Vitamin D-dependent Calcium-binding Protein

RESPONSE TO SOME PHYSIOLOGICAL AND NUTRITIONAL VARIABLES

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SUMMARY

The effect of some physiological and nutritional variables on the vitamin D-dependent calcium-binding protein content of duodenal mucosa of chicks was assessed. Calcium-binding protein, originally detected in vitamin D$_2$-treated rachitic chicks, is also present in normally raised chicks; however, the concentration of calcium-binding protein in the vitamin D$_1$-treated rachitic chick mucosa exceeded that in normal mucosa. $^{47}$Ca absorption by a duodenal loop and the calcium-binding protein content of duodenal mucosa decreased at a similar rate when chicks given a single dose of vitamin D$_1$ were replaced on a rachitogenic diet. Chicks adapted to a low calcium intake absorbed more calcium and had a greater concentration of duodenal calcium-binding protein than chicks on a normal diet. Further, content of mucosal calcium-binding protein was greater in younger than more mature birds, the same in pullets and cockerels of the same age, and greater in laying than in nonlaying hens. Estrogen (diethylstilbestrol), a hormone prominent in the process of egg laying, did not induce the formation of calcium-binding protein. Vitamin D$_2$ was more effective in calcium-binding protein formation than vitamin D$_1$ which fact coincides with their relative physiological effects. The relationship between these studies and the implication of a role of calcium-binding protein in calcium translocation is discussed.

Vitamin D, known to be required for the optimal absorption of calcium, has come under intensive investigation in recent years. One feature of vitamin D action that has required explanation is the lag period between the time when vitamin D is administered and the time when a physiological response can be observed (1-3). It was speculated that this lag was necessary for the transformation of vitamin D into an active form or to stimulate the synthesis of an unknown component of the calcium absorptive process (3). Recent evidence tends to confirm both hypotheses since Lund and DeLuca have shown that vitamin D$_2$ can be altered in vivo to yield a more polar metabolite having biological activity (4), and we have recently shown that one consequence of vitamin D$_1$ administration to a rachitic animal is the appearance in intestinal tissue of a calcium-binding protein not present in the rachitic animal (5). A possible role of vitamin D in a protein synthetic process is also derived from the several studies showing that inhibitors of protein synthesis (actinomycin D, puromycin, 5-fluorouracil) partially or completely inhibit its physiological effect (6-8), and that the vitamin stimulates the early incorporation of labeled nucleic acid precursors into intestinal mucosa RNA (9).

GENERAL METHODS

The experimental animals, variously treated as described below, were killed by decapitation. The handling of the tissue and the homogenization procedure were as previously described (5, 12). The duodenum was removed, cooled in 0.85% NaCl at $4^\circ$, everted, and the mucosal layer separated from the underlying muscle layers by the use of a glass slide. The mucosa was homogenized at $4^\circ$ with a Potter-Elvehjem homogenizer and Teflon pestle. The homogenate was centrifuged at 39,000 $\times$ g for 20 min in a refrigerated centrifuge and the supernatant retained. In all of these studies, the supernatant fluid was used.

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1 The abbreviation used is: CaBP, calcium-binding protein.
heated at 60° for 10 min, a procedure previously shown to remove about two-thirds of the inactive protein without appreciably influencing the calcium-binding activity of the supernatant (5).

The calcium-binding activity of the supernatant was assessed by the Chelex-100 assay procedure (5) except for one study which dealt with the alteration of calcium-binding capacity of the supernatants with time after vitamin D3 administration to rachitic chicks. This was done by a method employed earlier (11) in which, instead of resin, the homogenate debris served as the phase competing with the supernatant binding factor for added radiocalcium. The calcium-binding data are expressed as the percentage of added 45Ca remaining in the supernatant fluid and, in some cases, as (45CaPr:45CaR) which is the ratio of distribution of radiocalcium between the protein-bound form and the resin-bound form. This ratio is linearly related to the concentration of binding protein in the test sample and allows a quantitative correlation to be made between CaBP binding activity and a physiological or nutritional effect (10).

In several of the studies, the effect of the variables on the protein pattern of the supernatant fluid was assessed by slab acrylamide gel electrophoresis. This is described in detail elsewhere (12). With this procedure, a qualitative indication of CaBP content of the supernatant fluid can be obtained.

