XIV. STUDIES UPON THE MODE OF ACTION OF VITAMIN D

II. THE INFLUENCE OF VITAMIN D ON THE FAECAL OUTPUT OF ENDOGENOUS[1] CALCIUM AND PHOSPHORUS IN THE RAT

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THE chief metabolic symptom in rickets is reduced retention of Ca and P, due to increased loss of these elements in the faeces. The increased faecal output has been interpreted either as reduced absorption or increased excretion. The latter has always been supposed to take place in the large intestine. According to earlier experiments with calcium [Nicolaysen, 1934], with iron [Nicolaysen, 1935] and with magnesium [Nicolaysen, 1936] there is no evidence that the colon has any excretory function comparable with that of the kidney. The loss of minerals due to passage from the blood stream to the bowel takes place entirely proximal to the colon. As the author was unable to detect any increased loss of minerals in the faeces by those procedures which invariably increased their output in the urine, it was concluded that no regulated secretion of minerals takes place in the intestinal tract. Their loss with the faeces during the passage from the blood stream to the bowel must therefore—it was concluded—be due to diffusion or secretion of these minerals with the digestive juices, and a more or less complete precipitation in the bowel. According to this view the amount of mineral lost from the body via the intestine is the difference between the secreted amount and the reabsorbed amount.

With regard to what happens in the digestive tract to Ca and P in ordinary feeding experiments, we can distinguish between the primary absorption of the exogenous Ca and P, the secretion of these elements with the digestive juices and the secondary absorption of some part of these. The difference between the two latter will form the endogenous fraction of these elements in the faeces. The rest of the Ca and P in the faeces will be the unabsorbed food Ca and P, the exogenous fraction of the faeces.

Previous to a study of the absorption of Ca and P ingested with the food we must therefore make an estimation of the endogenous fraction of these elements in the faeces. When this fraction is known, the absorbed amount of Ca and P in any feeding experiment can be deduced.

The object of this and other papers in this series of publications has been to contribute to the knowledge of how vitamin D decreases the faecal output of Ca and P. As all experiments up to the present have been performed with con-

[1] By the term endogenous Ca and P is understood the amount of body Ca and P lost in the faeces.

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siderable amounts of Ca and P in the diet, there is no evidence concerning the mechanism of the increased loss of Ca and P with the faeces in vitamin D deficiency. This increased loss may be due partly to decreased absorption of ingested Ca and P, partly to decreased secondary absorption of the amount poured out in the digestive tract from the mouth to the small intestine. With regard to the last-mentioned fraction, full information is not available. To the survey given earlier [Nicolaysen, 1934] one can only add the results of Ågren [1935] which demonstrate that much larger amounts of Ca can be secreted with the pancreatic juice than earlier experiments had indicated. It is worth while mentioning that the amount of Ca passing into the digestive tract with the digestive juices may reach 1 g. daily in an adult human being: for calculation see Nicolaysen [1934]. As the loss of endogenous Ca by this route does not on the average exceed 0·2 g. daily in man according to Bauer *et al.* [1929], it is possible that the reduction in its reabsorption may play an important role in the origin of the increased faecal output of Ca in rickets.

As far as we know at present, there is little reason to believe that increased passage of Ca and P into the bowel will occur in vitamin D deficiency. Such an increase may be due either to an increased concentration of these elements in the digestive juices or to increased flow of the latter. The first event seems to happen only when the concentration in the blood is increased. This does not take place in rickets. Increased flow of digestive juices may be due either to increased appetite or to increased food intake. Neither of these exists in rickets.

A study of the endogenous fraction of Ca and P in the faeces can be performed only with a diet containing negligible amounts of these elements. Both human rickets and experimental rickets in animals, however, originate when diets containing considerable amounts of Ca and P are used. It is well known that, apart from the action which vitamin D may have on the fate of Ca and P in the bowel, there is an interaction between these two minerals in the intestinal tract. Telfer [1924] has demonstrated that almost all the P in the food is lost in the faeces when much more Ca than P is given. When the Ca content of the food is reduced far below the content of P, very little P will be lost with the faeces. Different levels of P in the food influence the utilization of Ca to a far less extent. According to Farquharson *et al.* [1931] excess P in the food has no influence upon the Ca which is lost in the faeces. According to the author's experiments [Nicolaysen, 1934] removal of almost all P from a diet rich in Ca increases the utilization of the food calcium only partially. In the above-mentioned experiments with a dog in balance with 660 mg. Ca in the daily ration, the increased utilization was 25–30 %.

At present we do not know if vitamin D influences primarily the absorption of Ca or P. It may well be thought that it acts on both. Knowledge is also lacking about how much of the Ca and P in the faeces in normal and vitamin D-deficient rats represents unabsorbed Ca and P and how much body Ca and P. If vitamin D has a direct action on the absorption of Ca (both from endogenous and exogenous sources), a secondary effect on P absorption (both from endogenous and exogenous sources) will be expected.

With regard to the origin of a possible increased output of endogenous Ca and P in faeces, we must therefore distinguish between the following mechanisms which have to be studied separately:

(1) Output of endogenous P in the faeces in normal and vitamin D-deficient animals as influenced by different levels of Ca in the food.

(2) Output of endogenous Ca in the faeces in normal and vitamin D-deficient animals as influenced by different amounts of P in the food.

