Binding Proteins from Animals with Possible Transport Function

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ABSTRACT Several proteins from various animal tissues with possible transport function have been briefly described, with emphasis given to a vitamin D-induced calcium-binding protein (CaBP) implicated in calcium translocation across epithelial membranes. The latter protein was shown to be present in the small intestine, colon, kidney, and the uterus (shell gland) of the chicken. CaBP was also found in the small intestine of the rat, dog, bovine, and monkey. This protein has been isolated in high purity from chick intestinal mucosa and some of its properties determined. Its molecular weight is about 28,000, its formation constant, about $2.6 \times 10^5 \text{ M}^{-1}$, and its binding capacity, 1 calcium atom per protein molecule. Correlative studies have shown that CaBP concentration in intestinal mucosa varies with the calcium absorptive capacity of the gut, thereby suggesting that CaBP is intimately involved in the process of calcium absorption. CaBP has been localized in the brush border region of the intestinal absorptive cell and within goblet cells. Among other proteins mentioned were the intrinsic factor required for vitamin $B_{12}$ absorption and the protein(s) associated with iron translocation.

Plasma membranes, to a large degree, determine the extent and rate of permeation of substrates into and out of the cytoplasmic mass. Contributing to their passive permeability and selectivity are various physical and chemical properties of the membrane, such as its lipoid nature, the presence of aqueous channels, and the electrical charges associated with the cell surface and the core of the channel. These and other important aspects have been well documented and have sometimes been amenable to detailed investigations by the use of artificially constructed membranes. Biological membranes, in addition, have the capacity to transport certain substrates against thermodynamic gradients (active transport) and to support reactions which accelerate the transfer of certain substrates down their electrochemical gradients (facilitated diffusion). Both of these latter two processes are hypothetically carrier-mediated and, in recent years, carrier-like molecules have been implicated in an increasing number of transport systems. These systems exhibit certain common characteristics including Michaelis-Menten kinetics, a high degree of struc-
tural specificity, and relative stereospecificity. Because of the specificity demanded, it is usually considered that the carriers are proteins, molecules having sufficient complexity to contain the required information to distinguish between chemically similar molecules. The de facto existence of membrane-associated proteins having a possible transport or carrier function is substantiated by the information given in the recent review article of Pardee (1) and the material presented at the present Symposium.

There appears to be at least two classes of proteins implicated in transport, those that are readily released from the cell or cell surface by the osmotic shock technique of Heppel (2) or by cell homogenization, and those that are firmly bound to membranous cell components and which can be solubilized by reagents such as Triton X-100 (3) and NaI (4). These may be designated as "soluble" and "insoluble" transport proteins, respectively, and the relation between these and solute movement, and between each other, is not clear at present. As an example of each, Anraku (5) has isolated a soluble protein from Escherichia coli K-12 with a high binding affinity for galactose; the insoluble "M" (membrane) protein of Kennedy and Fox (3), also from E. coli, appears to interact with β-galactosides during the transport reaction.

A few binding proteins with possible transport function have been detected in animal tissues. One of the first so designated is the membrane adenosine triphosphatase of the crab nerve and erythrocyte membrane; this enzyme is discussed in detail elsewhere in this volume. Others are the calcium-binding protein induced by vitamin D, the intrinsic factor related to vitamin B₁₂ absorption by the intestine, the galactose-binding component of intestinal brush border, a possible iron-binding protein of intestinal mucosa, the blood protein, transferrin, implicated in erythrocytic iron uptake, and a shockable protein from kidney that binds transportable organic acids. These will be subsequently discussed although emphasis will be given to the first mentioned.

**CALCIUM-BINDING PROTEINS**

The recognition of the existence of a calcium-binding protein (CaBP) in intestinal mucosa was derived from studies on the mechanism of action of vitamin D. It was early shown that the protein was not present in tissue derived from rachitic animals but began to appear in such animals after vitamin D had been administered. From the older, classical studies of Mellanby (6) and Nicolaysen (7), and as reviewed recently by Wasserman and Taylor (8), such treatment also restores the capacity of the intestine to absorb calcium and eventually causes a remission of the rachitic syndrome. Because of the close interconnections between calcium movement, vitamin D, and a possible transport protein, it was considered worthwhile to review briefly certain aspects of the effect of vitamin D on calcium absorption and on cell behavior before describing the investigations on CaBP.
Physiological Site of Vitamin D Action

