XV. STUDIES UPON THE MODE OF ACTION OF VITAMIN D

III. THE INFLUENCE OF VITAMIN D ON THE ABSORPTION OF CALCIUM AND PHOSPHORUS IN THE RAT

By RAGNAR NICOLAYSEN¹

*From the Nutritional Laboratory, University of Cambridge and Medical
Research Council*

(Received 19 November 1936)

THE influence of vitamin D on Ca and P metabolism has been studied in children by Telfer [1922], Findlay *et al.* [1920], Rominger *et al.* [1930; 1931]; in dogs by Jones [1927], Shohl & Bennett [1928], Skaar [1931]; and in rats by McClendon [1922], Boas [1926], Karelitz & Shohl [1927], Shohl & Bennett [1928]. The chief result about which all are agreed is that the Ca and P retention is reduced in vitamin D deficiency, and that the chief cause of the reduced retention is an increased output of these elements in the faeces. Retention is increased by administration of vitamin D. In a preceding paper [Nicolaysen, 1937] it has been shown that the output of endogenous Ca and P in the faeces in vitamin D deficiency plays only a small role compared with the decreased absorption of these elements from the ingested food.

As Ca interferes with the absorption of P [Telfer, 1924] and P to a certain extent with the absorption of Ca [Nicolaysen, 1934], it is unknown what is the cause of this reduced absorption of Ca and P. A great number of authors stress the importance of lack of P as the origin of rickets; a very short account only of these papers can be given here.

Rominger *et al.* [1930; 1931], investigating the changes in Ca and P metabolism in children, found that the change in P metabolism occurred earlier than that in Ca metabolism and thought it possible therefore that vitamin D acts primarily on the metabolism of P. By feeding salts of metals which form insoluble phosphates several authors have been able to provoke rickets in rats; Lehnerdt [1910] with strontium, Weltner [1927] with iron, Mouriquand *et al.* [1931] with magnesium, Rominger *et al.* [1931] with thallium and Guyatt *et al.* [1933] with beryllium. Rickets is both prevented and cured if more phosphate is added to the diet than can be precipitated by the ingested amount of metal.

The "cereal" rickets earlier believed of "toxic" origin has now been demonstrated [Bruce & Callow, 1934] to be due to the fact, not formerly recognized, that the chief source of P in the diets used to provoke "cereal" rickets is inositolhexaphosphoric acid, the P of which is utilized to a small extent only by the action of bacteria. That phytin is ineffective in the cure of rickets has been confirmed by Lecoq & Barban [1935].

Lecoq *et al.* [1932; 1934, 1, 2] have contributed further to our knowledge as to the effective and ineffective forms of P in the prevention and cure of rickets in rats. They find that the esters of phosphoric acid with aliphatic alcohols are as efficient in this respect as is inorganic phosphate, whereas esters of aromatic alcohols are inefficient. Pappenheimer *et al.* [1922] were able to cure rickets in

¹ Rockefeller Research Fellow.

rats by adding to 100 g. of their rachitic diet (high in Ca, low in P) 160 mg. P in the form of inorganic phosphate, lecithin P or meat P. Addition of 160 mg. P as caseinogen P was less effective.

The phosphoric esters ingested with the food are hydrolysed by phosphatases in the body. We do not know at present, however, if the phosphoric esters in the food are hydrolysed in the bowel, in the gut wall or elsewhere in the body. Kay & Skill [1934] have shown that glycerophosphate injected hypodermically can be used for bone formation in beryllium rickets. Heymann [1928] has demonstrated that injected glycerophosphate is hydrolysed in the body. As the gut wall, according to quantitative estimations performed, contains almost as much phosphatase as does any other tissue in the body, it seems possible that the phosphatases in the gut may play an essential role in the hydrolysis of phosphoric esters ingested with the food.

The results of Heymann [1930; 1931; 1933] and of Crimm & Strayer [1936] are of interest in this connexion. Heymann estimated the phosphatase activity quantitatively in the gut wall, in the intestinal content and in the faeces of rats and dogs and in the faeces of children. The phosphatase activity was much higher in the upper part of the small intestine than in the lower, insignificant in the colon. In vitamin D deficiency Heymann found that the activity of the phosphatases decreased in the gut wall and in the intestinal content as well as in the faeces. He followed the question up with diffusion experiments *in vitro* in loops from the small intestine. The results were that diffusion of P from phosphoric esters injected into the lumen was slower from loops of vitamin D-deficient rats than from loops of normal rats. Crimm & Strayer found that the phosphatase activity of the gut wall increased about 300% above the normal in rats given toxic amounts of vitamin D.