With regard to the second group of experiments the phosphorus should be given not only in inorganic form, but also in organic combinations. The reason for this, which will be discussed in more detail in the following paper, is that considerable evidence has lately been accumulated in favour of the interference of vitamin D with the hydrolysis of phosphoric esters in the gut.

Experimental

For the study of Ca and P excretion as outlined above a diet almost entirely free from Ca and P is needed. The author [1934] has, by the use of acid-extracted meat powder, prepared a diet free from Ca but adequate in other respects. The preparation of a diet low both in Ca and in P limited the source of protein to egg albumin. The necessity of mixing exact amounts of different salts in the diet made the use of fat in the diet extremely inconvenient. A diet deficient in several respects, and containing one toxic factor, viz. egg albumin, had to be used, and therefore a strictly limited experimental period was essential.

As intestinal contents may stay in the caecum of the rat over long periods, the caecum had to be amputated. The influence of this operation on the utilization of the food by the rat is almost negligible, as has been reported in a previous paper [Innes & Nicolaysen, 1937].

The experimental periods were limited to four days, with intervals of four to five days on the ordinary Steenbock-Black diet. The first of these four days was used to clear the intestinal tract from preceding Ca- and P-containing diet. Uncoloured Ca- and P-poor diet was used for this purpose. In the two following days—the experimental period proper—the rats were given the experimental diet containing 0·1 % carmine, which stains the faeces bright red. The fourth day, 2–3 hours after all coloured diet was eaten, a new portion of Ca- and P-poor diet was given. In order to get a sharp separation between the last 2 days' portion of faeces, it was necessary to administer 4 % of agar in the diet. When less agar was given some of the carmine stayed behind and the separation was not so good.

The commercial agar used contained 0·5 % Ca. This agar was therefore used only in experiments where more than 4·6 mg. Ca were given in the experimental period. The agar contains no P; the Ca can be completely utilized by the rat and is also readily extracted with acid. In the experiments with less than 4·6 mg. Ca in the diet, extracted agar was used. The extraction is performed as follows. To one portion of powdered agar about ten portions of 0·1 *N* HCl are added. After thoroughly mixing for about half an hour, most of the acid is neutralized by addition of a solution of NaOH. The fluid is filtered on a Gooch crucible and the now swollen agar is washed once with distilled water, dried at 100° and powdered again.

The constituents of the different diets were:

	% Ca	% P
Powdered egg albumin	0·04	0·07
Powdered sugar	0·001	0
"Ashless" extracted caseinogen (Glaxo)	0·02	0·900
Acid-extracted meat powder (for preparation see Nicolaysen [1934])	0·003	0·596
Powdered agar	0·5	0
Powdered extracted agar: preparation I	0·05	—
preparation II	0·12	—

The different salts used as sources of Ca and P were:

	% Ca	% P
Calcium gluconate *pro injectione* "Merck"	8·4	0
$CaCO_3$	40	0
NaH_2PO_4	0	25
KH_2PO_4	0	22·8
Disodium glycerophosphate	0	10·5

The following diets were made up, after the different constituents had been passed through a 40-mesh sieve:

Diet I

Agar	4%
Sodium chloride	1%
Egg albumin	20%
Sucrose	75%

Calcium content: 0·029% (2·3 mg. Ca in the daily ration of 8 g.).
Phosphorus content: 0·014% (1·1 mg. P in the daily ration of 8 g.).

Diet II

Extracted agar	4%
Sodium chloride	1%
Egg albumin	20%
Sucrose	75%

Calcium content: 0·014% (1·1 mg. Ca in the daily ration of 8 g.).
Phosphorus content: 0·014% (1·1 mg. P in the daily ration of 8 g.).

Diet III

Extracted agar	4%
Sodium chloride	1%
Meat powder	20%
Sucrose	75%

Calcium content: 0·0023% (0·2 mg. Ca in the daily ration of 8 g.).
Phosphorus content: 0·119% (9·6 mg. P in the daily ration of 8 g.).

Diet IV

Extracted agar	4%
Sodium chloride	1%
"Ashless" caseinogen	20%
Sucrose	75%

Calcium content: 0·009% (0·7 mg. Ca in the daily ration of 8 g.).
Phosphorus content: 0·18% (14·4 mg. P in the daily ration of 8 g.).

The diets were prepared in amounts up to 2 lb. each at a time and mixed thoroughly. As it was not possible to get the salts absolutely uniformly mixed into the diet, the salts used, except the sodium chloride, were weighed out for each animal, added to the 8 g. of food and mixed with this in a mortar. In the experimental period proper 0·1% carmine was added to the appropriate diet.

The normal rats were kept in exactly the same way as the rachitic, but received vitamin D in the form of a solution of calciferol given by pipette in amounts of 1 drop corresponding to 50 units daily, but administered during the periods of the Steenbock-Black diet only. This diet was given in unlimited amounts; the same was the case with distilled water given in the experimental period.

In most cases the animals finished the measured amount of food within the 48 hours. Sometimes a little was left, but was regularly finished in the following hours. Occasionally more food was left than was eaten during the first hours of the third day, and the experiment was then discontinued.

