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FEBRUARY 14-16, 1

1973

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MECHANISM OF CALCIUM ABSORPTION AND TRANSPORT: THE
INVOLVEMENT OF THE VITAMIN D-INDUCED CALCIUM-BINDING PROTEIN

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A basic understanding of the mechanism of Ca absorption is of nutritional importance because of the very significant role this element plays, in a variety of physiological processes: skeletal growth in the young, maintenance of skeletal structure in the adult, fetal Ca needs during pregnancy, lactation, egg shell formation in the avian species, muscle contraction, neurotransmission, etc. Transport of Ca from the intestinal lumen through the intestinal absorptive cells and into the body represents the initial step common to these processes and since it is the only route of input into the body it has received a considerable amount of attention.

From the thermodynamic standpoint there are two general means by which Ca can cross the barrier represented by the intestinal epithelial cells. First, Ca could gain entry to the blood supply by simple diffusion if the luminal concentration of Ca were high enough such that an electrochemical gradient existed downhill in the direction of the blood. In this situation there would be no theoretical need for an expenditure of metabolic energy since the Ca would be diffusing from a high to a low concentration. The other general class of transport, active transport, involves the input of metabolic energy in transporting substances from one compartment to another. This type of transport would be required if the luminal concentration of Ca were less than the serum concentration, i.e., an uphill gradient toward the blood. With charged species such as Ca^{++} , one has to also consider the electrical gradient which may exist between lumen and blood and therefore the combined electrochemical gradient is taken into consideration.

Experimental studies have indicated that Ca gains entry into the body by both simple diffusion and active transport (Schacter and Rosen, 1959; Wasserman and Kalifeltz, 1962) and recently an attempt was made to evaluate the relative importance of the two mechanisms (Wasserman and Taylor, 1969). It was concluded that Ca absorption occurred predominantly by simple diffusion if the luminal content of ionic Ca was greater than 2-5 mM, whereas if the concentration was less than 2-5mM then Ca could be absorbed only by the active transport process. In order to make use of this information, one must know the luminal concentration of Ca^{++} and technically this is often difficult to achieve with a high degree of reliability. However, two studies have been published using dogs on a 1% Ca diet (Cramer, 1965) and rats on a 0.15% Ca diet (Sernka and Borle, 1969). The Ca concentration of the intestinal contents of the rats was observed to be 0.2 - 1.0 mM, thus suggesting that active transport was involved. In the dogs which were receiving a higher level of Ca, the Ca content of the duodenum and jejunum was 2.0 - 2.4 mM, again suggesting active transport, while the ileum contained 8.1 mM Ca, a level at which simple diffusion most likely represented the major mechanism of Ca absorption.

Mention should be made of two other factors which influence the type of transport process involved in the absorption of Ca from the intestine: the intes-

tinal location of active transport processes and the significance of residence time of intestinal contents in the various segments of the small intestine. In general it has been noted that the active transport of Ca is much more prominent in the proximal as compared to the distal small intestine (Schachter and Rosen, 1959; Harrison and Harrison, 1960). Contrariwise, there is an increased residence time of intestinal contents in the distal intestine as compared to the proximal area (Cramer and Copp, 1959; Marcus and Lengemann, 1962). The net effect of these two factors, a shorter residence time in the proximal intestine containing a more efficient active transport system and a longer residence time in the distal intestine which has a less efficient active transport system, appears to be in favor of the latter, i.e., the distal small intestine is responsible for the absorption of more Ca than the proximal area.

Ca Absorption and Vitamin D

A variety of factors have been shown to alter the rate of intestinal Ca absorption, however, the one which appears to be quantitatively most important is vitamin D. A deficiency of this vitamin in the growing animal results in the faulty mineralization of bone and the development of rickets. Although an under mineralization of bone characterizes this disease, the primary cause was shown to be a defective absorption of Ca by the intestine (Nicolaysen, 1937) and thus an inadequate supply for the mineralization process. Although rickets is no longer a serious nutritional problem, the use of rachitic experimental animals and the subsequent supplementation with vitamin D has proven to be a very useful tool in investigating the mechanism of Ca transport in the intestine. In fact, in recent years a resurgence of interest has been noted in the study of vitamin D metabolism and its involvement in Ca transport reactions as substantiated by the number of publications in this field.

