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Vitamin D_3-Induced Calcium-Binding Protein in Chick Intestinal Mucosa

Abstract. *The administration of vitamin D_3 to rachitic chicks induces in intestinal mucosal tissue the formation or elaboration of a calcium-binding factor which is found in the supernatant of the mucosal homogenate. The enhanced binding of Ca by the "vitamin D" supernatant (in contrast to "rachitic" supernatant) was indicated by a slower rate of diffusion of Ca^{45} across a cellophane dialyzing membrane and by a lesser amount of Ca^{45} being bound to an ion-exchange resin (Chelex-100) in the presence of vitamin D_3 supernatant. The binding activity was only associated with the protein fraction from a Sephadex G-25 column and was destroyed by trypsin digestion. This and other evidence suggest that the soluble factor is a protein. The vitamin D_3–enhanced duodenal absorption of Ca^{47} in rachitic chicks occurred almost simultaneously with the appearance of the vitamin D_3–induced factor, and there was good correlation between the concentration of binding factor and the rate of absorption of Ca^{47}.*

The intestinal absorption of Ca is generally depressed in vitamin D–deficient animals and is restored to normal by vitamin D supplementation. Despite extensive investigations, the mechnism by which this vitamin elicits its response is not known, although several theories have been proposed (*1*). Among these was the suggestion that vitamin D may act by stimulating the synthesis or operation of a "carrier" that would facilitate the uphill or downhill transepithelial movement of calcium. Such a carrier could possibly be proteinaceous in nature with the intrinsic ability to form an association with the substrate being transported, in this case, Ca. That vitamin D may be involved, directly or indirectly, in protein synthesis was supported by the observations that actinomycin D inhibited vitamin D–induced hypercalcemia in rats (*2*) and inhibited the vitamin D–stimulated absorption of Ca in rachitic chicks (*3*, *4*) and rats (*5*).

We reported evidence (*3*, *6*) suggesting that there is a factor in the supernatant of intestinal mucosa homogenates from vitamin D_3–treated chicks which depresses Ca^{45} uptake by homogenate debris. The factor was not present (or to a lesser extent) in the supernatant of mucosal homogenates from untreated rachitic chicks, and appeared to be a protein or closely associated with one. We now report that the vitamin D–dependent supernatant factor is indeed a protein, that it forms a soluble complex with Ca, and that there is a close relation, in time after vitamin D_3 administration, between the appearance of the Ca-binding protein and the enhanced absorption of Ca^{47}.

White Leghorn cockerels (1-day-old) were fed a rachitogenic chick diet (General Biochemical, Inc.). After 4 to 5 weeks on this diet, severe rickets was evident. The chicks were given 500 international units of crystalline vitamin D_3 orally in vegetable oil, or vegetable oil only, and decapitated 72 hours later. The duodenum was excised immediately, cooled to 4°C, slit open, rinsed with cold 0.12*M* NaCl, and blotted. The mucosal tissue was scraped from the underlying muscle layers with a glass slide, and the harvest was homogenized in tris buffer (20 percent weight/volume) with a Potter-Elvehjem homogenizer with Teflon pestle. The composition of the tris buffer was $1.37 \times 10^{-2}M$ tris hydrochloric acid, $0.119M$ sodium chloride, $4.74 \times 10^{-3}M$ potassium chloride, $9.85 \times 10^{-5}M$ glucose; *p*H 7.4. The homogenate was then centrifuged at 38,000*g* in a refrigerated centrifuge for 20 minutes, and the supernatant was recovered for subsequent use. The supernatant was heated at 60°C for 10 minutes to remove extraneous proteins; this treatment decreased the protein content without greatly affecting Ca-binding activity. Protein was analyzed by the procedure of Lowry *et al.* (*7*).