Schachter and Rosen (9) showed several years ago that the intestinal epithelium of the rat contains a mechanism for actively transporting calcium from lumen to plasma. The transport capacity was dependent upon the vitamin D status of the animal, as well as on other factors, such as age, calcium intake, gestation, etc. (10, 11). Based upon several types of studies with isolated mucosal tissue, Schachter et al. (12) proposed that the Ca pump is located on the serosal aspect of the epithelial cell and that, in addition, a facilitated diffusion process is available in the brush border region for mediating the entry of calcium into the cell. The first step in the absorptive process, i.e. cell entry, is not an energy-dependent reaction whereas the second, i.e. active transport, requires an intact energy supply. The serosal Ca pump also becomes functionally inoperative when the concentration of Ca in the intestinal lumen is sufficiently high; absorption then occurs primarily by diffusion.

The important questions from our viewpoint are: Where in the transport path does vitamin D appear to act? Does it function at the first step, the second step, both steps, or elsewhere? Is it required for the functioning of the Ca pump or does it affect the pump only indirectly? Since, in Schachter's hands (13), the vitamin D response was less apparent in metabolically inhibited tissue, the view was expressed that the sterol was required for the operation of this unidirectionally oriented calcium transport system. Counter to this, Harrison and Harrison (14, 15) observed that, under the conditions of their experiments, inhibiting conditions did not nullify the increased rate of transfer of calcium due to vitamin D, and proposed that the vitamin decreased the passive resistance of the absorptive cell to calcium movement. This could be by way of an alteration of membrane structure or by way of the stimulation of the synthesis of a "diffusional facilitator." Some of our initial studies were designed to examine independently the physiological effect of the vitamin on calcium fluxes and in a manner such that the results would bear on the above controversy.

### Table I

**EFFECT OF VITAMIN D ON THE UNIDIRECTIONAL FLUXES OF CALCIUM ACROSS CHICK DUODENUM IN VIVO**

<table>
<thead>
<tr>
<th></th>
<th>Lumen → plasma flux ($J_{LP}$)</th>
<th>Plasma → lumen flux ($J_{PL}$)</th>
<th>Flux ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>–</td>
<td>0.72 ± 0.10</td>
<td>0.24 ± 0.08</td>
<td>0.30</td>
</tr>
<tr>
<td>+</td>
<td>1.54 ± 0.12</td>
<td>0.52 ± 0.16</td>
<td>0.30</td>
</tr>
</tbody>
</table>

*Perfused Ca concentration = 5.2 μM; values = mean ± SEM of five to six animals per group.*

*Table reprinted by permission from the Amer. J. Physiol. 1962, 203: 221.*

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It was reasoned that, if the Ca pump were stimulated or activated, the unidirectional flux ratio of rachitic chicks should be less than that of vitamin D-treated chicks. If the flux ratio remained the same, this would suggest that the permeability theory was probably correct. The data, part of which is presented in Table I, clearly showed that vitamin D treatment increased both the efflux (J_{ex}) and the influx (J_{in}) of Ca, and that the flux ratio remained essentially unchanged. The most obvious interpretation was that the sterol in some manner enhanced the permeability of the epithelial barrier of the intestine, allowing a greater movement of Ca in both directions. Of the two hypotheses previously offered, the one stating that the vitamin effect was by way of a nonenergy-requiring permeability alteration appeared to be the more likely one.

In respect to the cellular site of the response, other studies were done in which the uptake of either intraluminal ⁴⁷Ca or plasma ⁴⁸Ca by the mucosal tissue was determined. At the same time, the absorption of ⁴⁷Ca or the "secretion" of plasma ⁴⁷Ca into the lumen was also measured. From the data depicted in Fig. 1, it was apparent that vitamin D treatment enhanced ⁴⁷Ca absorption (per usual) and, at the earlier time periods, enhanced the quantity...
of $^{47}$Ca associated with the mucosa. The rate of release of $^{47}$Ca by the mucosa was also stimulated by the treatment. In regard to movement in the opposite direction, the transfer of plasma $^{47}$Ca to the intestinal lumen was greater in those chicks treated with the vitamin but, on the other hand, the transfer from plasma to mucosal tissue was unaffected (Fig. 2). That is, in both studies there was an apparent increase in Ca transfer across the mucosal border of the absorbing cell no matter whether Ca was derived from the lumen or the plasma and, from this, one may conclude that this region is at least one of the prominent sites of expression of the vitamin D effect. These and other aspects of calcium transfer across the intestine were discussed recently (18).

