1933] report a mean increase from a normal of 14 mg. to 31 mg. in the brains and from 4.5 to 30 mg. in the livers of dogs which were treated with large doses of ergosterol for periods varying from 8 to 73 days; the increases in the aorta, brain, muscle and spleen were statistically the most significant, but the largest increase was in the kidney. Smith & Elvove [1929] found 5 mg. in the livers of normal rabbits and 5–14 mg. after repeated excessive doses of ergosterol; the largest increases were found in the aorta, kidney and lungs. Hetenyi [1935] found 26.4 mg. in the brains and 22.4 mg. in the livers of normal dogs, and 27 and 31 mg. respectively after ergosterol; these increases he did not accept as significant. There were gross increases in the lungs, kidneys and muscles.

In the present series ergosterol was given in such doses that it appeared that all the animals would have died within a week, and many actually died before the end of the experiment. These were not included in the data because the organs would have contained much more blood than those of the rats killed by bleeding. The brains showed a definite increase in Ca which appears to have been directly due to the ergosterol and not to reduction of food consumed or to change in the water content of the tissue. The livers showed a definite increase but part of this was the result of reduced food consumption since normal rats with their diet restricted to that taken by the ergosterol-poisoned animals showed a significant increase in their liver Ca. However, after allowing for this there still remained a significant increase directly due to the ergosterol.

Although the absolute increase of Ca was not large, being 1.71 mg. in the brains and 1.69 mg. in the livers, the percentage increases, 36 for the brains and 51 for the livers, were quite considerable. They are, however, much smaller than those reported for prolonged experiments. Reed *et al.* [1933] found an increase of 122 % in the brain and 570 % in the liver after prolonged treatment and Smith & Elvove [1929] found increases up to 200 % in the liver after repeated excessive doses. Hetenyi [1935], the severity of whose treatment must have been comparable with that in the present experiments, found increases of 2 % in the brain and 38 % in the liver; these results are similar in magnitude to those reported here.

SUMMARY

1. The mean concentration of Ca in the brains of normal white rats was found to be 4.7 mg./100 g. of fresh tissue. In fresh liver it was 3.3 mg.
2. The brain Ca was decreased and the liver Ca unchanged in rickets produced by a high Ca low P diet.
3. The brain Ca was increased in acute ergosterol poisoning. The liver Ca was also increased, but part of the increase was the result of diminished consumption of food. The changes were smaller than those reported in less acute experiments.

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