The passage from the mouth to the anus in the rats without caecum generally took from 6 to 12 hours, i.e. when coloured food was given, the first portion of coloured faeces appeared 6–12 hours afterwards. When uncoloured diet followed the coloured, it was generally 6–12 hours before the first uncoloured portion of faeces was passed. Occasionally the passage lasted nearly 24 hours. It happened in a few of the 100 animals used in these experiments that diarrhoea occurred; the experiment was then discontinued. The distinction between coloured and uncoloured faeces was sharp and clear when 4% agar and 0·1% carmine were used.

The growth rate of the rats fed on the diets mentioned was about 1 g. daily during the whole time they were used for experiments. This was regular in the normal rats, whereas the vitamin D-deficient rats stopped growing after 4–7 weeks. A new lot of rats was then operated on, and new normal rats as well as vitamin D-deficient rats were taken for experiment. Rats of approximately the same size were therefore always used. They weighed about 50 g. when operated on and were taken for experiment about 10 days afterwards, during which time they were fed on the rachitogenic ration. Occasionally one rat was used twice for the same type of experiment. Most of the figures in each type of experiment conducted on 10–16 rats represent results from the same number of different rats.

In no case was any toxic effect observed with egg albumin. Querido [1935] who used 10% egg albumin regularly in the diet of growing rats also did not observe any toxic effect.

The animals were kept in large metabolism cages; the urine was absorbed on thick blotting paper. This leaves the faeces free from any contamination with urine. The collected faeces were ashed with nitric acid and 2 ml. of 60% perchloric acid. The ash solutions were transferred to volumetric flasks, and Ca and P were estimated in aliquot parts, Ca by oxidimetric determination of the oxalate, P by the Fiske and Subbarow colorimetric method.

I. Phosphorus

The excretion of P in Ca and P starvation

The daily ingestion of 1·1 mg. P together with very little Ca (see Table I) in the normal rat is followed by an output of 0·75–1·35 mg. P in the faeces per day, or roughly the same amount as ingested. Since it is uncertain whether the average output of 1 mg. P daily represented chiefly unabsorbed food P or endogenous P, the output of P was next studied on a P-free diet. The ration used for this purpose consisted of 95% sucrose, 4% agar and 1% sodium chloride, coloured as before. This diet was given in amounts of 8 g. daily to 10 normal rats in the experimental period. The results in these 10 rats were in mg. of P per 2 days: 1·7, 1·7, 1·8, 2·0, 2·0, 2·1, 2·1, 2·2, 2·3, 2·7, with an average of 1·0 mg. P per day. Although the diet used is extremely deficient and the results have to be interpreted with some care, it is clear that the P of the egg albumin is completely utilized. The P found in the faeces when egg albumin is the source of P is therefore endogenous P only.

With regard to the vitamin D-deficient rats fed on the diet poor both in Ca and P, we find a slightly increased output, as measured by the average of

16 experiments in this group. This higher average is caused by a definitely higher output in 5 out of the 16 experiments. A comparison between the single figures in the two series of experiments given in Table I leaves no doubt however about the significance of the higher average in the vitamin D-deficient rats.

Table I. *The output of endogenous Ca and P in faeces of normal and vitamin D-deficient rats*

Ca and P given in terms of mg. per 2 days.
Diet I (source of P: egg albumin). 4·6 mg. Ca, 2·2 mg. P in the food.

	50 units vitamin D daily		Without vitamin D	
	Ca mg.	P mg.	Ca mg.	P mg.
	0·5	1·5	2·0	2·6
	0·5	1·5	2·1	2·1
	0·5	1·8	2·2	2·5
	0·5	2·3	2·6	2·2
	0·6	2·0	3·2	2·4
	0·7	2·2	3·8	2·6
	0·7	2·5	3·9	3·1
	0·9	1·7	4·0	3·5
	0·9	2·7	4·1	2·0
	0·9	2·0	4·2	2·5
	1·0	2·2	4·5	2·5
	1·1	2·1	4·9	2·2
	1·2	1·6	5·6	2·8
	1·5	2·0	5·8	2·0
	1·7	2·0	7·2	4·1
	2·7	2·0	8·5	4·0
Av.	0·9	2·0	4·3	2·7
Daily av.	0·45	1·0	2·2	1·4

The excretion of P as influenced by increasing levels of Ca in the diet

The increased output of endogenous P in the vitamin D-deficient rats is intimately connected with an increased output of Ca in the faeces and is no doubt secondary to the increased amount of Ca in faeces. This follows from the experiments with increased levels of Ca in the food (Table II).

In the normal rats the ingestion of 15 mg. Ca daily is not followed by any increased output of P in the faeces, the 15 mg. being completely absorbed. Ingestion of 45 mg. Ca daily, an amount which is not completely absorbed, results in an average increase of 45 % in the output of endogenous P in the faeces. When 45 mg. Ca daily were given as Ca gluconate, severe diarrhoea occurred, and the rats stopped eating. When 90 mg. Ca are given in the daily ration, the output of endogenous P is increased by 75 %. The 90 mg. Ca administered as carbonate are more than the rats can dissolve in their stomachs and unchanged $CaCO_3$ can be observed in the faeces excreted in the experimental period. The maximum effect of ingested Ca upon the output of endogenous P which can be obtained in young normal rats is therefore an increase of up to nearly double the amount lost with faeces in Ca starvation. But the conclusion that incomplete absorption of Ca is followed by an increased output of endogenous P in faeces is well substantiated.

