Intestinal Membrane Calcium-binding Protein

VITAMIN D-DEPENDENT MEMBRANE COMPONENT OF THE INTESTINAL CALCIUM TRANSPORT MECHANISM*

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A particulate fraction of rat intestinal mucosal homogenates, termed the "calcium-binding complex," contains three vitamin D-dependent activities: calcium binding of high affinity, calcium-dependent adenosine triphosphatase, and p-nitrophenolphosphatase. These particulate activities vary concordantly with intestinal calcium transport, suggesting that they represent membrane components of the translocation mechanism. The particulate was solubilized with 1-butanol and the activities were resolved partially by gel filtration and by DEAE-cellulose and spheroidal hydroxylapatite column chromatography. The Ca-binding activity was separated from the enzymes and isolated as a protein of molecular weight ~200,000, as estimated by gel filtration in 0.1% Triton X-100. The membrane protein, named IMCal (intestinal membrane calcium-binding protein), was dissociated with sodium dodecyl sulfate to yield a monomer of molecular weight 20,500 which is clearly distinguishable from the soluble calcium-binding protein (molecular weight 11,500) of rat mucosa. The apparent dissociation constants for Ca** of IMCal and of the soluble calcium-binding protein were estimated as 0.37 µM and 2.25 µM, respectively. The vitamin D-dependent activities of the calcium-binding complex are present in isolated intestinal microvillus membranes and may mediate the translocation of calcium from the intestinal lumen to the cytosol.

Vitamin D is required for the intestinal absorption of calcium and a general understanding of the cellular and molecular basis of its action has emerged from the studies of many laboratories (1-35). The sterol vitamin is hydroxylated to activate metabolites in the liver and kidney (18, 27, 28, 33, 36, 37), and the resulting derivatives are transferred via the bloodstream to the mucosal cells of the small intestine (30, 31), where they regulate specific mechanisms of protein synthesis or turnover (11-13, 33-37). The specific mucosal proteins under control of the vitamin are essential for maintaining an active cation pump for calcium (2-10, 14, 21) which mediates and modulates the intestinal absorption (6-9, 21). Inasmuch as one or more of the vitamin D-dependent mucosal proteins are probably integral components of the cation pump, the identification and characterization of these molecules are of considerable physiological interest.

Prior reports have described two types of intestinal calcium-binding proteins dependent on vitamin D. A soluble calcium-binding protein of molecular weight 28,000 was identified by Wasserman and Taylor (15-17) in chicken mucosal homogenates and subsequently similar proteins in the molecular weight range 8,000 to 20,000 have been observed in the rat and other species (38-45). Although the total calcium-binding activity or mucosal content of the soluble CaBP1 may correlate generally with the level of intestinal calcium transport (16, 30, 39, 46-48), some discrepancies have been noted (49-52), and it is not established how the soluble protein participates in transmembrane transport of the cation. The second type of mucosal calcium-binding activity dependent on vitamin D resides in a membrane particulate isolated from rat intestinal homogenates, and termed the "calcium-binding complex" (30). The CaBC activity correlates well with the level of intestinal calcium transport as affected by vitamin D deficiency, the intestinal segment involved, dietary calcium content, rat age, and the effects of inhibitors of protein biosynthesis. Accordingly, we have suggested that it is a membrane component of the translocation mechanism for calcium (30).

The present report characterizes the rat CaBC preparation further and describes its solubilization and partial resolution. The particulate contains at least three activities dependent on vitamin D: calcium-binding of relatively high affinity (30), adenosine triphosphatase dependent on calcium (CaATPase), and p-nitrophenolphosphatase. After solubilization, the calcium-binding activity can be separated from the enzymes and isolated as a protein with a molecular weight of ~200,000 as determined by gel filtration. We have named the protein IMCal and shown that on dissociation with sodium dodecyl sulfate it consists of a monomer of molecular weight 20,500 which is clearly distinguishable from the soluble rat CaBP.

