CXC. STUDIES UPON THE MODE OF ACTION OF VITAMIN D.

I. INVESTIGATIONS UPON THE PHOSPHORUS COMPOUNDS IN MUSCLES, LIVER AND KIDNEYS AS INFLUENCED BY DIFFERENT LEVELS OF VITAMIN D AND PHOSPHORUS IN THE DIET.

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In recent years evidence has been obtained that vitamin D may play an important part in intermediary P metabolism. Thus in deficiency disease change in concentration of P compounds has been demonstrated not only in blood but also in tissue. The question as to whether vitamin D acts in a specific way on intermediary P metabolism and, if so, how it acts, is however far from solved, as will be seen from a short review of earlier investigations in this field.

Sharpe [1922] estimated the phospholipins in livers from normal and rachitic dogs. This work is perhaps open to the criticism that the livers had been preserved in formalin. This substance is often contaminated with formic acid, and it is noteworthy that the phospholipins are unstable in the presence of acids. Nevertheless it is difficult to understand how this could cause the striking decrease in concentration of phospholipins which he found in the rachitic dogs' livers. Cole & Koch [1931] estimated the acid-insoluble P (i.e. the protein and phospholipin-P in one fraction) in muscles from normal and from rachitic rats on McCollum diet. They found a reduction of the "muscle-P", and conclude that "the results suggest that the irradiated ergosterol simply hastened the transfer of muscle-P to bone formation at the expense of this type of muscle-P. However, the quantitative handling of this fraction was so difficult that the difference may not be significant". The total lipin- and protein-P in this series of investigations was sometimes lower than the usual P content of the muscle lipin alone, and it seems desirable to repeat the experiments before such an important suggestion can be accepted. McGowan [1933] suggests that vitamin D acts by setting free "nascent phosphoric acid" from the phospholipins of the liver. The experimental evidence for this mode of action of vitamin D is not given. Kay [1932] estimated inorganic P and acid-soluble P in liver and kidneys from normal and rachitic rats—partly using Steenbock's, partly McCollum's diet. The results, as Kay points out, are not very easy to interpret. However, they give the impression that the phosphoric esters are reduced to some extent in rickets. The clearest results were those of Skill & Kay [1934] in beryllium rickets where a great reduction was seen both in inorganic and in ester P. Cole & Koch also estimated the acid-soluble fraction of muscle-P; there was, however, no marked difference between normal and rachitic animals. Hentschel & Zoeller [1927] investigated total and inorganic P in the muscles of normal rats and rats on McCollum's

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diet, Sherman-Pappenheimer's diet and a modified Steenbock diet. They failed to find any regular alteration in the total P, but more often the inorganic P was greatly reduced, increasing towards normal on antirachitic treatment. They state, however, that the rachitic rat muscles generally contained more water than those from normal animals.

In addition to the well-known reduction of inorganic P in rickets other fractions of the blood-P are also stated to be affected. Skill & Kay [1934], in the experiments mentioned above, found a reduction of phosphoric esters in red blood cells in rachitic rats, particularly striking in the severe beryllium rickets. The withdrawal of beryllium from the diet was followed by an increase towards normal. Bakwin *et al.* [1932] found that in rachitic infants the fraction of the blood P easily hydrolysed by mineral acids—pyrophosphate—was reduced from 14 to 11 mg. per 100 ml. Jacobsen [1933] obtained analogous results in experimental rickets. Heymann [1931] claims that the reduction of inorganic P of the serum in rickets is accompanied by increased ester P. The concentration of ester P in serum is so small, however (about 0.5 mg./100 ml.), that analytical results cannot be very reliable.

It remains to be decided whether the abnormal values observed in different P compounds are due purely to P starvation, or to a disturbance in the correct balance between the various P compounds due to the absence of the regulating action of vitamin D. Although a complete analysis of the different P compounds in the various tissues is at present impossible, by estimation of inorganic P, phosphagen, readily and difficultly hydrolysable esters, lipin- and protein-P, on a diet containing various levels of P and vitamin D, one should gain a fairly good impression of what takes place.

