Vitamin D₃ and Calcium Absorption in the Chick

By E. S. HOLDSWORTH

Department of Biochemistry, University of Adelaide, South Australia

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1. An attempt has been made to locate the site of action of vitamin D₃ as it affects the translocation of calcium across the intestine. 2. Calcium appears to be pumped out of cells by a process dependent on energy from metabolism. 3. The effects of cold, inhibitors and vitamin D₃ on the translocation of calcium by everted sacs of intestine were studied and compared with results obtained *in vivo*. 4. A model was proposed to explain the results which suggests that vitamin D₃ inhibits a metabolically operated pump that returns calcium from the mucosal cell to the lumen. 5. Some observations on the effect of sodium lauryl sulphate on the translocation of calcium *in vivo* and *in vitro* are reported.

It has been firmly established that vitamin D increases the transport of calcium from the small intestine of many species (Harris & Innes, 1931; Nicolaysen, 1937; Greenberg, 1945; Albright & Reifenshtein, 1948; Harrison & Harrison, 1951; Migicovsky, 1957; Wasserman, Comar, Schooley & Lengeman, 1957; Schachter & Rosen, 1959; Coates & Holdsworth, 1961; Sallis & Holdsworth, 1962). From experiments with rat intestine *in vitro* Harrison & Harrison (1960) suggested that vitamin D increases the rate of diffusion of calcium through the intestinal mucosa, possibly by the alteration of the permeability of intestinal cells to calcium. A similar conclusion was arrived at by Wasserman & Kallfelz (1962), who also demonstrated that the usual criteria of active transport as developed by Ussing (1949) were unable to account for the facts of calcium transport. A model system for transcellular transport that has been developed by Parsons (1963) appears to be of general applicability, and the present paper uses this model in an attempt to explain the mode of action of vitamin D₃.

During an investigation into the role of bile and detergents on calcium transport, it was noticed that sodium lauryl sulphate improves calcium uptake in rachitic chicks (Webling & Holdsworth, 1965). The effect of this substance on calcium transport has been studied since it may provide a clue as to how vitamin D₃ achieves its effect.

EXPERIMENTAL

Birds and diet. White Leghorn cockerels were raised on a diet deficient only in vitamin D for a period of 4 weeks, as described by Sallis & Holdsworth (1962). To study the effect of vitamin D, the rachitic chicks were dosed with 200 i.u. of vitamin D₃ in arachis oil by stomach tube 16 hr. before the experiments.

Perfusions *in vivo*. Through the distal third segment of the small intestine of the chick was circulated 10 ml. of 0.15 M-NaCl containing glucose (0.02 M) and CaCl₂ (either 1 mM or 10 mM; 10 μ C of ⁴⁵Ca) by a peristaltic pump (Sallis & Holdsworth, 1962). Sodium lauryl sulphate, when added to the perfusion fluid, was used at a concentration of 8 mM. Samples of blood and circulating fluid were taken at intervals. At the end of the experiment the segment of gut was placed in ice-cold 0.15 M-NaCl and then washed through with a stream of ice-cold 0.15 M-NaCl. The segment was then cut open and pressed between soft tissue, and the mucosal cells and muscle were separated. The absorption of calcium was determined by measuring the ⁴⁵Ca in the carcass.

Transport of calcium *in vitro*. Segments of the distal third of the small intestine of the chick were everted and incubated as described by Sallis & Holdsworth (1962). For measurement of transport at 0°, the flasks containing the everted sacs were surrounded by ice. In some experiments the gut was not everted and on these occasions the serosal (outside) surface of the sac was immersed in the 5 ml. of Krebs-Henseleit bicarbonate buffer (Krebs & Henseleit, 1932) containing glucose (0.02 M) and ⁴⁵CaCl₂ (2 mM), and the sac (mucosal surface) contained 1 ml. of the same buffer containing non-radioactive CaCl₂ (2.25 mM). The flasks containing the sacs were shaken and gassed with O₂ + CO₂ (95:5).