![Figure 2. Effect of vitamin D$_3$ on the transfer of $^{47}$Ca from plasma to intestinal lumen and from plasma to mucosal tissue. The radiocalcium was injected intravenously into rachitic or vitamin D$_3$-treated chicks. Prior to this, 1 ml of 0.15 M NaCl was placed in a ligated loop of duodenum. At the times indicated, the duodenum was removed, and the radioactivity content of the duodenal fluid and the mucosa were measured, as described previously (20). The values represent the mean ± SEM of five to six chicks.](image)

**Molecular Basis of Vitamin D Action**

The administration of vitamin D to a rachitic animal does not cause an immediate restoration of the absorptive process but requires several hours, the time depending upon the dose level of the vitamin. This lag effect was noted by Lindquist (19) in 1952 and a typical time-response curve from our studies is shown in Fig. 3 (20). It is apparent that, at the dose level used (500 IU/chick), between 8–16 hr must transpire before a significant response occurs. Considerable attention has been given to the events which take place within this period. These have been tentatively identified as: (a) the time required for the sterol to localize in the target tissue and at the appropriate intracellular site; (b) the time for possible transformation of vitamin D to an active metabolite; and, finally, (c) time required for the induction of the synthesis of a necessary component of the calcium transport system (21, 22). The transformation of vitamin D to an active metabolite was first shown by Norman et al. (23), using radioactively labeled sterol. A metabolite was recently isolated and characterized...
by Blunt et al. (24) and found to be 25-hydroxycholecalciferol (Fig. 4). The involvement of vitamin D in protein synthesis was initially derived from the experiments of Eisenstein and Passavoy (25) in which it was demonstrated that the hypercalcemia produced by massive doses of vitamin D was prevented by pretreatment with actinomycin D. Actinomycin D and other inhibitors of protein synthesis, such as puromycin and cycloheximide, were later shown by several groups to inhibit the intestinal response of various species to vitamin D (26–28) and, from this, the most reasonable hypothesis would be that the synthesis of a specific protein is induced by the vitamin. In this same vein, the incorporation of $^3$H-orotic acid and $^3$H-uridine into RNA of intestinal mucosa was shown to be stimulated by the prior injection of vitamin D; this response was also inhibited by actinomycin D (29, 30).

The subcellular localization of the vitamin is also consistent with the protein synthetic hypothesis since both Haussler and Norman (31) and Stohs and DeLuca (32) find this sterol in highest concentration in the nucleus of the target tissue. There is disagreement as to whether it accumulates on the nuclear membrane or is bound by specific structures within the nucleoplasm. However, Haussler et al. (33) recently presented substantial evidence indicating that labeled vitamin D$_3$ associates with the chromatin of mucosal cells of
chick intestine and that there is competitive inhibition by nonradioactive vitamin D₂, D₃, tachysterol-2, or tachysterol-3, but not by ergosterol or cholesterol. Nearly all of the chromatin-bound vitamin was in the form of the active metabolite. Actinomycin D did not inhibit this reaction.

The observation that vitamin D administration to a rachitic animal causes a unique calcium-binding protein to be formed is direct evidence for the DNA-

![Diagram of vitamin D conversion]

**Figure 4.** Structural relationships between the precursor molecule, the parent molecule, and an active metabolite of vitamin D₃. This active metabolite of vitamin D₃ was recently characterized by Blunt et al. (24) and shown to be a hydroxylated form of activated dehydrocholesterol (vitamin D₂).

RNA-protein synthetic theory of vitamin D action. There is, on the other hand, data which does not appear to be consistent with this view. As an example, the release of Ca by isolated kidney mitochondria of the rat is accelerated when vitamin D is added to the in vitro preparation. The time course of this response is sufficiently short to eliminate protein synthesis as an intermediate step and, in addition, this effect is insensitive to actinomycin D (34). Another actinomycin D-insensitive response to vitamin D is the enhanced transfer of calcium across everted gut sacs of ileum from rats given extremely high levels of vitamin D (40,000 IU) and incubated under anaerobic conditions (34). In
order to explain these results, one may hypothesize that vitamin D has two mechanisms of action, one via the protein synthetic pathway and the other by way of a direct interaction with cellular membranes, the latter resulting in a selective permeability change. As an alternative hypothesis, it was suggested that the primary effect of the vitamin is to alter membrane permeability to calcium and, as a consequence of a change in the intracellular ionic environment, the synthesis of a specific protein is initiated. These aspects require further study. However, the bulk of the evidence derived from experiments with physiological levels of vitamin D and done under more physiological conditions, strongly indicate that the primary event is the direct interaction of the vitamin or its metabolite with the genetic machinery of the target tissue.