EXPERIMENTAL.

The experiments were carried out on rats. These animals were bred in the laboratory and fed on the same stock diet as their mothers from weaning until the commencement of the experiment, when they weighed about 50 g. They were then placed on one of the following experimental diets:

Steenbock-Black rachitogenic diet.

I.¹ 76 % maize meal.
20 % wheat gluten.
3 % CaCO_3 .
1 % NaCl.
Ca : P = 4.7.

II. As I with vitamin D (50 I.U. calciferol daily, dissolved in arachis oil and administered by pipette).

P-rich diet.

III. 78 % maize meal.
20 % wheat gluten.
1 % CaHPO_4 .
1 % NaCl.
Ca : P = 0.7.

IV. P-rich diet III with vitamin D (50 I.U. calciferol daily).

Overdosage of vitamin D.

V. Rats nos. 21, 22 and 23 were kept on the rachitic diet I until they developed typical rickets according to X-ray examination. They were then given massive doses of vitamin D over a very short period. No. 21 received 100,000 units 24 hours before killing; no. 22 received 50,000 units 48 hours and 24 hours

¹ Also called groups I, II, etc.

before killing; rat no. 23 received 50,000 units on each of the 3 days before killing. These experiments were carried out in this way to ascertain whether a rapid interchange could be induced between any of the P compounds. Rachitic animals were used as the effect might be expected to be more clearly demonstrated in these. The results, as will appear later, were mostly negative, and in consequence the same experiments were repeated on two normal rats on a P-poor diet in order to have less interference by P absorption from the gut. These two rats were treated in exactly the same way as rat no. 23, thus receiving in all 150,000 units in 3 days.

VI. As diet I but with addition of about 100,000 units vitamin D distributed over 10–14 days.

Condition of bones, etc. in various groups.

All the rats kept on diet I developed rickets according to X-ray observations in about 2 weeks. After killing the tibiae were cut by a longitudinal antero-posterior section. The bones were not well calcified, the proximal epiphyseal cartilage being about 1 mm. broad with irregular borders. The rats on diet II—normal according to X-ray—had epiphyseal cartilage about 0.5 mm. broad in upper tibiae with very regular sharp borders. The rats on diet III were all normal, the bones being better calcified than in group II and the epiphyseal cartilage only about 0.25 mm. broad.

The rats on diet IV were all normal, and had the most intensely calcified bones, with epiphyseal cartilage of the same shape and size as in group III.

Of the rats in group V the three rachitic animals had fresh deposits of bone salts in a broad epiphyseal cartilage; rat no. 23 however had very soft bones, indicating that, together with the deposit in the hypertrophic cartilage, a dissolution of already formed bone had taken place such as is well known in severe hypervitaminosis-D. In rats nos. 24 and 25 hardly any epiphyseal cartilage was recognizable, owing to fresh deposits of calcium salts; the bones, however, were not so fragile as in rat no. 23.

The rats on diet VI, also, had hardly any recognizable epiphyseal cartilage, but the sub-epiphyseal region seemed to be very sparingly calcified. Two rats in this group died; both had calculi in the pelvis of the kidneys. Thus the whole picture was that of typical hypervitaminosis-D. In Table III the body weight and average daily increase in body weight are given. These diets, well balanced with regard to Ca and P, seem to promote quicker increases in body weight than the P-poor Ca-rich diet. The average daily increase for the animals on diet V is given only until the commencement of overdosage, as this resulted in a loss in weight amounting to 5 g.

The rats were killed by decapitation and bled. During this period there occurred clonic convulsions, usually lasting 10–20 sec.; occasionally only a few spasms were seen.

Estimation of acid-soluble P.

As quickly as possible, samples of muscles (always the same muscles), liver and kidneys were weighed out. For extraction and analysis of the fractions of acid-soluble P in muscles the procedure of Eggleton & Eggleton [1929] was used with slight modifications in addition to those introduced by Mügge [1933] and King [1932]. The acid-soluble P of liver and kidney (one side for this purpose, the other for dry weight estimation), was extracted in the same way as for muscle, and inorganic, pyrophosphate and total P estimated in the usual way.