The measurement of the absorption of 45Ca from a ligated duodenal loop was described before (3). One milliliter of 0.85% NaCl containing 0.5 μC of 45Ca and 1 mg of calcium (as the chloride) was injected into the lumen of a tied-off loop of the duodenum of an anesthetized chick. At 30 min, the loop was excised and placed in a counting tube, and the residual radioactivity was measured with a well-type scintillation detector and γ-ray spectrometer. The spectrometer was set to eliminate any contribution of the radioactive daughter, 47Sc, to the count rate of the parent, 45Ca.

The cockerels, pullets, and hens used were of the White Leghorn breed. The cockerels were obtained from the Babcock Hatchery, Ithaca, New York, and the older birds from the Department of Poultry Science, Cornell University, Ithaca.

Protein was determined with a Technicon AutoAnalyzer by the procedure of Lowry et al. (13) with standardized human serum (Versatol, Warner-Chilcott Laboratories, Morris Plains, New Jersey) used as the protein standard.

**RESULTS**

**Normal Diet**—Day-old cockerels were raised on a commercially-available chick starter mash to the age of 4 weeks. At 72 hours before experiment, four chicks were given 500 i.u. of vitamin D3 orally in vegetable oil and the same number were given vegetable oil only. The calcium-binding activity and gel pattern of the duodenal mucosa supernatants were determined and, for comparative purposes, the calcium-binding activity and gel patterns of supernatants from rachitic and vitamin D3-treated rachitic chicks of the same age were also assessed.

The acrylamide gel pattern (Fig. 1) shows that the duodenal supernatant from vitamin D3-treated rachitic chicks and chicks raised on a vitamin D-adequate diet contains the CaBP protein band. Rachitic supernatant does not contain this band. This indicated that the formation of the protein is not merely an artifact consequence of the rachitic state subsequently modified by treatment with vitamin D. A comparison of the intensities of the stained bands on the gel further shows that the concentration of calcium-binding protein in the normal animal appears to be less than that in the vitamin D3-repleted rachitic chick. The 45Ca binding activities of these two groups indicated the same trend (Table I) and, further, that vitamin D3 administration to the normal animal did not alter the intensity of the gel band or enhance radiocalcium binding. A possible explanation for

![Fig. 1. Acrylamide gel electrophoresis (pH 8.3) of supernatant fluids from homogenates of rachitic (1), vitamin D-treated rachitic (2), normal (3), and vitamin D-treated normal chicks (4). The dark arrow designates the protein band associated with calcium-binding protein. The open arrow designates another band discussed in the text. Thirty-microliter samples were placed in the sample slots of the gel slab (top) and 200 volts were applied for 3 hours. Migration was toward the anode (bottom).](http://www.jbc.org/)

<table>
<thead>
<tr>
<th>Diet</th>
<th>Vitamin D&lt;sub&gt;3&lt;/sub&gt;</th>
<th>45Ca in supernatant fluid</th>
<th>Protein concentration</th>
<th>Specific activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal</td>
<td>−</td>
<td>29.3 ± 1.8</td>
<td>6.2 ± 0.3</td>
<td>4.7</td>
</tr>
<tr>
<td>Normal</td>
<td>+</td>
<td>29.0 ± 0.6</td>
<td>6.8 ± 0.8</td>
<td>4.3</td>
</tr>
<tr>
<td>Rachitogenic</td>
<td>−</td>
<td>11.2 ± 0.4</td>
<td>5.6 ± 0.3</td>
<td>2.0</td>
</tr>
<tr>
<td>Rachitogenic</td>
<td>+</td>
<td>47.4 ± 1.1</td>
<td>5.9 ± 0.1</td>
<td>8.0</td>
</tr>
</tbody>
</table>

* A normal diet consists of chick starter mash. Vitamin D<sub>3</sub> (500 i.u.) was given orally to those groups so designated. Values represent the mean of five determinations ± standard error of the mean.
**Supplemental Note:** The composition of the basal diet follows: cerealse (61.07%), C-1 soy protein (27%), corn oil (3%), non-nutritive fiber (3%), NaCl (1%), vitamin and amino acid supplement (0.425%), and salt mixture (3.501%). Vitamin and amino acid supplement supply the following per 100 pounds: glycine (136.2 g), methionine (817.8 g), nicotinol (11.35 g), inositol (2.27 g), calcium pantothenate (0.908 g), pyridoxine HCl (0.2043 g), folic acid (0.118 g), CuSO4.5H2O (0.758 g), CoSO4.7H2O (0.0908 g), sodium molybdate (0.377 g), and ZnCO3 (5.221 g). Supplemental calcium was added at level of 2.8 g of CaCO3 per 100 g of diet (cf. Footnote a to Table II for diet composition).