Calcium binding was determined in two ways. First, the rate of diffusion of Ca^{45} across a semipermeable membrane was measured in the presence of supernatant obtained from vitamin D–treated or rachitic chicks. The diffusion chamber consisted of a precursor-compartment containing 20 ml of supernatant from the mucosa of either rachitic or vitamin D–treated chicks, and the product compartment with 2.5 ml of "rachitic" supernatant only. The two compartments, separated by Visking cellophane dialysis membrane, were continuously mixed. After 6 μc of Ca^{45} was added to the precursor compartment, 0.1-ml samples were taken from the product compartment at 15-minute intervals over a period of

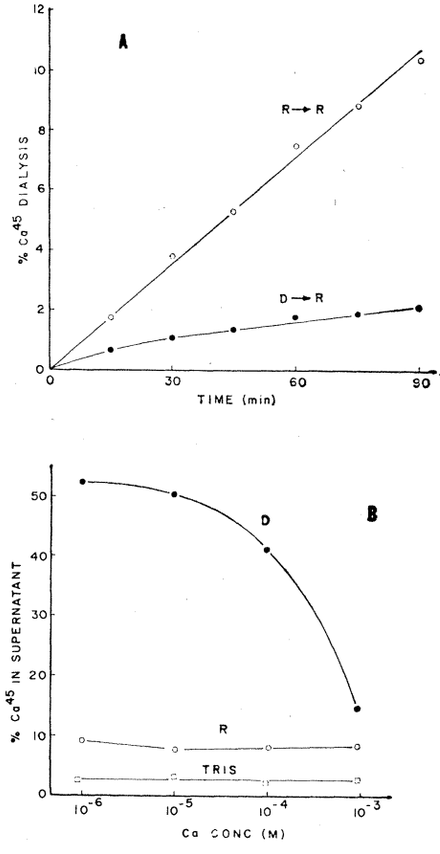

Fig. 1. (*A*) Diffusion of Ca^{45} across dialyzing membrane. $R \rightarrow R$ represents diffusion from rachitic supernatant to rachitic supernatant, and $D \rightarrow R$, from vitamin D supernatant to rachitic supernatant. Precursor volume = 20 ml; product volume = 2.5 ml. The protein concentrations of rachitic and vitamin D supernatants were 3.0 and 3.3 mg/ml. *B*, Influence of vitamin D supernatant on Ca^{45}-binding to Chelex-100. Each point represents the mean of three values. Each tube contained 0.1 ml of resin, 1 ml of vitamin D (*D*) or rachitic (*R*) supernatant or tris buffer, 0.1 ml Ca^{40} solution, and 0.1 ml Ca^{45} solution. After mixing and centrifuging, a portion of the supernatant was assessed for residual Ca^{45} activity. The protein concentrations of rachitic and vitamin D supernatants were 5.4 and 5.3 mg/ml.

1.5 hours. The sampled compartment was kept at constant volume by the addition of 0.1 ml. Each sample was dried on a stainless steel planchet, and Ca^{45} activity was detected by a Geiger-Muller counter; corrections were made for the dilution in the product compartment. Self-absorption corrections were unnecessary. The rate of diffusion of Ca^{45} across the semipermeable membrane was considerably less in the presence of vitamin D supernatant than with the rachitic supernatant (Fig. 1*A*). There were no significant differences in total protein in the two fluids. Therefore, these results could not be due to a mere difference in protein concentration *per se* nor to osmotic effects. However, the data obtained can be explained by the presence of a factor in the vitamin D supernatant that prevented or impeded Ca^{45} diffusion, presumably by complex or chelate formation.

