When it was revealed that the equalization of the phosphorus content of cereal grain rations did not equalize their antirachitogenic properties, it appeared probable that cereal phosphorus did not always have the same nutritive value as inorganic P (1). This was proved to be the case with high Ca, low P diets by Bruce and Callow (2). Krieger et al. (3) confirmed these findings with semisynthetic cereal-free diets to which phytic acid had been added. Later, with the same technique, the importance of the Ca:P ratio was stressed. An excessively high Ca intake depressed calcification, as was well known from the work of McCollum (4), but this effect was slight with inorganic P as contrasted with phytic acid P (5). Vitamin D improved calcification more with the latter than with inorganic P. Later Krieger et al. (6), using high calcium diets, demonstrated that phytic acid P was also inferior in value to the P of yeast nucleic acid and soy bean phosphatides. Vitamin D effected the most improvement with phytic acid, but the level of calcification never equaled that attained with P from other sources. Lately Boutwell et al. (7) found that bran P at a Ca:P ratio of 2:1 was poorly utilized, but that its utilization was improved by vitamin D to a level approaching that of inorganic P. Still more recently Spitzer et al. (8) obtained similar results with Ca phytate.

The nature of the mechanism whereby this improvement in the value of phytic acid phosphorus may be effected has been a challenge to investigators for some time. Mellanby (9) and Green and Mellanby (10) found that the anticalcifying action of cereals for dogs was reduced by boiling the cereals with 1 per cent HCl. Bruce and Callow (2) and Lowe and Steenbock (11) obtained similar results with rats on high Ca cereal diets. They reported that maize had its rachitogenic properties reduced in proportion to the extent that its phytic acid was hydrolyzed. Mellanby (12) found that germination and autolysis reduced the anticalcifying properties of oats. Templin and Steenbock (13) found that maize was improved antirachitically by germination, followed by autolysis. Lowe and Steenbock
(11) showed that this improvement followed an increase in inorganic P which they attributed to the activity of a phytase.

The recognition that hydrolysis \textit{in vivo} by an intestinal phytase might be responsible for the improvement effected with high calcium diets when supplemented with vitamin D emerged gradually. Plimmer (14) failed to find phytase in the intestinal extracts of the dog, sheep, ox, and rabbit. Lowe and Steenbock (11) similarly failed to find it in the intestinal extracts of rats and chicks. Patwardhan (15), however, found it in abundance in the intestinal extracts from rats, and Krieger (16) confirmed these results. Apparently Lowe and Steenbock failed in their experiments because they used phytin as a substrate which is precipitated at the pH optimal for the hydrolysis of Na phytate. Patwardhan and Krieger both used Na phytate in their experiments. Plimmer’s failure is not so easily explained, unless it was that his pretreatment of the intestinal extracts destroyed the enzyme. So far we have never failed to find some phytase in extracts from the small intestine of rats.

The work of Heymann (17) with glycerophosphatase suggested a possible phytase effect \textit{in vivo}. He found that the phosphatase activity of intestinal extracts from normal rats was higher than that from rachitic rats. Unfortunately he did not determine the direct effect of vitamin D with identical basal rations. Spitzer \textit{et al.} (8) performed such experiments for phytase activity but reported negative results. Crimm and Strayer (18) found a 3-fold increase in intestinal phosphatase with excessive amounts of vitamin D but Theopold (19) obtained the highest phosphatase values in rickets.

In view of the paucity of and negative nature of the data on phytase, it seemed desirable to make our recent results and those obtained some years ago (16) more accessible. While they reveal considerable variations in the effect of vitamin D, they do show a definite trend toward an increase in enzyme activity following the feeding of vitamin D.

\section*{EXPERIMENTAL}

Our first experiments were carried out with intestinal extracts from individual rats, guinea pigs, dogs, and chickens. These were designed to ascertain primarily the effect of variations in the technique of extraction, the effect of pepsin, trypsin, MgSO$_4$, and CaCO$_3$, the time of incubation, variations in pH, excess of substrate, excess of buffer, dilution of extract, etc. While these experiments were determinative in their specific objectives, they revealed too great a range in the activity of individual extracts to establish a relationship with vitamin D. It was not until a large number of animals were used under standard conditions, as later outlined, that the effectiveness of vitamin D was demonstrable. However, its importance in
the over-all antirachitogenic effect of vitamin D with cereal rations remains to be evaluated.

Chick Experiments—2 day-old white Leghorn chicks were fed in groups of five on two different rations; viz., Ration B, a practical poultry ration when supplemented with vitamin D, and Ration R, a strongly rachitogenic ration which is widely used for vitamin D assays. Ration B was composed of yellow corn 40, wheat middlings 15, wheat bran 10, meat scraps 8, dried skim milk 5, alfalfa meal 5, sodium chloride 0.5, soy bean oil meal 5, ground oats 10, and limestone grits 1.5. Ration R was composed of yellow corn 58, wheat middlings 25, casein 12, NaCl 1, Ca₃(PO₄)₂ 2, dried yeast 2, and MnSO₄·4H₂O 0.02 (20). After 4 to 8 weeks on these respective rations, the chicks were killed, the tibiae were removed for determinations of ash content after extraction with alcohol and ether, and the small intestines were excised for determination of phytase activity. For this determination the intestines were slit lengthwise, washed free of contents in cold running water, blotted on moist cheese-cloth, and weighed. After thorough maceration with sand in a mortar, the resultant mixture was allowed to autolyze with 5 times its weight of water in the presence of chloroform for 48 hours at room temperature. The digest was then centrifuged and the centrifugate, with washings, was diluted with water to 12 times the weight of the tissue.

