LIX. A METHOD FOR ESTIMATING THE RETENTION OF CALCIUM AND PHOSPHORUS IN YOUNG GROWING RATS.

BY MARGARET AVERIL BOAS

(Grocers' Company's Research Scholar).

From the Department of Experimental Pathology, Lister Institute.

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THE measure of calcium and phosphorus retention week by week during the period of active growth and the effect produced upon it by varying different factors is essential in the study of rickets and other disorders of calcium phosphate metabolism.

A diminished calcium retention will be demonstrable for some time before the summation of its effect will be sufficient to produce a lowered calcium content of the bones, outside the normal variation, and a determination of the calcium and phosphorus retention over a considerable period is therefore a more delicate and convincing test for abnormal calcium metabolism than any other available. A certain amount of work of this kind has been done on children by Birk [1910], Dibbelt [1910], Orgler [1911], Schabad [1910] and more recently by Findlay, Paton and Sharpe [1921] and Holt, Courtney, and Fales [1920]. Schabad [1910] pointed out that before any clinical signs of rickets occur the approach of the malady is indicated by a low positive, or even a negative, calcium balance.

The results of all these workers have been summed up by Korenchevsky [1922]. A lowered calcium retention was observed in most cases of rickets but as the period of observation was usually short, the general course of calcium metabolism is not apparent. Calcium retention has also been studied by Hart, Steenbock, and Hoppert [1921] on goats, by Meigs, Blatherwick and Cary [1919] and Hart, Steenbock, Hoppert and Humphrey [1922] on milking cows, but as these observations were carried out on adult animals no information as regards the metabolism during growth can be obtained from them.

In view of the large extent to which the rat has been used in the experimental investigation of rickets, it was thought that a series of studies of calcium and phosphorus metabolism in this animal upon various diets and over a considerable period of its growth would be of value. McClendon [1922] has estimated the calcium and phosphorus retention of a number of rats on

normal and rickets-producing diets, but as a rule his observations were carried on for one week only. He found in normal animals of about 30 g., a daily retention of calcium varying from 10 to 20 mg. and of phosphorus from 6 to 10 mg., whereas in the animals on deficient diets, both balances were much reduced. In one experiment on normal animals he observed the calcium and phosphorus balances over a period of one week when the animals were 28 days old and also during a period of three days when they were 57 days old. During the second period he found a daily retention which was double that found in the earlier period. At the same time it must be noted that the rate of growth was also much increased.

To arrive at a trustworthy balance sheet showing the weekly income and expenditure of calcium and phosphorus necessarily involves much labour and careful manipulation. The method described below has been evolved after a considerable amount of preliminary work. It has been repeatedly tested on rats receiving both normal and deficient diets and has proved satisfactory.

EXPERIMENTAL METHOD.

The diet used had the following composition:

Egg-albumin	41 g.	"Hardened" cotton-seed oil	5 g.
Wheaten starch	113 g.	Salt mixture	3.5 g.
Cod-liver oil	12 g.	Ferric citrate	0.14 g.
Marmite	12 g.	Distilled water	93 cc.
Orange juice	12 g.		

The salt mixture had the following composition:

Sodium chloride	13 g.
Sodium bicarbonate	53 g.
Magnesium carbonate	14 g.

The egg-white was soaked in the water over night. The starch and salts were mixed together and to them the cod-liver oil and marmite were added. The egg-white was cooked and stirred into the mixture; the hardened cotton-seed oil was melted and added, and last of all the orange juice was poured in. The whole was then mixed with extreme thoroughness. The diet was made once a week and kept in a refrigerator room.

Analyses of the freshly made diet gave a Ca content of 0.0261 % and a P content of 0.1402 %.

The experiments were usually started as soon as the rats were weaned, *i.e.* when they were between three and four weeks old and weighed 40 to 50 g. At first four rats of the same sex and litter were kept in one cage but it was afterwards decided to limit the number to two. Each experiment lasted five or six weeks and was divided into periods of one week each. The first week was used as a preliminary period during which no analyses were made, to enable the rats to become acclimatised to the basal diet and to the metabolism cages.