**Isolation and Some Properties of the Calcium-Binding Protein (CaBP)**

The first clue that vitamin D induces the formation of a unique protein was derived from studies on the distribution of $^{45}$Ca between components of homogenates of intestinal mucosa from rachitic and vitamin D-treated rachitic

![Figure 5](image_url)

**Figure 5.** Influence of vitamin D$_3$ on the $^{45}$Ca-binding activity of the supernatant fluid of homogenates of chick duodenal mucosa. The assay system consisted of Chelex 100 resin (0.04 ml in 0.2 ml of buffer mixture), 1 ml of supernatant fluid, and 0.1 ml radio-calcium solution. After mixing and centrifuging, an aliquot of the supernatant phase was counted. The symbols, D and R, refer to supernatant fluids from vitamin D-treated and untreated rachitic chicks, respectively. Each point represents the mean of three values. Figure reprinted by permission from Science, 1966, 152: 791.
chicks (35). Significantly more of the radiocalcium remained in the soluble phase of the material from vitamin D-treated versus that from rachitic animals and most or all of this effect appeared to be due to the presence of a factor in the vitamin D tissue with a capacity to bind Ca. The proteinaceous nature of the factor was shown by the fact that it was nondialyzable, heat labile, trypsin and Pronase digestible, and excluded by a Sephadex G-25 column (36).

The assay system for CaBP was based upon the ion exchange procedure of Briggs and Fleishman (37) and depended upon the competition of the resin and the soluble binding substance for added radiocalcium. With this assay procedure, the binding activities of the vitamin D and rachitic supernatant fluids and the buffer alone were determined and the results, as a function of calcium concentration in the system, are shown in Fig. 5. Clearly, the binding
activity of the vitamin D supernatant was considerably greater than that of the rachitic supernatant or the buffer alone and that, as the calcium concentration was increased, the proportion of radiocalcium maintained in the soluble phase of the vitamin D supernatant decreased, suggesting that a binding component of the latter fluid was being saturated. A comparison of acrylamide gel electrophoretic patterns also revealed that at least one protein was present in vitamin D supernatant which could not be visualized in the rachitic material (cf. arrow, Fig. 6). This band was later shown to be, or have associated with it, the vitamin D–induced calcium-binding protein.

The CaBP was obtained in high purity by the use of a three-step fractionation procedure which included, in order: (a) ammonium sulfate treatment; (b) gel filtration on Sephadex G-100; and (c) preparative disc acrylamide gel electrophoresis (38). The product yielded a single band on an analytical electrophoretic gel and a single Schlieren peak with the analytical ultracentrifuge. The molecular weight was estimated to be in the range of 25,000–28,000, as determined by the use of a calibrated gel filtration column and by sedimentation equilibrium. The association constant between Ca and the protein was determined by the procedure of Schubert et al. (39) and found to be about $2.6 \times 10^4 \text{M}^{-1}$. Those for Sr and Ba were observed to be about $3.0 \times 10^4$ and $5.8 \times 10^3 \text{M}^{-1}$, respectively. Analysis of the binding data indicated that 1 molecule of protein bound 1 atom of Ca.

**Tissue Localization of CaBP** The vitamin D–induced calcium-binding protein has been identified in all segments of the small intestine (40), the colon,1 and the kidney of the chick (40), and the uterus (shell gland) of the laying hen (41). Each of these are epithelial tissues across which calcium is translocated. CaBP has not been found in liver, muscle, pancreas, or blood. Although considerable effort was given to uncovering the presence of CaBP in bone, the data have proven negative thus far.

**Species Distribution of CaBP** A vitamin D–induced calcium-binding protein has been found in intestinal mucosa of the following species: chick (36), rat (42), dog (43), bovine, and monkey. There are some differences in the properties of these various proteins, such as electrophoretic mobility and sensitivity to proteolytic enzymes, but detailed studies in this direction have not yet been carried out.