Sodium phytate prepared from commercial Ca phytate was used as the substrate. 1 ml. of a solution containing 3.78 mg. of P, of which 3.72 mg. represented phytic acid P and the remainder inorganic P, was incubated with 0.5 to 2.0 ml. of intestinal extract, 5 ml. of Veronal acetate buffer, pH 7 (21), and 2 ml. of 0.012 M MgSO₄ in a volume of 20 ml. at 37°. After 48 hours, enzymic action was stopped by the addition of 2 ml. of 10 per cent trichloroacetic acid, and inorganic P was determined on 1 ml. filtered aliquots of 25 ml. volumes by the Fiske-Subbarow technique (22). The values were corrected for the inorganic P originally present, but not for the inorganic P liberated from tissue sources during the incubation. These were negligible in amount. The conditions provided for these assays gave us comparable values which were not necessarily the maximum obtainable. Table I reveals clearly that vitamin D increased the extractable intestinal phytase. That calcification had been proved in the chicks was indicated by the increase in bone ash.

Rat Experiments—Two sets of experiments with rats were carried out in sequence, one with excessive amounts of vitamin D and a cereal rachitogenic ration, and a second with approximately therapeutic amounts of vitamin D added to two non-rachitogenic rations and one semisynthetic rachitogenic ration. For the former experiment, use was made of rats which had been on Ration 2965 (23) for a few weeks and had received P³²
and relatively small amounts of vitamin D after 14 to 22 days of rachitogenesis in connection with other experiments (24). These doses were as follows: Group 1, 5, Group 2, 10, Groups 3, 4, 6, and 7, 100, and Group 5, 500 i.u. After the effect of vitamin D had been dissipated, that is 50 to 103 days after the administration of the vitamin, some of these various groups were given from 100 to 500 i.u. of vitamin D₂ daily for 20 to 30 days.

Phytase was determined essentially as already described. However, the extracts were limited in origin to the first 10 inches of the duodenum because, confirming the findings of Laskowski (25), Heymann (26, 27), and others, the highest concentration of enzyme was found in the part proximal to the stomach. These sections were allowed to autolyze for 7 days. The mg. of P liberated from a substrate of 3.85 mg. of phytic acid P were cor-

<table>
<thead>
<tr>
<th>Series No.</th>
<th>Ration</th>
<th>Phytase units per gm. wet tissue</th>
<th>Per cent bone ash</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>No vitamin D</td>
<td>Vitamin D</td>
</tr>
<tr>
<td>I</td>
<td>B</td>
<td>2.6 (1.9-3.6)</td>
<td>5.3 (4.1-7.1)</td>
</tr>
<tr>
<td>II</td>
<td>R</td>
<td>2.1 (1.0-4.7)</td>
<td>3.6 (2.7-5.0)</td>
</tr>
<tr>
<td>III</td>
<td>&quot;</td>
<td>2.8 (1.9-3.7)</td>
<td>4.1 (3.6-4.8)</td>
</tr>
</tbody>
</table>

Ration B was a practical poultry ration when supplemented with vitamin D (see the text). It was furnished here as irradiated yeast, 125 to 500 i.u. per 100 gm. of ration. Ration R was a rachitogenic ration (see the text) widely accepted for use in vitamin D assay. Vitamin D was given as 50 i.u. of vitamin D₂ twice weekly.

The results (Table II) reveal a very pronounced effect of vitamin D with the healing of the rachitic lesions. However, noteworthy in these experiments is the range in values obtained from the animals in the different
groups. Individually there was no evident relation between the severity of rickets and phytase activity.