EXPERIMENTAL PROCEDURES

Animals and Diets—Albino male rats of the Sherman strain were maintained on a complete pelleted diet (Cann Institute Research, Wayne, N. J.) plus water ad libitum and weighed 250 to 280 g when used for CaBC preparations. For vitamin D depletion studies, weaning male rats of the same strain, weighing 40 to 50 g, were maintained on a vitamin D-deficient diet containing 0.2% Ca and 0.4% P (Teklad Test Diets, Madison, Wis., diet number TD78174) for 4 to 5 weeks in cages shielded from the light, as previously described (30). For repletion, the deficient animals received 20,000 IU of vitamin D3 in ethanol/propylene glycol 1:1 (v/v) intramuscularly 18 h before death.

All rats were fasted 18 h in metabolism cages with water ad libitum prior to each experiment. To examine the effects of dietary Ca, weaning male rats were maintained for 5 to 6 weeks on a powdered basal diet (Nutritional Biochemicals, Cleveland, Ohio; Calcium-Deficient Test Diet) prepared to contain 0.5% P and either 1.2% Ca or 0.02% Ca.

The abbreviations used are: CaBP, soluble calcium-binding protein; CaBC, particulate calcium-binding complex; CaATPase, calcium-dependent adenosine triphosphatase; SDS, sodium dodecyl sulfate; IMCal, intestinal membrane calcium-binding protein.

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1 The abbreviations used are: CaBP, soluble calcium-binding protein; CaBC, particulate calcium-binding complex; CaATPase, calcium-dependent adenosine triphosphatase; SDS, sodium dodecyl sulfate; IMCal, intestinal membrane calcium-binding protein.
Calcium Transport Component

Preparation of CaBC—The method described previously for preparation of the CaBC was modified as indicated below. Starting material consisted of the pooled mucosal scrapings of small intestinal segments from 5 to 10 rats. The routine preparation was made from the "duodenal" segment, a 10-cm length measured from the pylorus; in some experiments the "jejunal" segment, a 15-cm length with the proximal end 25 to 30 cm from the pylorus was also studied. All specimens were frozen in 5 to 10 volumes of ice-cold "homogenizing buffer" (12 mM Tris buffer of pH 7.4 containing 119 mM NaCl plus 4.7 mM KCl) and homogenized for 25 s in a Vib-Tis homogenizer (Vib-Tis Co., Inc., Gardiner, N.Y.) at an intermediate speed (dial setting 70). The resultant homogenate was centrifuged at 140,000 g for 2 h to pellet the precipitate and the supernatant solution retained for assay and purification of the soluble CaBP (see below). The pellet was washed once by suspension in 3 ml of 5 mM sodium EDTA of pH 7.4 to remove endogenous cations, and then washed 5 times with 12 ml of homogenizing buffer to dissolve the EDTA (30). The washed pellet was then suspended in 15 to 30 ml of 1.5% Triton X-100 (Sigma Chemical) in 13 mM Tris of pH 7.4. Suspension was achieved in a Potter homogenizer using 15 strokes of a motor-driven Teflon pestle rotating at full speed. After storage overnight at 4°C, the suspension was centrifuged at 100,000 g for 30 min and the precipitate was removed by centrifugation in 1 to 3 ml of 0.1% Triton X-100 in 13 mM Tris, pH 7.4. The suspension could be stored frozen at −15°C for up to 30 days with no loss in activity.

In some experiments isolated duodenal or jejunal microvillus membranes were used instead of the starting material for the preparation of the CaBP. The membranes were prepared and characterized by marker enzymes as described previously (53), using modifications of the methods of Schmitz et al. (54) and Hopper et al. (55).

