Vitamin D Stimulation of Calcium-Dependent Adenosine Triphosphatase in Chick Intestinal Brush Borders*

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ABSTRACT: Vitamin D, *via* protein synthesis, gives rise to the active transport of calcium in the intestine of vitamin D deficient animals. Because the intestinal brush border may well be the site affected and membrane-bound adenosine triphosphatases have been related to active transport, the calcium-dependent adenosine triphosphatase activity of isolated intestinal brush borders from vitamin D deficient and repleted chicks was studied. Vitamin D gives rise to increased amounts of a calcium-dependent adenosine triphosphatase.

Substrate and cation requirements of this system were studied. The time course of the increase of calcium dependent adenosine triphosphatase activity following oral or intravenous vitamin D_3 repletion compares well with the time course of appearance of calcium absorption itself. It appears quite possible therefore that this calcium-dependent adenosine triphosphatase may be part of the vitamin D induced calcium transport system.

Vitamin D is required in a variety of species for adequate intestinal absorption of calcium and for bone mobilization. The fact that actinomycin D is able to block these functions of vitamin D (Eisenstein and Passavoy, 1964; Zull *et al.*, 1965, 1966; Norman, 1965; Schachter and Kowarski, 1965) indicates that RNA and protein synthesis is required for these responses. More recent observations of vitamin D action in intestine dealing with various aspects of the steps leading to protein synthesis have supported these concepts (Hallick and DeLuca, 1969; Stohs and DeLuca, 1967).

The major method of studying intestinal calcium transport and more specifically the effect of vitamin D thereon is the everted sac technique. Closed sacs are made from everted segments of small intestine, tracer calcium is placed in the sacs and in the bathing medium, and the ability of the intestinal segment to concentrate calcium inside of the sacs is measured (Wilson and Wiseman, 1954; Schachter *et al.*, 1966). It has been conclusively demonstrated that although sacs from normal rats can actively transport calcium against a concentration gradient, those from vitamin D deficient rats have poor transport activity. There is little agreement however on the locus at which the vitamin D effect is exerted (DeLuca, 1967).

Recently Martin and DeLuca (1969b) have compared the uptake of calcium by intestinal tissue at the mucosal surface and the unidirectional calcium fluxes across the entire intestinal wall of duodenum from vitamin D deficient and repleted rats. The mucosal to serosal:serosal to mucosal flux ratios of 5 observed in tissue from vitamin D repleted rats dropped to 0.8 when a nitrogen atmosphere and 2,4-dinitrophenol were utilized, showing the energy requirement of transporting calcium against a concentration gradient in agreement with previous conclusions (Schachter, 1963). Calcium uptake from the mucosal side was higher in intestine from vitamin D repleted rats than from deficient ones. This difference was observable as early as 1 min after the radioactive calcium was added and the uptake was saturable in intestine from both vitamin D deficient and repleted rats. This uptake was also inhibited by use of a nitrogen atmosphere. The short incubation time required to demonstrate the vitamin D effect on calcium uptake indicates that an early step in the transport, perhaps movement across the microvilli, is involved.

These data suggest that the vitamin D induced protein or proteins may somehow enable energy to be utilized to move calcium across the brush border of the intestinal cell. We have in fact reported the appearance of increased amounts of a calcium-stimulated ATPase in rat intestinal brush borders following vitamin D administration to vitamin D deficient rats (Martin *et al.*, 1969).

The present report deals with a calcium-dependent ATPase which appears in increased amounts in brush border preparations from vitamin D repleted chicks. Various parameters of this enzyme activity are studied and a possible relation to vitamin D induced calcium transport is considered.

Methods

White Rock standard chicks (1-day old) were obtained from the Sunnyside Hatchery, Oregon, Wis., and placed on a synthetic vitamin D deficient diet (Imrie *et al.*, 1967).