**Table II**

*Intestinal Phytase with Hypervitaminosis D in Rats on Ration 2965*

<table>
<thead>
<tr>
<th>Group No.</th>
<th>No. of rats</th>
<th>Final treatment of rats</th>
<th>Phytase units per gm. wet tissue</th>
<th>Metaphyseal measurements mm.</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>5</td>
<td>No vitamin D</td>
<td>1.16 (0.90-1.85)</td>
<td>0.40-1.20</td>
</tr>
<tr>
<td>2</td>
<td>5</td>
<td>&quot; &quot; &quot;</td>
<td>1.40 (0.93-2.17)</td>
<td>1.06-1.40</td>
</tr>
<tr>
<td>3</td>
<td>5</td>
<td>&quot; &quot; &quot;</td>
<td>1.51 (0.36-1.83)</td>
<td>0.54-0.86</td>
</tr>
<tr>
<td>4</td>
<td>3</td>
<td>2,450 i.u. vitamin D*</td>
<td>3.06 (2.92-3.01)</td>
<td>0.14-0.22</td>
</tr>
<tr>
<td>5</td>
<td>3</td>
<td>11,750 &quot; &quot; &quot;</td>
<td>3.27 (3.17-3.42)</td>
<td>0.14-0.26</td>
</tr>
<tr>
<td>6</td>
<td>8</td>
<td>3,450 &quot; &quot; &quot;</td>
<td>2.41 (1.54-3.14)</td>
<td>0.10-0.18</td>
</tr>
<tr>
<td>7</td>
<td>4</td>
<td>21,750 &quot; &quot; &quot;</td>
<td>2.52 (1.41-3.26)</td>
<td>0.12-0.16</td>
</tr>
</tbody>
</table>

* These large doses were given in 20 days for Groups 4 and 5, and in 30 days for Groups 6 and 7.

**Table III**

*Intestinal Phytase with Small Doses of Vitamin D in Rats on Various Rations*

<table>
<thead>
<tr>
<th>Experimental series No.</th>
<th>Ration No.</th>
<th>Phytase units per gm. wet tissue</th>
<th>Blood sera, inorganic P</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>No vitamin D</td>
<td>Vitamin D</td>
</tr>
<tr>
<td></td>
<td></td>
<td>mg. per cent</td>
<td>mg. per cent</td>
</tr>
<tr>
<td>P-10</td>
<td>23</td>
<td>0.99 (0.50-2.10)</td>
<td>1.29 (0.50-3.10)</td>
</tr>
<tr>
<td></td>
<td>11</td>
<td>0.80 (0.45-1.05)</td>
<td>0.90 (0.60-1.35)</td>
</tr>
<tr>
<td>P-14</td>
<td>23</td>
<td>1.48</td>
<td>3.20</td>
</tr>
<tr>
<td></td>
<td>11</td>
<td>1.59</td>
<td>1.94</td>
</tr>
<tr>
<td></td>
<td>2965W</td>
<td>1.07</td>
<td>2.13</td>
</tr>
<tr>
<td>P-14a</td>
<td>23</td>
<td>3.87</td>
<td>5.98</td>
</tr>
<tr>
<td></td>
<td>11</td>
<td>3.89</td>
<td>4.58</td>
</tr>
<tr>
<td></td>
<td>2965W</td>
<td>2.19</td>
<td>3.28</td>
</tr>
</tbody>
</table>

There were twelve rats in each group for Series P-10 and six for P-14 and P-14a. They were on their respective rations 14 days in Series P-10, 25 days for Series P-14, and 29 for P-14a. The doses of vitamin D were 400 i.u. for Series P-10, 600 for Series P-14, and 700 for P-14a.

In the second set of experiments there was fed a modified Ration 2965, *viz.* No. 2965W, which was no longer rachitogenic because its CaCO$_3$ and NaCl had been replaced with 4 per cent of a complete salt mixture, *viz.* Wesson's salt mixture (28). There was also fed a semisynthetic non-rachitogenic ration, *viz.* Ration 11 (29) composed essentially of egg white, cottonseed oil, glucose, a P-free salt mixture, and vitamins, identical with Ration 23 supplemented with a neutral mixture of K$_2$HPO$_4$ and KH$_2$PO$_4$. This gave
it a P content of 0.3 per cent and a Ca content of 0.47 per cent, the same as in Ration 23. The latter, however, contained only 0.017 per cent P, which made it strongly rachitogenic. Vitamin D, when supplied, was given in the form of a cottonseed oil solution of vitamin D₂ twice weekly in doses of 100 i.u. for the duration of the experiment; i.e., for 14 to 29 days.

The intestinal extracts were prepared as before, except that in most instances there were composite samples for each group. Furthermore, in the assay of the extracts, inorganic P was determined both before and after incubation. The resultant corrections for inorganic P were the same as in previous tests. As a check on the nutritional status of the rats, inorganic P was determined in the blood sera.

In this series as in the preceding one, but with a lower dose of vitamin D and variable ration composition, vitamin D increased the intestinal phytase (Table III). It is noteworthy that the increase was largest with the P-deficient rachitogenic Ration 23 with which vitamin D generally gave the largest increase in serum inorganic P, but also occurred with Ration 2965W, which was excessively rich in P, and with which vitamin D effected an actual reduction in serum P. Furthermore, a minimal effect, if any, on both phytase and serum P was obtained with Ration 11, which was approximately optimal in P and Ca content. In these results, Ca was not a direct determinant because all the rations contained Ca within the optimal range.

SUMMARY

Supplements of vitamin D tended to increase the extractable intestinal phytase of rats and chicks kept on cereal rachitogenic rations. Apparently this reaction was not limited to cereal rations or to the rachitic state, because the same trend was observed with rats kept on non-cereal rations which furnished approximately either optimal or excessive amounts of P and optimal amounts of Ca.

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