LXXV. THE PHOSPHORUS DISTRIBUTION IN BLOOD AND THE CALCIUM AND PHOSPHORUS EXCRETION DURING HYPERVITAMINOSIS D.

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It has been shown by several workers [Harris and Moore, 1928, 1, 2; 1929; Kreitmar and Moll, 1928; Pfannenstiel, 1927] that the feeding of doses of irradiated ergosterol far in excess of the therapeutic dose produces well-marked metabolic disturbance, a condition which has been called hypervitaminosis D. This condition is characterised by an extensive deposition of calcium salts in various organs of the body and is accompanied by an increased blood-inorganic phosphate [Harris and Stewart, 1929]. Hess and Lewis [1928] also observed signs of high blood-calcium and -inorganic phosphate in clinical cases of rickets, which were receiving 2·5–5 mg. of irradiated ergosterol per day. As phosphorus occurs combined in several different forms in blood, it was thought of interest to investigate whether the increase in inorganic phosphate, which in this condition can be maintained over a long period, produces any change in the blood content of acid-soluble organic phosphorus compounds. The work reported here was carried out to investigate this point.

In these experiments, the technique of Kay and Robison [1924] has been employed, and the following four types of phosphorus compounds have been studied.

(i) The inorganic phosphate.

(ii) The acid-soluble organic phosphorus compounds, almost all of which are present in the corpuscles. They are estimated by difference between total acid-soluble phosphorus and the inorganic phosphate.

(iii) That fraction of (ii) which is hydrolysed by Robison's bone-phosphatase, "the hydrolysable fraction."

(iv) That fraction of (ii) which is not hydrolysed by bone-phosphatase, "the non-hydrolysable fraction."

These four fractions, together with the "lecithin"-phosphorus, which has not been estimated, between them contain the whole of the phosphorus in blood.

¹ A short account of this work appeared in *Chem. Ind.* (1930), 49, 237.

EXPERIMENTAL.

Twelve Dutch rabbits of approximately the same size and age were used in this work. At the beginning of the experiment they were about 3 months old. At the end they were fully grown and adult. The data therefore are for both growing and adult rabbits. They were given a mixed diet of bran and roots. No attempt was made either to increase or to limit the intake of calcium or phosphorus as was done in the experiments of Hess, Weinstock and Rivkin [1928]. After a control period lasting a month, the rabbits were divided into four groups and the following additions made to their diets.

- A. 0.5 cc. arachis oil (2 rabbits);
- B. 0.5 cc. arachis oil, containing 10 mg. irradiated ergosterol (4 rabbits);
- C. 0.5 cc. arachis oil, containing 10 mg. ergosterol (2 rabbits);
- D. no addition (4 rabbits).

The irradiated ergosterol used was "B.D.H.—irradiated in alcohol." The oil was fed each day by means of a pipette. The rabbits were weighed at intervals, the onset of hypervitaminosis being shown by a cessation of growth followed in time by a loss of weight.

Both during the control period and after feeding had started small samples of blood were withdrawn at intervals from the ear. The experimental feeding always followed bleeding so as not to excite the animals. The blood samples were received into paraffined crucibles, containing a minimal amount of potassium oxalate. The volume of corpuscles was estimated by haematocrit, but as it varied very little from the average figure of 34 % no corrections have been made and all results are expressed as mg. per 100 cc. of whole blood. 2 cc. were measured by pipette and delivered into 5 cc. of 25 % trichloroacetic acid. After standing for $\frac{1}{4}$ – $\frac{1}{2}$ hour they were filtered into 25 cc. volumetric flasks, neutralised to phenolphthalein and made up to the mark. When not estimated immediately the filtrates were kept in the ice-chest. 5 cc. were taken for estimation of inorganic phosphate. Total acid-soluble phosphorus was estimated on 1 or 2 cc. by incineration with strong sulphuric acid and Merck's perhydrol. For the estimation of the hydrolysable fraction, 5 cc. were treated with 1 cc. of 5 % bone extract and adjusted to p_H 9. They were incubated for 24 hours (which was found to be the most suitable time) at 37° in the presence of a little chloroform. 1 cc. of *N* sulphuric acid was added to precipitate the proteins and, after standing overnight in the ice-chest, the precipitate was filtered off and the inorganic phosphate estimated, a correction being applied for the inorganic phosphate added in the bone extract. All estimations were carried out in duplicate by the method of Briggs [1924].