Solubilization of CaBP—Solubilization with recovery of CaBP activity was possible by treatment with either sodium deoxycholate (0.2%) or 1-butanol, but the latter proved more convenient and is now used routinely. Mucosal scrapings were treated as described for the CaBP preparation above, except that the EDTA treatment and succeeding washes were eliminated. After suspension in the 1.5% Triton X-100 homogenate, the material pelleted by centrifugation at 10,000 g for 30 min was discarded. To the supernatant suspension, containing the CaBP activity, sufficient ice-cold 1-butanol was added to give a final concentration of 30% of the mixture was shaken vigorously for 30 min at 5°C. After centrifugation at 100,000 g for 30 min, the aqueous (lower) phase contained the solubilized activities and was removed carefully, avoiding the interfacial float.

Assay Methods—Calcium binding was assayed routinely by using 45Ca bound to Chelex 100 (Bio-Rad Laboratories) as previously described (30), and the results were expressed in counts per minute/µg of protein. For assay of the CaBP derived from the isolated microvillus membrane, Ca binding was estimated by a direct procedure (30) in which the preparation is mixed with 45Ca, harvested by centrifugation and the radioisotope and protein content of the pellet are estimated. Methods for estimating CaATPase (29) and p-nitrophenylphosphatase (53) were reported previously. The apparent dissociation constants for Ca++ of rat CaBP and of purified IMCal were determined by a gel filtration (Waters Associates, Milford, MA) using a C-18 column under conditions for Ca++ of rat CaBP and of purified IMCal were determined by a gel filtration (Waters Associates, Milford, MA) using a C-18 column under conditions previously described (56). To minimize aggregation and to obtain reproducible electrophoretograms of the CaBP solubilized proteins in the absence of SDS, less than 10 µg of protein were applied per 5-mm sample slot and a protein solution containing 25 mM EDTA, 8.3 plus 1 mM dithiothreitol. The electrophoresis buffer (g of Tris base, plus 28.8 g of glycerol per liter, pH 8.3) also contained 25 mM EDTA, pH 8.3. Molecular weights were estimated by SDS-polyacrylamide gel electrophoresis (57) and by gel filtration through Sephacryl S-200 (Pharmacia Fine Chemicals). Proteins were estimated by the method of Lowry et al. (58) in the absence of Tris and by the modified procedure of Wang and Smith (59) in its presence using bovine serum albumin as the standard.

RESULTS

Vitamin D-dependent Activities in Rat CaBC—Several laboratories have identified vitamin D-dependent CaATPase and p-nitrophenylphosphatase activities in homogenates or brush border suspensions of rat (29, 60) and chicken (23, 25, 52, 61) mucosa. Accordingly, it was of interest to determine whether rat CaBC suspensions contain these enzyme activities, and duodenal preparations from each of seven groups of vitamin-deficient and -repleted rats were examined. As shown in Table I, CaBC suspensions do contain both enzyme activities in addition to the Ca-binding activity, and repletion with vitamin D increased the Ca-binding activity by 114% (p < 0.005). The CaATPase by 74% (p < 0.025), and the p-nitrophenylphosphatase by 60% (p < 0.005).

<table>
<thead>
<tr>
<th>Group</th>
<th>Number of experiments*</th>
<th>Calcium binding¹</th>
<th>CaATPase¹</th>
<th>p-Nitrophenylphosphatase¹</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>cpm µg⁻¹</td>
<td>nmol min⁻¹</td>
<td>µmol µg⁻¹</td>
<td>µmol µg⁻¹</td>
</tr>
<tr>
<td>- Vitamin D</td>
<td>7</td>
<td>44.7 ± 7</td>
<td>1.43 ± 0.20</td>
<td>0.91 ± 0.26</td>
</tr>
<tr>
<td>+ Vitamin D</td>
<td>7</td>
<td>94.14</td>
<td>2.49 ± 0.38</td>
<td>1.39 ± 0.28</td>
</tr>
<tr>
<td></td>
<td>(p &lt; 0.005)</td>
<td>(p &lt; 0.025)</td>
<td>(p &lt; 0.005)</td>
<td></td>
</tr>
<tr>
<td>Duodenal</td>
<td>2</td>
<td>206</td>
<td>0.76</td>
<td>2.09</td>
</tr>
<tr>
<td>Jejunal</td>
<td>2</td>
<td>96</td>
<td>0.26</td>
<td>0.19</td>
</tr>
<tr>
<td>Low-Ca diet</td>
<td>2</td>
<td>50</td>
<td>1.93</td>
<td>3.58</td>
</tr>
<tr>
<td>High-Ca diet</td>
<td>2</td>
<td>15</td>
<td>1.51</td>
<td>2.14</td>
</tr>
</tbody>
</table>

* Each experiment represents one CaBC suspension prepared from the pooled mucosa of five to seven rats.