Instead of calculating with a presumed water content of the tissue as Eggleton did, the same dilution calculation as for blood was used.

The acid extracts of kidney and liver were neutralized with sodium hydroxide to prevent a possible liberation of inorganic phosphate from the esters as described by De Toni [1929] for blood filtrates.

Estimation of lipin- and protein-P.

As soon as possible—always within 30 min.—the precipitates were washed twice with about 15 ml. water to each g. of tissue, centrifuged and the supernatant fluid filtered through the same filter paper as had been used for obtaining the trichloroacetic acid filtrate. The precipitates were then transferred quantitatively to 100 ml. volumetric flasks with the aid of repeated washings with 96% alcohol until a volume about 75 ml. was reached. They were then boiled 5–10 min. in a water-bath, cooled and made up to volume. The samples for lipin-P determination were obtained partly by filtration under watch glass and partly by centrifuging in tubes tightly covered with tinfoil. 15 ml. each of muscle and kidney filtrate and 5 ml. of liver filtrate were ashed with 1.5 ml. 60% perchloric acid, and the P analysis carried out as previously described for the ashed samples. The proteins were centrifuged from the alcoholic lipin extract, washed once with about 15 ml. of alcohol and thereupon quantitatively transferred back to the 100 ml. volumetric flask by several washings with water. 3–4 *N* NaOH was then added giving a final concentration of about 0.10 *N*. The flasks were placed in a beaker of boiling water and boiled gently until all proteins were dissolved, cooled and made up to volume. Aliquot parts were then taken for P determinations, which were performed in the same way as the lipin-P estimation. Duplicate estimations were run on one sample of tissue in all the above-mentioned analyses. This was considered quite safe as duplicate experiments on two samples of muscle, liver and kidneys from the same animal always gave concordant results.

DISCUSSION.

The results obtained on all animals are given in Tables I, II and III, which give the results of the analysis on the muscles, liver and kidneys respectively. The body weight and average daily increase in weight are given in Table III.

Phosphagen. The results for muscle demonstrate that there is too great a variability in the phosphagen to allow any comparison at all. The variation is at least to some extent due to the changing intensity in the convulsions following decapitation; the highest values were always obtained when only a few spasms occurred. It was thought safer, however, to carry on with this way of collection of the sample. It always provided organs with approximately the same low content of blood, thus giving more homogeneous results. Samples taken from living narcotized animals are subject to considerable variation in blood content, as in the case of muscles and particularly the liver. According to Bauer *et al.* [1932] the liver acts as a blood reservoir for the right heart, storing or giving up blood under the influence of various stimuli. As the concentration in the blood of some of the P compounds under investigation is entirely different from the concentration in the tissue, a varying blood content might constitute a serious source of error. In the samples taken from these experimental animals only traces of blood were seen when the portal vein was cut; in muscles and kidneys hardly any blood was detectable.

Phosphagen and inorganic phosphate. We may next consider the sum of inorganic P and phosphagen-P as one group, since the result of a breakdown of phosphagen is a corresponding increase in inorganic phosphate. There is no phosphagen in liver and kidney, and we find that, in the same experimental