![Table II](http://www.jbc.org/content/issue-725-1968/3989)

**Table II:** Effect of calcium intake on duodenal absorption of 47Ca and calcium-binding activity of duodenal supernatant

<table>
<thead>
<tr>
<th>Dietary calcium levela</th>
<th>Days on experiment</th>
<th>Duodenal absorption of 47Ca b</th>
<th>Supernatant calcium binding activity b</th>
<th>Protein concentration</th>
<th>Specific activity (Ca/P) mg/ml</th>
<th>Ratio of Ca*/Pr/Ca*/IZ</th>
<th>Protein concentration</th>
<th>Specific activity (Ca/P) mg/ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>Percent</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Low (0.1%)</td>
<td>4</td>
<td>78 ± 3 (6)</td>
<td>41 ± 1 (3)</td>
<td>0.65 ± 0.05</td>
<td>5.6 ± 0.3</td>
<td>0.12 ± 0.01</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Normal (1.2%)</td>
<td>4</td>
<td>57 ± 4 (6)</td>
<td>27 ± 1 (4)</td>
<td>0.33 ± 0.04</td>
<td>4.5 ± 0.4</td>
<td>0.68 ± 0.01</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Low (0.1%)</td>
<td>6</td>
<td>87 ± 3 (6)</td>
<td>41 ± 2 (4)</td>
<td>0.65 ± 0.04</td>
<td>5.6 ± 0.1</td>
<td>0.12 ± 0.01</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Normal (1.2%)</td>
<td>6</td>
<td>61 ± 4 (6)</td>
<td>34 ± 2 (4)</td>
<td>0.47 ± 0.04</td>
<td>5.7 ± 0.1</td>
<td>0.68 ± 0.01</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

a The composition of the basal diet follows: cerealse (61.07%), C-1 soy protein (27%), corn oil (3%), non-nutritive fiber (3%), NaCl (1%), vitamin and amino acid supplement (0.425%), and salt mixture (3.501%). Vitamin and amino acid supplement supply the following per 100 pounds: glycine (136.2 g), methionine (817.8 g), nicotinol (11.35 g), inositol (2.27 g), calcium pantothenate (0.908 g), pyridoxine HCl (0.2043 g), folic acid (0.118 g), CuSO4.5H2O (0.758 g), CoSO4.7H2O (0.0908 g), sodium molybdate (0.377 g), and ZnCO3 (5.221 g). Supplemental calcium was added at level of 2.8 g of CaCO3 per 100 g of diet at the expense of cerealse. Modified diet after Leach and Nesheim (14).

b Values represent mean ± standard error of the mean. Animals per group are given in parentheses.

This “overshoot” phenomenon could be that another factor is involved in CaBP synthesis in addition to vitamin D, a factor which may regulate the quantity of CaBP formed. Another feature of the acrylamide gel pattern should be pointed out. It may be noted that the band designated with the open arrow (Fig. 1) is much more intense in the normal chick (with or without excess vitamin D) than that in the rachitic and vitamin D-treated rachitic chick. Whether this is related to vitamin D action or due to different diets is not known at present.

**Figure 2:** Effect of redeveloped vitamin D deficiency on the binding capacity of supernatants of mucosal homogenates and the absorption of 47Ca by duodenum. The binding activity was determined by the earlier method of Taylor and Wasserman (11) in which the homogenate debris served as the competitive accumulator of 47Ca instead of resin. The absorptive capacity of the ligated duodenum was as previously published (3). One milliliter of a solution containing 1 mg of calcium (as the chloride), 0.9% NaCl, and 0.5 μC of 47Ca was injected into a tied-off loop of duodenum. At 30 min, the duodenum was excised and counted for 47Ca radioactivity with a γ-scintillation detector and γ-spectrometer set to eliminate the contribution from the 47Ca daughter. The dose was taken as 100%, and percentage of absorption of 47Ca calculated by difference.