As the above-mentioned experiments are either *in vitro* experiments, where the activity of the phosphatase is not measured under conditions identical with those in the cell, or diffusion experiments with excised loops, where the diffusion takes place through the whole of the gut wall instead of the epithelium only, their results must be interpreted with great care. The variation in the phosphatase activity may be significant, however, and a reduction of this activity might then be the cause of the reduced retention of P, the action on Ca being secondary.

If a decrease in the hydrolysis of ester P in the bowel, with subsequent slower absorption as indicated by Heymann's experiments is the cause of the increased loss of P in the faeces in rickets, it should be possible to demonstrate a difference in the output of P in the faeces as between normal and rachitic rats given the same amount of ester P. Such a study has therefore been the object of one part of the experiments presented here.

The increased outputs of Ca and P in the faeces in vitamin D deficiency might from a hypothetical point of view, however, be explained by a primary action of vitamin D on the absorption of Ca. This would then be followed by the well-known effect which increased amounts of Ca in the bowel have on the P: i.e. precipitation of phosphates causing increased output of P in the faeces. A study of the influence of vitamin D on the absorption of Ca was therefore the object of the second part of these experiments.

The author has previously stressed the necessity for separate studies of the output of endogenous Ca and P, and the results of these investigations have been reported in a preceding paper [Nicolaysen, 1937]. The experiments included also a study of the influence of different levels of Ca on the output of endogenous P and vice versa. By estimation of both Ca and P in the faeces in all experiments

these served a double purpose; they gave at the same time information (1) about the influence which different levels of Ca in the diet have upon the output of endogenous P, and (2) about the absorption of Ca from a ration free from P, and of P from a diet free from Ca.

The results to be discussed here are thus the outcome of the previously reported experiments in so far as they deal with: (1) the absorption of different amounts of Ca supplied in a diet containing negligible amounts of P, in normal and vitamin D-deficient rats; (2) the absorption of P from different sources of P supplied in a diet containing negligible amounts of Ca, in normal and vitamin D-deficient rats.

EXPERIMENTAL

With regard to the experimental conditions, the treatment of the animals, the diets used, collections of faeces etc., reference is made to the previous paper [Nicolaysen, 1937], where the output of endogenous Ca and P was reported.

The results with regard to the absorption of Ca are given in Table I, with regard to the absorption of P in Table II.

Table I. *The faecal excretion of Ca as influenced by vitamin D*

Ca expressed as mg. Ca in experimental period of 2 days.

Diet I. 4.6 mg. Ca, 2.2 mg. P in the food. Diet II. 30 mg. Ca, 2.2 mg. P in the food.

50 units vitamin D daily Ca mg.	Without vitamin D Ca mg.	50 units vitamin D daily Ca mg.	Without vitamin D Ca mg.
0.5	2.0	—	—
0.5	2.1	—	—
0.5	2.2	—	—
0.5	2.6	—	—
0.6	3.2	—	—
0.7	3.8	—	14.0
0.7	3.9	0.7	16.6
0.9	4.0	0.8	17.9
0.9	4.1	0.9	18.2
0.9	4.2	1.0	19.0
1.0	4.5	1.0	20.0
1.1	4.9	1.2	20.2
1.2	5.6	1.3	20.8
1.5	5.8	1.3	20.8
1.7	7.2	1.6	21.2
2.7	8.5	2.5	21.5
Av.	0.9	1.2	19.1
Daily av.	0.5	1.1	9.6

Diet III. 90 mg. Ca, 2.2 mg. P in the food. Diet IV. 180 mg. Ca, 2.2 mg. P in the food.