Means or means ± S.E. are listed.

Assayed as the release of P₃ in the presence of Ca²⁺ and Mg²⁺ minus the release in the presence of Mg²⁺ alone (29).

For differences between vitamin-deficient and -repleted preparations estimated by the paired t test.
Transport of Ca as influenced by vitamin D, localization in the small intestine, and dietary Ca.

CaBC from Isolated Microvillus Membranes—In a prior report (30) we noted that CaBC suspensions can be prepared from isolated intestinal brush borders. To determine more specifically whether the microvillus membrane, in contrast to the microvillus core material, is a source of the particulate activity, duodenal and jejunal microvillus membranes were prepared from normal rats and used as starting material for the CaBC preparation (see "Experimental Procedures"). The results summarized in Table II indicate that the Ca-binding activity of the duodenal preparation was approximately 3 times that of the jejunal (p < 0.025), as expected for a membrane activity related to Ca transport. In addition, the p-nitrophenylphosphatase activity was over 5-fold greater in the duodenal suspension.

Solubilization of CaBC—Suspensions of duodenal CaBC were prepared from each of 5 groups of vitamin D-depleted and -repleted rats and portions were solubilized with 1-butanol (see "Experimental Procedures"). Both the particulate and solubilized preparations were assayed for Ca binding, CaATPase, and p-nitrophenylphosphatase and the specific activities are shown in Table III. Values for Ca binding, CaATPase, and p-nitrophenylphosphatase were greater in the solubilized extracts as compared to the particulates, with relative increments of 52 to 59%, 16 to 18%, and 138 to 154%, respectively. Higher values owing to vitamin D were observed consistently for all three activities, and the percentage increase was similar

Table II
Calcium binding and p-nitrophenylphosphatase activities in CaBC suspensions prepared from isolated microvillus membranes

<table>
<thead>
<tr>
<th>Preparation</th>
<th>Calcium binding* (mean ± S.E.)</th>
<th>p-Nitrophenylphophatase*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>cpm µg⁻¹</td>
<td>nmol min⁻¹ µg⁻¹</td>
</tr>
<tr>
<td>Duodenal</td>
<td>174 ± 43</td>
<td>8.965 ± 0.58</td>
</tr>
<tr>
<td>Jejunal</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* Five preparations compared; p determined by t test of paired comparisons.

Table III
Vitamin D-dependent activities in particulate CaBC and in soluble extracts

Preparations were made from five groups of depleted and repleted rats and solubilized with 1-butanol (see "Experimental Procedures"). Values are means. p for the differences between depleted and repleted preparations was calculated by the t test for paired comparisons.

<table>
<thead>
<tr>
<th>Preparation</th>
<th>Group</th>
<th>Calcium binding</th>
<th>CaATPase</th>
<th>p-Nitrophenylphosphatase</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>cpm µg⁻¹</td>
<td>nmol min⁻¹ µg⁻¹</td>
<td>cpm µg⁻¹</td>
</tr>
<tr>
<td>Particulate</td>
<td>Vitamin D</td>
<td>44</td>
<td>0.82</td>
<td>0.37</td>
</tr>
<tr>
<td>CaBC</td>
<td>+ Vitamin D</td>
<td>&lt;0.025</td>
<td>&lt;0.025</td>
<td>&lt;0.025</td>
</tr>
<tr>
<td></td>
<td>p</td>
<td>60</td>
<td>1.07</td>
<td>1.37</td>
</tr>
<tr>
<td>Soluble</td>
<td>Vitamin D</td>
<td>91</td>
<td>1.48</td>
<td>1.37</td>
</tr>
<tr>
<td>extract</td>
<td>+ Vitamin D</td>
<td>&lt;0.015</td>
<td>&lt;0.015</td>
<td>&lt;0.015</td>
</tr>
</tbody>
</table>