After 3–4 weeks on this diet vitamin D deficiency was exhibited by leg weakness. The chicks were generally used for experimental purposes after 3.5–5 weeks on the diet. Vitamin D_3 was administered orally at a level of 400 or 500 IU in 0.20 ml of Wesson oil with a blunt needle affixed to a 0.5-ml syringe, while control chicks received oil only. On some occasions vitamin D_3 was administered intravenously in 0.05 ml of ethanol, in which case controls received ethanol alone. They were sacrificed by decapitation following a sharp blow on the head at various times following dosing. The small intestine was then removed and used for the prep-

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TABLE I: ATPase Activity of Intestinal Brush Borders from Vitamin D Deficient Chicks and Chicks Given 500 IU of Vitamin D_3 Orally 72 hr Earlier.[a]

	No. of Chicks		μmole of P_i/100 μg of Protein per 10 min					
			−D			+D		
Expt	−D	+D	No Ca^{2+}	$+Ca^{2+}$	Ca·ATPase	No Ca^{2+}	$+Ca^{2+}$	Ca·ATPase
1	2	2	0.25	0.27	0.02	0.25	0.50	0.25
2	1	1	0.42	0.57	0.15	0.56	0.82	0.86
3[b]	2	2	0.26	0.32	0.06	0.38	0.62	0.24
4	2	2	0.40	0.51	0.11	0.52	0.99	0.57
5	7	6	0.40	0.50	0.10	0.30	0.52	0.22

[a] Chick intestinal brush borders were prepared as described in the text and incubated in a medium containing 40 mM Tris·Cl (pH 7.4), 5 mM ATP (pH 7.4), and 2.5 mM $MgCl_2$ or 5 mM $MgCl_2$ + 10 mM $CaCl_2$. Each value is the average of at least duplicate determinations on the pooled preparation from the number of chicks indicated. [b] The data from expt 3 are in micromolesper tube. The Ca·ATPase of preparations from chicks given vitamin D averaged 0.34 μmole of P_i higher than those from vitamin D deficient controls ($p < 0.05$) while the noncalcium averaged 0.06 μmole of P_i higher ($p > 0.2$).

aration of brush borders. Although initially the entire length of the small intestine was used this was later reduced to the first 25–30 cm. The isolated intestinal segment was rinsed with ice-cold 5 mM EDTA (pH 7.4) slit lengthwise, blotted on tissue paper, and the mucosa were harvested by scraping with a glass slide. All the solutions used in these procedures were kept in an ice bath prior to use and centrifugations were performed at 0–5°. Essentially the method of Forstner *et al.* (1968) for the preparation of rat intestinal brush borders was used. Approximately 1.8% homogenates of the mucosal scrapings in 5 mM EDTA (pH 7.4) were made by means of 40-sec runs in an ice-jacketed Waring blender at high speed. The 450*g* precipitate (all centrifugations 12 min each) from this homogenization was washed three times with the same buffer. The final precipitate was suspended in a 90 mM NaCl–0.8 mM EDTA (pH 7.4) solution for 30 min and then filtered through a layer of glass wool in a Büchner funnel. The glass wool pad was then rinsed with an equivalent volume of 5 mM EDTA (pH 7.4). The 450*g* centrifugate from this step was then washed three times with a 100 mM Tris·Cl solution (pH 7.4) before the final suspension was made in the same medium.

Turbidity readings at 660 mμ were used to facilitate making brush border suspensions of approximately equal concentrations. The method of Lowry *et al.* (1951) was used to determine protein concentrations, with bovine serum albumin as standard. Because Tris interfered with this analysis it was necessary to centrifuge aliquots of the brush border preparations and resuspend in distilled water before measurements were made.

Incubations were performed in a water bath at 37°. The reactions were initiated by the addition of 0.40 ml of ice-cold brush border suspension containing about 100 μg of protein to chilled tubes containing the appropriate nucleotide (pH 7.4) and metal ions in a volume of 0.60 ml. They were terminated by the addition of 1.0 ml of ice-cold 5 N H_2SO_4 and placed in an ice bath until P_i measurements were made by the method of Gomori (1941). After all of the reagents had been added and mixed, the tubes were placed in a 37° water bath for 10 min before the OD_{660} was read.

For the study of pH effects, the brush border preparations were washed twice in Tris·Cl (pH 7.4) and then once in the various Tris·Cl solutions of various pH values before final suspension in these solutions or else washed three times in Tris·Cl (pH 7.4) and then merely suspended in the appropriate solution.

The solutions of divalent cations, as the chlorides, were prepared in different mixtures at the various levels required.

The nucleotides were Na·ATP, K·ATP, Na·ITP, and Na·ADP, in each case being brought to pH 7.4 with the appropriate base. Also in some cases Na·ATP or K·ATP was put through a column of Dowex AG-50W-X2 resin in the Tris form, made to pH 7.4 with Tris, and the desired concentration of sodium or potassium reached by the addition of NaCl or KCl, respectively.