**Correlation between CaBP and Calcium Absorption** Knowing that vitamin D increases Ca absorption and observing that a product of vitamin D interaction with the target tissue is a calcium-binding protein, is by itself suggestive evidence that the product (i.e. CaBP) is involved in the translocation process. However, other evidence is required to build a substantial case in favor of this hypothesis. For example, it must be shown that the appearance of

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CaBP in the rachitic chick after vitamin D administration precedes or at least accompanies the physiological change. This was shown to be approximately the situation in an earlier experiment (36) and was investigated in more detail recently (44). Part of the latter study was to examine the time required for the appearance of CaBP at two levels of vitamin D, one at a physiological level (100 IU) and one at a pharmacologic level (5000 IU). By resorting to the most sensitive technique available to us, i.e. double immunodiffusion, it was shown that CaBP is present in mucosal tissue at the same time that Ca absorption was

![Graph showing correlation between the appearance of CaBP and the restoration of the calcium-absorptive mechanism by vitamin D3.](image)

Figure 7. Correlation between the appearance of CaBP and the restoration of the calcium-absorptive mechanism by vitamin D3. At zero time, a group of rachitic chicks were given either 100 IU or 5000 IU vitamin D3. At the periods designated above, the absorption of radiocalcium from a ligated segment of duodenum was measured; the absorptive period was 1/2 hr. CaBP in the duodenal mucosa of other similarly treated chicks at these same time periods was also measured. Three procedures were used: G, detection of the characteristic band on an acrylamide gel electrophoretic pattern; I, immunodiffusion against a CaBP-specific antibody; and R, Chelex 100 ion exchange assay. In the above figure, the periods at which CaBP was first detected have been designated by a G, I, or R. The symbol, A, refers to the time at which a significant increase in absorption was first observed. Each point represents the group mean of five to six chicks ± SEM. Figure reprinted by permission from Amer. J. Clin. Nutr., 1969, 22: 431.
enhanced, in spite of the fact that the 5000 IU dose decreased the lag time by about 10 hr (Fig. 7).

Another criterion would be that the concentration of CaBP in mucosal tissue should vary directly with the efficiency of the calcium-absorptive mechanism, particularly when vitamin D is not limiting. This was achieved by resorting to

![Graph showing effect of dietary calcium level on ⁴⁷Ca absorption by the chick duodenum and on the calcium-binding activity of the supernatant fluid of duodenal mucosa homogenates. Chicks were raised on diets containing 0.08, 1.20, or 2.32% Ca. Absorption of ⁴⁷Ca by a ligated loop of duodenum was measured (20) and, in a comparable group of chicks, the calcium-binding activity of the supernatant fluid of homogenates of the duodenal mucosa was determined by the Chelex 100 ion exchange assay.\(^5\)](image)

the well-documented adaptation phenomenon, the latter defined as the ability of the animal to alter its capacity to absorb Ca in response to the dietary intake of that element. Animals raised on a low calcium diet absorb more Ca per unit time per unit length of intestine than similar animals raised on a normal calcium diet. As shown in Fig. 8, this was adequately demonstrated in the chick and, as depicted in the same figure, the concentration of CaBP increased in parallel fashion.\(^5\) Again a relationship between CaBP and Ca absorption was

In apparent. These and other correlations strongly suggested that the hypothesis is correct; i.e., CaBP functions in the translocation process.

Further it was demonstrated that CaBP synthesis is inhibited by actinomycin D (45), and that the concentration of CaBP in intestinal mucosa is greater in younger than older animals and greater in laying than in non-egg laying hens (8).

Two types of evidence, that would be unequivocal, have not been achieved successfully. One would be to place CaBP in rachitic mucosa and restore its absorptive capacity and, secondly, inhibit the response with a specific reagent such as the antibody to CaBP. Neither of these have yet been accomplished.

LOCALIZATION OF CALCIUM-BINDING PROTEIN  An antiserum shown to be...
specific for CaBP by Taylor (46) was used in the indirect fluorescent antibody method of Weller and Coons (47) for the purpose of localizing CaBP in intestinal tissue. Frozen sections of duodenum from rachitic and vitamin D3-treated rachitic chicks were used, and Fig. 9 shows the distribution of fluorescence obtained. This pattern of fluorescence was shown to be immunologically specific by the normal blocking reactions; i.e., substitution of either nonspecific or CaBP-adsorbed, specific antiserum in place of specific antiserum. The pattern observed with rachitic intestine provided an additional control, in that it contained no CaBP and likewise exhibited no specific fluorescence.

The correlation between the regions demonstrating specific fluorescence and the PAS (periodic acid–Schiff) positive areas is shown in Fig. 10. It can be seen that most PAS-positive goblet cells contain high concentrations of CaBP. In addition, specific fluorescence was observed to cover the surface of the villi.
indicating that CaBP is present in the surface coat–microvilli region of the cells lining the villar surfaces. The resolution obtainable with 6-μm frozen sections does not allow a definitive localization of CaBP in either the mucopolysaccharide surface coat or the brush border (microvilli); however, when viewed at high magnification, a surface coat localization is the more probable. These results are analogous to those obtained by Nakane et al. (48) in which it was shown that a leucine-binding protein was localized in the cell envelope of E. coli. In both cases, the binding proteins are located in “logical” positions to function as transport proteins.