The same effect is seen in the vitamin D-deficient rats. The ingestion of 15 mg. Ca in the daily ration increases the output of endogenous P by about 40 %, and 90 mg. of Ca by about 140 %. Ingestion of 45 mg. of Ca in the daily

Table II. *The output of endogenous P in the faeces, in normal and vitamin D-deficient rats, as influenced by different levels of Ca in the food*

Ca and P given in terms of mg. per 2 days.

	Diet I (added calcium gluconate). 30 mg. Ca, 2·2 mg. P in the food.				Diet I (added calcium carbonate). 90 mg. Ca, 2·2 mg. P in the food.			
	50 units vitamin D daily		Without vitamin D		50 units vitamin D daily		Without vitamin D	
	Ca mg.	P mg.	Ca mg.	P mg.	Ca mg.	P mg.	Ca mg.	P mg.
	—	—	—	—	—	—	54	2·6
	0·8	1·7	18	3·1	31	2·5	49	2·7
	0·9	1·8	21	3·1	33	2·5	52	2·7
	1·0	1·9	17	3·4	42	2·5	65	2·7
	1·6	1·9	19	3·4	28	2·6	58	2·8
	0·7	2·0	14	3·6	39	2·7	55	2·9
	1·0	2·0	18	3·6	42	2·8	57	2·9
	1·2	2·0	20	3·9	39	2·9	56	3·0
	1·3	2·2	20	4·2	43	3·0	59	3·0
	1·3	2·2	22	4·6	41	3·2	48	3·2
	2·5	2·5	21	4·8	38	3·8	71	4·1
Av.	1·2	2·0	19	3·8	38	2·9	57	2·9
Daily av.	0·6	1·0	9·5	1·9	19	1·5	28·5	1·5

Diet I (added calcium carbonate). 180 mg. Ca, 2·2 mg. P in the food.

	50 units vitamin D daily		Without vitamin D	
	Ca mg.	P mg.	Ca mg.	P mg.
	62	2·5	142	5·7
	102	3·0	122	5·8
	99	3·2	142	5·8
	96	3·3	143	6·2
	99	3·5	146	6·3
	94	3·6	148	6·4
	115	3·7	157	6·6
	101	4·0	162	7·0
	103	4·0	142	7·2
	100	4·2	150	7·4
Av.	97	3·5	144	6·4
Daily av.	43·5	1·8	72	3·2

ration resulted in these experiments, however, in a smaller extra output of P in faeces than did 15 mg. This paradoxical result needs explanation.

First it must be pointed out, however, that the main results found here fit in well with the explanation put forward already in the introduction about the effect of ingested Ca on the output of endogenous P in faeces, viz. one of precipitation and prevented reabsorption. Earlier investigators (for references see the following paper [Nicolaysen, 1937]) have repeatedly shown that the output of Ca and P in the faeces following ingestion of given amounts of these elements in the food is dependent on the stage of vitamin D deficiency. Shortly after the administration of a diet deficient in vitamin D, the output of Ca and P in the faeces is slightly increased, but it is largely increased when the animals have been given the vitamin D-deficient diet over a long period. The same observation has been made here with regard to the output of endogenous Ca and P, as well as exogenous Ca. The stage of vitamin D deficiency is therefore a determining factor with regard to the output in faeces. The rats given 15 and 90 mg. of Ca were in a late stage of vitamin D deficiency, the rats given 45 mg. Ca in an early stage. These experiments therefore establish the rather paradoxical fact that ingestion of a smaller amount of Ca is followed by a larger output of endogenous P in faeces in a late stage of vitamin D deficiency, than is the ingestion of a greater

amount of Ca when in an early stage of vitamin D deficiency, although the output of Ca in faeces is greater in the latter case. A more exact explanation cannot be given before more is known about the state of Ca and P in the intestinal contents.

A comparison of the output of Ca and P in the faeces in Ca and P starvation

It is seen in Table I that more Ca than P is excreted in the faeces in normal rats. If the output of endogenous P were a result of a precipitation by Ca, less P than Ca would appear in the faeces. As the diet contained only traces of other metals which could precipitate P, it may be that the part of the faeces formed in the gut (desquamated epithelium, growth of microbes, etc.) could account for this larger output of P than of Ca in the normal rats.

The partition of the extra loss of the endogenous phosphorus in the reduced retention of phosphorus in vitamin D deficiency

It has been pointed out in the preceding discussion that the effect of ingestion of larger amounts of Ca is chiefly dependent on the stage of vitamin D deficiency. Almost all diets used in experimental rickets contain more Ca than even the normal rat is able to absorb; accordingly the output of endogenous P in an early stage of vitamin D deficiency will be about the same in normal and in vitamin D-deficient rats. As rats fed on a rachitogenic ration generally develop rickets in a fortnight, and as according to the author's observation it takes at least 4–7 weeks to get the rats into what is here called a late stage of vitamin D deficiency, it is clear that the output of endogenous P in the faeces does not take any part in the reduced retention of P during the period in which rickets is developing in rats. Only in the case of rachitogenic rations with amounts of Ca limited to what the normal rat is able to absorb completely will an increased output of endogenous P start at once. As such rations are used only to produce the so-called "low calcium rickets" where the diet is much richer in P this border line case will be of no practical interest. In the maintenance of the reduced retention of P in the late stage of vitamin D deficiency, however, the increased output of endogenous P will participate to a certain extent. The rest of the reduced retention will be accounted for by the reduced absorption of ingested P, induced by the reduced absorption of ingested Ca. No general statement can be made about the partition between the increased faecal output of endogenous and exogenous P, as this will depend not only on the daily requirement, which again depends on the growth rate, but also on the amounts of Ca and P fed together with the Ca/P ratio. It is only in a late stage of vitamin D deficiency with a given amount of Ca in the diet that the output of endogenous P is increased above the level found in the normal rat. In this stage the growth rate is sinking towards zero, and the daily requirement is consequently reduced.