Calculations of the concentrations of Mg·ATP and Ca·ATP were based on the data of Taqui Khan and Martell (1966).

Statistical analyses were performed by the Students t test. Because of the variability of the activity of brush border preparations made on different days and from different batches of chicks each experiment was treated as a paired observation and treated as described by Mather (1947).

Calcium transport was evaluated by the isolated loop method. The parameter measured is the amount of [^{45}C]Ca appearing in 1 ml of blood 5 min after the radioactivity is injected into a tied-off loop of duodenum *in situ*. The exact method used has recently been described in detail by Harmeyer and DeLuca (1969) and was adopted from previously reported methods of Sallis and Holdsworth (1962). Aliquots (0.5 ml) of plasma were pipetted into shallow aluminum planchets, dried, and counted in a Nuclear-Chicago thin, end-window gas-flow counter.

Results

As evident from the results shown in Table I administration of an oral 500-IU dose of vitamin D_3 72 hr prior to sacrifice elicited an increase in ATPase activity of intestinal

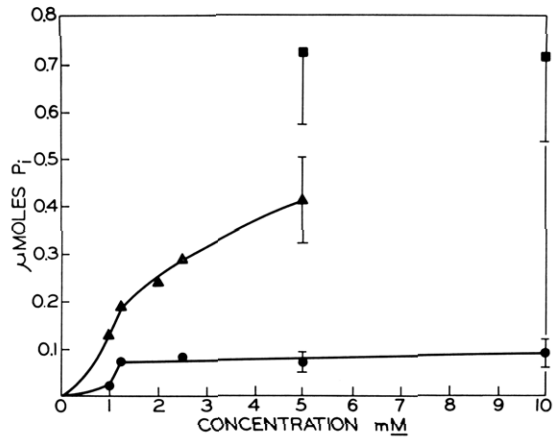

FIGURE 1: Effect of varying $MgCl_2$ and $CaCl_2$ concentrations on the ATPase activity of intestinal brush borders from chicks repleted with 500 IU of vitamin D_3 orally. The incubation conditions were described in Table I, except that incubations were performed with no $MgCl_2$ or $CaCl_2$ added and at the indicated concentrations of either or both. The P_i released as a result of added divalent cations above that in their absence is plotted *vs.* cation concentration. The data represent four similar experiments. The vertical bars indicate the standard errors and are used at the points which are common to all four experiments. (●—●) $CaCl_2$, (▲—▲) $MgCl_2$, and (■—■) 5 mM $MgCl_2$ + $CaCl_2$.

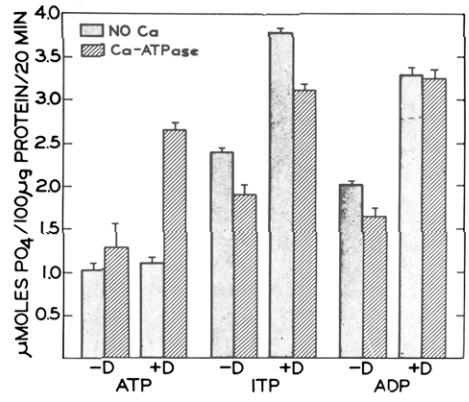

FIGURE 3: Nucleotide phosphatase activity of chick intestinal brush borders with various substrates. Brush borders (about 15 μg of protein/tube) were incubated for 20 min in a medium containing 40 mM Tris·Cl (pH 7.4), 5 mM $MgCl_2$, and 5 mM of nucleotide (pH 7.4 with NaOH) with or without 40 mM $CaCl_2$. Brush borders isolated from chicks dosed 23 hr earlier with 0.20 ml of oil or oil containing 400 IU of vitamin D_3 were used. Shaded bars represent phosphate released without calcium added to the incubation medium, while the hatched bars represent the additional phosphate released when 40 mM calcium chloride was present during the incubation. These data represent one of two experiments which utilized a total of five control and five vitamin D dosed chicks. Both experiments gave identical results. All values are the average of triplicate incubations. The short vertical lines indicate the standard error.

brush borders ($p < 0.05$). Addition of 10 mM calcium ions had little effect on ATPase of brush borders from vitamin D deficient chicks but it had a marked stimulatory effect on that from chicks which had received the vitamin D_3. Thus it appeared possible that vitamin D_3 might induce calcium-dependent ATPase which in turn might be related to the well-known action of the vitamin in increasing intestinal calcium transport.