Table IV
Recovery of activities and protein in solubilized CaBC extracts

Mean values ± S.E. for preparations from five groups of depleted and repleted rats are shown. For each assay the total quantity (units of activity or milligrams of protein) in the solubilized extract was compared to that in the particulate CaBC.

<table>
<thead>
<tr>
<th>Assay</th>
<th>Group</th>
<th>Recovery (mean ± S.E.) %</th>
</tr>
</thead>
<tbody>
<tr>
<td>Calcium binding</td>
<td>Vitamin D</td>
<td>89 ± 11</td>
</tr>
<tr>
<td></td>
<td>+ Vitamin D</td>
<td>91 ± 9</td>
</tr>
<tr>
<td>CaATPase</td>
<td>Vitamin D</td>
<td>78 ± 22</td>
</tr>
<tr>
<td></td>
<td>+ Vitamin D</td>
<td>77 ± 18</td>
</tr>
<tr>
<td>p-Nitrophenylphosphatase</td>
<td>Vitamin D</td>
<td>83 ± 31</td>
</tr>
<tr>
<td></td>
<td>+ Vitamin D</td>
<td>124 ± 42</td>
</tr>
<tr>
<td>Total protein</td>
<td>Vitamin D</td>
<td>62 ± 6</td>
</tr>
<tr>
<td></td>
<td>+ Vitamin D</td>
<td>62 ± 3</td>
</tr>
</tbody>
</table>

phenylphosphatase by 53% (p < 0.005). CaBC suspensions prepared from duodenal and jejunal segments of normal rats, maintained on the complete pellet diet, were also compared, since Ca transport is considerably greater in the duodenum (5, 8, 30). The mean values in Table I show that Ca binding, CaATPase, and p-nitrophenylphosphatase were all considerably greater in duodenal as compared to jejunal preparations. Lastly, intestinal Ca transport is greater in rats fed a low- as compared to a high-Ca diet (8, 30) and duodenal CaBC suspensions were prepared from each of two groups of animals maintained on the appropriate diets (see "Experimental Procedures"). The mean values for Ca-binding, CaATPase, and p-nitrophenylphosphatase were considerably greater in the low-Ca diet group (Table I). In summary, all three CaBC activities examined varied concordantly with the intestinal
in the extract as compared to the particulate. The vitamin-
dependent increment in Ca binding was 43% in the particulate
and 49% in the extract, and the corresponding values for
CaATPase were 55% versus 53% and for p-nitrophenolphos-
phatase 46% versus 56%.

Solubilization with 1-butanol yielded reasonable recoveries
of the total protein, approximately 62%, and of the vitamin D-
dependent activities, as shown in Table IV. Approximately
90% of the Ca binding, 77% of the CaATPase, and 80 to 120%
of the p-nitrophenolphosphatase activities were recovered,
with no significant differences between the vitamin-deficient
and-repleted preparations. The soluble extracts were consid-
ered suitable for further purification.

Partial Resolution of Solubilized CaBC—Suspensions of
duodenal CaBC prepared from vitamin D-depleted and -re-
pleted rats were solubilized with 1-butanol and the extracts
resolved by gel filtration through Sephadex G-150, followed
by ion exchange chromatography on DEAE-cellulose. Fig. 1
illustrates the results of the gel filtration experiments. The major vitamin D-dependent Ca-binding activity was eluted
shortly after the void volume (Fig. 1, fraction pools 2 and 3),
corresponding to a molecular weight range of approximately
200,000 to 250,000. A second, minor Ca-binding activity de-
dependent on the vitamin was eluted thereafter (fraction pool
5 and 6). The vitamin D-dependent CaATPase and p-nitro-
phenolphosphatase activities co-eluted with the higher molec-
ular weight Ca-binding activity. On the other hand, parallel
studies with these columns of the soluble CaBP yielded a
vitamin-dependent peak of Ca-binding activity in the molec-
ular weight range 14,000 to 24,000 (fraction pool 7).