50 units vitamin D daily Ca mg.	Without vitamin D Ca mg.	50 units vitamin D daily Ca mg.	Without vitamin D Ca mg.
28	48	61	122
31	49	94	140
33	52	96	142
38	54	99	142
39	55	99	143
39	56	100	146
41	57	101	148
42	58	102	150
42	59	103	157
43	65	115	162
—	71	—	—
Av.	38	97	144
Daily av.	19	49	72

Calcium

The absorption of calcium in normal rats in phosphorus starvation. The output of Ca in the faeces of the normal rat is about 0.5 mg. daily when 2.3 and 15 mg. Ca are given in the daily ration. As the output of Ca is the same also when only 0.2 mg. Ca is given in the daily ration (see experiments with meat powder in the previous paper [Nicolaysen, 1937], we must conclude that the 2.3 and 15 mg. Ca ingested daily are completely absorbed. When 45 and 90 mg. Ca are given in the daily ration, the absorption is no longer complete. The amount absorbed is the difference between the ingested amount and the amount lost with the faeces + the value previously found for the loss of endogenous Ca in the faeces. This value, on an average 0.5 mg. Ca daily, is almost negligible in normal rats, and is not taken into account. It will be seen that when 45 mg. are administered the average amount absorbed per rat by 10 rats is 26 mg., and when 90 mg. are administered 42 mg. are absorbed. With increased ingestion increased absorption follows. In the case of 90 mg. Ca fed in the form of CaCO_3 in the daily ration, undissolved white particles of CaCO_3 were observed in the faeces. So far as absorption is a function of solubility, the limit of absorption in the young rat seems to be about 42 mg. under the present experimental conditions. This does not exclude the possibility that a more rapidly growing rat, with a larger food intake and accordingly a richer flow of gastric and the other digestive juices, is able to dissolve more CaCO_3 and probably also absorb more. That the ability to dissolve is not the essential limiting factor in the absorption of Ca is clear from a comparison of the results with ingestion of 45 and 90 mg. Ca daily. In the latter case it is demonstrated that at least 42 mg. can be dissolved, but when approximately the latter amount is ingested, only a little more than half of it is absorbed.

The absorption of Ca in these normal rats is considerably higher than the absorption of Ca in rats given a Steenbock-Black diet containing about the same amount of Ca in the daily ration. The average daily absorption in these rats, also receiving 50 I.U. vitamin D daily, was as reported in a previous paper [Innes & Nicolaysen, 1937], 34 mg. of Ca. The Steenbock-Black diet contains considerable amounts of P and fat, as compared with the diet used in these experiments. That P ingested together with Ca prevents absorption of Ca to a certain degree has been demonstrated earlier [Nicolaysen, 1934]. A similar effect of fat has been reported by Westerlund [1934]. It is therefore probable that the difference between the two groups of experiments is due, at least to some extent, to the effect of P and fat on simultaneously ingested Ca.

The absorption of Ca in vitamin D-deficient rats in phosphorus starvation. When we compare the results obtained from the rats fed on a vitamin D-free diet with the results from the normal rats, a striking difference is seen. This is the case whether 2.3, 15, 45 or 90 mg. of Ca are given in the daily ration. In the preceding paper [Nicolaysen, 1937] it has been shown that the output of Ca in the faeces in the rachitic rat is the same whether 2.3 or 0.2 mg. Ca are given. It is therefore justifiable to consider as endogenous Ca all the Ca found in the faeces when 2.3 mg. are given daily. Although the difference between the normal and vitamin D-deficient rats is clear in all experiments, and greatest when 90 mg. are given, it is most striking when 15 mg. Ca are given. The normal rat can absorb this amount completely; the vitamin D-deficient rat loses 9.5 mg. Ca daily in the faeces. With increasing amounts of Ca in the food the rachitic rat also tends to absorb more Ca. If we include a correction for the fraction of endogenous Ca in the faeces we find that of 15 mg., $5.5 + 2.2 = 7.7$ mg. are absorbed; of 45 mg., $16.5 + 2.2 = 18.7$; and of 90 mg., $23 + 2.2 = 25.2$ mg.

The figures given here do not pretend to give a complete picture as to the extent to which the absorption of Ca is reduced in rickets. Thus the experiments with 45 mg. were performed in an early stage of vitamin D deficiency, the experiments with 15 and 90 mg. in a relatively late stage. Previous experiments, to which references have been made in the introduction, demonstrate that the absorption of Ca in vitamin D deficiency may vary from slightly below normal down to almost none, depending on the stage of vitamin D deficiency. The scope of this work, however, was to see how far vitamin D deficiency affected the absorption of Ca in the absence of P from the diet.