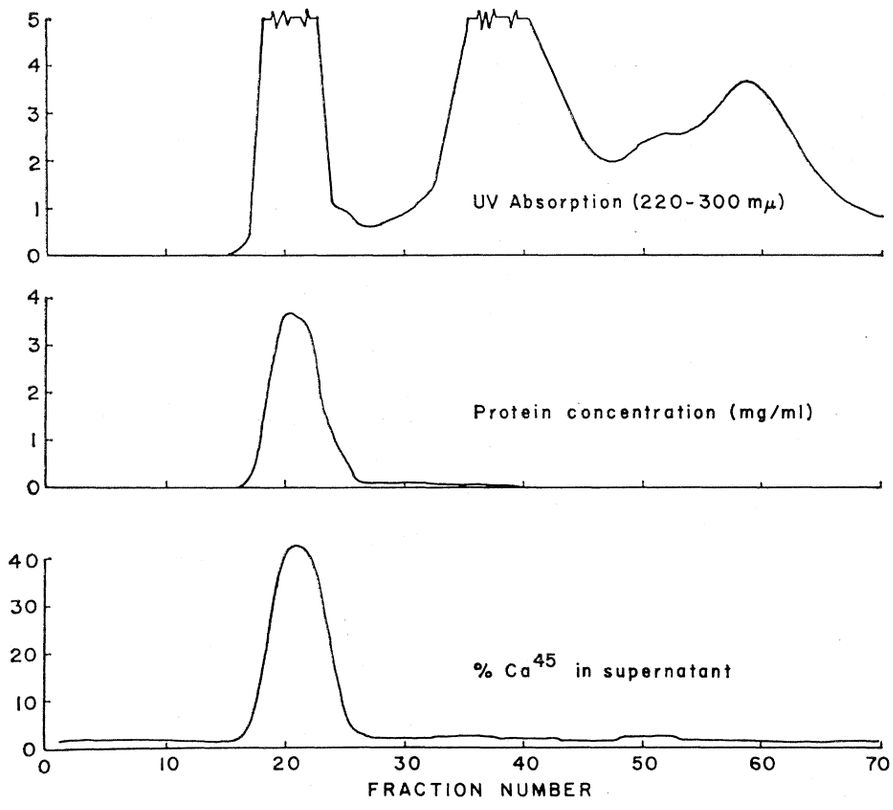

Fig. 2. Fractionation of Ca-binding activity in vitamin D supernatant by Sephadex G-25. The eluting solution was 0.08*M* NaCl–tris buffer (*p*H 7.4); 2.5-ml fractions were collected. The top curve represents ultraviolet absorption of the eluent (a continuous-flow ultraviolet scanner was used); the middle curve, protein content of the fractions (*7*); the bottom curve, the Ca-binding activity by the Chelex-100 assay.

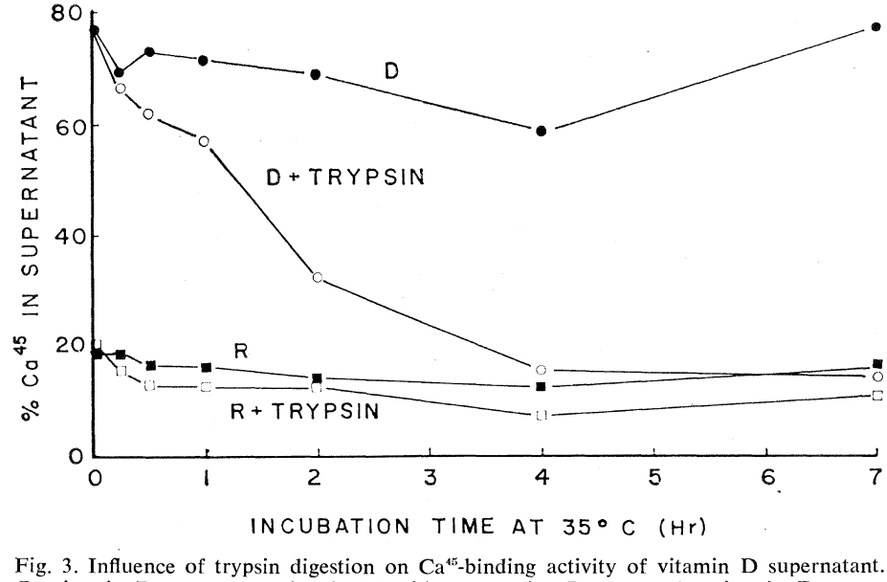

Fig. 3. Influence of trypsin digestion on Ca^{45}-binding activity of vitamin D supernatant. *D*, vitamin D supernatant incubated without trypsin; *D* + *trypsin*, vitamin D supernatant incubated with trypsin; *R*, rachitic supernatant without trypsin; *R* + *trypsin*, rachitic supernatant with trypsin. The protein concentrations of rachitic and vitamin D supernatants were initially 4.3 and 4.6 mg/ml.

