A LOW PHOSPHORUS DIET AND THE RESPONSE OF RATS TO VITAMIN D₂

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For the past decade or more the experimental production of rickets in rats has generally involved the use of cereal rachitogenic diets (Sherman and Pappenheimer (1), McCollum et al. (2), and Steenbock and Black (3)). Metabolic studies have revealed that the rickets thus produced is referable to a phosphorus deficiency in the growing animal, as indicated by a lowered inorganic P content of the blood and a lowered ash content of the bones. However, Bruce and Callow (4) and Lowe and Steenbock (5) have shown that the cereal, so called “low phosphorus” diets are so in effect only because of the low availability of their phytin phosphorus. Giri (6) reports that 50 to 70 per cent of the phosphorus of cereals is phytin phosphorus. Maize, the main constituent of the Steenbock-Black diet, is reported as having 69.1 per cent of its P present as phytin P. Giri (6) has also shown that the phytase activity of cereals is in inverse relation to the phytin content. Patwardhan (7) has demonstrated a low phytase activity in the intestine of the rat. Phytases evidently can introduce a factor of uncertainty in studies in phosphorus metabolism, for it is obviously essential that all sources of physiologically available phosphorus be known and controlled. Synthetic diets actually low in total phosphorus would offer obvious advantages over those composed of naturally occurring foods.

* A preliminary report of this investigation was presented at the Thirty-second annual meeting of the American Society of Biological Chemists at Baltimore, April 2, 1938.

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Basal Diet—In designing a low P diet otherwise nutritionally adequate the Ca content was fixed at a level commonly met with in synthetic diets; viz., 0.57 per cent. The vitamin D content was varied. Suitable proteins were available only from limited sources. Most proteins are either biologically incomplete and too high in P or too difficult of preparation in quantity. Jones (8) has recently suggested the use of alcohol-extracted blood fibrin in rachitogenic diets. Commercial egg white (Stein-Hall) was finally selected as the most suitable. Before use it was steamed for 3 hours to destroy toxic factors (Parsons (9)). The water-soluble vitamins were at first introduced in the form of water extracts of yeast in which the P content had been reduced by precipitation with iron salts. Supplements of alfalfa extracts were also used. Later these were replaced with vitab, a concentrated extract of rice bran and whey. Salts were provided as Wesson salts (10) from which the P-containing salts had been eliminated by substitution with KCl, Ca lactate, and Fe citrate.

The selection of the other components of the diet offered no particular problem, inasmuch as they were available in quantity and relatively pure. For most of the carbohydrate a commercial glucose of high purity (cerelose) was selected. It had a P content less than 0.01 per cent. For fat a commercial refined cottonseed oil (Wesson oil) was chosen. Vitamin A was supplied as the provitamin, β-carotene, 120 micrograms per rat per week, dissolved in Wesson oil. When vitamin D was added, it was supplied as a Wesson oil solution of calciferol. The Wesson oil itself was found to contain less than 0.001 per cent P.

For convenience the vitab was incorporated in the diet by evaporating an appropriate amount of a water solution on cooked starch at room temperature before a fan. When so prepared, no loss of growth-promoting properties was observed after a storage period of 3 months.

The basal diet (Diet R-14) had the following composition: cerelose (glucose) 49, egg white 18, cooked starch 20, vitab 4, P-free salts 4, cottonseed oil 5. It contained 0.04 per cent P. When supplemented with 1.80 parts of NaH₂PO₄·H₂O (Diet NR-14), it contained 0.41 per cent P. It was then identical in

1 Vitab, Type II, was kindly supplied by Vitab Products, Inc., Emeryville, California.
P content with Diet R-14 when supplemented with 4 per cent of Wesson salts (Diet WR-14). That the organic constituents were satisfactory for normal nutrition was demonstrated with Diet WR-14 supplemented with 600 i. u. of vitamin D per rat per week. On it growth was entirely normal; male rats gained on an average 4.0 gm. daily in 6 weeks. After 7 weeks, when two males and two females reared on the diet from a weanling weight of approximately 50 gm. each were mated, pregnancy was induced and normal young were born at term. The does nursed the young in litters of six. After weaning at 4 weeks, a second generation was also reared. The animals of this litter grew at approximately the same rate as the first. A male and a female of this second generation were bred and a third generation litter was born, suckled, and weaned.