The cages used were those designed by Ackroyd and Hopkins [1916] but a few modifications were introduced, the most important being a detachable wire floor, which greatly facilitated the cleaning of the cage. It was found that the glass separators used by Ackroyd and Hopkins did not ensure quantitative separation of urine and faeces but gave a rough separation which greatly assisted the subsequent analysis. When more than one rat was kept in one cage, it was found impossible to prevent food being brought back into the cage, and so the inner tunnels for the side arms were not used, as they served no useful purpose and only rendered the work of cleaning more complicated. Food was given in glass dishes in one or both of the side arms, and distilled water was placed in a bulb fixed in the roof of the cage.

Determination of Intake. Sufficient diet to last for six days was weighed out to the nearest g. in a wide-mouthed stoppered bottle. To this was then added sufficient calcium carbonate and potassium phosphate (K_2HPO_4) to give 0.7 to 0.9 % Ca and 0.6 to 0.8 % P in the final mixture. These were well mixed in, so that a diet of uniform constitution was obtained. The amounts of salts added were accurately weighed to the third place of decimals. The $CaCO_3$ was found on analysis to contain 40.0 % Ca. The K_2HPO_4 was heated at 110° for three hours before weighing; it contained 17.64 % P. Both carbonate and phosphate were kept in bottles with paraffined stoppers. On the seventh day of each week the basal diet only was fed to the rats, without addition of Ca and P salts. This was done in order to prevent as far as possible Ca given in one week being excreted during the subsequent one. Thus the total weight of Ca and P given in each week to each cage could be calculated from the known weights of fresh diet and of added $CaCO_3$ and K_2HPO_4 . From this amount was subtracted the total amount of Ca and P recovered from the cage in the course of the week (*i.e.* in excreta and any uneaten food). The remainder was therefore the amount retained in the bodies of the rats. It was most important that the method of recovery should be as efficient as possible, since all error would fall upon this difference, that is upon the retention which was being determined.

Estimation of Output. Although each metabolism period consisted of seven days, collection of excreta, etc. was made every second day. While this was being done the cage was removed from the funnel and placed over a large porcelain photographic dish, which could be afterwards rinsed into a large evaporating dish used to collect the urine. The solid matter consisting of faeces and uneaten food was put into a porcelain crucible. After each collection the crucible was put into a drying oven, and the contents when dry were partly ashed before the next collection of excreta.

In later experiments the amount of uneaten food was approximately determined. It was removed from the food-pots, any particles that had fallen into the faeces being separated by hand, and dried in a small porcelain crucible to constant weight, before adding it to the rest of the material to be ashed. When the seven day period was up, the weekly cleaning of the cage

took place. After the solid matter and urine had been removed as usual, the cage and glass parts were cleaned with scrupulous care with distilled water, a glass rod with a rubber tip and pieces of ash-free filter paper being used to ensure the removal of all adherent particles. These washings together with those from the diet bottle and the porcelain dish (on which the rats had been placed under a zinc cover during the cleaning) were added to the urine in the evaporating dish and the whole dried over a water-bath. When they were reduced nearly to dryness the contents of the evaporating dish were added to the partly ashed solids in the crucible and the whole dried in the oven at 105°–110°. As the dry material often amounted to as much as 100 g. in weight the ashing of it presented a problem of considerable difficulty. The large size needed put platinum crucibles out of the question, nickel was found to be attacked by some of the constituents of the ash, and silica was also unsatisfactory owing to the formation of calcium silicate. Porcelain crucibles were selected and proved satisfactory. They were used in the special furnaces designed by Prof. Martin [1924]. When the crucible was first placed in the furnace the silica and poilite plates were removed and only a very small flame was used. The material was allowed to ash slowly in this manner until the smoky stage was finished. Then the plates were replaced, the flame turned up gradually and a slow stream of air admitted. When a fairly white ash had been obtained, usually about three hours after the completion of the first stage, the crucible was allowed to cool slowly. When cool the ash was moistened with distilled water, strong HCl was added drop by drop until effervescence ceased and then 3–4 cc. in addition. The whole was allowed to stand for a few hours or over night, after which the supernatant fluid was pipetted off and filtered through ash-free filter paper into a calibrated litre flask. One more extraction with strong HCl was followed by repeated extractions with small quantities of distilled water. The well-washed filter paper and its contents were then replaced in the crucible, dried, re-ashed and extracted as before. This double ashing usually produced a completely soluble ash, but any insoluble matter still remaining was collected on a second filter paper, re-ashed and treated for a long time with strong HCl and water and then tested for the presence of calcium. Not more than the faintest trace was ever found.

