Intestinal CaBP: a new quantitative index of vitamin D deficiency in the rat

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Bronner, Felix, and Thomas Freund. Intestinal CaBP: a new quantitative index of vitamin D deficiency in the rat. Am. J. Physiol. 229(3) : 689 - 694. 1975.—Rats raised from weaning on regimens adequate in calcium and phosphorus but deficient in vitamin D will have no detectable intestinal calcium-binding proteins (CaBP), whether or not they show other signs of vitamin D deficiency, such as hypocalcemia. When hypocalcemic, vitamin D-deficient animals were treated with 25-hydroxycholecalciferol, a vitamin D metabolite, they showed a dose-dependent increase in plasma calcium and CaBP; both responses can be described by a single linear relationship, which appears to apply whether the metabolite is 25-hydroxycholecalciferol or dihydrotachysterol. Since vitamin D status is only one determinant of plasma calcium, whereas CaBP (or its expression) appears to depend on vitamin D quantitatively, CaBP may be used as an index of vitamin D status, provided calcium intake is controlled.

However, as shown by Hurwitz et al. (13), the actual plasma calcium level in the vitamin D-deficient rat on a normal mineral intake is a positive function of net calcium absorption. Therefore, hypocalcemia will supervene for certain only when the absolute calcium intake is low. This means that when rats are on intermediate or high-calcium (and phosphorus) intakes that are deficient in vitamin D, there is uncertainty as to their ultimate vitamin D status, since they may not become hypocalcemic and their bones on necropsy show no obvious signs of deficiency.

It is generally held that active duodenal calcium transport is vitamin D dependent (20,22). Therefore, the absence of active calcium transport in the duodenum could be used as a measure of vitamin D deficiency (21), but to our knowledge this has not been done systematically. Moreover, the most commonly used parameter of active calcium transport, the serosal-to-mucosal ratio in everted gut sacs, although a reliable index, has not been used to determine vitamin D status, except by Schaechter et al. (21).

The calcium-binding proteins (CaBP), found in the intestine of rats (9,11, 15), other mammals (see ref. 22 for review), and chicks (22,23), are vitamin D dependent in that their induction does not occur in deficient animals. We now report that this molecular expression of vitamin D action can be used as a quantitative index of the vitamin D status of rats.

MATERIALS AND METHODS

Animals and diets. Male Sprague-Dawley rats were used. Upon receipt, weanling animals were divided into lots and placed on either vitamin D-containing (2,200 IU/kg feed) or vitamin D-deficient regimens. Diets, purchased from General Biochemicals (now Teklad Mills), Chagrin Falls, Ohio, contained 28% vitamin-free test casein, 5% corn oil, 1% vitamin supplement (with or without vitamin D), 3% salt mixture, and 56% sucrose and cellophane spangles (a nutrient-free filler). Six diets were used: a low-calcium diet I, 0.06% Ca, 0.2% P; diet I without added vitamin D (I-D); an intermediate calcium diet, II, 0.5% Ca, 0.5% P; diet II without added vitamin D (II-D); a high-calcium diet, III, 1.5% Ca, and 1.5% P; and diet III

1 In the rat, CaBP occurs as two proteins of similar molecular weight (≈11,000–12,000 daltons) and specific calcium-binding capacity (11).

2 In an earlier publication (11), diet II with added vitamin D has been referred to as BCD and diet II without vitamin D as BC.
without added vitamin D (III-D). Ca and P contents were verified by analysis of dry-ashed samples (5, 13). Complete formulations are available from the supplier under the following codes: I- TD 67205A; I-D TD 73445; II TD 70387; II-D TD 70388; III TD 67907; III-D TD 73446.

Two to four weeks after the animals had been placed on their regimens, those on diets I-D and II-D had usually attained a level of hypocalcemia considered typical of vitamin D deficiency (<6.5 mg Ca/100 ml). The deficient animals (including those on diet III-D) and their controls were then utilized for the experiments.

A typical protocol involved measuring the plasma calcium at weekly intervals until the animals were considered hypocalcemic and/or deficient. Plasma calcium was then measured before and at varying intervals following the intraperitoneal injection of dihydrotachysterol (DHT3) or 25-hydroxyvitamin D3, a compound known (2) to have a more rapid action than vitamin D3 on calcium absorption and plasma calcium. Some animals received only vehicle. Thirty hours later the animals were decapitated, their intestines (15 cm beyond the pylorus) removed, rinsed in iced saline, everted, and the mucosal tissue removed. The material from six animals was pooled and fractionated.