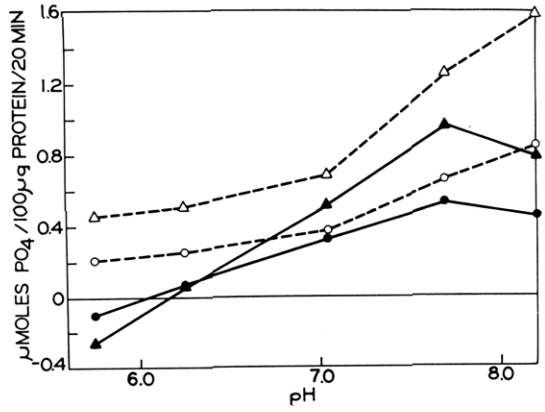

FIGURE 2: ATPase activity of chick intestinal brush borders at various pH values. Brush borders were prepared as described in the text from vitamin D deficient chicks and from chicks repleted with 400 IU of vitamin D_3 orally 23 hr earlier. These were incubated in a medium containing 5 mM $MgCl_2$, 5 mM Na·ATP (pH 7.4), and 40 mM Tris·Cl (pH 6.2, 6.8, 7.4, 8.0, or 8.6) with or without 40 mM $CaCl_2$ and phosphate release was determined. The measured pH values at the end of the incubations have been used in the figure. Each point represents the average of triplicate determinations on the pooled tissue from two animals. The data show one of two experiments which gave identical results. In all cases the standard errors are smaller than the symbols used in the graph. (○---○) −D preparation, no $CaCl_2$ added; (●—●) −D preparation, increase over $CaCl_2$-free level caused by addition of $CaCl_2$; (△---△) +D preparation, no $CaCl_2$ added and (▲—▲) +D preparation, increase over $CaCl_2$-free level caused by addition of $CaCl_2$.

The stimulatory effect of calcium on the brush border ATPase from vitamin D treated animals requires the presence of magnesium ions (Figure 1). On the other hand, magnesium by itself resulted in marked ATPase activity. However, magnesium by itself cannot achieve the stimulation of ATPase brought about by a combination of magnesium and calcium ions. When magnesium ions are added in excess of ATP, little change in ATPase is noted, for example, raising Mg^{2+} from 5 to 10 mM in the presence of 5 mM ATP increases ATPase by only 0.04 μmole of P_i/100 μg of protein per 20 min. On the other hand, when Mg^{2+} concentration is lowered below 5 mM in the presence of 5 mM ATP, the ATPase is markedly reduced. Thus it is likely that Mg·ATP is the actual substrate for the ATPase. In the standard incubation medium for determining calcium-dependent ATPase, a concentration of 5 mM Mg^{2+} and 10 mM Ca^{2+} is used. This mixture results in 2.24 mM Mg·ATP complex. Thus to determine the actual calcium-dependent ATPase, the ATPase in the presence of 2.3–2.5 mM Mg^{2+} is determined in one tube and the ATPase in the presence of 5 mM Mg^{2+} and 10 mM Ca^{2+} in another tube. The actual calcium-dependent ATPase is then calculated by subtracting the P_i released in the 2.3–2.5 mM Mg^{2+} tube from that released in the 10 mM Ca^{2+} and 5 mM Mg^{2+} tube.

The relationship of ATPase of brush borders to pH is illustrated in Figure 2. The release of P_i from the appropriate reaction mixture is plotted *vs. final* pH of the incubation mixture. Clearly calcium-dependent hydrolysis of ATP was optimal at pH 7.7 while ATP hydrolysis in the absence of calcium was highest at the highest pH studied.

TABLE II: Influence of Sodium and Potassium on the ATPase Activity of Intestinal Brush Borders from Vitamin D Deficient and Repleted Chicks.[a]

Expt		−D		+D	
		−Ca	Ca·ATPase	−Ca	Ca·ATPase
1					
	Na·ATP	0.51	1.04	1.17	1.53
	K·ATP	0.71	1.51	1.41	2.68
2					
	Na·ATP	1.05	1.13	1.13	2.40
	K·ATP	1.63	2.31	1.80	4.00
	Tris	0.42	0.46	0.84	1.25
3					
	Tris + Na	0.64	0.18	1.05	0.97
	Tris + K	0.52	0.40	0.97	1.67
	Tris	0.75	0.50	1.02	0.72
4					
	Tris + Na	0.87	0.30	1.26	0.48
	Tris + K	0.78	0.62	1.06	0.97