At the end of the incubation period, the outside of the gut was washed with a stream of ice-cold 0.15 M-NaCl and dried with tissue. The inside fluid was removed, the sac was rinsed with 1 ml. of 0.15 M-NaCl and the combined liquids were examined for ⁴⁵Ca content. The segment was either taken as such or split longitudinally, the mucosal cells were scraped off and the ⁴⁵Ca contents of the cells and muscle were measured.

To preload the everted sacs with ⁴⁵Ca they were incubated in the usual way either at 0° or at 38° for 30 min. The mucosal surface was washed with ice-cold 0.15 M-NaCl, and the serosal fluid was removed, rinsed out with 1 ml. of 0.15 M-NaCl and replaced with 1 ml. of Krebs-Henseleit buffer containing glucose (0.02 M) and non-radioactive CaCl₂ (2 mM). The sac was placed in 5 ml. of this same buffer

and reincubated at 0° or at 38° for 15 min. The serosal fluid, mucosal cells and muscle were treated as described above.

In all these experiments *in vitro* a reproducible length of gut was obtained (10–12 cm. long) by using chicks at the same stage of development and taking a segment of intestine from the point of attachment of the yolk-sac down to the caecum. The dry weight of cells and muscle from such segments differed little (see Table 3), and it was found that it made no difference to the results to correct for this slight variation in weight. For this reason all results are expressed as for a segment of intestine.

Incorporation into mucosal cells. The mucosal cells of the lower third of the small intestine were scraped off with the round end of a spatula and placed in ice-cold Krebs–Henseleit bicarbonate buffer containing glucose (0.02 M). The cells were washed once with the buffer and resuspended in the same medium to give a concentration of 50 mg. of protein/ml. To 2.5 ml. (in duplicate) of such a suspension was added $^{45}\text{CaCl}_2$ (1 mM; 0.2 μC) and the mixture incubated at 38° or kept on ice for 15 min. The cells were removed by a short high-speed centrifugation for approx. 2 min. at 30000g and the ^{45}Ca in the supernatant was determined. After being washed twice with ice-cold buffer, cells that had taken up ^{45}Ca at 0° were incubated in Krebs–Henseleit bicarbonate buffer containing glucose (0.02 M) but no calcium. The ^{45}Ca in the supernatant and cells was determined.

Incorporation into mitochondria. Mucosal cells prepared as above were homogenized in 0.25 M-sucrose with a Potter–Elvehjem homogenizer and the fraction sedimenting between 1000 and 10000g was obtained. The pellet was resuspended in 0.25 M-sucrose containing 10 μmoles of tris and 10 μmoles of succinate/ml. (final pH 7.2). The concentration of protein was adjusted to 2.5 mg./ml. and that of $^{45}\text{CaCl}_2$ to 1 mM (0.2 μC). After incubation for 15 min. at 38° in $\text{O}_2 + \text{CO}_2$ (95:5), the mitochondria were centrifuged down and washed once with ice-cold 0.25 M-sucrose and the ^{45}Ca was determined.

Preparation of cell fractions. Mucosal cells were scraped off the segments of intestine with the round end of a spatula. In most experiments ^{45}Ca was determined in the mucosal

cells and the residual muscle. In some experiments the mucosal cells were homogenized with 0.25 M-sucrose and fractions prepared by centrifugation at 600g for 10 min., 2000g for 10 min., 20000g for 20 min. and 100000g for 60 min. The pellet sedimenting between 600g and 2000g was resuspended in 20% (w/v) sucrose and centrifuged at 1000g for 10 min. and then at 3000g for 10 min. The ^{45}Ca was determined in all these fractions.

Determination of ^{45}Ca . (a) Blood. A 0.2 ml. sample of plasma was treated with 0.2 ml. of 10% (v/v) HClO_4 and centrifuged, and 0.2 ml. of the supernatant was added to 5 ml. of scintillator fluid (Bray, 1960).

(b) Serosal fluid. A 0.1 ml. sample was added directly to the scintillator fluid.