Effect of Low Phosphorus Diets without Vitamin D

Growth—When young growing rats weighing 50 to 60 gm. were placed on the low P diet (R-14), they grew at a much slower rate than rats on the same diet supplemented with adequate amounts of P (Diet NR-14) (Fig. 1). Growth on the low P diet reached its maximum at about the 4th week, followed by a rapid decline in weight and death. Most of the animals died by the 6th week. Only one animal out of 50 survived for 8 weeks.

Rachitogenesis—Diet R-14 was strongly rachitogenic. After 12 days the distal ends of the radii and ulnae revealed a rachitic metaphysis as wide as that produced in 21 days by a cereal rachitogenic diet (Ration 2965, Steenbock-Black). By the 4th week skeletal abnormalities were marked. Muscular tone was poor and a general lethargic condition prevailed. In advanced cases the hind limbs were paralyzed. Loss in weight occurred in spite of good food consumption. At autopsy, ventral spinal curvature, single or double, was easily recognized in the lumbar region. Sternal curvature also occurred. The costochondral junctions were enlarged. All bones were soft.

Other Symptoms—In the 3rd or 4th week an encrustation of dried blood and exudate accumulated around the nares. Breathing was labored and râles were audible. These symptoms were not observed in rats kept under identical conditions except for adequate supplements of P. Eppright and Smith (11) have reported
the production of nasal encrustations on diets low in mineral elements. They found the symptoms to disappear after dietary additions of calcium phosphate. These authors concluded that the respiratory difficulties were not referable to pulmonary infections, as postmortem examination failed to reveal any pulmonary lesions. In our own experiments the locus of the exudates was likewise confined to the nasal pharyngeal region.

Although an adequate supply of β-carotene was furnished in the diet, the possibility that a diet deficient in phosphorus might influence unfavorably its absorption or transformation to vitamin A was investigated. Two rats exhibiting marked signs of P deprivation after 4 weeks were killed and the vitamin A content of the livers determined by a modification of the method of Davies (12). A vitamin A content of 75 and 108 blue units respectively was found. A deficiency of this dietary essential was therefore excluded.

Hematopoiesis—Orten, Smith, and Mendel (13) have reported that salt-deficient diets produced a polycythemic anemia which yielded to dietary supplements of CaCO₃ and, less effectively, to FeCl₃. As their low salt ration contained casein as a source of P, there was a possibility that this P might have been responsible for the polycythemia. This was given some credence from the work of Swanson, Timson, and Frazier (14) who reported less drastic changes in the blood when edestin was fed in place of casein.

Determinations of the hemoglobin content of tail blood in rats on our low P ration, Diet R-14, made at weekly intervals by the method of Newcomer (15) failed to reveal that lack of P had any effect on hematopoietic activity. Four rats on the low P diet had an average Hb value of 14.65 gm. per 100 cc. at the end of the 4th week. Two of these died during the 5th week. The initial Hb value of the four rats was 13.15 gm.

Creatine Metabolism—It has already been remarked that the loss in weight after the 4th week on Diet R-14 was not due to an inanition. It has also been remarked that these rats on the low P ration were extremely weak. Since P is indispensable for the normal metabolism of muscle, it appeared desirable to determine the creatine excretion. Two rats were placed on Diet R-14 for 2 weeks in a metabolism cage for collection of urine and feces.
After the 14th day, collections of urine under toluene were made in 3 day periods. Creatinine was determined according to Shaffer (16) and creatine according to the method of Folin (17). Collections were made until the death of the animals.