**Supplementary Note:** The composition of the basal diet follows: cerelose (61.07%), C-1 soya protein (27%), corn oil (3%), non-nutritive fiber (3%), NaCl (1%), vitamin and amino acid supplement (0.425%), and salt mixture (3.501%). Vitamin and amino acid supplement supply the following per 100 pounds: glycine (136.2 g), methionine (817.8 g), nicotinol (11.35 g), inositol (2.27 g), calcium pantothenate (0.908 g), pyridoxine HCl (0.2043 g), folic acid (0.118 g), CuSO4.5H2O (0.758 g), CoSO4.7H2O (0.0908 g), sodium molybdate (0.377 g), and ZnCO3 (5.221 g). Supplemental calcium was added at level of 2.8 g of CaCO3 per 100 g of diet at the expense of cerealse. Modified diet after Leach and Nesheim (14).

Values represent mean ± standard error of the mean. Animals per group are given in parentheses.

(Ca*/Pr/Ca*/IZ) is defined in the text and was calculated by the procedure given in Reference 10.
one maintained on the normal calcium diet and the other on the same diet less the calcium supplement. The calcium content of the second diet was about 0.1%. At 4 and 6 days, the absorption of $^{46}$Ca by an isolated duodenal loop in vivo was determined and the calcium-binding activity of the heat-treated supernatant fluids assessed by the Chelex-100 procedure. In a similarly designed but separate experiment, the electrophoretic patterns of 30-μl aliquots of the supernatants of individual chicks were determined.

At both time periods, the duodenal absorption of $^{46}$Ca by the low calcium groups was significantly greater than that of the high calcium groups (Table II). Concomitant with this was the observation that the calcium-binding capacity of the duodenal supernatant fluid was similarly increased. When the binding data are expressed as $^{46}$CaPr/$^{46}$CaR, it was calculated that the factorial increase in absorptive capacity was similar to the increase in calcium-binding activity of the respective supernatants. For example, at 4 days $^{46}$Ca absorption and calcium-binding were enhanced by factors of 1.4 and 1.5, respectively.

The electrophoretic patterns of the mucosal supernatants (Fig. 3) showed clearly that the intensity of the CaBP band (dark arrow) was considerably greater in those chicks receiving a low calcium diet than in those maintained on the normal calcium intake. The open arrow (Fig. 3) designates another region in which differences were noted as a result of treatment. This is the same region that also differed between chicks given a normal diet and those raised on the rachitogenic diet (Fig. 1).

Again the relation of the proteins associated with this particular region with CaBP and calcium absorption is not known at present.

Age—Comparison of 3-week-old pullets with mature non-laying hens showed that the duodenal supernatant of the hens had about one-half the concentration of calcium-binding activity of the younger animals (Table III). The rapidly growing immature fowl requires considerably more dietary calcium than the mature individual and this coincides with their respective duodenal calcium-binding capacities.

Sex—No significant differences were noted in the calcium-binding activity of the supernatants of duodenal homogenates from 3-week-old pullets and cockerels (Table III).

Egg-laying versus Nonlaying Hens—During the egg-laying cycle, the requirement for ingested calcium increases in order to provide sufficient mineral for the shell (15). As shown in Table III, the binding activity of the duodenal supernatants of the layers was about 3 times greater than that of the nonlayers. There was thus an increase in intestinal CaBP in response to the physiological need for calcium. The direct stimulus to the protein-synthesizing mechanism of the duodenal cell could be due to hormones associated with egg laying, among which estrogen is prominent. The results of the experiment described below indicate that this hormone is not involved in CaBP formation.

Estrogen Effects—The administration of estrogen to cockerels was shown by others to increase plasma calcium and plasma protein levels (16, 17). In this study, the effect of estrogen on CaBP formation and calcium absorption was determined. Rachitic chicks were given either diethylstilbestrol or vitamin D$_3$ or both simultaneously (cf. Footnote a of Table IV for dosages and protocol). At 48 hours, the absorption of $^{46}$Ca by a ligated segment of duodenum in vivo and the calcium-binding activity by the supernatant fluids of duodenal homogenates were measured.