Plasma calcium was determined on tail vein samples collected in heparinized capillary tubes which were centrifuged. The resulting plasma was placed in tared tubes, diluted with 0.1% LaCl3, and analyzed by atomic absorption spectrophotometry (11, 13). Samples were analyzed individually and appropriate group averages computed.

Fractionation of calcium-binding protein. Mucosal tissue was removed by scraping the everted, rinsed duodenum with a glass slide. The pooled material from several animals (typically 6) was homogenized in a modified Tris-HCl buffer (0.013 M Tris-HCl, 0.12 M NaCl, 0.003 M KCl, pH 7.4), centrifuged (100,000 g for 60 min), and the resulting supernate lyophilized, reconstituted in 2 ml elution buffer (0.02 M ammonium acetate, 1 mM mercaptoethanol, pH 7.2), and chromatographed on a Sephadex G-50 column (2.5 x 90 cm). Peak B (Vp/Vo = 2.0) of the calcium-binding profile, which includes the intestinal calcium-binding proteins of the rat (11), was assayed quantitatively for calcium binding with the aid of a semimicro competitive binding assay utilizing Chelex resin (Bio-Rad Laboratories, Richmond, Calif.). Binding was determined as a function of the assay calcium concentration.

Typically, the calcium concentration of the sample was 0.1-0.2 mg/ml and the amount of added calcium was 0-100 mg/ml, so that the actual calcium concentration of the sample contributed negligibly to its calculated binding capacity. Free Ca2+ concentration in the assay varied from 0.2 to 15 μM. The binding data were analyzed by saturation kinetics. The binding capacity, n, reported as nanomoles calcium bound per milligram protein, is derived from a least-squares linearization of the Langmuir isotherm. It represents the maximum amount of calcium bound by the binding proteins at equilibrium and, as indicated above (see also ref. 11), is virtually independent of the calcium concentration of the pooled eluate. Consequently, n is a measure of the number of moles of CaBP in a given sample. For purposes of quantitative comparisons, equal amounts (200 mg) of protein (in terms of the reconstituted initial supernate) were loaded onto the column.

The precision of the estimate of n is determined by how well the calculated regression line (1/Cate vs. 1/Cabound) describes the experimental points. The percent standard error of the y intercept of each line can be taken as an expression of the goodness of fit of the line. In the experiments described here, this value, obtained typically on 10 points over a 100-fold calcium concentration, averaged 3%, with a range of 1-4.5%. The accuracy or replicability of this measure is given by the percent standard error of the mean value of n for each diet group. This value has been shown (11) to be less than 5% SE in analyses of 13-15 separate lots of animals and was similar in the experiments reported here (see below). See ref. 11 for further details concerning fractionation and assay procedures.

Protein was monitored at 280 nm. Calcium was determined by atomic absorption spectrophotometry, with suitable standards for each buffer. Ca2+ was analyzed by liquid scintillation.

Vitamin D metabolites 25-Hydroxyvitamin D3 (25-OH-D3) was a gift of The Upjohn Co. It was dissolved in propylene glycol-ethanol (1:1) and injected intraperitoneally in a volume of 0.1 ml. To verify the dose, the administered volume was weighed in sextuplicate and was found to weigh 0.1 g with a coefficient of variation of 1%. One unit = 0.026 μg. Dihydroxytachysterol (DHT3) was purchased from Philips Roxane Laboratories, Inc., Columbus, Ohio.

RESULTS

Time course of calcemic response to 25-OH-D3. In all animals that gave a calcemic response to 25-OH-D3, the time course was similar in that the measured peak response occurred at 24-25 h after intraperitoneal injection of the 25-OH-D3. When time courses were plotted for individual animals, the mean peak for all animals, regardless of dose, was estimated at 21.4 h (SD:1.5) after injection (13 animals). A time-response curve for one experiment is shown in Fig. 1. Somewhat similar response curves have been reported (rat, ref. 2; chick, ref. 25).

Extent of calcemic response. Figure 2 shows a plasma calcium dose-response curve to 25-OH-D3 in animals that showed an appropriate time-response curve. As can be seen, the response was dose related and can be represented as a linear function of the dose raised to the power of 2.