To get an approximate idea however, we may correlate the increased output of endogenous P, which ranges around 1·5 mg. a day, with the daily requirement of the growing rat. According to Sherman & Quinn [1926] rats between 30 and 60 days of age need about 5 mg. P daily per 1 g. increase in body weight. The average normal growth rate is about 3 g. daily, but on Steenbock-Black diet it is about 1 g. daily only. In the first case the rat needs 15 mg. P daily, in the second about 5 mg. It is thus seen that the extra output of endogenous P is rather small compared with the daily requirement.

An objection against these conclusions is that the rations on which rickets generally develops contain both P and fatty acids, both of which by combining

with the Ca present may reduce the amount of endogenous P excreted with the faeces. The extra output observed may therefore be larger than when the usual rachitogenic rations are used.

II. Calcium

The output of Ca in Ca and P starvation

With regard to the output of Ca on a diet poor both in Ca and in P (Table I), a significant difference is observed between the normal and the vitamin D-deficient rats. The daily output of Ca varies in the normal rats between 0·25 and 1·35 mg. in the rats deficient in vitamin D between 1 and 5 mg. The experiments in which there was practically no Ca in the diet, as when the acid-extracted meat powder was used (Table III), leave no doubt that the Ca in the faeces is almost entirely endogenous Ca. The variation in the faecal Ca, however, is much greater

Table III. *The output of endogenous Ca in the faeces, in normal and vitamin D-deficient rats, as influenced by different forms of P in the food.*

Ca and P given in terms of mg. per 2 days.

Diet II (added NaH_2PO_4). 2·2 mg. Ca, 30 mg. P in the food.

	50 units vitamin D daily		Without vitamin D	
	Ca mg.	P mg.	Ca mg.	P mg.
	0·5	2·2	2·1	2·1
	0·6	1·8	2·5	3·1
	0·7	1·7	2·6	3·3
	0·8	2·0	2·7	2·6
	0·8	2·1	2·8	2·6
	0·9	2·2	2·9	2·2
	0·9	2·3	3·0	2·9
	1·4	1·8	3·1	2·9
	1·8	2·2	3·2	2·8
	2·7	2·6	4·7	3·1
Av.	1·1	2·1	3·0	2·8
Daily av.	0·6	1·1	1·5	1·4

Diet III (source of P: meat powder). 0·4 mg. Ca, 19·2 mg. P in the food.

	50 units vitamin D daily		Without vitamin D	
	Ca mg.	P mg.	Ca mg.	P mg.
	0·6	4·4	4·5	6·6
	0·8	4·5	4·8	5·8
	0·8	4·8	4·9	5·0
	1·1	4·6	5·7	5·6
	1·1	5·2	5·8	5·3
	1·2	4·7	5·9	6·1
	1·2	4·9	6·3	5·7
	1·4	4·4	7·0	5·0
	1·5	4·8	8·1	5·2
	2·4	5·7	10·1	6·8
Av.	1·2	4·8	6·3	5·7
Daily av.	1·1	2·4	3·2	2·9

Diet II (added alkaline sodium glycerophosphate). 2·2 mg. Ca, 30 mg. P in the food.

	50 units vitamin D daily		Without vitamin D	
	Ca mg.	P mg.	Ca mg.	P mg.
	0·8	1·7	2·1	2·0
	0·8	1·9	2·4	2·3
	0·8	2·0	2·5	2·7
	0·9	2·3	2·9	2·8
	1·3	2·2	3·0	2·6
	1·5	1·9	3·1	3·0
	1·6	1·9	3·3	2·8
	1·6	2·4	3·6	2·2
	1·8	2·6	3·9	3·1
	2·0	2·1	4·1	3·3
Av.	1·3	2·1	3·1	2·7
Daily av.	0·7	1·1	1·6	1·4

Diet IV (source of P: "ashless" caseinogen P). 1·4 mg. Ca, 28·8 mg. P in the food.

	50 units vitamin D daily		Without vitamin D	
	Ca mg.	P mg.	Ca mg.	P mg.
	0·6	2·0	2·4	2·3
	0·7	2·3	2·5	2·0
	0·8	2·1	2·6	2·5
	0·9	2·1	2·8	2·5
	1·1	1·8	2·9	2·1
	1·1	1·9	3·0	2·4
	1·1	2·2	3·2	3·4
	1·2	1·9	3·7	3·3
	1·3	2·2	3·9	3·3
	1·4	2·2	4·1	3·7
Av.	1·0	2·0	3·1	2·7
Daily av.	0·5	1·0	1·6	1·4

than in the faecal P. The author [Nicolaysen, 1934] found about the same variability in experiments with dogs. These were kept under the most constant experimental conditions and received a constant amount of vitamin D. A