As expected from the papers cited above (16, 17), the injection of diethylstilbestrol alone into cockerels was shown to increase both plasma calcium and plasma protein concentrations (Table IV). The simultaneous administration of vitamin D$_3$ and diethylstilbestrol increased plasma calcium to a greater extent than when estrogen was given alone, whereas plasma protein concentration was not significantly affected. Vitamin D$_3$, in the absence or presence of estrogen, enhanced radio
calcium.
TABLE IV

Effect of diethylstilbestrol and vitamin \( \text{D}_3 \) on plasma calcium and protein levels, duodenal absorption of \( ^{40}\text{Ca} \), and on calcium-binding activity of supernatants of duodenal homogenates

<table>
<thead>
<tr>
<th>Group</th>
<th>Treatment</th>
<th>Plasma Calcium mg/100 ml</th>
<th>Protein g/100 ml</th>
<th>Supematant calcium-binding activity</th>
<th>Specific activity mCi/mg protein</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vitamin D3</td>
<td>Estrogen</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>–</td>
<td>–</td>
<td>7.1 ± 0.8</td>
<td>3.2 ± 0.2</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>+</td>
<td>–</td>
<td>11.2 ± 0.7</td>
<td>3.1 ± 0.2</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>+</td>
<td>+</td>
<td>26.3 ± 4.0</td>
<td>3.8 ± 0.3</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>–</td>
<td>+</td>
<td>18.4 ± 2.2</td>
<td>4.3 ± 0.2</td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th></th>
<th>Vitamin D3 absorption %</th>
<th>( ^{40}\text{Ca} ) in supernatant fluid mg/ml</th>
<th>Supematant protein %</th>
<th>Specific activity mCi/mg protein</th>
</tr>
</thead>
<tbody>
<tr>
<td>10.3 ± 1.1</td>
<td>4.8 ± 0.5</td>
<td>2.9 ± 0.4</td>
<td>1.7 ± 0.3</td>
<td></td>
</tr>
<tr>
<td>71.4 ± 3.3</td>
<td>20.4 ± 0.9</td>
<td>2.7 ± 0.2</td>
<td>7.6 ± 1.1</td>
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</tr>
<tr>
<td>72.4 ± 2.5</td>
<td>18.0 ± 1.5</td>
<td>2.6 ± 0.2</td>
<td>6.8 ± 1.2</td>
<td></td>
</tr>
<tr>
<td>12.9 ± 0.8</td>
<td>7.0 ± 0.2</td>
<td>3.2 ± 0.7</td>
<td>2.3 ± 0.7</td>
<td></td>
</tr>
</tbody>
</table>

* Rachitic chicks at 3 weeks of age were divided into four groups; six chicks per group. Group 1 was given single doses of 0.2 ml of propylene glycol intramuscularly and subcutaneously; Group 2, 500 i.u. of vitamin \( \text{D}_3 \) in propylene glycol intramuscularly; Group 3, 500 i.u. of vitamin \( \text{D}_3 \) in propylene glycol intramuscularly, 9 mg of diethylstilbestrol in propylene glycol subcutaneously; and Group 4, 9 mg of diethylstilbestrol in propylene glycol subcutaneously. The experiment was performed 48 hours after dosing. Values represent mean ± standard error of the mean.

**Fig. 4.** Acrylamide gel electrophoresis (pH 8.3) of supernatant fluids from duodenal homogenates of rachitic chicks (1), rachitic chicks given 500 i.u. of vitamin \( \text{D}_3 \) in propylene glycol intramuscularly (2), rachitic chicks given vitamin \( \text{D}_3 \) in propylene glycol intramuscularly and 9 mg of diethylstilbestrol in propylene glycol subcutaneously (3), and rachitic chicks given diethylstilbestrol only (4). The arrow designates the protein band associated with CaBP. The conditions of the electrophoresis are as given in Fig. 1.