Throughout the experiment creatinine excretion remained constant when referred to body weight, an average of 1.27 mg. of creatinine N being excreted per 100 gm. per day. This constant excretion was observed both during growth and the eventual loss in weight as the animal declined. However, creatine excretion was observed to be fairly constant only during the growth of the rats and rose sharply when loss of weight began. The creatine excretion during the period of weight loss rose to 1.11 mg. per rat per day from a previous level of 0.08 mg. per rat per day. It is an open question whether this outpouring of creatine may be attributed directly to some aberration in the metabolism of creatine-phosphate or whether it is merely referable to a general tissue disintegration.

**Phosphorus Content of Tissues**—P was determined in various tissues of rats on Diet R-14. It is well known that low P diets of the cereal type result in a lowered blood P and a diminished P content of bone, but, in general, soft tissues have been found to maintain a constant P content. Thus Hentschel and Zoeller (18) found no difference in the total P content of muscle of normal rats and rats on the rachitogenic diets of McCollum, Sherman and Pappenheimer, and Steenbock and Black.

Three diets were fed; viz., a synthetic low P diet (R-14), Diet R-14 with its P content increased to adequate levels with Na2PO4 (Diet NR-14), and a cereal "low P" diet (Ration 2965). 50 to 60 gm. weanling rats from our stock colony were placed on these diets and at suitable intervals several rats were killed in each group after they were etherized and blood withdrawn with a syringe from the abdominal aorta. Inorganic P and total Ca were determined in the sera. The liver, heart, brain, the left femur, and the right gastrocnemius were dissected out, dried at 102° for 48 hours and analyzed for total P by ashing under Mg(NO3)2 in a muffle furnace by a modified Fiske-Subbarow method (19).

From the resultant data (Table I) it is apparent that blood and bone were the only tissues which showed any decrease in phos-
phorus content. Even after 45 days, when the rats of Diet R-14 were in extremis, liver, heart, and brain showed no significant alteration in P content. Although a low level of 3.01 mg. of P per 100 cc. of serum was reached after 14 days, and occasionally a level as low as 1.35 per 100 cc. after 21 days, after 14 days the P sometimes increased as the rats began to lose weight. It is also interesting to note the occurrence of a hypercalcemia on Diet R-14 similar to that observed on the cereal rachitogenic diet, Ration 2965. This is notable, since the Ca content of Diet

<table>
<thead>
<tr>
<th>Group and diet</th>
<th>Days on experimental diet</th>
<th>Mg. total P per gm., dry weight</th>
<th>Mg. per 100 cc. Blood serum</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Liver</td>
<td>Bone</td>
</tr>
<tr>
<td>Group 0. Stock rats, 50-60 gm.</td>
<td>0</td>
<td>9.65</td>
<td>77.0</td>
</tr>
<tr>
<td>Group 1. Low P diet, R-14</td>
<td>14</td>
<td>11.0</td>
<td>50.0</td>
</tr>
<tr>
<td></td>
<td>35</td>
<td>11.1</td>
<td>40.5</td>
</tr>
<tr>
<td></td>
<td>45</td>
<td>11.3</td>
<td>39.2</td>
</tr>
<tr>
<td>Group 2. Diet R-14 + NaH₂PO₄</td>
<td>14</td>
<td>11.5</td>
<td>82.9</td>
</tr>
<tr>
<td></td>
<td>35</td>
<td>11.8</td>
<td>87.6</td>
</tr>
<tr>
<td></td>
<td>45</td>
<td>11.6</td>
<td>88.8</td>
</tr>
<tr>
<td>Group 3. Ration 2965</td>
<td>14</td>
<td>10.6</td>
<td>61.5</td>
</tr>
<tr>
<td></td>
<td>35</td>
<td>11.6</td>
<td>41.1</td>
</tr>
<tr>
<td></td>
<td>45</td>
<td>11.4</td>
<td>37.8</td>
</tr>
</tbody>
</table>

R-14 was at the "normal" level of 0.57 per cent as compared with the surfeit of Ca, 1.2 per cent supplied by Ration 2965.

A diet of adequate P content (Diet NR-14) was observed to maintain the high serum inorganic P, as observed in rats on a stock diet by Bethke, Steenbock, and Nelson (20).