[a] Repleted chicks received 400 IU of vitamin D_3 orally 24 hr before sacrifice. Intestinal brush borders were prepared as described in the text. In expt 1 and 2 the incubation medium contained 40 mM Tris·Cl (pH 7.4), 5 mM $MgCl_2$, 5 mM Na·ATP or K·ATP (pH 7.4), with or without 40 mM $CaCl_2$. In expt 3 and 4 the incubation medium contained 40 mM Tris·Cl (pH 7.4), 10 mM $MgCl_2$–Tris·ATP (pH 7.4), or 20 mM KCl or NaCl in addition to Tris (pH 7.4), with or without 20 mM $CaCl_2$. All values are the average of triplicate determinations on the pooled preparation from two or more chicks. Data are expressed as μmoles of Pi/100 μg of protein per 20 min.

From Figure 3 it is evident that brush borders can hydrolyze ITP and ADP more rapidly than ATP. However, the calcium-dependent release of P_i is evident most clearly when ATP is the substrate.

The specificity of the ATPase was examined because of the well-known fact that vitamin D has smaller but significant effects on Sr^{2+} and Mg^{2+} but not on Mn^{2+} absorption. These experiments were carried out with a medium of 40 mM Tris-Cl (pH 7.4), 5 mM Na·ATP, and 5 mM Mg^{2+}; 10 mM of either Ca^{2+}, Mg^{2+}, Sr^{2+}, Ba^{2+}, or Mn^{2+} was used to study their effect on ATPase. Figure 4 reveals that Mg^{2+} and Sr^{2+} exhibit 30% and Ba^{2+} 15% of the effect of Ca^{2+} on ATP hydrolysis while Mn^{2+} actually inhibits ATPase. Clearly a high degree of specificity for calcium is evident and the relative specificities correlate with the well-known effects of vitamin D on absorption of these ions (Worker and Migicovsky, 1961; Wasserman, 1963).

Both potassium and sodium are known to affect calcium transport (Martin and DeLuca, 1969a; Schachter, 1969a; Harrison and Harrison, 1963). Thus it is of considerable interest to determine what effect the presence of these ions has on ATP hydrolysis by brush borders. The results show that both calcium and noncalcium ATPase is increased when the K^+ salt of ATP is used as substrate (Table II). ATP was converted into the Tris salt and then used alone or with 20 mM K^+ or 20 mM Na^+ as substrate in the ATPase determination. The results in the lower portion of Table II show clearly the K^+ increases the Ca^{2+}·ATPase especially in brush borders from chicks given vitamin D.

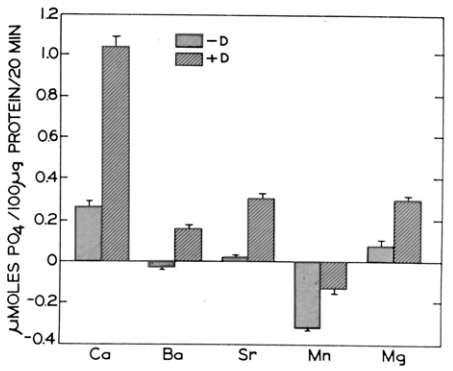

FIGURE 4: The effect of a variety of divalent cations on ATPase activity of intestinal brush borders from vitamin D deficient and repleted chicks. Brush borders were prepared as described in the text and incubated in a medium containing 40 mM Tris·Cl (pH 7.4), 5 mM $MgCl_2$, and 5 mM Na·ATP (pH 7.4), with or without the indicated ions added as the chlorides at a concentration of 10 mM. Without the addition of these ions the phosphate release was 0.69 ± 0.00 μmole by the preparations from vitamin D deficient chicks and 0.79 ± 0.00 from chicks given 400 IU of vitamin D_3 orally 24 hr earlier. The data given in the graph are the changes from these values caused by the addition of the indicated ions. These data represent one of two experiments which utilized a total of five control and five vitamin D dosed chicks. Both experiments gave identical results. All values are the average of triplicate incubations. The vertical lines indicate the standard errors.

Table III demonstrates that inhibitors of either Na^+/K^+·ATPase or oxidative phosphorylation have little or no effect on the ATPase of brush borders both in the presence and absence of calcium.