The chief result of these experiments with steadily increasing amounts of Ca in a food containing very little P is that vitamin D must affect the absorption of Ca directly and not secondarily to an action on P.

If vitamin D were to act through the hydrolysis of organic P compounds ingested with the food, the effect would be the following. Phosphoric acid ingested in ester form is liberated in the form of phosphoric acid by the action of phosphatases. An increased acidity follows, more Ca goes into solution and forms Ca phosphate. The kind of Ca phosphate formed will depend on the pH in the intestinal content. In the small intestine this may be assumed to be about 6. This will favour the formation of a mixture of primary and secondary Ca phosphates. If we assume that secondary phosphate is formed the maximum effect of the 2.2 mg. P in the food would be to combine with 2.9 mg. Ca in the form of secondary phosphate. As the absorption of P was the same in the normal and vitamin D-deficient rats, the absorption of Ca in these experiments must have been independent of the fate of the ingested P.

Phosphorus (see Table II)

With egg albumin as the sole source of P, about the same amount of P is lost with the faeces as is ingested with the food. To decide further as to the actual amount of endogenous P in the faeces in these experiments, 10 normal rats were given a diet free from P (95% sucrose, 4% agar, 1% sodium chloride) and experiments performed on them in the same way as before. The amount of P in the faeces in these experiments varied between 1.7 and 2.7 mg. with an average of 1.0 mg. P per day. Thus the P in the faeces in the experiments with egg albumin is all endogenous P.

The object of the present work, however, was to investigate if there was any difference between the normal and vitamin D-deficient rats in their ability to utilize P added in different forms to the food. That this is not the case is clearly demonstrated by the experiments given in Table II. Inorganic P is completely absorbed in both groups, this is the case also when sodium glycerophosphate and caseinogen are the sources of P in the food. When acid-extracted meat powder is given, a certain amount is not absorbed, but this amount is the same in both vitamin D-deficient and normal rats. This is made clear by a subtraction of the figures found for the daily P excretion in P starvation in normal and vitamin D-deficient rats (1.0 and 1.4 mg. respectively) from the values found in the faeces when meat powder is given (2.4 and 2.9 mg. respectively). The differences obtained are 1.7 and 1.5 mg., i.e. practically the same.

The ability to utilize inorganic phosphate and P in meat powder, caseinogen and glycerophosphate is thus not reduced in the rat in vitamin D deficiency. It might be objected against the conclusiveness of these experiments that only moderate amounts of P have been ingested with the diet. It was impossible, however, to feed more. The animals then refused to finish their measured ration completely. The cause seemed to be latent tetany, the limbs were more rigid than usual, and the rats seemed more irritable. It is well known that P injected

Table II. *The faecal excretion of P as influenced by different forms of P in the food in normal and vitamin D-deficient rats*

P expressed as mg. P in experimental periods of 2 days.

Diet I. (Source of P, egg albumin.) 4.6 mg. Ca, 2.2. mg. P in the food.

Diet II. (Source of P, NaH_2PO_4 .) 2.2 mg. Ca, 30 mg. P in the food.

Diet III. (Source of P, acid-extracted meat powder.) 0.4 mg. Ca, 19.2 mg. P in the food.

50 units vitamin D daily P mg.	Without vitamin D P mg.	50 units vitamin D daily P mg.	Without vitamin D P mg.	50 units vitamin D daily P mg.	Without vitamin D P mg.
1.5	2.0	—	—	—	—
1.5	2.0	—	—	—	—
1.6	2.1	—	—	—	—
1.7	2.2	—	—	—	—
1.8	2.2	—	—	—	—
2.0	2.4	—	—	—	—
2.0	2.5	1.7	2.2	4.4	5.0
2.0	2.5	1.8	2.5	4.4	5.0
2.0	2.5	1.8	2.6	4.5	5.2
2.0	2.6	2.0	2.7	4.6	5.3
2.1	2.6	2.1	2.7	4.7	5.6
2.2	2.8	2.2	2.8	4.8	5.7
2.2	3.1	2.2	2.9	4.9	5.8
2.3	3.5	2.2	3.1	4.9	6.1
2.5	4.0	2.3	3.1	5.2	6.6
2.7	4.1	2.6	3.3	5.7	6.8
Av.	2.0	2.1	2.8	4.8	5.7
Daily av.	1.0	1.1	1.4	2.4	2.9

Diet II. (Source of P, disodium glycerophosphate.) 2.2 mg. Ca, 30 mg. P in the food.