SPECULATION ON THE MECHANISM OF ACTION OF CaBP

The exact function of CaBP in the translocation process (if this is actually the case) is, of course, unknown. Some speculation perhaps is warranted if only to serve as a starting point for subsequent experimentation.

From physiological information, it seems that vitamin D–dependent calcium absorption is not necessarily dependent on an intact energy supply and, equating the vitamin D mechanism with CaBP function, one may tentatively conclude that CaBP does not function as part of an active transport system. Pertaining to this same point, the Ca pump appears to be associated with the basal membranes of the epithelial cell whereas CaBP is localized in highest concentration at the opposite pole of the cell, and most likely within the surface coat matrix. Further, the mucosal border has been identified as a region of the cell where vitamin D manifests an important part of its effect, and across which the translocation of calcium in both directions is enhanced.

Assuming that these features are the most pertinent, at least three models can be offered, these being depicted in Fig. 11. First, CaBP may serve as a “diffusional facilitator,” enhancing the transfer of calcium across the microvillar membrane and/or through the sea of anionic charges that exists within the surface coat (cf. Borle (49) for a description of the relationship between the extraneous surface coat of isolated HeLa cells and calcium binding). Because of the large binding affinity of CaBP, this molecule could successfully compete for luminal Ca with respect to the anionic groups in the surface coat. However, two difficulties arise with this first suggestion. The protein must be able to penetrate through the core of the membrane in order to act as a membrane facilitator and, secondly, some mechanism must be available to release Ca from the protein (unless a Ca sink of sufficient magnitude is maintained).

A second possibility is that CaBP serves as an “intracellular carrier,” facilitating the movement of calcium through the cytoplasmic milieu. At the basal aspect of the cell, the transported Ca could be come attached to a component of the Ca pump and then be actively extruded from the cell or, in conjunction with a dumping system, leave the cell by diffusion. This type of mechanism appears unlikely because it was estimated that insufficient CaBP is available to

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complex all of the calcium in transit when a relatively high concentration of calcium is placed in the intestinal lumen; thus, CaBP appears to function catalytically.

The third possibility is that CaBP may act primarily as a "microvillar concentrator" of calcium, being analogous to an ion exchange resin and accumulating Ca in the region adjacent to the plasma membrane. The bound Ca is then available for the next step in the chain, the transfer of the element across the membrane into the cell cytoplasm. The latter step might involve another carrier of calcium.

![Diagram](image)

**FIGURE 11.** Diagrammatic representation of speculative ways by which CaBP may function. The solid circle represents CaBP; X represents an intracellular, soluble complex of calcium, such as citrate; the open circle with squiggle, the Ca pump.

**OTHER POSSIBLE TRANSPORT PROTEINS**

There are several other animal proteins that appear to be involved in the transport of specific substrates into the cell. One of these, "intrinsic factor" (IF) has been studied for over 35 yr and its existence was suggested by Castle in 1930 (50). He observed a hematopoietic response in pernicious anemia patients after normal human gastric juice, previously incubated with beef muscle, was introduced into the patient's stomach. Castle was able to conclude on the basis of this and other experiments that normal gastric juice contained an "intrinsic factor" lacking in pernicious anemia patients that was essential for the absorption of some substance generated by the interaction between it and an "extrinsic factor" contained in certain foods, such as beef. It is now known
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that the "extrinsic factor" is vitamin B₁₂ which was first isolated in pure form in 1948 (51, 52) and fully characterized in 1955 (53). Complete characterization of IF has not yet been realized, but it appears to be a glycoprotein of molecular weight 50,000–60,000 (54) and formed in the stomach, perhaps, in the chief cells (55).

The exact mechanism of IF-mediated B₁₂ transport is unknown but certain features have been elucidated. It is known that IF, though produced in the stomach, promotes B₁₂ absorption in the small intestine (56). It is generally accepted that B₁₂ must combine with IF prior to absorption (57, 58) and that calcium is necessary for optimal absorption (58). Very recent evidence has indicated that there is a specific receptor for the IF-B₁₂ complex in the intestinal surface coat. In one study, fluorescein-labeled rabbit antibodies to hamster microvillous membranes showed specific reaction with hamster intestine brush borders (59). In a related study, IF-mediated attachment of B₁₂ to brush borders isolated from hamster small intestine was shown to be prevented by incubation in vitro with rabbit antiserum to hamster microvillous membranes (60). Once attached to the luminal surface of the intestinal cell, the IF-B₁₂ complex may penetrate the cell as a unit but may be altered in transit since, in one study, the vitamin entering intestinal lymph, though not dialyzable, was devoid of IF activity (61). Serious doubt has been cast on this concept by a very recent study, however, which showed that IF was not absorbed during the process of IF-mediated vitamin B₁₂ absorption (62).