**Fig. 5.** Acrylamide gel electrophoresis (pH 8.3) of supernatants from rachitic chicks given cottonseed oil alone (1), 5000 i.u. of vitamin \( \text{D}_3 \) in cottonseed oil (2), and 500 i.u. of vitamin \( \text{D}_3 \) in cottonseed oil (3). The arrow designates the protein band with which CaBP is associated. The electrophoretic conditions are given in Fig. 1.
absorption from the ligated duodenum of the rachitic chicks; estrogen alone had no significant effect. As may be also seen in Table IV, vitamin D₃ greatly increased the calcium-binding capacity of the duodenal supernatant; estrogen slightly influenced calcium-binding activity but certainly not to the same extent as vitamin D₃. Contamination of Group 4 mucosa with plasma having a higher binding affinity for calcium than that of Group 1 may have contributed to some of the apparent difference. The acrylamide gel pattern readily revealed the presence of the CaBP band in those groups given vitamin D₃; however, this band was undetectable in the groups not given vitamin D₃, including Group 4 to which estrogen alone was administered (Fig. 4).

From these studies with the cockerels, it is suggested that differences in estrogen output by laying and nonlaying hens cannot account for differences in CaBP content of the duodenal mucosa of these birds. It is suggested that another factor is elaborated that influences the degree of CaBP formation when vitamin D is not limiting.

Comparison of Effect of Vitamin D₂ and Vitamin D₃—Rachitic chicks aged 3 weeks were given a single dose of either vitamin D₂ (5000 i.u.), vitamin D₃ (500 i.u.), or vitamin carrier alone (cottonseed oil). About 10 times as much vitamin D₂ was administered because in the chick the vitamin D₃ form has considerably less biological activity (18, 19). It was observed that the binding activity of the supernatants from the duodenum of the vitamin D₂-treated chicks was 1.55 times greater than that of the vitamin D₃ chicks. This difference is reflected in the acrylamide gel pattern shown in Fig. 5. With CaBP formation as a criterion of biological effectiveness, it appears that under the conditions of the present experiment, vitamin D₂ has more than 10 times the potency of vitamin D₃. Chen and Bosmann (18) clearly point out that the potency ratio of vitamin D₃ to vitamin D₂ is highly dependent upon the absolute quantities administered and the parameter measured.

DISCUSSION

It has been established that the administration of vitamin D₂ to a rachitic chick leads to the appearance of an intestinal mucosa protein, a protein which has a high affinity for calcium (5). Since vitamin D₂ also enhances calcium absorption, a not unreasonable hypothesis would be that calcium-binding protein is in some manner intimately involved in the translocation of calcium across the intestinal epithelial cell. If this hypothesis were correct, the amount of CaBP in intestinal mucosa should vary with the calcium absorptive capacity of the gut and the calcium needs of the animal under different situations. This was examined in this and previous reports, and the findings are summarized, as follows.

1. The appearance of CaBP in rachitic chick intestine after vitamin D₃ administration occurs at about the same time as when a stimulated absorption of calcium is seen with the conditions used (5).

2. When rachitic chicks, given a single dose of vitamin D₂, are continued on a vitamin D₃-deficient diet, the rate of decrease of CaBP is about the same as the rate of decrease of calcium absorption (this report).

3. In 1 and 2, the degree of calcium absorption at any time correlates with the amount of mucosal CaBP present (Reference 5 and this report).

4. The CaBP content and calcium absorptive efficiency of various segments of the small intestine vary in the same direction, i.e. duodenum > jejunum > ileum (12).

5. Actinomycin D inhibits the vitamin D-stimulated absorption of calcium and similarly depresses CaBP formation.²

6. Younger, more rapidly growing chicks have more mucosal CaBP than more mature individuals (this report).

7. Laying hens, which require about 2 g of calcium for eggshell formation, have more mucosal CaBP than nonlaying hens of the same age, and this difference is not due to the effect of estrogen (this report).

8. Chicks that have adapted to low calcium intakes absorb more Ca and have more intestinal CaBP than chicks maintained on a normal calcium diet (this report).

9. The relative absorption of three alkaline earth cations varies directly with the formation constant, K₂, of CaBP with these cations, i.e. calcium > strontium > barium (3, 10).

10. Vitamin D₃ is known to exert a greater physiological response in the chick than vitamin D₂, and was shown to stimulate the formation of a higher concentration of CaBP (this report).