The higher molecular weight material containing the Ca-
binding, CaATPase, and p-nitrophenolphosphatase activities
was resolved further by DEAE-cellulose column chromatog-
raphy, and the results are illustrated in Fig. 2. Here a clear
separation of the vitamin D-dependent Ca-binding activity
from the p-nitrophenolphosphatase was obtained. The Ca-
ATPase co-eluted with the p-nitrophenolphosphatase activity
data not shown). The fractions containing the Ca-binding
activity were examined by polyacrylamide slab-gel electrophor-
esis, and a major band difference between the vitamin D-
depleted and -repleted preparations was observed (Fig. 3, A
and B). To obtain sufficient starting material for further
purification of the Ca-binding activity, we turned next to the
intestinal mucosa of normal rats maintained on the complete
stock diet.

Purification of the Duodenal Calcium Binding Activity—
Exploratory studies showed that adsorption chromatography
on columns of spheroidal hydroxyapatite could separate the
Ca-binding activity from the p-nitrophenolphosphatase and
yield highly purified Ca-binding activity as judged by poly-
acrylamide gel electrophoresis. The results summarized in
Table V show that stepwise elution with 0.01, 0.1, and 0.2 M
sodium phosphate of pH 6.8 separates the enzyme activity,
present in the 0.01 M eluate, from the Ca-binding activity in
the 0.2 M fraction. Moreover, as expected for these transport-

![Diagram](image-url)

**Fig. 3.** Polyacrylamide slab gel electrophoreograms of par-
tially and fully purified preparations. A and B show the results
with preparations from vitamin D-depleted and -repleted rats,
respectively, partially purified as described in the text and resolved in
a 5% polyacrylamide gel. The arrow indicates the major band differ-
ence observed consistently. The electrophoreogram obtained with
purified IMCal resolved in a 4 to 30% polyacrylamide gradient gel is
shown in C, D, E, and F illustrate electrophoreograms of SDS-
polyacrylamide gradient gels (4 to 30%). D shows the SDS monomer
of IMCal, E that of a crystalline myoglobin reference (molecular
weight 17,200), and F that of the soluble CaBP. The other reference
proteins were chymotrypsinogen (molecular weight 25,700) and oval-
bumin (molecular weight 43,000).

**Table V**

<table>
<thead>
<tr>
<th>Preparation</th>
<th>Elution buffer*</th>
<th>Calcium binding</th>
<th>p-Nitrophenolphosphatase</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>M</td>
<td>cpm µg⁻¹</td>
<td>nmol min⁻¹ µg⁻¹</td>
</tr>
<tr>
<td>Duodenal</td>
<td>0.01</td>
<td>39 ± 5</td>
<td>0.70 ± 0.20</td>
</tr>
<tr>
<td></td>
<td>0.10</td>
<td>93 ± 12</td>
<td>0.03 ± 0.00</td>
</tr>
<tr>
<td></td>
<td>0.20</td>
<td>224 ± 43</td>
<td>0.01 ± 0.00</td>
</tr>
<tr>
<td>Jejunal</td>
<td>0.01</td>
<td>22 ± 7</td>
<td>0.17 ± 0.02</td>
</tr>
<tr>
<td></td>
<td>0.10</td>
<td>71 ± 13</td>
<td>0.01 ± 0.01</td>
</tr>
<tr>
<td></td>
<td>0.20</td>
<td>40 ± 6</td>
<td>0</td>
</tr>
</tbody>
</table>

* Sodium phosphate of pH 6.8.
related activities, the duodenal specific activities considerably exceeded the jejunal values.