The second method for measuring Ca-binding was similar to that used by Briggs and Fleishman (*8*) to detect a soluble Ca-binding substance in rabbit muscle. The procedure depends upon the competition between a cation-exchange resin and a Ca-binding substance for added radiocalcium; in the presence of a chelator or a soluble complexing factor, less Ca^{45} is sequestered by the resin. The resin, Chelex-100, was washed several times in tris buffer and diluted in tris to a resin concentration of 0.1 ml of resin per 0.2 ml of suspension. With the resin maintained in suspension by vigorous mixing with a magnetic stirrer, 0.2 ml was pipetted into a 15-ml centrifuge tube. This was followed, in turn, by 1 ml of rachitic or vitamin D supernatant or tris buffer and 0.1 ml Ca^{45} (about 0.5 μc) solution in tris buffer. The tube was mixed thoroughly on a vibrator (Vortex) for 15 seconds and then centrifuged in order to quickly separate the resin from the supernatant. In each case, Ca^{45} was determined on 0.2 ml of the supernatant by the aforementioned method. With counting standards and appropriate volume corrections, the percentage of Ca^{45} not bound to resin was calculated. When extra Ca^{40} was added, this was done in a volume of 0.1 ml before the addition of the Ca^{45}. There was considerably less Ca^{45} sequestered by the resin in the presence of vitamin D supernatant than in the presence of either rachitic supernatant or buffer (Fig. 1*B*). With increasing Ca^{40} concentrations in the test system, the percentage of Ca^{45} bound by the unknown factor decreased as expected if a binding substance were approaching saturation. This behavior provides substantive support for the occurrence of a Ca-binding factor in vitamin D supernatant. Added Ca^{40} was without effect on the percentage of Ca^{45} not bound to resin in the presence of rachitic supernatant or tris buffer.

The distribution of the supernatant factor in fractions from a Sephadex G-25 column was determined, with the cation-exchange resin procedure as an assay for binding activity. The eluting solvent was 0.08*M* NaCl-tris buffer, *p*H 7.4. To determine ultraviolet-absorbing material, the effluent was passed through an inline scanner; then 2.5-ml samples were collected and assessed for binding activity and total protein content, the latter by the method of Lowry *et al.* (*7*). Calcium-binding activity was associated with the first ultraviolet-absorption peak (Fig. 2), and only the fractions collected under this peak contained measurable protein. In fact, the percentage of unbound Ca^{45} observed in a given fraction by the Chelex-100 assay was directly proportional to the protein contained therein.

Further proof of the protein nature of the unknown factor was sought by determining the effect of trypsin digestion on binding activity. Here, 2.5 mg of crystalline trypsin (Worthington, 2× crystallized) was added to 25 ml of vitamin D supernatant or 25 ml of rachitic supernatant and incubated at 35°C. Samples were periodically taken, cooled immediately to 4°C, and assayed for Ca-binding activity. The binding activity of the vitamin D supernatant was destroyed by trypsin (Fig. 3), the probable protein nature of the unknown factor again being indicated. Trypsin had little or no effect on the binding activity of the rachitic supernatant.

The Ca-binding factor (resin-assayed) was destroyed by heat at 86°C for 10 minutes but not at 70°C; it was not dialyzable through Visking cellophane casing. This behavior coincides with that of the supernatant factor (*3, 6*) and that expected of a protein.