Studies with inhibitors of protein synthesis indicated that the enhanced absorption of Ca due to vitamin D could be inhibited by administration of agents which blocked transcription or translation (Norman, 1965; Schachter and Kowarski, 1965; Zull, *et al.*, 1965). Thus at least part of the lag time, the time after vitamin D administration before a physiological effect was noted, could be accounted for as being due to the synthesis of protein. Since the concept of active transport invokes the existence of some sort of membrane carrier, the protein(s) being synthesized was speculated to be such a membrane carrier for Ca (Schachter and Kowarski, 1965). At about the same time, studies in our laboratory indicated that vitamin D stimulated the appearance of a soluble factor in homogenates of intestinal mucosa which altered the distribution of Ca between the subcellular fractions and the supernatant phase (Wasserman and Taylor, 1963). The protein nature of this factor was suggested from the early studies (Taylor and Wasserman, 1965) and confirmed later in investigations of the Ca-binding property of the protein (Wasserman and Taylor, 1966).

The Vitamin D-Induced Calcium Binding Protein

The question of whether or not the vitamin D-induced Ca-binding protein (CaBP) represents a "carrier" for Ca has not been resolved, however, there is an abundance of evidence which indicates that CaBP plays a significant role in the mechanism of intestinal Ca absorption. This evidence is derived mainly from experiments in which a good correlation has been noted between CaBP content of intestine and the capacity of the intestine to absorb Ca under a variety of conditions.

The absence of CaBP in intestinal tissue from rachitic chicks which have a poor Ca absorptive capacity and the appearance after vitamin D administration concomitant with an increased efficiency of Ca absorption was the first such correlation noted (See Figure 1). The fact that CaBP had such a high Ca-binding affinity ($2.6 \times 10^5 \text{ M}^{-1}$ to 10^6 M^{-1}), as measured *in vitro*, also suggested an involvement of the protein in Ca metabolism (Wasserman, *et al.*, 1968; P. J. Bredderman, personal communication). A crucial point in considering the potential involvement of any factor in the mediation of the vitamin D enhanced Ca absorption is the time course of appearance of that factor in relation to the time of increased Ca absorption which signals the end of the lag period, i.e., the factor should appear concomitant with the increased absorption or slightly prior to the physiological effect. A very early publication from our laboratory and also some from other labs indicated a poor correlation between onset of physiological effect and the appearance of CaBP. However, it is now accepted that those results were due to the relative insensitivity of the methods used to detect CaBP and a good correlation can be seen between first appearance of CaBP and initial increase in Ca absorption. Figure 2 illustrates this point when the *in vitro* ^{45}Ca uptake by mucosal tissue is used as an index of Ca transport. CaBP is first detectable 10 hours after vitamin D administration and the base level ^{45}Ca uptake present in the rachitic animal can be seen to also increase at this time, although a statistically significant increase was not noted until the next time period, 14 hours. When *in vivo* absorption of ^{47}Ca was compared to time of appearance of CaBP, a good correlation between these parameters was also observed (Ebel, *et al.*, 1969). In both of these experiments a radial immunoassay was used to measure CaBP. Using a different but still very sensitive measure of specific protein synthesis MacGregor *et al.* (1970) were able to demonstrate specific incorporation of labeled amino acids into CaBP a few hours before an increased Ca absorption was detectable.

Figure 1. Disc gel electrophoresis (7% acrylamide, pH 8.4) of soluble proteins present in supernatants derived from homogenates of intestinal mucosa from rachitic (-D) and vitamin D treated (+D) chicks. Migration was toward the anode (bottom) and the arrow indicates CaBP in the +D sample while none is visible in the rachitic sample.

Figure 2. Time course of events following vitamin D₃ (500 I.U.) administration to rachitic chicks. *In vitro* ⁴⁵Ca uptake by mucosal tissue was determined by the method of Martin and DeLuca (1969). The CaBP content of adjacent sections of tissue was determined by radial immunodiffusion. Each point represents the mean $\pm$ SEM of tissue from 5 chicks.

CaBP was initially found only in vitamin D-dosed or normal chicks and in tissues which were considered to be target organs of the vitamin, intestine and kidney (Taylor and Wasserman, 1967). Also, the concentration in different segments of the small intestine correlated with the relative efficiencies of Ca transport in those segments, duodenum > jejunum > ileum. Later, CaBP was detected in the uterus (shell gland) of the laying hen, a tissue not previously thought of as a target organ for vitamin D, but certainly one involved in the transport of large amounts of Ca (Corradino, *et al.* 1968).

