Citrate and action of vitamin D on calcium and phosphorus metabolism

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GUROFF, GORDON, HECTOR F. DELUCA, AND HARRY STEENBOCK. Citrate and action of vitamin D on calcium and phosphorus metabolism. Am. J. Physiol. 204(5): 833-836. 1963.—Pantothenic acid and pyridoxine deficiencies in rats greatly minimized the rise in serum citrate following vitamin D administration without affecting the rise in serum calcium, serum phosphorus, and femur ash. In addition, pyridoxine deficiency reduced the response of bone citrate to the administration of vitamin D. The administration of cortisone acetate to rats on a low-calcium diet completely prevented the response of serum and bone citrate to vitamin D administration while having no effect on the response of serum calcium to vitamin D. These findings make it unlikely that an elevated citrate content of extracellular fluid and bone mediates the basic effects of vitamin D on mineral metabolism.

The discovery of large amounts of citric acid in bone and its relative decrease in rickets (1) opened the question of the relationship of citric acid to calcification. It is well established that both vitamin D and parathyroid hormone increase bone and serum citrate (2,19). Vitamin D administration raises the citrate content of urine and of certain soft tissues as well (2, 5, 11). Earlier studies in this laboratory revealed that the antirachitic action of dietary citrate was due to an increase in the availability of dietary phytic acid phosphorus and not through a metabolic action (12). However, the exact role, if any, played by metabolically produced citrate in calcium metabolism is as yet not understood.

The well-known ability of citrate to complex calcium ions has been of the basis of many suggested mechanisms in calcium metabolism. For example, it has been suggested that vitamin D has a calcium-mobilizing effect on bone which is mediated by citric acid (8). On the other hand, it has been argued on the basis of evidence obtained with model systems that the occurrence of citric acid in bone is adventitious (13).

Recently it has been shown that cortisol prevents the elevation of citrate in bone and serum of rats given vitamin D (14, 15). This agent did not prevent the effect of vitamin D on serum calcium and phosphorus or on the deposition of calcium in bone (14, 15).

Previous work in this laboratory (16) revealed that deficiencies of pantothenic acid and pyridoxine but not of vitamins A, B1, or biotin interfere with the effect of vitamin D on urine and tissue citrate. In this paper, it will be shown that, although pantothenic acid and pyridoxine deficiencies interfere with the action of vitamin D on citrate, they do not interfere with its action on serum calcium and phosphorus or on bone ash accretion. Furthermore, it will be shown that cortisone, added to a low-calcium diet, completely prevents the action of vitamin D on serum and bone citrate but has no effect on the vitamin D-induced elevation of serum calcium.

EXPERIMENTAL METHODS AND RESULTS

Male Sprague-Dawley weanling rats weighing 60 g were used in all experiments. They were housed in screen-bottomed cages and given food and water ad libitum.

The basal diet, as described by Bellin and Steenbock (11), was composed of the following in per cent: glucose monohydrate 67 (Cericlose), vitamin-free casein 18 (General Biochemicals, Inc.), cystine 0.2, cottonseed oil (Wesson) 10, roughage (Celluloflor) 3, calcium and phosphorus-free salts 2 (11), and vitamins. Each kilogram of diet supplied the following quantities (mg) of crystalline, water-soluble vitamins: thiamine hydrochloride 5, riboflavin 5, pyridoxine hydrochloride 5, calcium pantothenate 28, nicotinamide 20, inositol 200, folic acid 0.2, vitamin B12 0.02, biotin 0.1, and choline chloride 500. In addition, the rats received a supplement of fat-soluble vitamins in cottonseed oil solution. The supplement provided the following quantities (µg) per week: β-carotene 20, α-tocopherol 875, and 4-methyl 2,4-naphthoquinone (Menadione) 105. Crys-
talline vitamin D₂ was administered orally, in the indicated amounts, in cottonseed oil solution. Cortisone acetate, where used, was incorporated in the diet at a level of 30 mg/100 g diet. The respective deficiencies were produced by omitting the appropriate vitamin from the diet.

The desired levels of calcium and phosphorus in the diet were obtained by the addition of Ca₃(PO₄)₂ and an equimolar mixture of KH₂PO₄ and K₂HPO₄, respectively, at the expense of the Cerelose. The nonrachitogenic diet (no. 11) contained 0.47% calcium and 0.3% phosphorus; the high-calcium rachitogenic diet (no. 24) contained 1.5% calcium and 0.12% phosphorus; and the low-calcium diet (no. 11D) contained 0.016% calcium and 0.3% phosphorus.

In the experiments in which the nonrachitogenic diet (no. 11) was employed (Table 2), the animals were maintained for 3 weeks on diets deficient in either vitamin D or the appropriate water-soluble vitamin. The control group received the complete diet.

In the experiments in which the high-calcium rachitogenic diet (no. 24) (Table 1) was used, the animals were depleted of vitamin D and the appropriate water-soluble vitamin for 21 days. At the end of this depletion period one group was killed for o-time values. A second group was given 75 IU of vitamin D every other day for 10 days on the doubly deficient, rachitogenic diet (no. 24). The response values are from animals given 75 IU of vitamin D₂ every 2 days during the 10-day period following o-time. There were at least 4 rats in each group.