Because the intestinal brush border calcium·ATPase is obviously increased following vitamin D repletion, it was of great interest to determine if this enzyme activity is involved

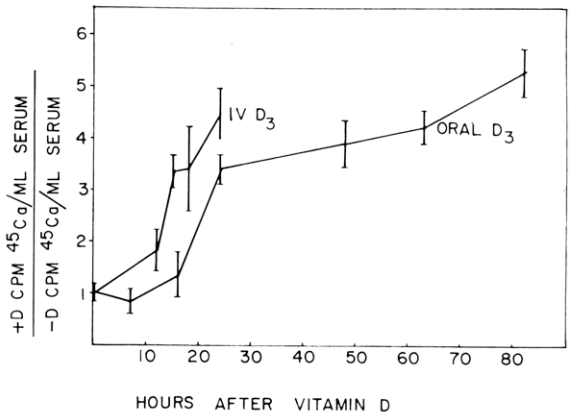

FIGURE 5: Time course of response of duodenal calcium absorption of the vitamin D deficient chick to oral and intravenous vitamin D_3. Calcium absorption was measured by the isolated loop technique as described in the text at the indicated times following 500 IU of vitamin D_3 orally (Harmeyer and DeLuca, 1969) or 100 IU of vitamin D_3 intravenously.

TABLE III: Influence of a Variety of Inhibitors on Brush Border ATPase.[a]

	Per Cent of Control			
	−D		+D	
	No Ca^{2+}	Ca·ATPase	No Ca^{2+}	Ca·ATPase
Ouabain (1 mM)	95	88	86	88
2,4-Dinitrophenol (1 mM)	102	92	94	78
Oligomycin (1 μg/ml)	99	94	87	98
Mersalyl acid (1 mM)	88	106	86	100
4-Mercuribenzoate (1 mM)	118	83	105	121

[a] Chick intestinal brush borders were prepared as described in the text. Vitamin D repleted animals received 400 IU of vitamin D_3 orally 23 hr before sacrifice. Approximately 100 μg of brush border protein was incubated in a medium containing 40 mM Tris·Cl (pH 7.4), 5 mM K·ATP (pH 7.4), 10 mM $MgCl_2$, with or without 20 mM $CaCl_2$, and inhibitors as indicated. Values represent the average of six determinations.

TABLE IV: ATPase Activity of Intestinal Brush Borders from Vitamin D Deficient Chicks and Chicks Repleted with 400 IU of Vitamin D_3 Orally 24 hr Earlier.[a]

	No. of Chicks		μmoles of P_i/100 μg of Protein per 20 min			
			No Ca^{2+}		Ca·ATPase	
Expt	−D	+D	−D	+D	−D	+D
1[b]	2	2	0.60	0.70	0.30	0.62
2[b]	2	2	0.48	0.74	0.14	0.50
3[c]	2	2	0.36	0.70	0.34	1.19
4[c]	2	2	0.43	0.57	0.10	0.56
5[d]	2	2	0.33	0.69	0.38	0.60
6[d]	2	2	0.37	0.75	0.33	0.55
7[d]	2	2	0.34	0.38	0.74	1.42
8[d]	2	2	0.68	1.41	1.55	2.70
9[d]	2	2	1.38	1.80	2.54	4.00
10[d]	2	2	0.17	0.86	0.04	1.84
11[e]	2	2	0.52	0.97	0.40	1.67
12[e]	2	2	1.41	1.37	0.11	0.73
13[e]	2	2	0.82	1.07	0.66	1.15
14[e]	2	2	0.60	0.90	0.34	0.66
15[e]	2	2	0.60	0.74	0.18	0.46

[a] Chick intestinal brush borders were prepared as described in the text and incubated in a medium containing 40 mM Tris-Cl (pH 7.4), 5 mM ATP (pH 7.4), and $MgCl_2 \pm CaCl_2$. Na·ATP was utilized in expt 3–6 while K·ATP was used in the others. Each value is the average of duplicate or triplicate determinations on the pooled preparations from the numbers of chicks indicated. Following vitamin D administration noncalcium ATPase was increased by an average of 0.30 μmole of P_i/100 μg of protein per 20 min ($p < 0.01$) and Ca·ATPase was increased by an average of 0.80 μmole of P_i/100 μg of protein per 20 min ($p < 0.001$). [b] 5 mM $MgCl_2 \pm$ 10 mM $CaCl_2$. [c] 5 mM $MgCl_2 \pm$ 20 mM $CaCl_2$. [d] 5 mM $MgCl_2 \pm$ 40 mM $CaCl_2$. [e] 10 mM $MgCl_2 \pm$ 20 mM $CaCl_2$.

with vitamin D induced calcium transport. If such is indeed the case, this increase in Ca·ATPase must respond at least as rapidly to vitamin D as does calcium transport itself. The data in Figure 5 show the response of calcium transport by isolated duodenal loops to orally administered vitamin D_3 and to intravenous vitamin D_3 as determined in the current study.