**Effect of Low Phosphorus Diets with Vitamin D**

**Therapeutic Effects**—Thirty weanling rats, weighing 50 to 60 gm., were divided into three groups of ten each. Group 1 was fed Ration 2965 for 3 weeks; Group 2, Diet R-14 for 3 weeks; Group 3, Diet R-14 for 2 weeks. At the end of these periods the
animals were fed 3.3 i. u. of vitamin D as crystalline calciferol dissolved in Wesson oil in 8 days according to the u. s. p. XI technique. On the 10th day the rats were killed and the distal ends of the radii and ulnae sectioned and stained with silver nitrate. It was found that rats made rachitic on Diet R-14 showed approximately the same response to vitamin D as those given the cereal rachitogenic diet, Ration 2965, as indicated by the line test. But the rats on Diet R-14 did not increase in body weight during the period of supplementation, and in fact lost on an average of 1.8 gm. Rats on Ration 2965 averaged a gain of 6.6 gm. It is recognized that healing may occur spontaneously if the animal fasts. In this instance the animals did not fast, but as rats on Diet R-14 suffer a rapid loss in weight beginning in the 4th week, no particular significance was attributed to the loss in weight when first observed.

Prophylactic Effects—The early administration of vitamin D prevented the incidence of rickets on Diet R-14. Skeletal abnormalities did not appear, enlarged costochondral junctions were not observed, and examination of sections of the wrists by staining with silver nitrate failed to reveal any sign of rickets. The nasal discharge which was characteristic of rats on Diet R-14 without vitamin D did not appear. But what was most striking were the growth effects. Without vitamin D, it will be recalled, growth on Diet R-14 was subnormal but none the less definite, a gain of 35 gm. during a 3 week rachitogenic period being common. However, when 1200 i. u. per week of vitamin D as calciferol were fed, a loss in weight occurred (Fig. 1), and in 8 weeks the rats had not regained their initial weight. Similar results were obtained with smaller amounts of vitamin D (Fig. 1), although the loss in weight was less. Ultimately growth was resumed, but the rate was approximately one-third of that shown by rats which were developing rickets on the same diet in the absence of vitamin D. The same inhibitory action of the vitamin was demonstrated when vitamin D was added to Diet R-14 after varying periods of rachitogenesis.

To our knowledge there is no previous account of vitamin D causing a loss in weight or acting as an inhibitor of growth. Thus Shohl et al. (21) in studying the effects of a "low P" diet used Ration 2965 and supplemented it with 2 per cent cod liver oil.
The growth curves he reported gave no indication of any inhibition. Similarly, in our own laboratory when Ration 2965 was supplemented with 1200 i. u. of vitamin D as calciferol per rat per week, there was no indication of such an effect. The inhib-
itory influence of the vitamin could not be attributed to toxicity, since the same amount of vitamin D when fed as a supplement to Ration 2965 was without effect, and the same type of inhibition was obtained on Diet R-14 when amounts as little as 4 I. u. per rat per week were fed (Fig. 1). By supplementation with vitamin D, rats were maintained, at the inhibited growth rate, for as long as 20 weeks. Pathological changes observable after such long periods of maintenance will be reported in a subsequent paper (22).

**Ca and P Retention, Growth and P Content of Tissues**—Twelve rats weighing from 50 to 60 gm. were fed the following diets: Group 1, Ration 2965; Group 2, Ration 2965 plus vitamin D; Group 3, Diet R-14; Group 4, Diet R-14 plus vitamin D. During the entire experiment food consumption was controlled daily by the paired feeding method. It was soon evident that the rats on the supplemented diets would be the pace-makers; especially marked was the diminished and slow consumption of Diet R-14. On the cereal rachitogenic diet the tendency to restrict consumption with the addition of supplements was less evident. After 3 days on the diets without vitamin D the excreta were collected for 1 week; vitamin D additions were then made, with collection of excreta in a similar manner during the 2nd and 3rd weeks. Groups 2 and 4 received 50 I. u. per day as calciferol in Wesson oil, by dropper. The combined urine and fecal collections were dried and ashed for Ca determinations by the oxalate-permanganate method; P determinations were made by a modified Fiske-Subbarow procedure. At the conclusion of the feeding trials the animals were killed and tissues analyzed as described before. The growth record and Ca and P balances are presented in Table II, tissue and blood serum analyses in Table III.