Femurs were removed from the carcasses and freed of adhering tissue. For citric acid analysis they were extracted with diethyl ether in a Soxhlet extractor for 24 hr, dried at 100°C, ground in a mortar, and weighed. The bone powder was extracted for four 1-hr periods with 1 N H₂SO₄ and the extracts combined for analysis. For ash determination, the bones were extracted for 24 hr with 95% ethanol and 24 hr with diethyl ether. They were finally ashed at 1,200°F for 8-12 hr.

Citric acid analyses were by the method of Speck, Moulder, and Evans (17). Serum phosphorus was determined by the procedure of Fiske and SubbaRow (18), and serum calcium was estimated by the method of Wang (19).

Since it was found previously that pantothentic acid and pyridoxine deficiencies interfered with the vitamin D-induced rise in citrate of serum, kidney, urine, and to a limited extent of bone, the first series of experiments were designed to determine whether these deficiencies would also interfere with the action of vitamin D on bone ash accretion and on serum P in rachitic rats. It was quite evident (Table 1) that neither deficiency interfered with femur ash accretion or the rise in serum phosphorus brought about by the administration of vitamin D. Nevertheless, it was evident from previous (16) and present experiments that they greatly interfered with the effect of vitamin D on serum citrate. In addition, pyridoxine deficiency reduced the rise in bone citrate following vitamin D as well. In agreement with previous results (16), pantothentic acid had little or no effect on bone citrate. It should be noted that prolonged deficiency of these B vitamins was avoided since it has been shown that calcification is adversely affected under these conditions, perhaps by interference with matrix synthesis (20, 21). The data from these experiments then indicated that the rise in serum citrate and to some degree of bone citrate was not necessary for the rise in serum P and bone ash deposition in response to vitamin D. These results agree closely with those of Harrison et al. (15) in which cortisol was used to prevent the vitamin D-induced rise in serum and bone citrate.

### Table 1. Response of rachitic rats deficient in either pantothentic acid or pyridoxine to vitamin D administration

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Femur</th>
<th>Serum</th>
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<tbody>
<tr>
<td>Dry wt., mg</td>
<td>Citrate, mg</td>
<td>Ash, mg</td>
</tr>
<tr>
<td>o-time</td>
<td>95.3</td>
<td>0.391 ± 0.001</td>
</tr>
<tr>
<td>Plus vitamin D</td>
<td>123.8</td>
<td>0.630 ± 0.010</td>
</tr>
<tr>
<td>Plus vitamin D and pantothetic acid</td>
<td>117.9</td>
<td>0.688 ± 0.015</td>
</tr>
<tr>
<td>o-time</td>
<td>102.4</td>
<td>0.290 ± 0.015</td>
</tr>
<tr>
<td>Plus vitamin D</td>
<td>120.4</td>
<td>0.527 ± 0.006</td>
</tr>
<tr>
<td>Plus vitamin D and pyridoxine</td>
<td>129.6</td>
<td>0.740 ± 0.017</td>
</tr>
</tbody>
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Values are means ± SEM. The o-time values are taken from animals after 21 days on the doubly deficient, rachitogenic diet (no. 24). The response values are from animals given 75 IU of vitamin D₂ every 2 days during the 10-day period following o-time. There were at least 4 rats in each group.
DISCUSSION

It has been known for many years that vitamin D consistently increases serum calcium. This was again reemphasized by Steenbock and Herting (22), who showed that this effect of vitamin D is found regardless of the Ca and P content of the diet. When an adequate calcium and phosphorus diet was fed, vitamin D had a rather striking effect on the serum calcium (Table 2). Again it is obvious that vitamin B deficiencies were inadequate in this regard since they would not prevent the rise in bone citrate (Table 1). By utilizing the finding of Harrison et al. (15) that cortisol administration prevents the effect of vitamin D on bone citrate, it was possible to carry out such an experiment. The results (Table 3) show clearly, in agreement with Harrison et al. (14, 15), that cortisol completely prevents the action of vitamin D on bone and serum citrate. However, it had no effect on the vitamin D-induced rise in serum calcium. These results then do not support the hypothesis that bone citrate mediates the vitamin D-induced rise in serum calcium even when diets very low in calcium are fed. It may be of interest to note the change in serum citrate noted in the three experiments presented. Serum citrate values were found low with low calcium diets (Table 3) and high with high calcium diets (Table 1). This is not unexpected and may provide some insight into the accumulation of citrate following vitamin D administration.

Our over-all results agree closely with those of Harrison et al. (14, 15). A clear separation of the effects of vitamin D on citrate and mineral metabolism can be achieved by means of pantothenic acid and pyridoxine deficiencies or cortisol, which indicates that citrate per se does not play a role in the basic actions of vitamin D on calcium and phosphate metabolism. However, the citrate phenomenon should not be discarded completely since it may nevertheless serve as an indicator or may be the direct result of the basic biochemical action of vitamin D. It is quite possible that citrate accumulation following vitamin D administration is a reflection of changes in cell membranes or subcellular particulate membranes. For example, the decreased oxidation of citrate by kidney mitochondria from rats fed vitamin D now appears related to structural changes of these particles (24). However, the elucidation of the exact mechanism of citrate accumulation following vitamin D administration must await further investigation.

REFERENCES