Table I. *Muscles.*

mg. P in 100 g. fresh tissue in

Rat no.	I Inor- ganic phos- phate	II Phos- phagen	I + II	Readily hydro- lysable esters	Diffi- cultly hydro- lysable esters	Phospho- lipins	Pro- teins	% Water	Diet
1	36	21	57	33	58	55	44	75	Without D, I (Ca : P = 4·7)
2	36	23	59	33	57	56	39	75	
3	38	24	62	35	50	46	39	74	
4	33	29	62	31	60	47	40	76	
5	39	21	60	35	56	46	40	73	
6	46	22	68	35	54	50	37	76	With D, II (Ca : P = 4·7)
7	50	16	66	38	64	51	41	74	
8	54	13	67	32	60	53	44	73	
9	52	25	77	39	54	54	40	73	
10	42	39	81	37	51	50	41	73	
11	47	14	61	34	66	50	40	73	Without D, III (Ca : P = 0·7)
12	37	28	65	37	61	49	39	71	
13	51	20	71	31	56	52	43	78	
14	53	14	67	31	53	51	44	71	
15	54	16	70	35	65	46	44	74	
16	51	18	69	32	66	49	44	74	With D, IV (Ca : P = 0·7)
17	50	20	71	34	54	51	46	74	
18	54	16	70	36	66	48	43	74	
19	43	27	70	33	50	48	40	76	
20	56	19	75	32	53	51	43	74	
21	32	33	65	37	59	49	42	76	Hypervita- minosis-D, V (Ca : P = 4·7)
22	38	35	73	41	51	49	40	71	
23	48	20	68	36	54	48	38	73	
24	42	31	73	36	55	45	39	71	
25	43	37	80	37	57	44	40	73	
26	53	27	80	36	56	48	42	71	Hypervita- minosis-D, VI (Ca : P = 4·7)
27	32	40	72	32	53	47	41	75	
28	40	31	71	38	52	45	42	76	
29	38	31	69	30	59	46	42	74	

group, the values for inorganic P in liver and kidneys are much more regular than those for muscles. By comparing the inorganic phosphate plus phosphagen content of muscles, and the inorganic phosphate in liver and kidneys from animals on a rachitic diet (group I) with those from animals on the same diet but receiving a daily addition of vitamin D as protection against rickets (group II), we find a definite reduction in these compounds in tissue from the rachitic animals. Vitamin D therefore increases the inorganic P in tissues just as it has been shown to do in blood.

When the Ca content of the diet is reduced and the P content increased so that the diet is well balanced with regard to Ca and P, as in diet III, the animals do not develop rickets even when vitamin D is not added to the diet, and the inorganic phosphate in the tissue is kept at the same level as when vitamin D is added to a diet badly balanced with regard to Ca/P. The bones of these animals were even better calcified than the bones of the rats on the P-poorer diet with added vitamin D. Diet IV (addition of vitamin D to diet III) had no further effect on the inorganic P in the tissue than had diet III, but bone calcification was still better in this group. This is in agreement with the well-known fact that even with a good Ca/P balance in the diet, addition of vitamin D increases the retention of Ca and P. As there is no increase of these minerals in the soft tissue or in blood, it is clear that the increased deposition is confined to the bones.

Table II. *Liver.*

mg. P per 100 g. fresh tissue in

Rat no.	Inorganic phosphate	Readily hydrolysable esters	Difficultly hydrolysable esters	Phospholipins	Proteins	% Water	Diet
1	25	14	51	102	104	72	Without D, I (Ca : P = 4.7)
2	26	12	54	101	106	71	
3	25	14	59	123	121	71	
4	23	16	55	107	106	72	
5	24	14	58	124	129	67	
6	35	14	54	146	126	72	With D, II (Ca : P = 4.7)
7	29	18	62	160	132	67	
8	32	12	49	135	130	67	
9	32	13	54	149	136	71	
10	30	18	54	130	119	72	
11	28	13	61	130	120	67	Without D, III (Ca : P = 0.7)
12	27	12	64	134	124	67	
13	30	12	61	127	127	69	
14	27	15	64	130	124	69	
15	29	13	60	138	128	69	
16	27	16	61	137	127	70	With D, IV (Ca : P = 0.7)
17	29	12	63	126	115	69	
18	28	11	61	126	120	70	
19	35	10	59	157	140	71	
20	27	13	50	120	114	70	
21	29	15	53	130	130	67	Hypervitaminosis-D, V (Ca : P = 4.7)
22	29	14	52	118	117	71	
23	28	13	61	121	116	70	
24	29	14	57	122	128	70	
25	30	13	57	137	128	70	
26	30	13	56	130	136	71	Hypervitaminosis-D, VI (Ca : P = 4.7)
27	29	13	60	115	115	67	
28	26	13	55	119	110	68	
29	26	15	66	116	114	69	