The results are shown in Fig. 1. The three types of control A, C and D, have been treated together as they behaved in the same manner. It will be seen that, although the inorganic phosphate was raised by nearly 100 % at one time when the rabbits were young, and at another by nearly 50 % when

the rabbits were adult, no significant change in the content of acid-soluble organic phosphorus compounds was detected. The hydrolysable and non-hydrolysable fractions are shown in Table I. It can be seen that there are no significant differences between the control and experimental periods.

Fig. 1. Inorganic and acid-soluble organic phosphorus of control and experimental animals.

Table I. *Average values during control and experimental periods. All values mg. phosphorus per 100 cc. whole blood.*

Rabbit No.	Total organic		Hydrolysable		Non-hydrolysable	
	Control period	Experimental period	Control period	Experimental period	Control period	Experimental period
3	33.48	31.42	6.04	4.84	27.06	26.58
4	29.44	29.80	5.80	6.15	23.64	23.65
6	32.15	32.05	5.76	5.41	26.39	26.64
7	30.82	31.90	5.80	5.67	25.02	26.13

Calcium.

It has already been mentioned that Hess and Lewis [1928] observed hypercalcaemia in clinical cases of rickets receiving 2.5–5 mg. of irradiated ergosterol per day. Hess, Weinstock and Rivkin [1928] also observed that, in adult animals with a low serum-calcium and a very low intake, the level was rapidly raised to normal by large doses of irradiated ergosterol. Harris and Stewart [1929] reported a very slight rise in the adult rabbit and a 25 % rise in the case of rats. It was therefore thought desirable to carry out some estimations of serum-calcium, and I am greatly indebted to Miss E. Watchorn for being so kind as to do these. The method used was that of Kramer and Tisdall [1921]. The results are shown in Table II. It will be seen that the experimental animals have, on the whole, a higher serum-calcium than the controls, although the control values are rather low for rabbits. It should be mentioned that the experimental animals were in a condition of hyper-

vitaminosis during the whole period and at that time were adult. Rabbit No. 7, when killed on March 11, had a serum-calcium of 21 mg. per 100 cc. and an inorganic phosphate of about 8 mg. The symptoms were very severe and the animal had practically stopped eating and had fallen in weight from 3 lbs. to 2 lbs. in 10 days. Extensive calcification was visible to the naked eye in the aorta and kidneys, which were enlarged. Rabbit No. 6, showed the same symptoms and had a serum-calcium of 18.8 mg. per 100 cc. on March 30. It would seem that the rise in serum-calcium is a somewhat slower process than the rise in inorganic phosphate.

Table II. *Serum-calcium; mg. per 100 cc.*

Rabbit No.	Experimental				Controls						
	Arachis oil + irradiated ergosterol				Arachis oil		Arachis oil + ergosterol		No addition		
	3	4	6	7	1	2	10	12	5	8	9
Feb. 12	—	—	13.9	15.2	—	12.9	12.7	—	12.6	—	—
" 19	14.4	13.1	—	—	—	—	—	10.1	—	10.1	10.8
Mar. 4	13.9	11.4	13.7	13.3	—	—	—	—	—	—	—
" 11	—	—	—	21.0	—	—	—	—	—	—	—
" 30	—	—	18.8	—	—	—	—	—	—	—	—

Table III. *Daily urinary output of calcium in mg.*

	Experimental				Controls						
	Arachis oil + irradiated ergosterol				Arachis oil		Arachis oil + ergosterol		No addition		
Rabbit No.	3	4	6	7	1	2	10	12	5	8	9
Feb. 15	37	—	—	83	—	34	Trace	—	Trace	—	—
„ 23	67	—	—	—	—	11	—	—	—	—	—
„ 24	51	—	—	29	—	—	Trace	—	Trace	—	—
„ 26	—	37	Trace	—	—	—	—	Trace	—	Trace	—
„ 27	—	—	72	—	Trace	—	—	—	—	—	—
Mar. 1	—	45	161	—	Trace	—	—	Trace	—	Trace	—
„ 3	—	108	85	—	Trace	—	—	Trace	—	Trace	—
„ 6	—	83	39	—	Trace	—	—	Trace	—	19	—
„ 7	—	130	—	—	—	—	—	16	—	53	—

Urinary calcium and phosphate.