systematic analysis of the faeces revealed nothing as to the cause of this variability. With regard to the rats on a vitamin D-free diet, some part of the variability is due to vitamin D being stored in different amounts in the animal organism previous to the experiments. It is seen that the rats fed on diet III (see Table III) with 0·2 mg. Ca in the daily ration lost definitely more Ca in the faeces than did the rats fed on diet I with 2·3 mg. Ca in the daily ration. The output of P in the experiments with diet III was definitely higher than when diet I was fed, but this increased output of P was not connected with the increased output of Ca, since the same increase in the output of P was seen also in the normal rats fed on meat powder, but the output of Ca in these rats was not at all affected. That the amount of P ingested in the meat powder is not connected with the increased output of Ca in these rachitic rats is further substantiated by the fact that ingestion of larger amounts of P does not affect the output of Ca in faeces. This is the case both when inorganic P, glycerophosphate and caseinogen are given in the diet.

The output of Ca in early and late stages of vitamin D deficiency

The rats given 0·2 mg. Ca (see Table III) daily were in a late stage of vitamin D deficiency, the experiments having been performed in the sixth week of vitamin D starvation. The rats given 2·3 mg. Ca daily belonged to two different groups of animals. In the one group, the experiments were performed in an early, in the other group in a late, stage of vitamin D deficiency. The experiments with the first group gave the following outputs of Ca: 2·0, 2·1, 2·2, 2·6, 3·2, 3·8, 3·9, 4·0, 4·5, 5·6, with an average of 3·4, whereas the results in the second group were 4·1, 4·2, 4·9, 5·8, 7·2, 8·5, with an average of 5·8 mg.

The explanation is that the animals put on a rachitogenic ration have small stores of vitamin D in their bodies. These stores are gradually exhausted, probably completely when the animals stop growing. That the increased output is not due to the effect of mineral starvation on the cells is demonstrated by the fact that the animals in a late stage of vitamin D deficiency react immediately to a dose of vitamin D with a return to the normal output of Ca in the faeces.

Four of the animals presumably completely deprived of their stores of vitamin D were given 150 units vitamin D and put on experiment next day. Diet I was used; the average output of Ca in the faeces was 1 mg. in the experimental period, as compared with about 5 in the preceding period.

It seemed possible that the variability found in the late stage of vitamin D deficiency was due to a variation in the time different rats take to exhaust their previously acquired stores of vitamin D. This cannot be the case however as experiments on rats completely deprived of their previous stores of vitamin D showed the same variability.

To illustrate this, experiments were performed on rats which were kept alive on a diet which was vitamin D-free but well balanced with regard to Ca/P. Of a batch of 12 rats, 5 were alive 50 days after they had stopped growing, and in a good enough condition to pass through an experiment. A repetition of experiments made in the same way as those given in Table I gave the following figures for the output of Ca in the faeces in the two days' experiment: 4·3, 4·5, 4·8, 6·8, 8·6 mg. Ca.

The effect of different forms of phosphorus on the output of endogenous calcium in the faeces

The results of experiments to test the ability to absorb P from different sources are given in Table III. It has already been mentioned that, although ingestion of acid-extracted meat powder is followed by an increased output of P

in the faeces, the output of endogenous Ca is unaffected. Ingestion of inorganic P, glycerophosphate or of caseinogen was likewise without effect upon the output of endogenous Ca. That this is the case in the normal rats is beyond doubt, for the outputs of Ca and P in the faeces are the same as on rations poor in Ca and P. With regard to the rachitic rats, the present experiments are readily enough interpreted in the same way. More care is needed however in the interpretation of results obtained with rachitic rats, as the results in these vary according to the length of the period during which they have been fed on a diet free from vitamin D. The experiments, except those with meat powder, were all performed within the first three weeks of vitamin D starvation, and the results are seen to correspond as well as can be expected with those obtained in Ca and P starvation experiments in an early stage of vitamin D deficiency. It can therefore be justifiably concluded that the amounts of P ingested in these experiments did not affect the output of endogenous Ca in the rachitic rats.

It is seen that the amounts of P ingested are moderate. It was impossible to increase the amount ingested, however, and at the same time feed a measured amount of food. Even with the amounts here ingested more rats than usual refused to eat their food completely, and several experiments had to be discontinued. The cause seemed to be latent tetany. The limbs were rigid and the rats more irritable than usual.

The amount of sodium glycerophosphate ingested corresponded to 0·175% P in the diet. Whereas this is a diet low in phosphate, it must be remembered that a considerable amount of the P in the usual rachitogenic rations is not present in ester form. The only ration on which rickets can develop, containing more ester P than corresponds with the amount ingested as glycerophosphate in the above experiment, is a pure milk ration, on which rickets occasionally develops in children. The rations used for experimental rickets always contain less organic phosphate than was present in the above experiment. The present test has therefore been performed with at least as much organic phosphate as is found in almost all rachitogenic rations, and it must be concluded that organic phosphate ingested with the food does not influence the faecal output of endogenous calcium in vitamin D deficiency.