(c) Intestinal samples. Organic matter from intestinal segments, mucosal cells, muscle and mitochondria was destroyed by wet oxidation with conc. HNO_3 and HClO_4 . After evaporation to dryness, the residue was dissolved in water and a portion added to the scintillator fluid.

(d) Carcasses. To determine the ^{45}Ca taken up during perfusion, the carcass, after removal of the perfused loop, was ashed in a silica dish, converted into chlorides by evaporation with conc. HCl and dissolved in 100 ml. of water. The radioactivity in 0.5 ml. portions was determined by scintillation counting.

(e) Scintillation counting. All samples were counted in the scintillation fluid developed by Bray (1960) by using an Ekco N664A counter. An internal standard of ^{45}Ca was added after counting the sample and suitable corrections were made for quenching.

RESULTS

Perfusions in vivo. It was found that the only reliable method of measuring the amount of calcium absorbed from the perfusion fluid was to determine ^{45}Ca in the carcass. Estimation of calcium uptake by estimating ^{45}Ca in the circulating fluid presupposes an accurate knowledge of the volume change of this fluid, which could not be determined with accuracy. The results obtained at two concentrations of

Table 1. *Absorption of calcium by small intestine during perfusion in vivo*

Distal segments of small intestine from rachitic or vitamin D_3 -treated chicks were perfused with 10 ml. of 0.15 M-NaCl containing CaCl_2 (1.0 or 10 mM; 10 μC of ^{45}Ca) and glucose (0.02 M). Perfusion was for 30 min. at 38° and blood samples were taken at 10 and 20 min. The results are mean values $\pm$ s.e.m. for six chicks.

Concn. of CaCl_2 (mM)	Treatment	^{45}Ca in carcass ($\mu\text{g./30 min.}$)	^{45}Ca in mucosa ($\mu\text{g./30 min.}$)		^{45}Ca in plasma (counts/min./ml.)	
			Cells	Muscle	10 min.	20 min.
1.0	Rachitic	3.1 ± 1.7	2.8 ± 1.1	2.0 ± 0.8	8300*	7700
	Vitamin D_3	38 ± 9	3.5 ± 1.5	1.9 ± 0.6	38900	55900
10	Rachitic	22 ± 8	17 ± 3.9	11 ± 2.5	4100	5200
	Vitamin D_3	190 ± 43	21 ± 5.1	15 ± 4.1	16900	18700
	Rachitic + sodium lauryl sulphate (8 mM)	290 ± 67	48 ± 92	16 ± 4.5	29000	26300

* The ^{45}Ca absorbed at mM- CaCl_2 had 10 times the specific activity of that at 10 mM- CaCl_2 .

Ca²⁺ are shown in Table 1. Vitamin D₃ greatly increased the translocation of calcium into the chick but there was no significant increase in the amount of calcium bound by the mucosal cells under the steady-state condition reached after 30 min. perfusion. Sodium lauryl sulphate also increased the translocation of calcium in the rachitic chick and in this instance there was a marked increase in the calcium taken up by the mucosal cells (Table 1).

Studies in vitro. (a) Everted sacs were kept at 0° and the amount of ⁴⁵Ca transferred to the serosal fluid and taken up by the mucosa was measured. Vitamin D₃ had no effect on these processes (Table 2). When the everted sacs were preloaded with ⁴⁵Ca in this way and then rinsed and placed in medium containing non-radioactive Ca²⁺, the exchange or transport of ⁴⁵Ca could be measured in the mucosal and serosal fluids. Table 2 shows that, for mucosa preloaded at 0° and reincubated at 0°, the process of exchange with the mucosal fluid or transport into the serosal fluid was the same for gut from both rachitic or vitamin D₃-treated chicks. Table 2 also shows that cells preloaded at 0° and reincubated at 38° transported or exchanged more ⁴⁵Ca at the higher temperature, but again there is no observable effect of vitamin D₃.

