LXXII. THE ABSORPTION AND EXCRETION OF CALCIUM AND PHOSPHORUS BY RATS RECEIVING EXCESSIVE DOSES OF IRRADIATED ERGOSTEROL.

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It has been shown by Kreitmair and Moll [1928], Harris and Moore [1928, 1929], and Kreitmair and Hintzelmann [1928], and has since been confirmed by many other workers, that the administration of excessive doses of irradiated ergosterol to animals results in the production of calcium deposits, notably in the kidney and aorta, and in a tendency to hypercalcaemia and hyperphosphataemia. There have been two theories put forward to account for the source of this extra calcium in the blood and in certain tissues. One, first suggested by Harris and Moore, postulates an increased absorption from the gut together with an increased retention. Smith and Elvove [1929] suggested diminished excretion of calcium, though they did not indicate whether this decreased elimination is by way of the gut or the kidney, or both. The second theory is that the source of hypercalcaemia and depositions is the calcium of the bone substance. This has been suggested by Hess, Weinstock and Rivkin [1929–30], Baumgartner, King and Page [1929], Light, Miller and Frey [1929] and others.

Hoyle and Buckland [1929] made a study of the urinary phosphorus in hypervitaminosis D, but did not estimate the calcium. Light, Miller and Frey [1929] have given figures for calcium and phosphorus balances, but owing to the fact that no figures for food intake, urine or faeces were published it is not possible to judge what processes were at work to produce the results obtained by them. An investigation of the urinary calcium in some detail (as was done by Hoyle and Buckland for the phosphorus) followed by total balance experiments was therefore undertaken, and is described below. Just as the work was completed a paper by Brown and Shohl [1930] was published, in which they showed a definitely decreased retention of calcium after large doses of irradiated ergosterol, and a shift in the excreted calcium from the faeces to the urine. It was felt that independent confirmation of their results was probably worth publication, especially as some new points have emerged.

I. Urinary excretion in hypervitaminosis D.

Methods.

Calcium. Shohl and Pedley's [1922] method was used, with slight modifications. As rats' urine seems to have given trouble to other workers (see Hoyle and Buckland for a discussion of the difficulties) the final routine adopted is described in full. Urine and washings from the receiving flask, approximating to 100 cc., is treated with 10 cc. concentrated sulphuric acid and 4 g. ammonium persulphate, as described by Shohl and Pedley. As the urine is heated a large bulky precipitate separates. This precipitate also occurs, though to a lesser degree, in human urine, whereas there is no trace of it in rabbit urine. By continuing the heating for 2 or 3 hours (instead of 1 hour as recommended by Shohl and Pedley), adding more ammonium persulphate from time to time whenever the urine begins to darken in colour, the precipitate becomes much reduced in amount, but does not entirely disappear. If the calcium is precipitated in its presence the final titration with permanganate gives results which are much too high. It must, therefore, be filtered off and washed twice with small quantities of hot hydrochloric acid, the washings being added to the treated urine. This washed precipitate when dissolved in a small quantity of alkali gives no reactions when tested for urates, calcium or inorganic phosphate. The precipitation of calcium can now be carried out as described in the original method. If small amounts of calcium are present, as in normal rats' urine, it is advisable to allow 24 hours for complete precipitation.

Phosphorus. The filtrate and washings from the calcium precipitate were made up to 250 cc. and used for the colorimetric estimation of phosphate, after further suitable dilution. The amount of oxalic acid present in the final dilution is too small to interfere with the colour development. The Bell-Doisy reagents [1920] were used instead of those of Briggs.

Experimental.