The partition of the increased output of endogenous Ca in the decreased retention of Ca in vitamin D deficiency

The first question which arises is as to the figure to be used for calculation. Both the output of endogenous Ca and the absorption of ingested Ca depend on the stage of vitamin D deficiency. The 16 experiments summarized in Table I were performed partly in an early and partly in a late stage; we can therefore use the daily average of these experiments, i.e. 2·2 mg., as an approximately correct expression of the output of endogenous Ca. The normal rats lose on the average 0·5 mg. per day. The extra output is thus 1·7 mg. Ca daily. The absorption of Ca in rickets varies, according to all earlier experiments, from slightly below normal to zero. The author found in metabolism experiments with Steenbock-Black diet, as reported in a previous paper [Innes & Nicolaysen, 1937], that the net amount absorbed in the normal rats was 34 mg., in the vitamin D-deficient rats 15 mg. The amount absorbed in the normal rats is then 34+0·5 mg. Ca daily, and in the rachitic rats 15+2·2 mg. daily. The experiments with the Steenbock-Black diet were performed from the tenth to the twenty-fourth day of the experiment, i.e. in the period between an early and a late stage of vitamin D deficiency. Karelitz & Shohl [1927] found during 5 weeks' experiments about the same average absorption of Ca in rachitic rats fed on Steenbock-Black

diet modified by addition of 10% of fat. No experiments were performed on normal rats. As these two series of experiments have given the same results with rachitic rats, the difference (34·5 − 17·2 = 17·3 mg. Ca) can be accepted as an approximately correct expression of the average difference in the absorption of Ca as between normal and vitamin D-deficient rats.

The difference in the net amount absorbed between the two groups was 19 mg. 17·3 mg. of this, or 91%, is due to extra loss of ingested Ca, and 1·7 mg. or 9% to extra loss of endogenous Ca. If in an analogous calculation we use the figures obtained for the average absorption in the experiments with 15, 45 and 90 mg. Ca in the daily ration, the following figures result:

	Extra loss of endogenous Ca %	Reduced absorption of food Ca %
Experiments with 15 mg. Ca	18	82
Experiments with 45 mg. Ca	14	86
Experiments with 90 mg. Ca	7	93

It is assumed here that the output of endogenous Ca is not increased in the absorptive and post-absorptive periods. To prove or disprove this is impossible. The author in experiments with dogs has shown, however, that no extra excretion takes place in the faeces even after large amounts of Ca are injected hypodermically. The same experiments repeated in normal rats gave analogous results, whereas in vitamin D-deficient rats a slight extra output was seen in some cases. (For a further discussion of this see below.) Since any extra output of Ca with the digestive juices, according to our present knowledge, must be deemed to be due to an increase in the Ca concentration of the blood, and as all experiments up to the present leave doubt as to whether ingestion of even large amounts of Ca in the food is followed by any increase in the concentration of Ca in the blood, it is justifiable to make the above assumption.

Although such a calculation may be liable to a considerable error, this error is not of such a magnitude as to invalidate the conclusion to be drawn: i.e. the reduced absorption of food Ca is a much more important factor in the reduced retention of Ca in vitamin D deficiency than is the output of endogenous Ca in the faeces. It must be added that all authors agree in finding a reduced output of Ca in the urine in rickets, so that this path of excretion endeavours to compensate for the increased loss of Ca in the faeces.

Parenteral injections of calcium and phosphorus

Nicolaysen [1934] has reviewed earlier experiments and also reported on experiments with parenteral injections of Ca in dogs. The results of these experiments were that roughly 30% of the amount injected was excreted in the urine, whereas no appreciable extra loss took place in the faeces.

Although increase in blood Ca tends to increase the output of Ca in the digestive juices, the increase is neither constant nor large. The extra output which may follow an injection must in any case be of moderate amount, and according to experiments with normal dogs will be completely reabsorbed. Similar experiments have never been performed with rats, however, and were needed therefore both in normal and vitamin D-deficient rats where the reabsorption is assumed to be decreased.

The experiments with ingestion of P without any Ca in the diet demonstrate that the P is completely absorbed, and that there is no re-excretion of P ingested without Ca, either in normal or in vitamin D-deficient rats. It has been claimed

by earlier authors, however [see Klinke, 1931], that Ca and P are re-excreted in the intestinal tract when ingested together. The reason given for this claim was that an increased output of these elements was observed in the faeces when they had been ingested together in the food. The present author interpreted this result not as re-excretion but as precipitation and prevented re-absorption. This view could be substantiated by making a combined injection of Ca and P.

Pappenheimer [1924] has shown that injection of 5 mg. P as KH_2PO_4 daily will cure rickets in rats. Kay & Skill [1934] were able to cure beryllium rickets with about the same amount of P injected as glycerophosphate. This amount of P was therefore chosen as the daily dose of P to be injected. The author [Nicolaysen, 1934] was not able to inject more than 300 mg. Ca daily, in the form of gluconate, into dogs of 8 kg. In preliminary experiments with rats, the largest amount of Ca which was found to be tolerated without any reduction in the food intake was 8 mg. daily.

Sterile solutions of the potassium phosphate, containing 5 mg. P per ml. and of Ca gluconate, containing 4 mg. Ca per ml., were injected. The solution of Ca gluconate contained 5% of this salt. A 10% solution was not tolerated so well by the rats, infiltration and necrosis occurred occasionally. The injections were given each morning for 3 days, including the two experimental days and the day before. Each injection was given hypodermically, Ca gluconate in one place, potassium phosphate in another, under the skin. The injections seemed to upset the rats for most of the day; during the night they compensated for it by a greater food intake. More experiments than usual had to be discarded owing to the inadequate food intake.