The considerable response of brush border ATPase 72 hr following vitamin D repletion has been shown in Table I. Although the yield of mucosal scrapings was increased by about 50% 72 hr after vitamin D repletion, as compared with vitamin D deficient controls, this was not true at shorter response times. Twenty-three to twenty-four hours following vitamin D repletion (Table IV) noncalcium ATPase was increased by an average of 0.30 μmole of P_i/100 μg of protein per 20 min ($p < 0.01$) and Ca·ATPase was increased by an average of 0.80 μmole of P_i/100 μg of protein per 20 min ($p < 0.001$). The data presented in Table V show that this vitamin D effect on Ca·ATPase was not usually obtained at times shorter than 19.5 hr.

The data in Table VI show the time course of response of ATPase to intravenous vitamin D_3. At 18–18.5 hr following vitamin D, noncalcium ATPase was increased by an average of 0.07 μmole of P_i/100 μg of protein per 20 min ($p > 0.05$), while Ca·ATPase was increased by an average of 0.23 μmole of P_i/100 μg of protein per 20 min ($p < 0.001$). At 16–16.5 hr following vitamin D repletion noncalcium ATPase was increased by an average of 0.11 μmole of P_i/100 μg of protein per 20 min ($p > 0.1$), while Ca·ATPase was increased an average of 0.17 μmole of P_i/100 μg of protein per 20 min ($p < 0.05$). The vitamin D induced stimulation of brush border Ca·ATPase was not usually observed at times shorter than 16 hr.

Discussion

The mechanism of sodium transport out of cells has been widely investigated. Considerable effort has been devoted to the study of membrane-bound, sodium-stimulated ATPase activities which have been obtained from a large variety of cell types. Because in many cases ATP is required for sodium transport, it appears that such membrane-bound sodium ATPases may couple the energy released during ATP hydrolysis to sodium transport and in that regard supply a convenient means of measurement.

ATP can also be utilized for the movement and accumulation of the divalent cations Mg^{2+} and Ca^{2+} (Davis, 1969). It would seem reasonable therefore that a membrane-bound, calcium-stimulated ATPase might be involved with calcium transport. The existence of such an enzyme, with considerable

TABLE V: ATPase Activity of Intestinal Brush Borders from Vitamin D Deficient Chicks and Chicks Repleted with 400 IU of Vitamin D_3 Orally.[a]

		μmoles of P_i/100 μg of Protein per 20 min					
		No. of Chicks		No Ca^{2+}		Ca·ATPase	
Time (hr)	Expt	−D	+D	−D	+D	−D	+D
19.5	2	2	2	0.19	0.26	0.10	0.21
18	3	2	2	0.26	0.64	0.39	0.68
	4	2	2	0.40	0.52	0.69	0.70
16.5	5[b]	2	2	0.17	0.22	0.11	0.23
15	3		2	0.26	0.45	0.39	0.32
	4		2	0.40	0.60	0.69	0.73
12	3		2	0.26	1.13	0.39	0.13
	4		2	0.40	0.43	0.69	0.48
	5A[b]		2	0.19	0.09	0.04	0.00

[a] Chick intestinal brush borders were prepared as described in the text and incubated in a medium containing 40 mM Tris-Cl and 5 mM ATP, both pH 7.4 and 5 mM $MgCl_2$ ± 20 mM, $CaCl_2$. The time of incubation was 20 min. All values represent multiple determinations on the pooled preparations from the indicated numbers of chicks. Vitamin D was administered at the indicated time before sacrifice. [b] 5 mM Mg ± 40 mM Ca.