During the latter part of the experiment urine was collected from control and experimental animals. The first fact which emerges is the absence of the diuresis which Hoyle and Buckland [1929] reported with rats. Fig. 1 shows that, if anything, the daily volumes are slightly less, which is probably accounted for by the decreased intake of food and hence water. Fig. 2 shows that the daily excretion of inorganic phosphate (estimated by the uranium acetate titration method) is consistently higher than in the controls. The urines were very alkaline (about p_H 8.5) as were also those of the controls, but, unlike the latter, they contained a white precipitate of calcium salts. Calcium estimations were again carried out by Miss Watchorn. The results (Table III) show a large excretion of calcium salts by the kidney, from a mere trace in the case of the controls to as much as 100 mg. per day

in the experimental animals, which were in a condition of hypervitaminosis during the whole period. It is interesting that a sample of urine (about 25 cc.) taken from the bladder of one of the animals after killing contained 156 mg. of calcium per 100 cc.

Fig. 2. Daily urinary volumes of control and experimental animals.

Recovery from hypervitaminosis.

Feeding of irradiated ergosterol to one animal was discontinued on February 26. A fortnight later, however, this animal was still excreting large amounts of calcium and a high level of inorganic phosphate by the kidney. Even on April 2 the urine contained much calcium and phosphate. When the animal was killed and examined on this date, calcification in the aorta was entirely absent and the kidneys appeared normal. It would appear from the urine, then, that recovery is very much slower than the rapidly falling blood-inorganic phosphate would lead one to suppose. On the other hand, the serum-calcium was still high, 16.6 mg. per 100 cc., while the blood-inorganic phosphate was 4 mg. per 100 cc.

DISCUSSION.

It was shown by Wigglesworth and Woodrow [1923], that, when the blood-inorganic phosphate was raised by ingestion of phosphate, no increase in the organic esters took place. On the other hand, Haldane, Wigglesworth and Woodrow [1924] and Kay [1924] observed a rise in blood-inorganic phosphate in acidosis, accompanied by a fall in the content of organic acid-soluble phosphorus compounds, which Kay traced to the non-hydrolysable fraction. He suggested that this fall was necessary to help to neutralise the free acid, and there was an increased phosphate excretion by the kidney. Conversely, alkalosis caused a diminution in free phosphate. In the experiments reported

Fig. 3. Total daily urinary excretion of inorganic phosphorus by control and experimental animals.

here the p_H of the plasma was not determined, but there was no sign of ketosis in the urine, in spite of the increased phosphate excretion by the kidney. It seems unlikely that the relationship between the inorganic and organic phosphorus compounds of blood can be a simple one, because, if such were the case, a change in one, unaccompanied by reaction changes, should produce a change in the other. A probable explanation of the findings reported here is to be found in the work of Jost [1927] and of Martland, Hansmann and Robison [1924]. Jost showed that by far the greater portion of the acid-soluble organic esters of the red corpuscles consists of a diphosphoglyceric

acid, and that glycolysis in blood is associated with a synthesis of this ester. Martland, Hansmann and Robison showed the presence of an esterase in blood which attacks the organic esters under suitable conditions of p_H . Both these processes appear to be independent of the inorganic phosphate level within the limits obtaining in the blood, and hence no change in the organic esters was detected in the experiments described here. The system, in fact, is somewhat analogous to that obtaining in the synthesis and hydrolysis of hexosephosphate in alcoholic fermentation. Any accumulation of organic phosphorus esters in the plasma is probably prevented by hydrolysis in the kidney, which Eichholtz, Robison and Brull [1925] showed to be able rapidly to break down added esters.

With regard to the origin of the increase in blood-inorganic phosphate and calcium in hypervitaminosis there is no certain knowledge. It has been suggested that one of the functions of vitamin D is to regulate absorption from the gut, and the increased phosphate and calcium excretion by the kidney might suggest that this is the case. On the other hand, Hess, Weinstock and Rivkin [1929] suggest that the calcium comes from the bones. Total balance experiments in rats carried out by Light, Miller and Frey [1929], Brown and Shohl [1930] and Watchorn [1930] seem to support this view. Similar experiments under different levels of calcium and phosphate, which are being carried out in this laboratory at the present time, may serve to throw additional light on the two theories outlined above. In the experiments reported here, however, histological examination of the bones by Mr J. R. M. Innes hardly suggested that the calcium came from the bones, as these were hard and well calcified.

SUMMARY.

1. The increase in blood-inorganic phosphate, which accompanies hypervitaminosis D, does not lead to any change in the content of organic acid-soluble phosphorus compounds.
2. A rise in serum-calcium during hypervitaminosis is confirmed.
3. Hypervitaminosis produces an increase in inorganic phosphate excretion by the kidney, probably with a slight decrease in the daily urinary volume.
4. There is a greatly increased excretion of calcium by the kidney.
5. Animals recovering from hypervitaminosis D maintain a high phosphate and calcium excretion by the kidney, when, apparently, other symptoms have disappeared.

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