Eight rats, four males and four females, were kept in Hopkins's metabolism cages, two rats in each cage. The urine from each cage was kept and analysed separately, three or four days' output being mixed together. The weights of the animals were between 100 and 168 g. The diet was identical with that used by Harris and Moore [1928]. Full synthetic diet was given for 14 days before administration of the irradiated ergosterol, which was then fed at 0·1 % level. Urine was collected for 1 week only during the preliminary period. It was found essential that the animals should be kept in the metabolism cages for a few days before collecting urine, as, until they became accustomed to the change of cage, metabolism was apt to be appreciably disturbed. After 20 days of the irradiated ergosterol administration the animals were put back on to normal synthetic diet for the "curative" period. During the course of the experiment the females showed greater loss of weight than the males; two of them died at the beginning of the "curative" period. Post-mortem examination revealed the usual lesions associated with hypervitaminosis D in an advanced form. Four of the rats had some diarrhoea during the intake of irradiated ergosterol; consequently one or two samples of urine had to be discarded as they were badly contaminated with faeces.

The results of this experiment are given in Table I.

Table I. *Urinary excretion of calcium and phosphorus (mg. per rat per day).*

	Group I					Group II			
	Females		Males			Females		Males	
No. of days	Ca	P	Ca	P	No. of days	Ca	P	Ca	P
				Preliminary period.					
4	1·3	25·7	0·8	26·5	4	0·1	21·8	0·1	26·7
4	0·3	23·9	0·7	24·3	3	0·1	30·0	0·1	28·9
				Irradiated ergosterol period.					
3	5·9	25·4	5·9	27·3	4	4·5	17·2	5·3	18·5
3	5·3	17·9	7·9	24·6	4	8·3	24·5	8·9	22·6
4	2·3	15·1	3·2	10·5	4	9·9	23·5	10·1	23·8
					4	8·8	23·7	8·9	28·3
					4	8·1	23·5	8·4	25·9
				"Curative" period.					
4	—	—	2·3	17·8	3	7·3	22·4	7·0	19·1
3	—	—	1·2	30·9	3	4·3	20·4	3·8	24·2
3	—	—	1·4	28·2	2	2·2	16·8	2·0	21·3

Discussion.

These two groups of rats agreed in showing the great increase in urinary calcium as a result of the massive doses of irradiated ergosterol; this was more marked in the second group. This group did not show the sudden decrease in the calcium excretion towards the end of the irradiated ergosterol intake. The calcium in the urine of both males and females of the second group showed a rise to a maximum occurring at about the tenth day, and then followed a slow and steady decline. The concentration of calcium rose steadily throughout the period of irradiated ergosterol administration in the case of the females, whereas in the case of the males the concentration was roughly parallel to the total daily amount of calcium excreted. There was a sharp drop in the calcium concentration about 3 days after the initiation of the "curative" period (Figs. 1 and 2). The diuresis found by Hoyle and Buckland [1929] was not shown by any of these rats. The worse physical condition of the animals in the first group may explain the drop in the urinary calcium towards the end of the irradiated ergosterol period; kidney function was probably failing rapidly, as the phosphate showed the same decline and the volume of urine passed was very small. There was no increase in phosphorus excretion produced by the hypervitaminosis. This is in agreement with Hoyle and Buckland.

It is thus evident that decreased excretion of calcium by the kidney is not responsible for the hypercalcaemia or for the calcification of tissues.