From Table II it is apparent that on Diet R-14, as on Ration 2965, administration of vitamin D resulted in an improved retention of Ca and P. Particular attention is called to Groups 3 and 4. In the 3rd week the rats in Group 3 were continuing in negative balance of these elements, although they were increasing in weight. When vitamin D was added as in Group 4, the balance became positive; yet the rats gained only slightly in weight. If as in Group 3 an animal is in negative P balance and at the same time is growing, then the P used for growth must have come from tissues other than those responsible for the growth.
Vitamin D$_2$ Response in Rats

increments. Analyses indicated that blood and bone were the tissues thus drawn upon. However, when vitamin D was given,

\[ \text{TABLE II} \]

Ca and P Balances of Rats on Low P Diets; Averages of Three Rats,
Paired Feeding

<table>
<thead>
<tr>
<th>Group No.</th>
<th>Wk.</th>
<th>Gain in weight</th>
<th>Intake</th>
<th>Retention</th>
<th>Remarks</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>gm.</td>
<td>Ca mg.</td>
<td>P mg.</td>
<td>Ca mg.</td>
</tr>
<tr>
<td>1</td>
<td>1st</td>
<td>13.3</td>
<td>626</td>
<td>141</td>
<td>85</td>
</tr>
<tr>
<td></td>
<td>2nd</td>
<td>5.7</td>
<td>557</td>
<td>126</td>
<td>-0.3</td>
</tr>
<tr>
<td></td>
<td>3rd</td>
<td>4.7</td>
<td>475</td>
<td>107</td>
<td>32.3</td>
</tr>
<tr>
<td>2</td>
<td>1st</td>
<td>14.6</td>
<td>Same as</td>
<td>82</td>
<td>38.7</td>
</tr>
<tr>
<td></td>
<td>2nd</td>
<td>5.0</td>
<td>Group 1</td>
<td>40.7</td>
<td>39.1</td>
</tr>
<tr>
<td></td>
<td>3rd</td>
<td>4.7</td>
<td></td>
<td>95.6</td>
<td>39.5</td>
</tr>
<tr>
<td>3</td>
<td>1st</td>
<td>12.0</td>
<td>172</td>
<td>20</td>
<td>-4.4</td>
</tr>
<tr>
<td></td>
<td>2nd</td>
<td>4.3</td>
<td>142</td>
<td>16</td>
<td>-40.5</td>
</tr>
<tr>
<td></td>
<td>3rd</td>
<td>3.3</td>
<td>145</td>
<td>16</td>
<td>-17.9</td>
</tr>
<tr>
<td>4</td>
<td>1st</td>
<td>10.4</td>
<td>Same as</td>
<td>-4.7</td>
<td>9.0</td>
</tr>
<tr>
<td></td>
<td>2nd</td>
<td>-0.7</td>
<td>Group 3</td>
<td>-10.5</td>
<td>6.9</td>
</tr>
<tr>
<td></td>
<td>3rd</td>
<td>1.3</td>
<td></td>
<td>17.6</td>
<td>10.9</td>
</tr>
</tbody>
</table>

\[ \text{TABLE III} \]

Tissue Analyses of Rats on Low P Diets, with and without Vitamin D$_2$; Averages for Three Rats

<table>
<thead>
<tr>
<th>Group and diet</th>
<th>Vitamin D</th>
<th>Mg. total P per gm., dry weight</th>
<th>Mg. per cent</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Liver</td>
<td>Base</td>
</tr>
<tr>
<td>Group 1. Ration 2965</td>
<td>None</td>
<td>11.3</td>
<td>13.0</td>
</tr>
<tr>
<td>Group 2. Ration 2965</td>
<td>50 i. u. per day, for 2 wks.</td>
<td>10.2</td>
<td>64.5</td>
</tr>
<tr>
<td>Group 3. Diet R-14</td>
<td>None</td>
<td>11.3</td>
<td>36.9</td>
</tr>
<tr>
<td>Group 4. Diet R-14</td>
<td>50 i. u. per day, for 2 wks.</td>
<td>11.22</td>
<td>44.5</td>
</tr>
</tbody>
</table>

although Ca and P retention were improved, growth was halted, and P went to blood and bone to restore them to their normal
phosphorus levels (Table III). While P was being diverted to blood and bone, growth became static, for, as already apparent from our analyses, the P content of the soft tissues tends to remain constant.