Based on the above, the following purification procedure was adopted and Table VI indicates the results of two preparations illustrative of a total of six. Approximately 25 to 30 g wet weight of duodenal mucosa was obtained from 30 rats and the particulate CaBC was prepared and solubilized with 1-butanol to yield 40 to 80 mg of soluble proteins. The material was resolved by Sephadex G-150 gel filtration and the active fraction containing 26 to 35 mg of protein was concentrated and applied to a column of spheroidal hydroxylapatite. On stepwise elution, as described above, the 0.2 M phosphate fractions contained 0.6 to 0.8 mg of protein with a specific activity for Ca binding approximately 7 to 11 times that of the 1-butanol extract. Based on the specific activity of the crude mucosal homogenate, the overall purification achieved in different preparations varied from 32- to 110-fold.

**Characteristics of the Purified Protein**—The purified protein (IMCal) migrated as a single band on polyacrylamide gel electrophoresis (Fig. 3C), and after treatment with SDS, one band was observed on SDS-polyacrylamide gel electrophoresis (Fig. 3D). Based on the migration rates in the gels (57), the molecular weight of the SDS monomer was estimated to be 20,450 ± 450 (mean ± S.E.; six preparations). Purified rat CaBP, on the other hand, migrated more rapidly in the SDS-gel filtration and the active fraction containing 32- to 35 mg of protein was concentrated and applied to a column of spheroidal hydroxylapatite. On stepwise elution, as described above, the 0.2 M phosphate fractions contained 0.6 to 0.8 mg of protein with a specific activity for Ca binding approximately 7 to 11 times that of the 1-butanol extract. Based on the specific activity of the crude mucosal homogenate, the overall purification achieved in different preparations varied from 32- to 110-fold.

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**TABLE VI**

**Purification of the solubilized intestinal membrane calcium-binding protein (IMCal)**

Two preparations illustrative of a total of six are shown. Procedures are described in the text.

<table>
<thead>
<tr>
<th>Preparation</th>
<th>Fraction</th>
<th>Protein Total</th>
<th>% of Total</th>
<th>Calcium binding</th>
<th>Specific Activity</th>
<th>% of Total</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>mg</td>
<td>cpm µg⁻¹ 10⁻⁶</td>
</tr>
<tr>
<td>1</td>
<td>Butanol extract</td>
<td>51</td>
<td>100</td>
<td>27</td>
<td>1377</td>
<td>100</td>
</tr>
<tr>
<td></td>
<td>Sephadex G-150</td>
<td>31</td>
<td>61</td>
<td>33</td>
<td>1023</td>
<td>74</td>
</tr>
<tr>
<td></td>
<td>Hydroxylapatite</td>
<td>1</td>
<td>1</td>
<td>265</td>
<td>171</td>
<td>12</td>
</tr>
<tr>
<td>2</td>
<td>Butanol extract</td>
<td>40</td>
<td>100</td>
<td>22</td>
<td>880</td>
<td>100</td>
</tr>
<tr>
<td></td>
<td>Sephadex G-150</td>
<td>31</td>
<td>78</td>
<td>29</td>
<td>899</td>
<td>102</td>
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<tr>
<td></td>
<td>Hydroxylapatite</td>
<td>1</td>
<td>2</td>
<td>154</td>
<td>123</td>
<td>14</td>
</tr>
</tbody>
</table>

**TABLE VII**

**Amino acid analysis of IMCal and CaBP**

Results are for two preparations of CaBP and one of IMCal. Examinations utilized a Beckman 121MB amino acid analyzer and a four-buffer single-column methodology provided by Beckman Instruments.

<table>
<thead>
<tr>
<th>Amino Acid</th>
<th>IMCal</th>
<th>CaBP</th>
</tr>
</thead>
<tbody>
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* Moles residue per 100 mol of amino acid residues.
working hypothesis that the vitamin D-dependent activities of the membrane CaBC, including the Ca-binding component isolated as the protein IMCal, are functional components of the active transport mechanism which regulates Ca absorption.