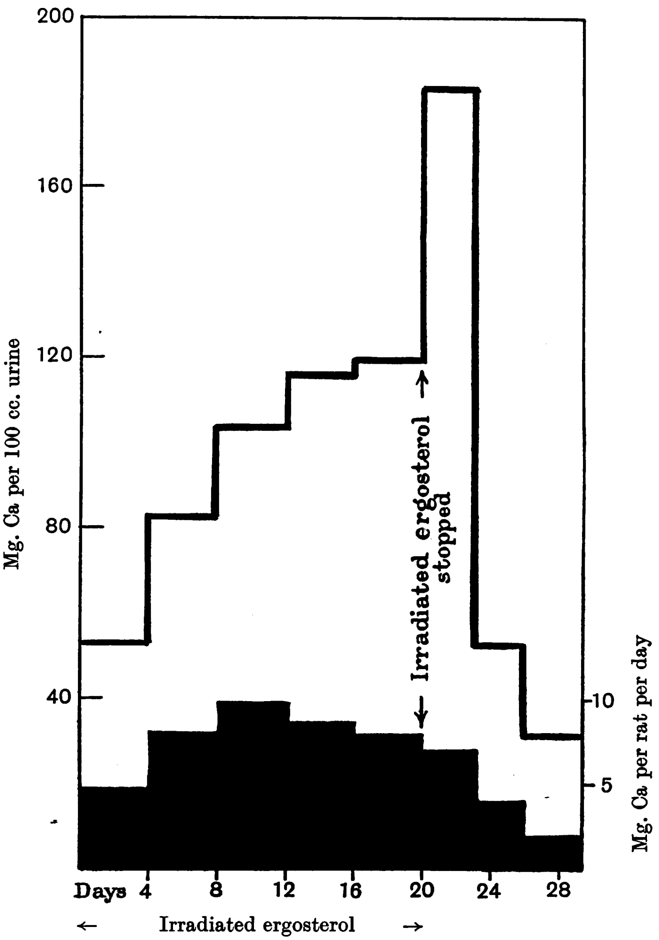

Fig. 1. ♀ Daily excretion of Ca.

II. Total balance experiments.

Methods.

The technique for the urine analyses was as described above. Calcium in the faeces was determined by a slight modification of McCrudden's method [1910] rendered necessary by the fact that there was sometimes insufficient faeces to admit of a separate phosphorus estimation. The following technique was therefore adopted in all cases to ensure uniformity. After ashing, the ash was dissolved in 5 cc. concentrated sulphuric acid, and heated with about 10 drops of perhydrol[1]. This gave a clear solution, and converted any pyrophosphate present as the result of the ashing process into orthophosphate,

[1] This method of treating the ash was suggested to me by Mr E. G. Holmes, to whom my thanks are due.

which could be estimated colorimetrically. The dissolved ash in the sulphuric acid was diluted to 100 cc. with distilled water, and the calcium estimated as described by McCrudden. The filtrate and washings from the calcium precipitate were made up to 250 cc. and the phosphorus determined as in the case of urine.

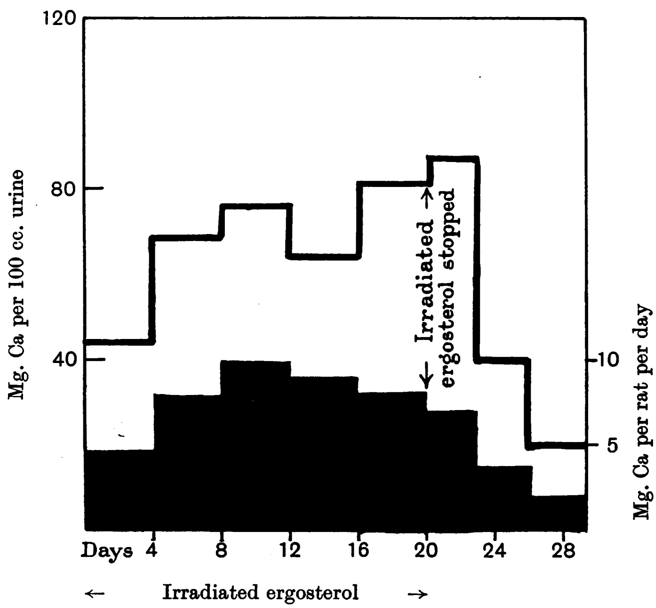

Fig. 2. ♂ Daily excretion of Ca.

Experimental.

Six albino male rats of approximately the same age were used. Each animal was kept in a separate metabolism cage, and faeces and urine were collected separately in 3 or 4 day lots. The daily amount of food fed was weighed, the dry weight of an equivalent amount determined, and any uneaten residues collected, dried and weighed. In this way the exact quantity eaten could be obtained by subtraction. Distilled water was given for drinking. The experiment as before was divided into three parts, (1) preliminary period, (2) irradiated ergosterol period and (3) "curative" period. The irradiated ergosterol was given at 0·05 % level, this avoids the complication of soft faeces or diarrhoea, yet is sufficient to cause loss of weight and pathological lesions. The weights of the animals (g.) are given below.