The solution in the flask was made up to 1 litre and aliquot portions taken for calcium analysis, the quantities being measured in a calibrated flask. The method of analysis adopted was based on those of McCrudden [1909, 1911] and Shohl [1922]. The final determination was, however, gravimetric. It was as follows. To the measured volume of solution which was strongly acid with HCl, 10 cc. of 10 % oxalic acid solution and three drops of methyl red were added, and afterwards strong ammonia drop by drop until the solution was alkaline; the solution was then made definitely acid again with 2 *N* HCl and heated on a water-bath until the precipitate was granular. When cool, 20 % sodium acetate solution was added until a p_H of 5.0 was reached. (The danger limits are 4.0 and 5.6.) After standing over night the

precipitate was filtered through ash-free filter paper and washed with 0.5 % ammonium oxalate solution. The paper and contents were then transferred to a weighed platinum crucible and converted to a white ash. When quite cool about 0.3 cc. of strong H_2SO_4 was added very cautiously drop by drop down the side of the crucible, the lid being kept as far as possible over the top. The crucible was then placed in a slanting position on a pipeclay triangle and the edge of the lid heated with a small flame, till all free H_2SO_4 had been driven off; it was then placed upright and heated to a dull red heat; it was afterwards cooled, three drops of strong H_2SO_4 were added and a cautious heat was applied as before, except that this time the crucible was afterwards heated to a cherry red heat, for 10 mins. It was then cooled in a desiccator and weighed, heated again for 10–15 mins., cooled and again weighed. It was found that from 0.2 to 0.4 g. of calcium sulphate, which was the amount obtained from about 50 cc. of the solution, was the most satisfactory amount to deal with. Analyses were always made in duplicate, and estimations were repeated, if the results obtained differed by more than 1 mg. from one another. This gave an error of less than 0.5 %. In the majority of cases the difference was less than 0.5 mg.

For the phosphorus estimations the original solution was diluted 10 times and the 50 cc. taken for analysis were placed in a Kjeldahl distilling flask, 10 cc. of strong H_2SO_4 added, and boiled till fumes of H_2SO_4 appeared. The estimation was then carried on by Neumann's method [1902]. All results which did not fall within 0.1 cc. of one another in the titration against $N/2$ NaOH were repeated.

EXPERIMENTS ON NORMAL RATS.

Three experiments were carried out on normal rats to which the above basal diet was fed. At the time these experiments were performed, the poor biological quality of egg-white as a protein was not suspected [see Boas, 1924] and the loss of hair and accompanying decline in weight were attributed to an infection. However, although in two cases considerable loss of weight occurred during the last week of the experiment, it will be seen that this had little or no effect on the growth and calcification of the skeleton.

Exp. I. Four female rats of the same litter were put in a metabolism cage at the age of 27 days and kept there for a preliminary period of five days, followed by three periods of a week each, during which the retention of Ca and P was estimated. During the last week loss of hair, blepharitis and decline in weight appeared and the experiment was terminated abruptly as it was thought that the animals were infected with mange.

Exp. II. Four female rats all belonging to the same litter were observed during a preliminary period of one week followed by five consecutive periods of one week each, during which estimations of Ca and P retention were made. At the end of the first period the four animals were divided between two cages and the experiment continued thus. Slight signs of coat defect appeared

during the last week and the weight curve flattened but no loss of weight occurred. The amount of P fed to the rats as K_2HPO_4 was doubled during the fifth week.