Differences have also been noted in the intestinal content of CaBP in animals maintained on vitamin D-replete diets and these altered CaBP levels have also been correlated with Ca absorptive capacity. The ability of animals to adapt to a lowered dietary intake of Ca by increasing their efficiency of intestinal absorption of Ca was originally studied by Nicolaysen *et al.* (1953), and it was later noted that the intestinal content of CaBP in adapting animals increased in a manner similar to the increased Ca absorption (Wasserman and Taylor, 1968; Morrissey and Wasserman, 1971). Such an increase in intestinal CaBP and efficiency of Ca absorption can be seen in figure 3. When all or part of the dietary Ca is replaced with Sr the syndrome of strontium rickets soon develops (Shipley, *et al.*, 1922) and it was thought to be due to an interference of Sr at the calcification site (Sobel, 1954). However, it would now appear that

Figure 3. Slab gel electrophoresis (7% acrylamide, pH 8.3) of supernatant proteins from intestinal mucosal homogenates from chicks raised on a normal diet (I) or one deficient in Ca (II). Both diets were normal with respect to vitamin D content. The ^{47}Ca absorption represents % of dose absorbed from *in situ* ligated duodenum during a 30 minute period.

one primary defect is the decreased efficiency of Ca absorption from the intestine and it was noted at the same time that the intestinal CaBP content was also severely diminished (Corradino and Wasserman, 1970; Corradino, *et al.*, 1971). Another correlation noted in animals raised on a normal diet is the effect of age, i.e., in young animals where there is a large need for Ca for growing bones, there is more intestinal CaBP than in older more mature animals (Wasserman and Taylor, 1968). In the avian species a higher intestinal CaBP content was observed in laying hens than in non-laying hens (Wasserman and Taylor, 1968; Bar and Hurwitz, 1972). These correlations and other observations indicate that once the initial vitamin D requirement has been satisfied for the synthesis of CaBP, some other factor(s) regulates the amount of CaBP present in the intestinal mucosa. The identity of this unknown factor is presently under investigation but it would appear not to be serum Ca concentration or parathyroid hormone.

Localization of the intestinal site of CaBP was accomplished using fluorescent antibody techniques (Taylor and Wasserman, 1970). Two distinct locations were observed: the PAS positive goblet cells and the microvillar-surface coat region of all of the intestinal epithelial cells. The latter site of CaBP, the absorptive surface, has been indicated as a membrane site primarily affected by vitamin D on the basis of Ca-flux experiments (Wasserman and Taylor, 1969).

Since goblet cells are secretory in nature, it was speculated that CaBP might be synthesized therein and subsequently relocated at the absorptive surface via the mucus secretion (Taylor and Wasserman, 1970). In such a scheme the CaBP at the microvillar surface would then represent the functional CaBP mediating in some manner the vitamin D-enhanced Ca transport process. The mechanism by which CaBP might function at this location is not known although two proposals which are based on the high *in vitro* Ca-binding property of the protein have been suggested: (1) if CaBP is located in the surface coat material, it could act as an ion trap sequestering Ca ions from the intestinal lumen and thus providing the absorptive cells with a higher available concentration of Ca, or (2) it might act as a diffusional facilitator for Ca across either the surface coat material or the microvillar membrane.

In addition to indicating the mucosal facing surface of the absorptive cells as a site directly affected by vitamin D, the analysis of the Ca-flux experiments (Wasserman and Taylor, 1969) also suggested that the permeability of the basal or lateral membranes of the epithelial cells was increased by vitamin D treatment. It was not clear, however, if this was a direct effect of the sterol or a secondary effect resulting from the increased permeability at the absorptive surface, i.e., more Ca was simply being made available to the metabolically dependent Ca pump which has been proposed to be present at the basal pole of the absorptive cell (Schachter, *et al.*, 1966).

The initial findings concerning CaBP utilized the chick as an experimental animal. Subsequently intestinal CaBP has been detected in a variety of species throughout the animal kingdom: rat (Kallfelz, *et al.*, 1967), hamster (Schachter, *et al.*, 1967; Kallfelz and Wasserman, 1972), dog (Taylor, *et al.*, 1968), monkey (Wasserman and Taylor, 1971), cow (Fullmer and Wasserman, 1972), pig and human (Piazola, *et al.*, 1971; Hitchman and Harrison, 1972). Also, immunological evidence exists for the presence of intestinal CaBP in some of the lower species: duck, turtle, toad, and fish. Although the vitamin D dependence has not been established in some of these species, the widespread distribution would suggest a significant involvement of the protein in Ca metabolism.

The polar metabolites of vitamin D₃, 25-hydroxycholecalciferol and 1,25-dihydroxycholecalciferol also stimulate the formation of CaBP. They do so relatively more rapidly than the parent vitamin which is consistent with the concept (Boyle, *et al.*, 1972) that vitamin D first undergoes hydroxylation at the 25- then the 1- position before exerting its biological effect.