No. of rat	F5	F6	F7	F8	F21	F22
Beginning of irradiated ergosterol intake	135	139	159	155	160	157
End of irradiated ergosterol intake	116	123	141	142	144	143
End of "curative" period	144	150	167	164	155	160

Table II.

No. of rat	Ca intake Periods I	II	III	Faecal Ca Periods I	II	III	Urinary Ca Periods I	II	III	Total Ca excretion Periods I	II	III	Ca retention Periods I	II	III
F5	24·2	18·0	30·1	7·2	6·9	19·8	1·5	6·6	4·9	8·7	13·5	24·7	15·5	4·5	5·4
F6	29·4	25·9	38·4	3·8	1·4	6·2	1·6	9·4	5·8	5·4	10·8	12·0	24·0	15·1	26·4
F7	33·4	21·9	36·4	9·7	6·5	21·2	2·3	7·4	3·3	12·0	13·9	24·5	21·4	8·0	11·9
F8	26·8	26·7	34·6	8·1	6·8	11·4	1·3	5·9	4·9	9·4	12·7	16·3	17·4	14·0	18·3
F21	28·6	20·2	35·2	16·7	10·1	23·9	0·1	5·3	3·6	16·8	15·4	27·5	11·8	4·8	7·7
F22	28·1	18·9	32·1	16·9	5·9	19·9	0·2	6·4	4·4	17·1	12·3	24·3	11·0	6·6	7·8

Figures represent mg. per rat per day.
Period I. Preliminary.
„ II. Irradiated ergosterol intake.
„ III. "Curative" period.

Table III.

No. of rat	P intake Periods I	II	III	Faecal P Periods I	II	III	Urinary P Periods I	II	III	Total P excretion Periods I	II	III	P retention Periods I	II	III
F5	49·3	36·6	64·7	7·2	7·1	16·2	39·8	31·0	36·6	47·0	38·1	52·8	2·3	−1·4	11·9
F6	59·6	52·6	78·0	5·6	3·8	4·8	36·0	36·2	39·7	41·6	40·0	44·5	18·0	12·6	33·5
F7	67·9	44·7	74·0	10·4	8·5	18·4	40·4	35·2	36·4	50·8	43·7	54·8	17·1	1·0	19·2
F8	54·4	54·2	70·1	9·4	8·9	16·0	29·2	37·4	40·7	38·6	46·3	56·7	15·8	7·9	13·4
F21	58·1	41·0	71·2	11·0	10·1	23·6	28·6	38·2	39·7	39·6	48·3	63·3	18·5	−7·3	7·9
F22	57·0	37·7	65·4	12·4	5·2	19·2	29·2	28·1	36·9	41·6	33·3	56·1	15·4	4·4	9·3

Figures represent mg. per rat per day.
Period I. Preliminary.
„ II. Irradiated ergosterol intake.
„ III. "Curative" period.

The intake, output, and retention of calcium and phosphorus are given in Tables II and III. The following points are shown by an examination of the figures. (1) The urinary excretion was much the same as in the case of the rats receiving the higher dose of irradiated ergosterol. (2) The irradiated ergosterol period was characterised by a decrease (sometimes very marked) of the faecal calcium. Only two cases (F6, F22) showed a marked change in the phosphorus. (3) The urinary calcium did not return to normal in the "curative" period during the time of the experiment (9 days generally). Evidently the recovery from the effects of the hypervitaminosis is only gradual, as one would expect. (4) The faecal calcium in the recovery period tended to be very high—much higher than it was in the normal preliminary period, so that the total excretion of calcium was the highest of all in the "curative" period. The calcium intake was also highest in this period, yet even so, in two cases (F5, F7) the extra intake compared with that of the preliminary period was 6 and 3 mg. per day, respectively, while the extra output in the faeces was 12 and 11 mg. per day respectively. (5) The faecal phosphorus was definitely increased in the "curative" period in all cases except one (F6). (6) The balance of calcium was never negative when averaged for the whole period of irradiated ergosterol administration (see however later remarks on this point). In every case the balance was materially reduced by the hypervitaminosis, and in three cases was not restored to normal during the observed period of recovery. (7) The phosphorus balance was also considerably reduced by the irradiated ergosterol.