The results are given in Table IV. It will be seen that the output of Ca in the normal rats is the same as in the Ca and P starvation experiments, both with regard to the range of variability and the average. The output of P is increased

Table IV. *The output of Ca and P in the faeces in normal and vitamin D-deficient rats, as influenced by injections of Ca and P*

Ca and P given in terms of mg. per two days.

Diet I. Injected 8 mg. Ca as gluconate and 5 mg. P as KH_2PO_4 daily, on the two experimental days and the day before. 2·3 mg. Ca, 2·2 mg. P in the food.

	50 units vitamin D daily		Without vitamin D	
	Ca mg.	P mg.	Ca mg.	P mg.
	—	—	3·0	2·0
	0·7	2·2	3·1	2·5
	0·7	2·2	3·1	2·6
	0·7	2·4	3·4	2·4
	0·8	1·8	3·5	2·1
	0·8	2·1	3·7	3·3
	0·8	2·3	4·1	2·1
	0·9	2·7	7·0	2·6
	1·1	2·3	7·7	2·8
	1·1	2·0	12·7	3·2
	1·2	2·5	13·1	4·1
Av.	0·9	2·3	6·1	2·7
Daily av.	0·5	1·2	3·1	1·4

by an average of 15%, but as no figure exceeds the maximum obtained in the starvation experiments, the slight increase must be considered as insignificant. This conclusion is substantiated by the previously mentioned fact that a double amount of P ingested with the food does not give rise to any extra output of P in the faeces.

The same argument as above can be used also about the output of P in the vitamin D-deficient rats, and it can be concluded that the injection of enough P to cover the daily requirement does not lead to any extra output of P in the faeces.

The interpretation of the results with regard to the output of Ca in the faeces of vitamin D-deficient rats meets with more difficulties. The range of variation is wider than in any previous experiments and two figures definitely exceed any earlier figures obtained in Ca starvation, including those obtained in the experiments with meat powder (Table III). The rats used in these experiments had been fed on a diet deficient in vitamin D for 6 weeks when the experiments were performed; they were thus in a relatively late stage of vitamin D deficiency. A comparison must therefore be made with the results of Ca starvation experiments performed in a late stage of vitamin D deficiency. These results range from 4·1 to 10·1 mg., with an average of about 6 mg. Of the figures found in the injection experiments only two exceed the maximum found in Ca starvation, and the average is about the same. The results appear to indicate, although it is difficult to obtain direct proof, that only rats which have the largest output of endogenous Ca show an additional increase of Ca in the faeces when Ca is injected. Assuming that the cause of the large output is that the secondary absorption is most seriously affected in these cases, the extra output would be explained by the extra output in the digestive juices, as the result of the injection of Ca, and a faulty reabsorption.

Even the rats which certainly have suffered an extra loss of Ca in the faeces as a consequence of Ca injection do not lose more endogenous P than do rats in Ca and P starvation. According to the earlier opinion, as expressed by Klinke [1931], an injection experiment as performed here would be followed by an extra output of Ca phosphate from the blood stream into the bowel. The present experiments furnish a further argument in favour of the view of the writer: the fate of Ca and P in the digestive tract, both from endogenous and exogenous sources, is determined by limited primary and secondary absorption, and any increased faecal output is chiefly determined by these factors.

Summary

The outputs of Ca and P in the faeces on a Ca- and P-free diet have been studied (*a*) in rats receiving 50 I.U. vitamin D daily, (*b*) in vitamin D-deficient rats.

(1) The output of Ca in Ca and P starvation varies between 1 and 5 mg. daily in vitamin D-deficient rats, as compared with 0·25–1·35 mg. in the normal rats. The output of Ca in the former group is partly dependent on the stage of vitamin D deficiency, the larger figures being obtained in a late stage only.

(2) The output of Ca in Ca starvation is not affected by ingestion of inorganic phosphate, sodium glycerophosphate, acid-extracted meat powder or caseinogen.

(3) The output of Ca in the faeces of vitamin D-deficient rats is occasionally increased to a slight degree by parenteral injections of Ca. This is not the case in normal rats.

(4) The output of P in Ca and P starvation varies between 1·0 and 2·1 mg. daily in vitamin D-deficient rats, as compared with 0·75–1·35 mg. in normal rats. The largest figures are observed only when the output of endogenous Ca is largely increased.

(5) The output of P in P starvation is increased both in normal and vitamin D-deficient rats by ingestion of Ca. The percentage increase depends on the amount of Ca given and is the same in both groups in an early stage of vitamin D deficiency. In a late stage, however, the increase is larger in the vitamin D-deficient rats and reaches about 140 % above the average in Ca starvation, when large amounts of Ca are given, as compared with an increase of 75 % above the starvation level in normal rats.

(6) The output of P in the faeces is not affected by simultaneous injections of Ca and P.

Thus these experiments in the rat demonstrate (*a*) that the output of endogenous Ca in the faeces is increased in vitamin D deficiency; occasionally it may be slightly further increased by injections of Ca, but is not influenced by ingested P; (*b*) that the output of endogenous P is increased by ingestion of Ca in all rats, is increased more in vitamin D-deficient than in normal rats, in a late stage of vitamin D deficiency, but is not affected by injections of Ca and P.

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