The lack of effect of added Ca^{40} and trypsin on Ca-binding by rachitic supernatant (Figs. 2 and 3) suggested that the unknown factor is not present therein or only in relatively small quantities. Therefore, vitamin D may be inducing the *de novo* synthesis of the supernatant factor or the alteration of the binding characteristics of existing proteins. This may also represent the release of the factor from bound intracellular sites.

The time between the increased absorption of Ca^{47} and appearance of the calcium-binding protein was determined as follows: Rachitic chicks were injected intramuscularly with 500 I.U. of vitamin D_3 in propylene glycol. After 0, 5, 10, 15, 25, and 40 hours the absorption of Ca^{47} by the ligated duodenum over a 30-minute test period was measured (*9*). In another group of the same batch of rachitic chicks and at about the same time periods after intramuscular vitamin D_3, the presence of supernatant binding factor was determined by the Chelex-100 procedure. An increase in Ca^{47} absorption was first seen at 10 hours with the rate of absorption increasing over the 40-hour period (Fig. 4), a trend similar to that of orally given vitamin D_3 (*9*). Calcium-binding activity was slightly less at 5 hours than at zero time but increased initially at 10 hours and continuously thereafter. There was, then, a similar relation with respect to time between the occurrence of the vitamin D_3-stimulated absorption of Ca^{47} and the detection of the vitamin D_3-induced binding factor. Also, at any particular time, the rate of Ca^{47} absorption was generally proportional to the amount of binding factor present in the mucosal tissue, suggesting that this factor may be involved in the process of calcium absorption (*10*).

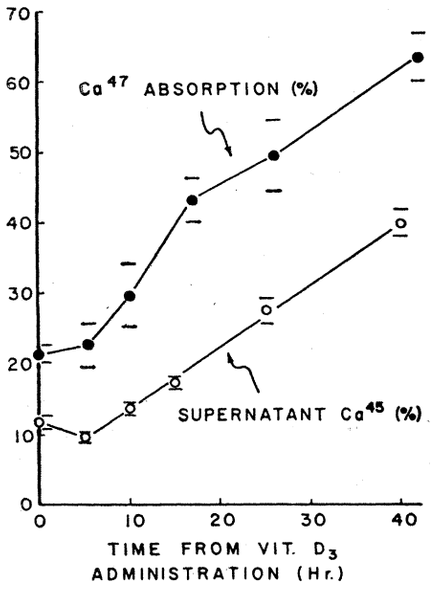

Fig. 4. Increased duodenal absorption of Ca^{47} and supernatant factor with time after administration of vitamin D_3 to rachitic chicks. At zero time, 500 I.U. of crystalline vitamin D_3 in propylene glycol was injected intramuscularly into rachitic chicks. At the time specified, the absorption of Ca^{47} from a 10-cm ligated segment of duodenum was measured over a 30-minute absorption period. In other chicks from the same batch, homogenates of duodenum were prepared, and the Ca-binding activity of the supernatant fluid was determined by the Chelex-100 method. Each point represents the mean ± standard error of the mean of values from five chicks.

The physiological and biochemical function of the vitamin D–induced Ca-binding factor is not known, but it could enhance ion transfer in several ways. For example, the factor might conceivably be identical with, or related to, an intracellular carrier of Ca, operating in a facilitated diffusion or an active transport system. The factor might also function in the vascular transport of Ca, since it has been observed that the tibia deposition of Ca^{47} absorbed by vitamin D–treated chicks was greater than that absorbed by rachitic chicks, whereas intravenously injected Ca^{47} was handled similarly (*11*). We had suggested that Ca^{47} might be absorbed as a complex which, in some fashion, enhances its uptake. Furthermore, if this Ca-binding factor is present in skeletal tissues, it could possibly be involved in the bone-resorption and -accretion effect of vitamin D (*12, 13*) and perhaps may be close to the site at which parathyroid hormone and thyrocalcitonin act.

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