VITAMIN D AND PLASMA PHOSPHATASE IN THE RAT*

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It is well established that in man plasma phosphatase is greatly increased in rickets and in some other bone diseases (1–6). A similar relationship has been found in various experimental animals, viz. chicks (7–9), sheep (10), dogs (11–13), and calves (14, 15). In rats, however, the reports have been contradictory. Sure and coworkers (16) found no difference between rachitic and vitamin D-treated rats when fed Steenbock and Black's rachitogenic diet 2965. Patwardhan's group (17, 18) reported a decrease in rats made rachitic with a low P diet similar to the one used by Schneider and Steenbock (19). However, Tuba et al. (20) and Scoz (21) observed an increase in rickets. Very recently Iber (22) reported no increase after feeding a low P, high Ca rachitogenic diet, and no response to vitamin D additions. Crimm and Strayer (23) and also Taylor et al. (24) found a reduction in activity after giving toxic doses of vitamin D.

In view of the above conflicting reports, experiments were carried out with rats to determine the effect of vitamin D with diets differing widely in Ca and P content, and therefore different in their effect on the structure and mineralization of bone.

EXPERIMENTAL

30 day-old male Sprague-Dawley rats weighing 70 to 80 gm., housed in individual overhanging wire cages, were fed the various rations ad libitum with and without vitamin D$_2$. A semisynthetic ration of Bellin and Steenbock (25), composed of glucose, egg white, cottonseed oil (Wesson), roughage, vitamins, and salts, was used as the basal ration. To this, CaCO$_3$, an equimolar mixture of Na$_2$HPO$_4$ and NaH$_2$PO$_4$, and Ca phytate were added to obtain the desired levels of Ca, inorganic P, and phytic acid P. Vitamin D was supplied when required by the experimental plan as an oral dose of 75 i.u. of vitamin D$_2$ in 0.1 ml. of cottonseed oil solution per rat every 3 days.

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937
The Ca and P content of the rations was determined, respectively, by the methods of Wang (26) and Fiske and Subbarow (27), following digestion with nitric and perchloric acids. Phosphatase determinations were made on blood collected from ether-anesthetized rats by heart puncture with a 22 gage needle and a 5 ml. syringe moistened with a 20 per cent potassium oxalate solution. After centrifugation, 0.2 ml. of the plasma in 2 ml. of 0.9 per cent NaCl solution was incubated for 2 hours at 37° in 0.0267 M Na-β-glycerophosphate (Eastman Kodak Company), 0.0190 M Veronal-acetate buffer (pH 9.2), and 0.0012 M magnesium sulfate solution. The final volume was 15 ml.

Preliminary tests revealed that the stated amounts of substrate and magnesium sulfate gave optimal activity, and the buffer concentration maintained the desired pH (9.2) during incubation with only a slight inhibitory effect on the enzyme. A pH of 9.2 was selected since higher pH values, although giving somewhat greater activity, resulted in enzyme instability. The preliminary studies also revealed that the pH-dependence curve in the alkaline area was due, at least in part, to enzyme denaturation.

Both prior to and following incubation, aliquots of the reaction mixture were removed, treated with an equivalent volume of 20 per cent trichloroacetic acid, and centrifuged. Phosphatase values were determined within 48 hours after the collection of blood, although the enzyme was found stable for at least 2 weeks in the diluted plasma at 5°. Inorganic P in the supernatant solution was determined by the method of Fiske and Subbarow (27). The P liberated was converted to phosphatase units by reference to a standard curve, since dilution and addition studies failed to reveal the presence of either activators or inhibitors and since the enzyme was found to be stable during incubation. 1 unit of plasma phosphatase was defined as the amount of enzyme which liberated 0.8 mg. of inorganic P in the 15 ml. solution mentioned before in 2 hours under the conditions described.

In the first series of experiments, a stock ration which contained vitamin D was compared in its effect with that obtained with semisynthetic rations which were either normal, rachitogenic, or osteoporogenic. These rations were fed with and without vitamin D. On the terminal day of the experiment, a blood sample was drawn early in the morning, before the animals started eating, for the determination of phosphatase. This technique was found to be adequately successful in reducing the well known variations induced by food ingestion (28). The rats were sacrificed with ether immediately following the collection of the sample. The right femur was removed, extracted for 24 hours with alcohol and ether, and then ashed. The distal ends of the radii and ulnae were sectioned and the cartilaginous metaphyses examined after staining with silver nitrate.
The high phosphatase activity found with rats on some of the basal rations and the large reduction of activity effected by vitamin D led us to test for the possible presence of activators or inhibitors in the plasma. For this purpose, plasma was obtained, respectively, from rats on the stock ration, on the rachitogenic Rations 711-B and 23, and on the osteoporogenic Ration 11-K, fed with and without supplements of vitamin D. On these samples both dilution and addition experiments were carried out. With the exception of a difference in the volume of diluted plasma, the technique used was the same as described earlier.