There is a B₁₂-binding factor in the blood, appearing both in normal humans and in pernicious anemia patients having no gastrointestinal IF. This factor facilitates uptake of B₁₂ by reticulocyte-rich erythrocyte preparations by an adsorption mechanism quite similar to the IF-mediated B₁₂ uptake by the intestine (63). The binding factor involved appears to be a β-globulin and has been called transcobalamin II (64). It is possible to speculate that both the "intrinsic factor" and transcobalamin II–mediated B₁₂ uptake are components of a sequential mechanism operating to assure optimal availability of B₁₂ for erythrocyte production and/or maturation.

Specific proteins have also been implicated in the intestinal absorption and erythrocytic uptake of iron. In the former case, apoferitin was considered to function in the transepithelial movement of iron (65). Little support for this concept has developed and, in fact, the evidence on which it was based was highly circumstantial. Ferritin was qualitatively estimated in guinea pig intestine by histological means and shown to increase with iron feeding (65). It was speculated that this stored iron could be called upon as needed to maintain blood levels (66). This proposal has been discounted and apoferitin is not even considered per se in a recent model of intestinal iron transport although the data presented are consistent with the presence of some carrier within the intestinal cell (67).
A second iron-binding protein, gastroferrin, appears to be involved in intestinal iron transport (68) but in a negative sense. This protein is found in gastric juice and has the capacity to complex iron, perhaps rendering it unavailable for absorption by the intestinal mucosa. Supporting this concept are two observations. In hemochromatosis, in which there is excessive iron absorption, gastroferrin is absent (69). Iron-deficiency anemia caused by severe blood loss was associated with low gastroferrin levels which returned to normal after restoration of normal blood levels by transfusion (70).

With regard to erythrocytic iron translocation, the case for the participation of a specific protein, transferrin, is quite good. Transferrin is the major nonheme, iron-binding protein of serum. In studies of human red cell suspensions, the uptake of iron-laden transferrin was much greater than the uptake of apotransferrin (71). Once attached to the red cell membrane, the iron is released into the red cell while the apotransferrin molecule becomes detached. The transferrin-mediated uptake of iron was prevented by metabolic inhibitors with the iron accumulation process being far more sensitive to inhibition than the transferrin attachment step (71).

Proteins have been suspected of being involved in other transport systems in animals but little is known of their nature. Transport of glucose by intestine (72, 73) and by the erythrocyte (4, 74, 75), for example, have been related to binding by proteins or protein-like substances. Similarly, work is proceeding on the purification of a carrier-like protein for organic bases that was released by osmotic shock from the kidney (76).

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REFERENCES


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Discussion from the Floor

Felix Bronner (University of Louisville, Louisville, Ky.): Dr. Maddaiah and I, stimulated by Dr. Wasserman's studies, have investigated the occurrence of this calcium-binding protein in rat mucosa and have studied the amount of binding activity as a function of calcium intake. We find, in agreement with what Dr. Wasserman has shown, that there is an inverse relationship between the amount absorbed and the amount bound; i.e., the amount of binding activity.

When animals that have been on a high calcium diet are placed on a low calcium diet, within 24 hr the amount of binding activity increases as shown on the slide (Fig. 1). As can be seen, there was a marked increase in calcium-binding activity, expressed as moles Ca × 10⁻¹⁰/mg protein, assayed by the Wasserman method. This increase was marked, then decreased in intensity, and stabilized at about the level

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you see on the right-hand side of the slide. The animals on diet III were absorbing roughly 100 mg calcium per day, the animals on diet I approximately 5 mg calcium per day.

When you treat animals with an antibiotic such as puromycin you can inhibit the increase of binding activity shown on the left-hand side of the slide. We have not done the reverse. However, when you place animals that are on the low calcium diet and shift them to a high calcium diet you see there is a decrease in activity. The increase in activity, which can be inhibited by puromycin, is not parathyroid dependent, as animals that have been parathyroidectomized show behavior similar to that of intact animals.