11. Further, three tissues having epithelial cells across which calcium is transferred were shown to contain CaBP, these being intestine, kidney (12), and hen shell gland (20). Vitamin D is required for the formation of the protein in each of these tissues.

The above evidence tends to support the aforementioned hypothesis, but the types of experiments that would conclusively show that calcium-binding protein has a transport function have yet to be undertaken. As was previously pointed out (10), if CaBP can be introduced into CaBP-deficient cells and the transport capacity restored, or if the transport capacity of the epithelial cells could be inhibited by a specific antibody against CaBP, these would represent convincing proofs. Aside from evidence of this nature, the present hypothesis must depend upon the correlative type of data thus far obtained.

It is important to point out that we are not dealing with a macromolecule found only in the chick. Vitamin D-dependent calcium-binding proteins have been detected in the intestinal mucosa of the rat (21), dog,⁴ and monkey.⁴ Further, since CaBP is present in intestinal mucosa of normally raised chicks, we can eliminate the possibility that CaBP arises only in rachitic chicks after treatment with vitamin D.

The nature of the interaction of vitamin D with the mucosal cell that leads to calcium-binding protein formation has not been delineated. The suggestion that vitamin D is involved in some synthetic process was derived from studies by Schachter and Kowarski (6), Zull, Czarowska-Misztal, and DeLuca (7), and Norman (8), in which it was shown that various inhibitors of protein synthesis partially or completely inhibit vitamin D function. Labeled vitamin D, when administered to animals at physiological levels, appears in high concentration in the nucleus, which may also reflect its molecular site of action (22). Whether this nuclear interaction represents a direct effect on the DNA-mRNA complex or an indirect alteration of the permeability characteristics of the nuclear membrane is not known.

³ R. H., Wasserman, unpublished results.
(7, 23). Whichever is the mechanism, the product of this interaction has been proposed to be a translocase or a transport enzyme (24).

The present studies also indicate that when vitamin D is not limiting at least another factor controls the amount of CaBP formed. This is clearest in the experiment comparing the effect of normal and low calcium intakes on the CaBP content of duodenal mucosa. At 4 and 6 days after chicks originally raised on a normal calcium intake were placed on a low calcium diet, the efficiency of absorption of a test dose of 45Ca by duodenum was significantly increased. At these same periods it was further shown that the CaBP content of the mucosa of the low calcium groups was greater than that of the normal calcium group. The fact that animals and man can adapt to the dietary level of calcium has been known for several years, and the earlier studies of Nicolaysen, Eeg-Larsen, and Malm (25) indicated that this adaptation phenomenon was dependent upon the presence of vitamin D. The present study, however, is the first to demonstrate a molecular change that is associated with this alteration in efficiency of calcium absorption. Similarly, the CaBP content of mucosal tissue is greater when additional calcium is required for growth or egg laying. The nature of the controlling factor is unknown but is unlikely to be the calcium ion per se, either in terms of the transient calcium content of the mucosal cell during absorption or the ambient calcium concentration. This is brought out by the fact that the egg-laying hen requires and absorbs more calcium and has a higher mucosal CaBP content than chicks maintained on the normal calcium diet. It is reasonable to suppose that the control of CaBP formation is due to a factor elaborated by a tissue or gland that can “sense” the calcium status or requirements of an individual. Consideration must be given to the parathyroid glands; however, Kimberg, Schachter, and Schenker (26), using calcium transported by everted gut sacs of rat intestine as their criteria, showed that thyroparathyroidectomized rats can adapt to low calcium intakes. They also showed that hypophysectomized and adrenalectomized rats could also adapt. Another possible source of the unknown factor is the cellular elements of the skeleton, as proposed by Nicolaysen et al. (25) and recently discussed by Stanbury (27), although direct evidence in this direction has not been obtained.

The vitamin D-dependent calcium-binding protein has not been found in all tissues but, by the techniques thus far available, has been detected in small intestine (5), kidney (12), the uterine shell gland of the laying hen (20), and not in liver or muscle. It is of special interest that CaBP is found only in those tissues having a mechanism for moving calcium across the total epithelial cell. It may be inferred that CaBP, if it does have a transport function, is specifically involved with the transmural flux of calcium rather than with those calcium transport mechanisms found in many cell types and which may be concerned with maintaining a low intracellular calcium concentration (28).

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