It was not felt that the average balances for the whole of the irradiated ergosterol and "curative" periods respectively gave an adequate picture of what was taking place. These balances have therefore been further analysed in Tables IV and V. Only two 3-day collections of excreta were made from rats F5 and F7, as they were in such bad condition at the end of this short time that it was not advisable to push the experiment further. Rats F6 and F8 were observed for only 6 days instead of 9 in the recovery period. In these tables also are given the so-called "percentages of absorption" of the two elements. These have been calculated tentatively as follows: the faecal excretion was subtracted from the intake and the remainder counted as the amount absorbed, and expressed as a percentage of the intake. This is not strictly accurate, because the faecal calcium consists both of calcium which has never been absorbed and of some which has been absorbed and passed back into the gut. There is no way at present of distinguishing these two fractions, so that the above method of calculating the amount absorbed is the only one possible in the circumstances.

Table IV.

	Retention of calcium (mg. Ca per rat per day)							Calcium absorption (%)						
No. of rat	I	II			III			I	II			III		
F5	15·5	10·5	−3·5	—	−1·7	13·7	1·6	70	70	34	—	33	55	7
F6	23·4	21·7	12·1	13·8	27·6	24·6	—	87	95	94	95	95	72	—
F7	21·3	17·3	−3·2	—	3·7	17·8	12·3	71	86	30	—	36	49	36
F8	17·4	18·0	11·6	9·2	22·7	12·3	—	70	80	71	68	81	45	—
F21	11·8	−8·2	11·1	5·8	−0·5	18·1	5·7	42	9	81	70	25	49	20
F22	6·5	10·9	8·4	0·6	13·1	8·2	2·4	40	66	83	57	70	50	12

Period I. Preliminary.
„ II. Irradiated ergosterol.
„ III. Recovery.

Table V.

	Retention of phosphorus (mg. P per rat per day)							Phosphorus absorption (%)						
No. of rat	I	II			III			I	II			III		
F5	2·3	6·3	−11·9	—	11·9	14·3	10·4	85	85	62	—	81	80	65
F6	18·0	14·6	7·9	14·7	29·4	38·9	—	91	94	92	93	96	92	—
F7	17·1	10·8	−11·9	—	13·8	23·1	19·5	85	91	60	—	78	75	73
F8	15·8	12·9	1·3	7·8	15·0	11·3	—	83	87	80	81	80	73	—
F21	18·5	−6·7	14·5	2·9	11·0	12·0	2·4	81	56	90	85	66	77	57
F22	15·4	10·1	8·6	−5·2	19·6	5·8	2·4	78	87	90	80	85	66	60

Period I. Preliminary.
„ II. Irradiated ergosterol.
„ III. Recovery.

It will be seen that the balance of calcium during the period of irradiated ergosterol intake may fall quickly, as in rats F5 and F7, or more steadily as in F8, or very abruptly after several days on the diet as in the case of F22. The rapidity and extent of the fall ran closely parallel to the physical condition of the animal. Two rats developed negative balances while taking the irradiated ergosterol. In most cases the amount of calcium retained was rapidly increased as soon as the excess vitamin was removed from the diet, but it is surprising to find that this improved retention was not maintained, and in five of the rats the positive balance was actually lower than normal after a few days on the normal diet. The phosphorus retention in general tended to follow similar lines.

The percentage of calcium apparently absorbed in the preliminary period was in most cases already so high that no very marked results from the irradiated ergosterol could be expected. There is however some evidence of increased absorption in all cases but one (F 5), and it is interesting to note that with rats F 21 and F 22, where the initial absorption was lower, there is very definite evidence of the action of irradiated ergosterol in this respect. But the increased absorption was not maintained, and in every instance in the recovery period there was ultimately a definitely reduced percentage of absorption. A similar mechanism occurs in the case of the phosphorus, especially during the "curative" period.