The effects of excessive amounts of vitamin D on the inorganic P and phosphagen. Vitamin D was administered in excess in two ways as previously described. In the first series of experiments rats received massive doses for 1-3 days. As several authors have suggested that vitamin D might act by inducing a sudden liberation of inorganic P from organic compounds, this should be best demonstrated in experiments of short duration by administering very large doses of the vitamin to animals on a P-poor diet, under which conditions the absorption of P should influence the results to a small extent only. This experiment was first carried out on three rachitic animals; the only noteworthy result was an increase in the inorganic phosphate up to the level found in normal rats. Similarly this was the only effect obtained on two animals kept before the experiment on diet II (the same diet with normal amount of vitamin D).

Secondly, experiments were carried out on rats kept on a rachitic diet but given a more moderate overdosage of vitamin D over about 2 weeks. No effects were obtained on the liver and muscles beyond those induced by small additions of vitamin D to this diet or by balancing the same diet correctly with regard to Ca and P with no vitamin D added. An immense increase occurred in the kidneys; this was due to the deposition of calcium phosphate both in the tubule and in the pelvis of the kidneys, as is well known from the experiments of other authors, e.g. Harris & Moore [1929].

Table III. *Kidneys.*

mg. P per 100 g. fresh tissue in

Rat no.	Body weight g.	Average daily increase in wt. g.	Inorganic phosphate	Difficultly hydrolysable esters	Phospholipins	Proteins	% Water	Diet
1	62	1.0	30	51	100	89	79	Without D,
2	71	0.9	27	50	102	81	78	I
3	75	1.0	28	66	102	77	79	(Ca : P = 4.7)
4	68	1.0	30	55	102	77	79	
5	78	1.1	30	53	91	70	79	
6	68	1.2	36	62	111	92	77	With D,
7	57	0.8	33	57	107	87	78	II
8	57	0.9	37	46	111	84	79	(Ca : P = 4.7)
9	71	1.0	35	60	104	87	80	
10	72	1.0	33	61	109	85	76	
11	77	1.7	37	69	103	84	77	Without D,
12	80	2.0	35	52	115	91	79	III
13	78	1.5	38	62	108	82	78	(Ca : P = 0.7)
14	82	1.7	38	61	104	85	78	
15	79	1.4	39	62	110	95	77	
16	74	2.0	37	61	111	87	78	With D,
17	76	1.6	37	58	106	86	76	IV
18	73	1.5	39	64	116	80	—	(Ca : P = 0.7)
19	78	1.5	39	65	117	85	79	
20	63	1.4	38	60	108	84	76	
21	68	0.8	29	57	105	86	78	Hypervita-
22	73	1.0	39	56	101	73	79	minosis-D,
23	75	1.2	37	61	107	77	79	V
24	73	0.9	47	55	104	80	79	(Ca : P = 4.7)
25	74	1.0	49	64	103	80	79	
26	52	0	82	66	106	87	79	Hypervita-
27	61	0	63	56	111	88	78	minosis-D,
28	65	0	73	52	97	85	80	VI
29	66	0	53	61	107	86	79	(Ca : P = 4.7)

Variations in the esterified forms of P. In the readily hydrolysable esters—adenosinetriphosphate—we find the same variation in all six groups of rats, without any striking difference between one group and another. Occasionally we find a low value for inorganic P with a high pyrophosphate value in the same organ and inversely (compare livers from animals 16 and 19). This might appear to be due to analytical error. The pyrophosphate-P is the difference between P estimations after 7 min. hydrolysis by boiling with acid and a direct estimation of inorganic P in the same sample. When such values as mentioned above occurred, new analyses were made to ensure the correctness of the estimation. The same analytical results were always obtained. We know, however, that adenosinetriphosphate is formed by phosphorylation of adenylic acid. This may explain why an inverse variability of inorganic and pyrophosphate-P occurs in some animals.