The comparative rapidity of response to the administration of vitamin D in rachitic and osteoporotic rats was investigated by feeding to forty rats Ration 711-B and to an additional forty rats Ration 11-K. Eight rats on each ration were given vitamin D on the 21st day and four rats on the 26th day of feeding. Phosphatase was determined in these rats 2 or 5 days after the administration of the vitamin, and also on an equal number of controls. In six others, phosphatase levels were determined on the 21st day before vitamin D was given. Eight rats were given prophylactic doses of vitamin D throughout the experiment.

Results

The values on bone ash and the metaphyseal widths obtained from the rats on the various rations previously mentioned, fed with and without vitamin D, show that the expected effects on bone structure and composition had been attained (29).

With all of the basal rations (Table I), plasma phosphatase was increased to levels higher than those found in stock rats; however, although it is evident that Ca and P were important factors in determining the final levels, there is a wide variation in the degree of increase which cannot be accounted for by differences in the Ca and P content of the rations. Low Ca rations, either rachitogenic (Ration 34-B) or osteoporogenic (Ration 11-K), gave greatly increased values, while rations optimal in Ca and either low in P (Ration 23) or optimal in P (Ration 11) gave only slightly increased values. Also, when Ca was supplied in excess and the amount of available P was low (Ration 711-B) (30), phosphatase was greatly increased.

The results also show clearly that the level of phosphatase was reduced by the addition of vitamin D to any of the basal rations, whether they were approximately optimal in Ca and P content (Ration 11), rachitogenic (Rations 23, 711-B, 34-B), or osteoporogenic (Ration 11-K). While the resultant phosphatase level approximated that found in rats on the stock ration, it is clear that rations low in Ca gave higher levels than did rations normal or high in Ca, and that the high Ca rations gave the lowest levels.

Although phosphatase increased with Ration 711-B with the increase in
### Table I

**Effect of Vitamin D on Plasma Phosphatase with Rations Varying Widely in Ca and P Content**

<table>
<thead>
<tr>
<th>Ration No.</th>
<th>Days fed</th>
<th>Weight increase</th>
<th>Bone ash</th>
<th>Phosphatase</th>
<th>Decrease in phosphatase</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>gm.</td>
<td>gm.</td>
<td>mg.</td>
<td>per cent</td>
</tr>
<tr>
<td>Initial</td>
<td>0</td>
<td></td>
<td>Normal</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Stock</td>
<td>21</td>
<td>47</td>
<td>60</td>
<td></td>
<td></td>
</tr>
<tr>
<td>11 (0.4% Ca, 0.3% P)</td>
<td>21</td>
<td>47</td>
<td>60</td>
<td>99.0</td>
<td>53.9</td>
</tr>
<tr>
<td>34-B (0.016% Ca, 0.016% P)</td>
<td>19</td>
<td>53</td>
<td>67</td>
<td>53.6</td>
<td>44.3</td>
</tr>
<tr>
<td>23 (0.47% Ca, 0.016% P)</td>
<td>15</td>
<td>41</td>
<td>11</td>
<td>Very rachitic in 9 days</td>
<td>47.8</td>
</tr>
<tr>
<td>711-B (1.2% Ca, 0.294% phytate P)</td>
<td>21</td>
<td>44</td>
<td>23</td>
<td></td>
<td></td>
</tr>
<tr>
<td>11-K (0.016% Ca, 1.2% P)</td>
<td>21</td>
<td>6</td>
<td>83</td>
<td>Severely osteoporotic</td>
<td>62.5</td>
</tr>
</tbody>
</table>

75 i.u. of vitamin D were given to each rat every 3 days, beginning with the 1st day of the experiment. Usually there were six to ten rats in each group, but in those marked with an asterisk there were only four. Standard deviation = $\sqrt{\frac{\sum x^2}{n} - \overline{x}^2}$.
the severity of rickets (Fig. 1), with Ration 23 this was not evident (Table I). Also, there was no evident correlation between the variations in phosphatase levels with the relative amounts of cartilage and osseous tissue found in the skeleton. The level was high when cartilage was produced in limited amounts with one ration (11-K) but not with another (Ration 11). It was also high when cartilage was produced in abundance on Rations 34-B and 711-B, but only moderately so when produced on Ration 23.