Studies are now in progress to further characterize the nature of this calcium-binding substance which, in agreement with Dr. Wasserman, we believe is a protein. We have preliminary evidence to this effect as $^{44}$Ca activity coincides with the first protein peak when the material is passed through Sephadex G-25 gel. Moreover, the activity disappears when the extract is digested with trypsin. The protein does not occur in the brush borders of the mucosal cells (prepared by the method of D. Miller and R. K. Crane. 1961. *Anal. Biochem.* 2:284). Rather, most of the activity is localized in the microsomal fraction of the mucosal cells (as determined by ultracentrifugal fractionation studies).
Dr. Wasserman: It is not surprising that CaBP was not observed to be associated with isolated brush borders since the protein is easily disengaged from the mucosal tissue by simple homogenization. The fluorescent antibody study, however, does tend to show that the protein is localized in the brush border region in vivo, but again the bonding between CaBP and the brush border material must be quite labile.

Dr. Geoffrey W. G. Sharp (Massachusetts General Hospital): In connection with your suggestion that the calcium-binding protein is situated in the mucus layer and may be synthesized in the goblet cells, do you have evidence of specific nuclear localization of vitamin D in the goblet cells, or is it localized in most of the cells?

Dr. Wasserman: We have no evidence on this point.

Dr. Pardee: Dr. Roseman asks whether it has carbohydrate in it.

Dr. Wasserman: We concluded from our previous analysis that CaBP would contain 1% or less of a glucose equivalent on a weight basis. This was done when limited amounts of purified protein were available.

Mr. Arthur B. Chausmer (State University of New York, Downstate): The other mediator of calcium absorption which springs to mind is parathyroid hormone. Have you looked for this binding protein under the influence of parathyroid hormone? The second question I have goes back to whether your actinomycin itself will lower serum calcium in a parathyroidectomized rat. I didn't notice whether you had given actinomycin to an animal which was essentially normal, and whether you had noted any changes in serum calcium levels.

Dr. Wasserman: No, we do not have any specific information on the influence of parathyroid hormone on the calcium-binding protein. The hormone, I'm sure, would not induce the synthesis of the protein. However, if vitamin D is not limiting, it may possibly alter the concentration of CaBP in the target tissue.

Regarding the actinomycin D response, several groups (Norman, A. W. 1965. Science. 149:134; Zull, J. E., et al. 1965. Science. 149:182; and Bosmann, H. B., and P. S. Chen, Jr. 1966. J. Nutr. 90:405) showed that the increment in Ca absorption in rachitic animals due to vitamin D treatment was prevented when they were given actinomycin D before or simultaneously with the vitamin. If the antibiotic were administered several hours after the vitamin was given, little or no inhibition was noted, indicating that there was a direct effect on the vitamin D-mediated process. However, H. E. Harrison and H. C. Harrison (Proc. Soc. Exp. Biol. Med. 1966. 121:312) observed that actinomycin D decreased Ca absorption by rachitic rats and this suggested that the antibiotic acted on the "transport system itself rather than on the enhancing effect of vitamin D on this process."

Dr. Joseph F. Gennaro (New York University): Is the anticalcium-binding protein species-specific?

Dr. Wasserman: The antibody formed against chick CaBP does not interact with the dog protein.

Dr. Gennaro: Will the anti-mammalian antibodies react with the chick CaBP?

Dr. Wasserman: The chick CaBP antibody is the only antibody we have in hand.

Dr. Gennaro: If you treated this tissue on the mucosal surface with neuraminidase, is it possible that you could eliminate this calcium-binding effect? Secondly, in your first electrophoresis slide you mentioned the increase in the staining intensity of the
furthermost band, but the two above it also showed variations. Do you have any comments on that?

Dr. Wasserman: In regard to your first question, those treatments have not been attempted as yet.

You are quite correct in noting that there are other alterations in the gel electrophoretic pattern of proteins due to vitamin D treatment or to a low calcium intake. In the past, however, we have given attention only to CaBP and its characteristic band, but eventually will attempt to characterize or at least gain some information on the other proteins.

Dr. George Nichols, Jr. (Harvard Medical School): Dr. Wasserman, I have two questions. The first was somewhat similar to the comments of the last gentleman, namely what are the effects of proteolytic enzymes on the presence of the protein? The second is: Have you yet had a chance to see how the presence or absence of this protein on the surface may effect the actual transfer of calcium 45 across the mucosal membrane? Can you put the two together yet?

Dr. Wasserman: You mean restore the system?

Dr. Nichols: Yes.

Dr. Wasserman: We have made some attempts in this direction but thus far we have not been successful.