TABLE VI: ATPase Activity of Intestinal Brush Borders from Vitamin D Deficient Chicks and Chicks Repleted with 100 IU of Vitamin D_3 Intravenously.[a]

		μmoles of P_i/100 μg of Protein per 20 min					
		No. of Chicks		No Ca^{2+}		Ca·ATPase	
Time (hr)	Expt	−D	+D	−D	+D	−D	+D
	1[b]	3	3	0.36	0.42	0.16	0.26
18–18.5	2[b]	3	3	0.53	0.60	0.34	0.70
	3[b]	2	1	0.40	0.44	0.01	0.25
	4[c]	3	3	0.78	0.94	0.06	0.32
	5[c]	4	4	0.20	0.17	0.04	0.24
	6[c]	4	3	0.35	0.33	0.38	0.48
16–16.5	7[b]	2	1	0.36	0.63	0.30	0.56
	8[d]	2	2	0.38	0.36	0.25	0.36
	9[d]	3	3	0.28	0.36	0.22	0.36
	10[e]	2	2	0.38	0.50	0.08	0.24
15	6		3	0.35	0.32	0.38	0.38
14	8		2	0.38	0.40	0.25	0.38
	6		3	0.35	0.26	0.38	0.40
12	8		2	0.38	0.36	0.25	0.21
	9		3	0.28	0.36	0.22	0.20
	10		3	0.38	0.48	0.08	0.12

[a] Chick intestinal brush borders were prepared as described in the text and incubated in a medium containing 40 mM Tris-Cl (pH 7.4), 5 mM ATP, and $MgCl_2$ ± $CaCl_2$. Each value represents the average of triplicate or quadruplicate determinations on the pooled samples from the number of chicks indicated. [b] 2.33 mM $MgCl_2$ or 5 mM $MgCl_2$ + 10 mM $CaCl_2$. [c] 5 mM $MgCl_2$ ± 10 mM $CaCl_2$. [d] 2.5 mM $MgCl_2$ or 5 mM $MgCl_2$ + 10 mM $CaCl_2$. [e] 5 mM $MgCl_2$ ± 20 mM $CaCl_2$.

specificity for calcium, has been clearly demonstrated in this and a previous report (Martin *et al.*, 1969). The fact that vitamin D, which has as its major physiological effect the movement of calcium, gives rise to increased amounts of calcium-dependent ATPase in both chicken and rat, lends considerable weight to such an assumption. The similarity between the time courses of appearance of calcium transport and increased Ca·ATPase activity following vitamin D

repletion of chicks indicates that these two responses may be intimately related. The observations of Martin and DeLuca (1969b) and Schachter and Rosen (1959) which implicate an energy-requiring movement of calcium across the intestinal brush border in the vitamin D induced intestinal transport of calcium also lend support to this concept. Furthermore the K_m for Ca^{2+} in both systems is about 2 mM. In addition, the lack of sodium dependence of this Ca·ATPase corresponds to the lack of a sodium requirement for movement of calcium across the mucosal surface (Martin and DeLuca, 1969a).

Another factor which may be of importance in vitamin D induced calcium transport is the calcium binding protein (CaBP) which has been discovered and isolated by Wasserman and coworkers (Wasserman and Taylor, 1963; Wasserman *et al.*, 1968). This protein appears in chick intestinal mucosa homogenates following vitamin D repletion. It has also been found in the intestinal homogenates of rats (Kallfelz *et al.*, 1967; Schachter *et al.*, 1967), dogs (Taylor *et al.*, 1968), and monkeys (Wassermen *et al.*, 1968), in chick kidney (Taylor and Wasserman, 1967), and the uterus of the laying hen (Corradino *et al.*, 1968).

Studies in this laboratory (Harmeyer and DeLuca, 1969), however, have shown a lag in appearance of CaBP following vitamin D repletion in chicks as compared with calcium transport. This lag was even more pronounced when a low calcium diet was utilized. With this diet the time of appearance of calcium transport was reduced by about 10 hr whereas the time of appearance of CaBP was not affected.

Ebel *et al.* (1969) have reported finding small amounts of CaBP by immunological techniques at shorter times following vitamin D repletion when it is not yet possible to detect increased calcium binding activity by resin methods. The fact that CaBP is present in such small amounts at a time when substantial vitamin D induced transport is occurring suggest that if CaBP is an essential part of a calcium transport system, it must perform in some special way, for example, as a component of intestinal brush border Ca·ATPase.

Clearly much remains to be established before the role of either the CaBP or the calcium ATPase in intestinal calcium transport can be delineated. It is hoped that the ATPase may yield another perspective from which the calcium transport problem of intestine can be approached.

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