The observations mentioned above raised the question as to the extent to which our values of phosphatase activity were really indicative of the phosphatase content of the plasmas. While the effect of initial dilution could not be determined, inasmuch as all plasmas were diluted with saline for convenience, further dilution of various samples from normal, rachitic, and osteoporotic rats, and from rats which had received vitamin D, gave values which were lowered in proportion to the degree of dilution. Mixing boiled plasma with untreated plasma, or plasma from vitamin D-treated rats with plasma from rats which had not received vitamin D, gave results equivalent to the sum of their activities (Table II). The results of the dilution experiments are not presented in detail in order to conserve space.

The importance of the general decrease in phosphatase activity following the administration of vitamin D is hard to assess. It might be assumed that this decrease would be in harmony with a shift in the metabolic balance from the hydrolytic to a preponderance of the synthetic and this in turn might be correlated with the growth-promoting activity of vitamin D. But inspection of our data (Table I) shows that, while the decrease in activity with stimulation of growth (Ration 11-K) was great, it was almost equally great when growth was inhibited (Ration 711-B).
TABLE II

*Effect of Additions of Low Activity Plasma on Activity of Plasma from Normal, Rachitic, and Osteoporotic Rats*

<table>
<thead>
<tr>
<th>Active plasma</th>
<th>Plasma additions</th>
<th>Total phosphatase</th>
<th>Deviation per cent</th>
</tr>
</thead>
<tbody>
<tr>
<td>Source</td>
<td>Phosphatase units</td>
<td>Source</td>
<td>Phosphatase unit</td>
</tr>
<tr>
<td>Normal rat (stock ration)</td>
<td>1.32</td>
<td>Boiled plasma (stock ration)</td>
<td>0.00</td>
</tr>
<tr>
<td>Rachitic rat (Ration 711-B)</td>
<td>1.65</td>
<td>Vitamin D-treated rat (Ration 711-B)</td>
<td>0.34</td>
</tr>
<tr>
<td>Osteoporotic rat (Ration 11-K)</td>
<td>2.01</td>
<td>Vitamin D-treated rat (Ration 11-K)</td>
<td>0.76</td>
</tr>
<tr>
<td></td>
<td>1.85</td>
<td>Rachitic rat (Ration 23)</td>
<td>0.93</td>
</tr>
</tbody>
</table>

The diluted plasma (1 ml. diluted to 10 ml. with 0.9 per cent NaCl) plus additions were incubated with 0.0267 M Na-β-glycerophosphate, 0.0012 M MgSO₄, and 0.0190 M Veronal-acetate buffer (pH 9.2) in a total incubating volume of 15 ml. for 2 hours at 37°.

![Graph](http://www.jbc.org/)

**Fig. 2.** Decrease in plasma phosphatase with the administration of vitamin D to rats made rachitic on Ration 711-B (1.2 per cent Ca, 0.294 per cent phytate P). Each point represents an average of the values obtained on four to eight rats.

**Fig. 3.** The response of plasma phosphatase to the administration of vitamin D in rats made osteoporotic on Ration 11-K (0.016 per cent Ca, 1.2 per cent P). Each point represents an average of the values obtained on four to eight rats.
Some experiments were carried out to determine whether the rate of reduction in phosphatase content following the administration of vitamin D to rats made rachitic on Ration 711-B was the same as with rats made osteoporotic on Ration 11-K. To our surprise, while a reduction in phosphatase occurred quite promptly in the rachitic rats (Fig. 2), an initial increase resulted in those which were osteoporotic (Fig. 3). However, in confirmation of the data obtained when vitamin D was given from the beginning of the experiment (Table I and Fig. 3), the values were reduced when a second dose of vitamin D (100 i.u.) was given after the rats had been on the ration 26 days and were sacrificed 5 days later. The phosphatase activity was then reduced to 200 units per 100 ml. of plasma, a level similar to that obtained when 1000 i.u. of vitamin D were given daily to some of the rats which had been on the ration for 21 days. We have no explanation for the initial increase.

SUMMARY

The alkaline phosphatase content of plasma in rats on various semi-synthetic, vitamin D-free rations, whether they produced approximately normal, severely rachitic, or porotic bone, was always higher than it was in rats which were fed a vitamin D-containing stock ration. The highest values were obtained with a high P, low Ca osteoporogenic ration. The administration of vitamin D prophylactically or therapeutically reduced the values to approximately those found in stock rats. The amount of phosphatase in the plasma could not be related to the severity of rickets or to the amount of cartilage present in bone.

BIBLIOGRAPHY