The absolute calcemic response to a given dose varied in different experiments. Thus, the overall mean calcemic response to 15 U 25-OH-D3 in three experiments (13 animals on diet II-D) was 2.78 mg Ca/100 ml (SE: 0.38), as opposed to the value of 2.25 shown in Fig. 2. The variability may be related in part to the fact that pretreatment values differed in different experiments. As also reported by Cuisinier-Gleizes et al. (8), a statistically significant inverse relationship was observed between the plasma calcium response, ΔCa, and the initial plasma calcium value, [Ca0], namely

\[
\Delta [Ca] = 6.17 (\pm 1.82) - 0.45 (\pm 0.23) [Ca0] \quad (1)
\]
CABP—INDEX OF VITAMIN D DEFICIENCY

FIG. 1. Time course of mean plasma calcium response, $\Delta [Ca]$, of 4 vitamin D-deficient, hypocalcemic rats to an intraperitoneal injection of 15 U 25-hydroxycholecalciferol. $\Delta [Ca]$ was calculated for each of 4 rats for each time period, averaged, and mean values of $\Delta [Ca]$ are shown with associated standard errors. Absolute plasma calcium values were:

<table>
<thead>
<tr>
<th>Body Wt, g (SE)</th>
<th>Plasma Calcium, mg/100 ml (SE)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0 5 15 25 30</td>
</tr>
<tr>
<td>116.1</td>
<td>4.79 4.92 8.26 9.30 8.46</td>
</tr>
<tr>
<td>(6.1)</td>
<td>(0.15) (0.24) (0.48) (0.28) (0.31)</td>
</tr>
</tbody>
</table>

where dose is units, administered intraperitoneally.

FIG. 2. Plasma calcium response ($\Delta [Ca]$) of hypocalcemic, vitamin D-deficient rats to varying doses of 25-hydroxycholecalciferol. Responses were determined 24 h after intraperitoneal injection. Equation of line, derived by a least-squares method is:

$$\Delta [Ca] = 0.88 (\pm 0.8) + 0.32 (\pm 0.16) \log_2$$

where dose is units and the values in parentheses are 95% confidence limits. $r = 0.88$. Response curve was obtained from a single lot of 9 animals (150 U: 4; 15 U: 3; 1.5 U: 2) that had been raised on diet II-D from weaning and whose time course of response in all cases except one (starred) was like that shown in Fig. 1.

where the units are milligrams Ca/100 ml and the values in parentheses are 95% confidence limits. The value of $r$, the correlation coefficient, for equation 1 is 0.79.

**CaBP response.** Figurc 3 shows a doce-response curve for CaBP in three groups of six animals each (single lot), treated with 1.5, 15, and 150 U 25-OH-D$_3$ 30 h before sacrifice. The 30-h interval was chosen because in experiments in which the amount of CaBP was increased as a result of dietary maneuvers (11), CaBP reached a maximum between 24 and 48 h after the animals had been shifted to a low-calcium intake. As can be seen, the response was dose related and can be described as a function of the administered dose raised to the power of 2.

**CaBP and $\Delta [Ca]$ responses combined.** Because both calcemic and CaBP responses to 25-OH-D$_3$ were a function of the dose of metabolite raised to the power of 2, CaBP levels were plotted as a function of $\Delta [Ca]$, Fig. 4. As can be seen, the responses to vehicle, 1.5, 15, and 25 U 25-OH-D$_3$, can be described by a single line, the equation of which is given in the legend of Fig. 4. Moreover, when in separate experiments with hypocalcemic rats made vitamin D deficient on the same regimen (II-D) doses of dihydrotachysterol or 25-hydroxycholecalciferol were administered, the plasma calcium and CaBP responses are equally well described by the equation. Absolute values of $\Delta [Ca]$ and CaBP varied in different experiments by about 10% SE, a value that probably can be reduced by further replication. It would thus appear that CaBP may be used as a measure of the vitamin D status of a group of animals.

**CaBP as an index of vitamin D status.** Further to test the validity of the conclusion that CaBP may be used as an index of vitamin D status, animals were placed on either vitamin D-deficient or D-supplemented diets that contained either low (I) or high (III) concentrations of calcium and phosphorus. Table 1 shows the results of two separate experiments. As can be seen, when animals were on the low-calcium diet from weaning their plasma calcium was somewhat depressed as compared to that of animals on the high-calcium diet (9.7 mg/100 ml vs. 10.3 or 10.1), but the differences failed to be statistically significant ($P > 0.05$).
Animals raised from weaning on diet II-D usually develop hypocalcemia, but the severity is variable and unpredictable (13). In one experiment some animals that were becoming hypocalcemic reversed themselves so that 2 wk later their plasma calcium had become near normal (10.00 mg/100 ml, SE: 0.09). Rather than discard these animals, we divided them into two groups of six and four each and treated the first with 15 U 25-OH-D3 and the second with vehicle. The treated group responded by developing hypercalcemia (mean Δ[Ca+] after 24 h: 1.89; SE: 0.36; Fig. 5), whereas vitamin D-replete, normocalcemic animals do not respond to either 15 or 150 U 25-OH-D3 (Table 2). Analysis of the mucosal scrapings of the normocalcemic animals fed a vitamin D-deficient diet revealed that the vehicle-treated group had no CaBP, while the 25-OH-D3-treated group showed a level of CaBP that corresponded to the level predicted from the graph in Fig. 4 for the measured increment in Δ[Ca+] (starred value, Fig. 4). Again CaBP was a qualitative and quantitative index of vitamin D status.