(b) Everted sacs incubated at 38° show that vitamin D₃ increases the amount of ⁴⁵Ca transferred to the serosal fluid and also increases the ⁴⁵Ca found in the mucosal cells and muscle. These results (Table 3) are in agreement with our previous findings and those of other workers (Sallis & Holdsworth, 1962; Harrison & Harrison, 1960; Schachter & Rosen, 1959). When everted sacs were preloaded with ⁴⁵Ca at 38° and then reincubated in medium containing non-radioactive Ca²⁺ at 0°, the ⁴⁵Ca was transferred to the serosal and mucosal fluids in amounts proportional to the initial ⁴⁵Ca content of the sac. This last value was obtained by summing the ⁴⁵Ca contents of mucosal and serosal fluids and mucosal cells and muscle. Thus the ratio of ⁴⁵Ca obtained by dividing the uptake of the sacs from vitamin D₃-treated chicks by the uptake of the sacs from rachitic chicks was 3.1, and the ratio of transfer to serosal fluid by sacs from vitamin D₃-treated and rachitic chicks was 5.5 during the loading period and 3.9 on reincubation. Thus the vitamin D₃ effect was obtained during reincubation at 0° but appears to be related to the initial uptake of ⁴⁵Ca under the influence of vitamin D₃ at 38° (Table 4).

Everted sacs preloaded at 38° and reincubated at 38° showed greater exchange and transfer to serosal fluid than those reincubated at 0°, but again the greater transfer to serosal fluid observed with sacs from vitamin D₃-treated chicks appears to be due to the greater uptake during the preloading

Table 2. *Uptake and transport of calcium at 0° by everted sacs of small intestine and exchange of ⁴⁵Ca at 0° and 38°*

Everted sacs of small intestine from rachitic or vitamin D₃-treated chicks were incubated for 30 min. at 0° in Krebs-Henseleit bicarbonate medium containing ⁴⁵CaCl₂ (2 mM) and glucose (0.02 M). After being rinsed, the sacs were reincubated for 15 min. at 0° or 38° in the same medium but containing non-radioactive CaCl₂. The results are mean values ± S.E.M. for ten sacs (first incubation) or five sacs (for each temperature during second incubation).

	First incubation (30 min. at 0°)				Second incubation (15 min. at 0°)				Second incubation (15 min. at 38°)			
	⁴⁵ Ca accumulated (μg.)		⁴⁵ Ca efflux (μg.)		Percentage exchange of mucosal cellular uptake		⁴⁵ Ca efflux (μg.)		Percentage exchange of mucosal cellular uptake		⁴⁵ Ca efflux (μg.)	
	In mucosal cells*	In serosal fluid	In mucosal medium	In serosal fluid	Mucosal medium	Serosal fluid	In mucosal medium	In serosal fluid	Mucosal medium	Serosal fluid	In mucosal medium	In serosal fluid
Rachitic	4.58 ± 1.46†	0.468 ± 0.152†	1.17 ± 0.53	0.348 ± 0.084	25.6	7.6	1.87 ± 0.51	0.632 ± 0.190	40.9	13.8	2.16 ± 0.67	0.756 ± 0.213
Vitamin D ₃ -treated	5.36 ± 1.30†	0.540 ± 0.258†	1.30 ± 0.47	0.396 ± 0.106	24.3	7.4			40.2	14.1		

* Calculated from the sum of the final ⁴⁵Ca in the gut plus that in the mucosal and serosal fluids after the second incubation.

† No difference between rachitic and vitamin D₃-treated groups at *P* = 0.1 level.

Table 3. *Uptake and transport of calcium at 38° by everted sacs of small intestine*

Everted sacs of small intestine from rachitic or vitamin D₃-treated chicks were incubated for 30 min. at 38° in Krebs-Henseleit bicarbonate medium containing ⁴⁵CaCl₂ (2mM) and glucose (0.02M). The ⁴⁵Ca uptake by the cells and muscle of the mucosa and of the serosal fluid was determined. Accumulated results (means ± S.E.M.) of several experiments (32 sacs) are given. Only six sacs were used in the experiments with added sodium lauryl sulphate.