Diet IV. (Source of P, ashless caseinogen.) 1.0 mg. Ca, 28.8 mg. P in the food.

50 units vitamin D daily P mg.	Without vitamin D P mg.	50 units vitamin D daily P mg.	Without vitamin D P mg.
—	—	1.8	—
1.7	2.0	1.8	2.0
1.9	2.2	1.9	2.1
1.9	2.3	1.9	2.3
1.9	2.6	2.0	2.4
2.0	2.7	2.1	2.5
2.1	2.8	2.1	2.5
2.2	2.8	2.2	3.1
2.3	3.0	2.2	3.3
2.4	3.1	2.2	3.4
2.6	3.3	2.3	3.7
Av.	2.1	2.0	2.7
Daily av.	1.1	1.0	1.4

parentally can give rise to tetany. It is therefore easily understood how ingested P, which is completely absorbed, can produce the same symptoms in the absence of Ca. The amount of P in the diet containing glycerophosphate corresponds to a diet with 0.188% P of which at least 0.174% is phosphoric ester P. This is a diet low in P but rich in available P, when compared with the different rachitogenic rations used for rats and dogs. It may therefore be said that the *in vivo* test of the intestinal phosphatases has been performed in these experiments with as much available phosphoric ester as is present in any experimental rachitogenic ration.

The absorption of phosphorus in a late stage of vitamin D deficiency. The experiments with glycerophosphate and caseinogen presented in Table II were all performed in an early stage of vitamin D deficiency. This is also evident from the results with regard to the output of endogenous Ca in these experiments. It was therefore decided to perform some experiments with rats completely depleted of vitamin D. The rats used for this purpose had been kept on a vitamin D-free ration for nearly three months, they had stopped growing, but were kept alive by giving them a food well balanced with regard to Ca/P ratio. These rats were about 150 days old and weighed 75–115 g. Of a lot of 12 only 5 had survived. One experiment was done on each of these five rats, with glycerophosphate as the source of organic phosphate, in the same way as in the experiments given in Table II. The food intake was delayed in one rat but not so long that the experiment had to be discarded. All the other rats finished their food completely in the two days. The outputs of P in these five rats were as follows (the corresponding values for Ca are given in brackets): 2.3 (4.5), 2.8 (4.8), 2.9 (4.3), 3.3 (6.8), 3.4 (8.5) mg. These results correspond exactly with the results found in Ca and P starvation in a late stage of vitamin D deficiency [Nicolaysen, 1937]. As rats completely depleted of their stores of vitamin D are also able to utilize more phosphoric esters than exist in almost any rachitogenic ration, it must be concluded that the ability to hydrolyse phosphoric esters in the gut is not impaired in vitamin D deficiency and is therefore not connected with the increased output of P in the faeces in rickets.

This conclusion is in good agreement with the results of Lecoq & Villus [1932], who found that the phosphoric esters which are hydrolysed by phosphatases are as efficient in the prevention and cure of rickets as is inorganic phosphate. The experiments with caseinogen demonstrate that the reason why Pappenheimer *et al.* [1922] did not find caseinogen P as efficient in the cure of rickets as inorganic phosphate is not due to incomplete hydrolysis of the phosphoserine in the caseinogen. The caseinogen used by Pappenheimer *et al.* ("commercial casein") is very rich in Ca. The inefficiency of the "casein" can therefore easily be explained by the effect which a surplus of Ca has on the P in the intestine. The acid-extracted meat powder however contains about 15% P which is not utilized in the gut. This may partly be ascribed to the fact that a certain portion will be tendinous tissue, which is not digested, but this cannot account for the whole deficit. Some part of the acid-insoluble P is thus not completely utilized. This can hardly be due to the preparatory procedure (extraction with dilute hydrochloric acid, followed by dialysis against running water). The question has not yet been followed up further.

It will be remembered that Pappenheimer *et al.* found meat P as efficient as inorganic P in the cure of rickets in rats. The acid-insoluble P is about one-third of the total P of the muscles, the non-utilized part therefore not more than 5%, and this difference could hardly be expected to be recognized in biological assays.