**DISCUSSION**

*Vitamin D deficiency and hypocalcemia.* Table 1 shows that in animals raised on vitamin D-deficient diets the intestinal CaBP was undetectable, regardless of the plasma calcium level of these animals. In rats, the plasma calcium level is regulated by bone and, to a minor degree, by kidney (1, 3), with the proportion of circulating plasma calcium that is bone derived varying inversely with calcium absorption (4, 19). The capacity to maintain plasma calcium even on a very low-calcium intake is virtually unimpaired in normal animals (refs. 10, 19; Table 1), but compromised in parathyroidectomized (19) or vitamin D-deficient (ref. 13; Table 1) animals. The degree to which calcium homeostasis is impaired is a complicated function of intake, ability to absorb calcium, the degree of calcification and metabolic capacity of the skeleton, and of renal function. Nevertheless, if one plots the calcium plasma levels of vitamin D-re-

If these animals were also deprived of vitamin D (I-D), they became severely hypocalcemic, as expected (4.9 and 4.8 mg/100 ml). On the other hand, animals raised on diet III-D from weaning in one case showed no and in the other a statistically significant, but modest hypocalcemia compared to the animals on diet III. Thus, a dietary maneuver, high-calcium intake, obscured the only sign of vitamin D deficiency, viz. hypocalcemia. Yet both groups of animals on the vitamin D-deficient diets, whether hypocalcemic or not, were devoid of detectable levels of CaBP. It must therefore be concluded that CaBP can be a more reliable index of the vitamin D status of an animal than its plasma calcium level.

**TABLE 1. Plasma calcium and intestinal CaBP levels of rats on low- and high-calcium regimens**

<table>
<thead>
<tr>
<th>Exp</th>
<th>Regimen</th>
<th>Vitamin D Content, U/kg Diet</th>
<th>Body Wt, g</th>
<th>Plasma Ca [Ca+, mg/100 ml]</th>
<th>CaBP nmol Ca Bound per mg Protein</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>I</td>
<td>0.06 0.2 2,200</td>
<td>139±2</td>
<td>9.7±0.2</td>
<td>66±0.3</td>
</tr>
<tr>
<td></td>
<td>D</td>
<td>0.06 0.2 0</td>
<td>115±3</td>
<td>4.9±0.2</td>
<td>None</td>
</tr>
<tr>
<td></td>
<td>III</td>
<td>1.5 1.5 2,200</td>
<td>154±3</td>
<td>10.3±0.3</td>
<td>53±1.0</td>
</tr>
<tr>
<td></td>
<td>III-D</td>
<td>1.5 1.5 0</td>
<td>140±7</td>
<td>10.2±0.2</td>
<td>None</td>
</tr>
<tr>
<td>B</td>
<td>I</td>
<td>0.06 0.2 2,200</td>
<td>107±2</td>
<td>9.7±0.3</td>
<td>74±1.2</td>
</tr>
<tr>
<td></td>
<td>I-D</td>
<td>0.06 0.2 0</td>
<td>77±2</td>
<td>4.8±0.3</td>
<td>None</td>
</tr>
<tr>
<td></td>
<td>III</td>
<td>1.5 1.5 2,200</td>
<td>102±4</td>
<td>10.1±0.5</td>
<td>48±1</td>
</tr>
<tr>
<td></td>
<td>III-D</td>
<td>1.5 1.5 0</td>
<td>101±4</td>
<td>8.0±0.5</td>
<td>None</td>
</tr>
</tbody>
</table>

Values are means ± SE. Animals raised on regimens from weaning. Each group consisted of six male rats. None means none detected.

**FIG. 4. Intestinal calcium-binding protein levels (n; nmol Ca bound/mg protein) as a function of increment in plasma calcium (Δ[Ca+], mg/100 ml).** Data from which least-squares regression line was obtained were from 4 groups each of 6 hypocalcemic, vitamin D-deficient rats (diet II-D) treated with vehicle, 1.5, 15, and 150 U 25-hydroxycholecalciferol (- -). Plasma calcium levels were measured 24 h after dosing, CaBP levels 30 h after dosing. Horizontal standard error bars apply to plasma calcium measurements. Note that values obtained in separate experiments with hypocalcemic, vitamin D-deficient animals that had been dosed with 2.5 and 25 µg DHTZ (- O-) or 15 U 25-hydroxycholecalciferol (- -) 30 h earlier or normocalcemic animals on same vitamin D-deficient regimen (- -) given 15 U 25-hydroxycholecalciferol arc also predicted by equation, viz.  