Since most theories of the mode of action of vitamin D have been evolved from experimental data accumulated with the use of Ration 2965, the question arises, how do the findings here presented fit in with current theories? Bills (23) in his review points out that the view was once held that the action of vitamin D was confined to the gut. Nicolaysen (24) revived this older theory when he found that in isolated loops of the intestine the administration of vitamin D increased the rate of absorption of Ca salts, but that no effect was observed when phosphates were used. Nicolaysen arrives at the conclusion that the primary effect of vitamin D is to increase the rate of Ca absorption from the gut. In view of the plethora of Ca in the gut and blood it is difficult to attribute the calcifying effects of vitamin D to an increase in the absorption of what already must be furnished in adequate amounts. Certainly any theory of the mode of action of vitamin D in rachitogenic diets must consider and include effects on P as well.

A distinction should be made between the demands of bone and the soft tissues for phosphorus. Nicolaysen (25) has calculated the weekly P requirement of a rat growing 1 gm. per day as 31.5 mg. Assuming that the P requirements are proportionate to the amounts retained by the various tissues, since approximately 20 per cent of the P of the body is found in the soft tissues, 6.3 mg. of P could be accepted as the weekly requirement for soft tissue development and 25.2 mg. of P as the weekly bone requirement. It is obvious from Table II that supplementing Ration 2965 with vitamin D resulted in a retention of P ample for the requirements of both soft and bone tissue. This shows why a rachitogenic diet composed of cereals cannot be used to demonstrate the diversion of P to bone by a supplement of vitamin D.

From examination of the relation of the blood changes to these differences in growth it is apparent that without vitamin D the inorganic P content of the blood was low (2.2 mg. per cent), but growth proceeded; with vitamin D the inorganic P content of the blood was raised (5.20 mg. per cent), but growth was halted.
As the soft tissues were seeking P, and the P supply in the blood was increased, it follows that either the increase in inorganic P in the blood represented a form of P not available for growth, or that the growth of the tissues was inhibited by the presence of vitamin D directly. The latter is improbable, since adequate P in the diet gave normal growth in the presence of identical amounts of vitamin D.

The hypothesis that the increased inorganic P in the blood is not simple inorganic P has been suggested. Fractionation of the forms of Ca and P in the blood by Benjamin and Hess (26) and Benjamin (27) indicate that a diffusible, adsorbable, Ca-P complex provides the substance for bone calcification. Vitamin D has been shown to increase the amount of this adsorbable P.

**SUMMARY**

A synthetic diet low in phosphorus and free of vitamin D but adequate in other nutritional requirements of the rat has been devised. When supplemented with sufficient phosphorus and vitamin D, it sufficed as the sole source of nutrients for three successive generations of rats. Without these supplements it produced rickets in from 12 to 14 days and brought on severe skeletal deformities in 6 weeks. Growth stopped after 4 to 5 weeks, followed by a rapid decline in weight and death by the 6th week. Phosphorus analyses of tissues and data on creatine and creatinine excretion did not provide the explanation.

When vitamin D was added to the low P diet, the resultant metaphyseal healing was identical with that observed when like amounts were fed in cereal diets. However, when the low P diet was supplemented with vitamin D, a cessation or inhibition of growth occurred which did not result under the same circumstances with cereal rachitogenic diets. Calcium and phosphorus balances and tissue analyses revealed that vitamin D induced the utilization of P by bone, thereby depriving the soft tissues of their supply of P, which in turn inhibited growth.

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