Exp. III. Two female rats of the same litter were kept in metabolism cages for a preliminary period of one week followed by four periods of one week each, during which the Ca and P metabolism was studied. Loss of hair and loss of weight again occurred during the last week. One rat was killed and the long bones, kindly analysed by Dr Goldblatt, gave a Ca content equal to 11.22 % of the wet weight and 20.80 % of the dry weight; the average for normal rats of the same age in the investigations of Korenchevsky was 11.50 % wet weight, and 20.50 % dry weight. The other rat was put on a diet of bread and milk, cabbage and carrot, and in a short time completely recovered. In this experiment also the amount of K_2HPO_4 given was doubled during the last week.

The results of these experiments are shown in the accompanying table. As the amounts of uneaten food were not estimated, the actual amounts of Ca and P ingested cannot be given. From the amounts fed a rough estimate can be made. In the first week of each experiment it was of the order of 0.6 g. Ca and 0.4 g. of P per rat per week, and it rose by about 0.1 g. Ca and 0.08 g. P every succeeding week except where in Exps. II and III a larger rise has been already stated.

Retention of Ca and P in young female rats aged four weeks at the beginning of the first week. In Exp. I four rats were in one cage; in Exp. II four rats were in one cage during the first week and after that in two cages; in Exp. III only two rats were observed.

Exp. No.	Week of Exp.	Initial	Final	Ca retained per rat (average) g.			P retained per rat (average) g.		Ca/P retained
		body wt (mean of 4 animals) g.	body wt (mean of 4 animals) g.						
I	1	41	54			0.1519		0.1018	1.72
	2	54	73			0.1227		0.0575	
	3	73	65			0.1254		0.0620	2.02
	Totals				0.4000			0.2213	1.77
II				Cage A	Cage B		Cage A	Cage B	
	1	56	77			0.2182		0.1043	1.91
	2	77	100	Lost	0.2409	0.2409	Lost	0.1361	
	3	100	115	0.2119	0.1914	0.2017	0.1452	0.1184	1.55
	4	115	127	0.1439	0.1662	0.1551	0.1153	0.0828	
	5	127	135	0.1305	0.1429	0.1367	0.0803	0.0181	2.71
	Totals				0.9526			0.5205	1.83
III		Mean of 2 animals	Mean of 2 animals						
	1	45	70			0.2126		0.1754	1.24
	2	70	87			0.2000		0.1578	
	3	87	98			0.1643		0.0964	1.18
	4	98	94			0.0895		0.1184	
	Totals				0.6664			0.5480	1.22

The retention of Ca and P decreases week by week, and though by a mere consideration of the figures this decrease seems to be somewhat irregular this

Fig. 1 (Exp. I). Curves of average increase in body weight and of average Ca and P retention per rat of the four female rats in Exp. I, plotted against the time in days.

Fig. 2 (Exp. II). Curves of average increase in body weight and of average Ca and P retention per rat of the four female rats in Exp. II, plotted against the time in days.

Fig. 3 (Exp. III). Curves of average increase in body weight and of average Ca and P retention per rat of the two female rats in Exp. III plotted against the time in days.

is due to the arbitrary periods into which the observation was divided. If x is taken as the unknown amount of Ca or P in the body of the rat at the beginning of the experiment and the successive increments are plotted against the time, a curve showing the weekly additions of Ca or P to the body is obtained. Figs. 1, 2 and 3 show the curves given by the above experiments together with the corresponding growth curves, and it will be seen that both Ca and P give smooth curves. The decline in weight at the end of the experiments, shown to be due to the inadequacy of egg-white as a source of nitrogen, does not seem to have affected the Ca retention.

The Ca : P ratio of the retention during successive periods in these experiments, which is given in the last column of the table, shows some variation. In the first two experiments it is higher during the last period than during the first, and in the third experiment it is approximately constant. This rise was shown in a more marked degree in the experiments described in the following paper [Boas and Chick, 1924] where the significance of it is fully discussed so that it need only be mentioned here.

SUMMARY.

A method for estimating the retention of Ca and P in young growing rats for periods of three to seven weeks is described.

Rats on normal diet showed a retention of Ca and P which declined week by week, and curves plotted from the figures obtained gave smooth curves similar in shape to the curve of normal growth.

In conclusion I wish to express my gratitude to Prof. C. J. Martin for suggesting this line of research to me, and for his invaluable help and advice throughout.

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