\( n = 10.1 (±14.4) + 9 (±7.7) \Delta[Ca+] \)

with 95% confidence limits in parentheses. \( r = 0.91. \)

**FIG. 5. Time course of mean plasma calcium response, Δ[Ca+], of 6 vitamin D-deficient, normocalcemic rats to an intraperitoneal injection of 15 U 25-hydroxycholecalciferol. Δ[Ca+] was calculated as described in legend for Fig. 1. Absolute plasma calcium values were:**

<table>
<thead>
<tr>
<th>Body Wt, g (SE)</th>
<th>Plasma Calcium, mg/100 ml (SE)</th>
<th>h after dosing</th>
</tr>
</thead>
<tbody>
<tr>
<td>125.2 (4.4)</td>
<td>9.76 (0.20)</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>9.79 (0.16)</td>
<td>5</td>
</tr>
<tr>
<td></td>
<td>10.79 (0.12)</td>
<td>15</td>
</tr>
<tr>
<td></td>
<td>11.65 (0.27)</td>
<td>25</td>
</tr>
<tr>
<td></td>
<td>10.50 (0.13)</td>
<td>30</td>
</tr>
</tbody>
</table>
plete and vitamin D-deficient animals as a function of net absorption (see Fig. 5 in ref. 13; Cuisinier-Gleizes, personal communication), one obtains two curvilinear functions. In other words, for a given value of absorbed calcium, the plasma calcium is always higher in the vitamin D-replete than in the deficient animals. Experimentally speaking, however, the difference in plasma calcium at high levels of absorption may be too small to be detectable, especially if measured without reference to absorption and expressed as the vitamin D metabolite. This seems unlikely, however.

The diminution of the calcemic response with higher species as well (22). Moreover, CaBP synthesis is one of the earliest events following the administration of 1, 25-dihydroxyvitamin D$_3$, whose potency for CaBP induction in the chick appears to be twice as great as its calcemic potency (17). Therefore, the presence of CaBP seems to be a more reliable index of vitamin D status than is the plasma calcium level, which, as discussed above, is influenced by many factors, not all of which relate directly to vitamin D.

Moreover, our quantitative studies (Fig. 4 and the increase in CaBP following treatment of normocalcemic, vitamin D-deficient animals), although not exhaustive, indicate that CaBP may serve as a quantitative index of vitamin D deficiency or restitution.

With the aid of the regression equation in Fig. 4, one can predict that in hypocalcemic animals on the vitamin D-deficient diet II (0.5% Ca; 0.5% P) the plasma calcium level that corresponds to zero CaBP activity is 5.2 mg/100 ml. Moreover, one would predict that animals whose plasma calcium level lies between 5.5 and 6.0 mg/100 ml would have detectable amounts of CaBP. Whether CaBP values of 0 < n < 8 reflect merely the lower level of detection or a small, but significant amount of CaBP must await further work.

Usefulness of CaBP test for vitamin D status. At present the test as practiced by us is destructive and requires at least six animals. If a quantitative immunoassay is developed for rat intestinal CaBP, it may be possible to test individual animals. The possibility of an intestinal biopsy test also exists and would have importance for human studies, provided the quantitative relationships demonstrated here for rats also apply to humans or other animals.

Implications for vitamin D metabolism. Work is needed to demonstrate whether the vitamin D requirement for CaBP...
synthesis differs from that for expression of vitamin D function at the bone level. The linearity of the relationship between CaBP and the increment in [Ca\textsubscript{0}] obtained in vitamin D-deficient, hypocalcemic animals (Fig. 4) argues against significant quantitative differences. So does the report by MacGregor et al. (16), who studied CaBP synthesis in partially deficient chicks and from whose data one can derive a reasonably linear correlation between plasma calcium and the logarithm of intestinal CaBP activity. On the other hand, until each process has been studied in vitro and in vivo, it is difficult to evaluate quantitative differences in detail.

We thank Janet Cuddiloo and Ethan S. Bronner for devoted technical assistance.

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Since submission of this article, Bar and Wasserman published on the use of the duodenal calcium-binding protein as a bioassay for vitamin D in the chick (J. Nutr. 104: 1202-1207, 1974).

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REFERENCES