The organelar distribution of IMCal, and of the particulate CaBC in the mucosal cell is unknown, but the results in Table II indicate that one site is the microvillus membrane. We have previously described evidence from transport experiments that Ca absorption across the mucosal cell involves at least two steps: entry into the cell across the microvillus membrane, followed by exit across the contraluminal, basolateral membrane (9, 14, 21, 63). Neither step involves simple diffusion alone. The transport observations indicate that the exit step is dependent on cellular metabolism and results in cation pumping; the entry step is rate-limited; and both entry and exit are dependent on vitamin D. The localization of the CaBC activities to the microvillus membrane suggests that at this site they mediate the entry of Ca across the luminal surface into the cytosol. This proposed function would elucidate electron microscopic observations (24) which indicate that calcium accumulates at the luminal surface of the mucosa in vitamin D-deficient rats and that treatment with the vitamin results in translocation of the cation to the cytosol. It seems reasonable to propose that the membrane-bound, vitamin D-dependent activities of the CaBC, i.e. the high affinity calcium binding to IMCal and the CaATPase, act in concert to mediate the translocation process. (The p-nitrophosphatase and CaATPase activities may reside in the same enzyme, as suggested previously (25, 26, 29), inasmuch as the activities have not been separated to date.) Although the precise molecular interactions in such a mechanism are unknown, it is noteworthy that the interplay of a Ca-binding protein and a CaATPase underlies a number of important physiological processes, including the contraction of skeletal and cardiac muscle myofilaments (64), the sequestration of calcium by the sarcoplasmic reticulum (65, 66), and the transport function of erythrocyte plasma membrane CaATPase (67, 68).

Recent publications emphasize the pivotal roles of calcium-binding proteins such as calmodulin (68, 69) in the regulation of a variety of cellular functions. IMCal has an apparent affinity constant for Ca2+ which is similar to that of many of these proteins (69), but it differs from known members of the group in that it is a vitamin D-dependent, intrinsic membrane protein of relatively high molecular weight. In this respect, it is noteworthy that several investigators have reported evidence for membrane-bound, vitamin D-dependent components in chicken intestinal mucosa. Moriiuchi and DeLuca (52) prepared 1-butanol extracts of chicken mucosal brush borders and observed greater calcium binding in preparations from vitamin D-treated as compared to -deficient animals. They related this finding to a change in electrophoretic mobility of a brush border protein, which exhibited p-nitrophosphatase activity and migrated in SDS-polyacrylamide gel electrophoretograms with an apparent molecular weight of 210,000. Wilson and Lawson (70) studied the incorporation of radioactive leucine into chicken brush border proteins after administration of 1,25-dihydroxycholecalciferol to vitamin D-deficient animals. Enhanced leucine incorporation dependent on the vitamin was observed for two SDS-proteins, corresponding to molecular weights of 45,000 and 84,000. Fehers and Wasserman (71) obtained evidence of a membrane-bound calcium-binding activity dependent on vitamin D in whole homogenates of chicken mucosa. Treatment with Triton X-100 released the bound activity, which was then found to be identical with the soluble chicken CaBP, molecular weight 28,000, on the bases of electrophoretic mobility, gel filtration, and immunological reactivity. In summary, studies in the chicken point to the occurrence of vitamin D-dependent, membrane-bound components of the calcium transport mechanism, but a protein similar to IMCal has not yet been isolated.

Lastly, Miller et al. (72) have reported the identification of a vitamin D-dependent calcium binding protein (M, ~ 18,500) which is pelleted with rat duodenal brush borders but does not require detergents for solubilization. This entity differs from IMCal, which is an intrinsic membrane protein of higher molecular weight, and its relationship to calcium transport deserves further investigation.

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REFERENCES
Calcium Transport Component

1204
44. Alpers, D. H., Lee, S. W., and Avioli, L. V. (1972) Gastroenterology 63, 559–564