The ester-P hydrolysable with difficulty—adenylic acid and hexosephosphates—varied from about 50 to 60 mg./100 g. in all organs and in each group of animals. Slight differences between the various groups were observed, but it is rather doubtful if the variations are significant.

That ester-P content can be influenced by further restriction of the P supply to the body is however shown impressively by Kay & Skill in their experiments

with beryllium rickets. In the animals where very severe rickets occurred there was a depression in ester-P (sum of readily and difficultly hydrolysable ester-P) from 80 to about 30 mg./100 g.

In the lipin-P of the muscles there is, in all groups, the same slight variability, without any difference from one group to another; the same can be said about the protein-P in the muscles. The variability in these P compounds in the kidney is about the same as in the muscles but an evident though slight reduction in the rachitic animals is apparent when the groups are compared. In the liver the variability of phospholipin- and protein-P is greater, but here also there is a definite reduction in the rachitic animals. Contrary to expectation slightly lower values for these compounds were given by diet IV as compared with diet II which demonstrates that the normal variability is not properly registered by using only five animals in each group. The conclusion must therefore be drawn with some care. It seems, however, justifiable to conclude that on a P-poor diet both the phospholipin- and protein-P in liver and kidneys will be decreased to some extent. Why this does not take place in the muscles cannot be decided from these experimental results. We know, however, that both the liver and kidneys are far more susceptible to pathological anatomical changes than muscles. This is observed both in acute infections and intoxications. When the noxious agent disappears from the body a rapid recovery to a normal anatomical picture takes place. This may explain why rats given excessive doses of vitamin D for long periods had lower acid-insoluble P in the liver than the other animals, with the exception of the rachitic rats.

It has been suggested by earlier authors that vitamin D acts by a sudden liberation of inorganic P from the acid-insoluble P compounds in tissue. If such a conclusion were to be justified the results should have been low acid-insoluble P in normal animals with an increase in rachitic animals. The inverse results have been obtained.

With regard to the acid-soluble P it is noteworthy that in the present work a decrease in the inorganic fraction was observed, which is in contrast with the decrease in the ester fraction reported by Kay and other workers. This apparent discrepancy may be attributable to the fact that the variability in normal and rachitic animals, as determined not only by body weight and daily increase in body weight but also by the length of experiment and P retention, has not yet been established.

When we find, as in these experiments, a depression in the tissue-P only after deprivation of vitamin D, and that this depression is eliminated by addition of P to the diet, the conclusion that the action of vitamin D in P metabolism, at least in rats, is to increase the supply of P from the intestinal contents to the blood stream, and thence to the tissue, seems well justified. There appears to be no evidence to support the view that vitamin D acts by maintaining a balance between the different forms of P in the organism.

SUMMARY.

1. In rachitic rats compared with rats on the same diet but with addition of therapeutic amounts of vitamin D there was a slight but definite reduction in the sum of inorganic P+phosphagen in muscles and in inorganic P in liver and kidneys. A reduction was also seen both in lipin-P and protein-P in the liver and kidneys from rachitic rats, but not in muscles. No definite change was observed in readily or difficultly hydrolysable esters in muscles or liver or in the difficultly hydrolysable esters in kidneys.

2. When P was added to the rachitic diet and the Ca content reduced, the animals remained normal and gave the same results for all P compounds as did animals on the rachitic diet with added vitamin D.

3. Addition of vitamin D to this P-richer diet, although giving better calcified bones, had no influence upon the amount of the different P compounds in muscles, liver and kidneys.

4. Overdosage by massive doses of vitamin D for short periods to rachitic rats increased the inorganic P to the normal level in muscles, liver and kidneys. The same experiments on rats given a rachitogenic diet, but protected from rickets by therapeutic amounts of vitamin D, showed only a slight increase of inorganic P in the kidneys.

5. The same massive amounts of vitamin D administered over 2 weeks to rats on a rachitogenic diet resulted only in an increase of inorganic P in the kidneys up to double the normal content, presumably through the formation of deposits.

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