It has to be remembered that these rats were losing weight. In order to get some idea of what effect, if any, loss of weight *per se* has upon calcium and phosphorus metabolism two male rats were given full synthetic diet in gradually decreasing amounts. The loss of weight experienced was 26 and 23 g. respectively, but the animals remained in good condition. The calcium excretion by the kidney ceased entirely, but in spite of this the retention declined rapidly (Tables VI and VII). The urinary phosphorus also became reduced in amount, roughly 50 %, and the amount retained decreased, rat F 24 actually having a negative balance for the last three days. The absorption of both calcium and phosphorus was apparently much reduced by the low food intake, but again it must be emphasised that this may not be real, but may only mean that the path of excretion has been diverted from kidney to gut.

Table VI. *Calcium balance on low food intake.*

Mg. Ca per rat per day.

No. of rat	Intake	Faeces	Urine	Total	Retention	% absorption
F 23	47·6	5·3	0·9	6·2	41·4	87
	26·5	9·2	0·9	10·1	16·4	65
	17·3	6·8	Nil	6·8	10·5	60
F 24	50·8	14·2	1·1	15·3	35·5	72
	26·5	11·5	0·9	12·4	14·1	57
	17·3	9·9	Nil	9·9	7·4	43

Table VII. *Phosphorus balance on low food intake.*

Mg. P per rat per day.

No. of rat	Intake	Faeces	Urine	Total	Retention	% absorption
F 23	96·7	6·7	57·5	64·2	32·5	93
	53·9	8·8	38·2	47·0	6·9	84
	35·2	5·2	26·7	31·9	3·3	85
F 24	103·3	4·8	56·4	61·2	42·1	95
	53·9	10·3	41·7	52·0	1·9	81
	35·2	8·0	29·3	37·3	−2·1	77

Discussion.

Brown and Shohl [1930] have adequately stated the difficulties and dangers attendant upon any attempt to determine from urinary and faecal examinations whether the absorption has been altered or not. The figures given above must be accepted with reserve. It would appear from them that large doses of irradiated ergosterol tend at first to increase absorption of calcium and later to decrease it. We are on surer ground when dealing with retention, and here there is no doubt that excessive doses of irradiated ergosterol, combined with the resultant low food intake and loss of weight, reduce this amount very gravely. Recovery from the deleterious effects is evidently slow, for nine days after restoration to normal diet the balances were still gravely disturbed. During this period the calcium deposits are disappearing from the body, and the fact that the urinary excretion is declining, whereas the faecal elimination is unusually high, points to the probability that much of this deposited calcium is being eliminated by the gut rather than by the kidney. Whatever the effect of irradiated ergosterol may be on absorption it is clear that the hypercalcaemia and calcification of various tissues is not due entirely, if at all, to increased absorption, since the amounts retained in the body are less than is normally the case. The source of this calcium must be looked for elsewhere, and unlikely as it appears *a priori* that the action of large doses of a substance which normally aids calcification of the bones should be in the reverse direction, yet it is difficult to see where the deposited calcium comes from if not from the bones.

The phosphorus retention was greatly reduced as the result of the irradiated ergosterol, if anything more so than was that of the calcium. Yet regarded as a whole there was less disturbance of phosphorus metabolism, for the urinary phosphorus was not increased, or at most very slightly, and there was in general a quicker settling down to normal in the "curative" period. The reduced balance during the irradiated ergosterol period must not be attributed entirely to the effect of the hypervitaminosis, for loss of weight due solely to reduced food intake produces equally severe reduction in retention.

Summary.

1. Calcium and phosphorus retention in rats is decreased by excessive doses of irradiated ergosterol.

2. The urinary calcium is greatly increased, but not the phosphorus.

3. The faecal content of both calcium and phosphorus decreases, but not always in proportion to the intake; either the absorption is diminished in these cases, or increased amounts of absorbed material are being excreted by the gut.

4. Even graver disturbances in retention and absorption occur during the recovery period.

5. The low food intake and loss of weight characteristic of the hypervitaminosis probably contribute to some of these results.

6. The deposits of calcium found in certain tissues as the result of large doses of irradiated ergosterol cannot be accounted for on the grounds of increased absorption and retention.

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