With regard to the contradiction between the *in vivo* test of phosphatase activity in the intestine, as performed in these experiments and the *in vitro* test by Heymann, the present author cannot suggest any explanation. It must be remembered, however, that the *in vitro* estimation of phosphatases, valuable as it has been in many respects, has yielded many surprising and at present inexplicable results. It is necessary only to mention the results which Crimm & Strayer [1936] obtained in hypervitaminosis D. They found that the intestinal phosphatase activity increased 3-fold, as compared with no change in the bones, a 4-fold reduction in the blood and in the kidneys, and a 20-fold reduction in the liver.

SUMMARY

The absorption of Ca from a diet nearly free from P, and the absorption of P from a diet nearly free from Ca, have been studied in normal and vitamin D-deficient rats.

In P starvation the absorption of 15, 45 and 90 mg. Ca in vitamin D-deficient rats was 50, 36 and 28 %, as compared with 100, 57 and 47 % in the rats receiving 50 I.U. of vitamin D daily.

In Ca starvation the absorption of the combined P of acid-extracted meat powder is equally impaired (about 15 %) in both normal and vitamin D-deficient rats, whilst the absorption of P from inorganic phosphate, glycerophosphate and caseinogen is complete both in normal and vitamin D-deficient rats. This is the case also in rats completely deprived of their previous stores of vitamin D.

The indication from these experiments in the rat is that the action of vitamin D in the gut is confined to a direct action on the absorption of Ca. The well-known reduced absorption of P in vitamin D deficiency is due to a precipitation by the increased amount of Ca in the bowel.

My thanks are due to the Medical Research Council and Dr L. J. Harris for their kind hospitality during this work.

REFERENCES

- Boas (1926). *Biochem. J.* **20**, 153.
 Bruce & Callow (1934). *Biochem. J.* **28**, 517.
 Crimm & Strayer (1936). *J. biol. Chem.* **112**, 511.
 Findlay, Paton & Sharpe (1920). *Quart. J. Med.* **14**, 352.
 Guyatt, Kay & Branion (1933). *J. Nutrit.* **6**, 315.
 Heymann (1928). *Z. Kinderheilk.* **45**, 232.
 — (1930). *Z. Kinderheilk.* **49**, 748, 761.
 — (1931). *Z. Kinderheilk.* **51**, 673.
 — (1933). *Z. Kinderheilk.* **55**, 92.
 Innes & Nicolaysen (1937). *Biochem. J.* **31**, 101.
 Jones (1927). *Amer. J. Physiol.* **79**, 694.
 Karelitz & Shohl (1927). *J. biol. Chem.* **73**, 655, 665.
 Kay & Skill (1934). *Biochem. J.* **28**, 1222.
 Lecoq & Barban (1934, 1). *C.R. Acad. Sci., Paris*, **199**, 1255.
 — (1934, 2). *C.R. Soc. Biol., Paris*, **117**, 671.
 — (1935). *C.R. Soc. Biol., Paris*, **118**, 867.
 — & Villus (1932). *C.R. Soc. Biol., Paris*, **110**, 687.
 Lehnardt (1910). *Beitr. path. Anat.* **47**, 215.
 McClendon (1922). *Amer. J. Physiol.* **61**, 373.
 Mourigand, Leulier & Roche (1931). *Pr. méd.* 769.
 Nicolaysen (1934). *Skand. Arch. Physiol.* **69**, Suppl.
 — (1937). *Biochem. J.* **31**, 107.
 Pappenheimer, McCann & Zucker (1922). *J. exp. Med.* **35**, 447.
 Querido (1935). *Arch. néerl. Physiol.* **20**, 487.
 Rominger, Meyer & Bomskov (1930). *J. exp. Med.* **73**, 343.
 — (1931). *Z. exp. Med.* **78**, 259.
 Shohl & Bennett (1928). *J. biol. Chem.* **76**, 633; **78**, 181.
 Skaar (1931). *Acta paediatr. Stockh.* **12**, Suppl.
 Telfer (1922). *Quart. J. Med.* **16**, 7.
 — (1924). *Quart. J. Med.* **17**, 245.
 Weltner (1927). *Biochem. Z.* **188**, 381.
 Westerlund (1934). *Lantbruks-Högskolans Ann.* **1**. Uppsala.