Vitamin D and Brush Border Enzymes

Other events occur at the molecular level in the intestine when vitamin D is administered to rachitic animals. Various enzyme activities associated with the brush borders (isolated microvilli) have been shown to increase: alkaline phosphatase (Haussler, *et al.*, 1970), Ca dependent adenosine triphosphatase (Martin, *et al.*, 1969) and leucine aminopeptidase (Taylor and Wasserman, 1970a). These enzyme activities can be demonstrated in rachitic animals and their activity is subsequently increased by vitamin D administration. This is unlike CaBP which is not present in intestines of rachitic animals. The inter-relationship, if it exists, of these enzymes with CaBP in the overall Ca absorptive mechanism is not apparent from data presently available.

The Effect of Other Factors on Ca Absorption

The phenomenon of adaptation to a low dietary Ca level which was mentioned previously can also be noted with low intakes of dietary phosphorus (Carlsson, 1953), i.e., in both cases an increased efficiency of intestinal Ca absorption was noted. Therefore, the dietary levels of both Ca and P affect quite significantly the efficiency of the Ca transport mechanism. Nicolaysen, who first investigated the adaptation to low Ca diets, postulated a humoral factor from bone, the endogenous factor, which supposedly detected an undersaturation of bone mineral due to the lowered Ca intake and subsequently signaled the intestine to increase the efficiency of the absorptive mechanism (Nicolaysen, *et al.*, 1953). The existence of the postulated endogenous factor has not been definitively proven although considerable research effort has been expended in that direction. A possible practical application of this phenomenon is illustrated by the studies of Boda and Cole (1954). They effectively prevented parturient paresis (milk fever) in cows by feeding a low Ca diet for one month prior to parturition and the dietary regime did not appreciably alter the subsequent lactation.

Certain sugars such as lactose, mannose, raffinose, xylose and others when included in the diet have been shown to increase the absorption of Ca. Also, mannitol, sorbitol and inositol were effective in increasing Ca absorption (see reviews by Dupuis and Fournier, 1963; Wasserman and Taylor, 1969). Various hypotheses have been suggested to explain the effect: an increased solubilization of luminal Ca, an interaction with available metabolic energy, and intestinal bacterial metabolism of the sugars causing an altered intestinal pH. However, the mechanism by which these compounds act to increase Ca transport is not agreed upon, but it apparently does not work via a vitamin D-mediated pathway because the effect can be seen in rachitic animals.

Certain amino acids are also effective in increasing Ca absorption (reviewed by Wasserman and Taylor, 1969). Therefore, the protein intake can theoretically have a bearing on Ca utilization. Like the "lactose effect", the mechanism involved is not clearly understood although it has been suggested that the amino acids may act to solubilize luminal Ca thus making it more available for absorption. Another similarity is the fact that vitamin D is not required to demonstrate the effect and the increase of absorption is noted with little or no lag period involved.

Summary

Although vitamin D deficiency is no longer regarded as a significant nutritional problem, an understanding of the mechanism of normal Ca absorption requires knowledge of how this vitamin and/or its metabolites function. This is true because the greater part of the Ca absorptive process is lost in most species in the absence of adequate vitamin D. In recent years considerable progress has been achieved toward this end. Although there is not complete agreement on the mechanism of vitamin D action among the investigators who are active in this field, the following observations have been made and may some day lead to a clear picture of the Ca absorptive process: vitamin D either from dietary sources or from U. V. irradiation of skin is hydroxylated to 25-hydroxycholecalciferol in the liver; 25-hydroxycholecalciferol is then hydroxylated to 1,25-dihydroxycholecalciferol in the kidney; *in vitro* evidence exists which indicates vitamin D does not require hydroxylation prior to acting on the intestine, therefore, vitamin D and/or the metabolites next act to induce

protein synthesis; one protein, CaBP, found in vitamin D target organs and located at the absorptive surface of intestinal cells is probably involved in enhancing Ca uptake by intestinal cells; the activity of various brush border enzymes is increased and they may interact in some manner with the Ca absorptive process or their increased activity may be a secondary consequence of vitamin D action; the increased amount of Ca taken into the cells either diffused and/or is pumped by a metabolically driven Ca pump into the blood supply; additional nutritional factors may then act to modulate the basic vitamin D-mediated mechanism or they may act independent of vitamin D in certain cases, i.e., lactose and amino acids.

This rather abridged review of recent developments in the study of Ca absorptive mechanisms has emphasized the end result of vitamin D administration, the mediation of the Ca absorptive process. For a current discussion of the metabolism of vitamin D and the factors affecting the metabolism refer to the paper by H. F. DeLuca in these proceedings.

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