	Mucosal cells		Muscle layer		Serosal fluid
	Dry wt. (mg./mg.)	⁴⁵ Ca accumulated (μg./30 min.)	Dry wt. (mg./mg.)	⁴⁵ Ca accumulated (μg./30 min.)	⁴⁵ Ca accumulated (μg./30 min.)
Rachitic	113 ± 13	2.5 ± 0.95	253 ± 38	3.1 ± 1.1	1.9 ± 0.59
Vitamin D ₃ -treated	120 ± 17	6.9 ± 1.7	241 ± 43	11.4 ± 2.1	8.2 ± 2.1
Rachitic + sodium lauryl sulphate (8mM)	—	10.4 ± 1.9	—	5.4 ± 1.8	2.2 ± 0.40
Vitamin D ₃ -treated + sodium lauryl sulphate (8mM)	—	18.4 ± 2.9	—	11.5 ± 2.0	3.9 ± 0.58

period by the sacs from vitamin D₃-treated chicks. This can be seen from the value for the transfer of ⁴⁵Ca as a percentage of the ⁴⁵Ca content after the preloading period and in Table 4 referred to as transfer as a percentage of total uptake.

(c) The transport of ⁴⁵Ca and the uptake of ⁴⁵Ca by everted sacs was studied in the presence and absence of 2mM-iodoacetate (Table 5). Iodoacetate had only a slight effect on the intracellular ⁴⁵Ca of the mucosal cells of sacs from vitamin D₃-treated chicks but it did prevent the further translocation of this ⁴⁵Ca to the serosal fluid. With everted sacs from rachitic chicks, iodoacetate caused an accumulation of ⁴⁵Ca in the mucosal cells, and again further transport of the ⁴⁵Ca was blocked. The amount of ⁴⁵Ca found in the muscle layer of sacs from rachitic chicks was increased by iodoacetate whereas iodoacetate caused a decrease in the amount of ⁴⁵Ca found in the muscle layer of the sacs from vitamin D₃-treated chicks.

(d) When sodium lauryl sulphate was added to the mucosal fluid there was a great increase in the amount of calcium taken up by the mucosal cells. Only a small proportion of this calcium was transported to the serosal fluid even when segments from vitamin D₃-treated chicks were studied (Table 3).

(e) The effect of increasing the concentration of mucosal and serosal Ca²⁺ on the translocation of ⁴⁵Ca was studied. With increasing Ca²⁺ concentration the ratio of transfer to serosal fluid by sacs from vitamin D₃-treated and rachitic chicks steadily decreased (Table 6).

(f) Translocation of ⁴⁵Ca in the opposite direction to that usually studied was investigated by having sacs of intestine treated in usual manner except they were not everted. Transfer from serosal to mucosal surface is shown in Table 7, together with results obtained with the same batch of chicks but

by using everted sacs. As expected, there is less translocation of ⁴⁵Ca from serosal to mucosal fluids than in the opposite sense. The most important observation is that transport with non-everted sacs was not affected by vitamin D₃ treatment.

Distribution of ⁴⁵Ca in the small intestine. Table 8 shows the ⁴⁵Ca content of broken mucosal cells and muscle after preloading the segment either *in vivo* or *in vitro*. The greatest concentration of ⁴⁵Ca in mucosal cells always occurred in a fraction that sedimented between 1000 and 3000g in 20% sucrose. The large increase of calcium taken up by mucosal cells in the presence of sodium lauryl sulphate is evident (Table 8), and again the fraction sedimenting between 1000 and 3000g contained most of the radioactivity. Attempts to identify this fraction have not been successful. Electron-microscope pictures (kindly produced by Dr G. Rogers, University of Adelaide) showed the presence of structures resembling swollen and distorted brush borders, but the preparation also contained mitochondria and cell-membrane fragments. When intestine was preloaded with ⁴⁵Ca, it was found that mucosal cells and muscle from everted sacs always contained more calcium than tissue loaded by circulating ⁴⁵Ca *in vivo* even though the same solution of ⁴⁵Ca was used for the two preparations.

Uptake of ⁴⁵Ca by mucosal cells. Cells scraped from the small intestine of rachitic or vitamin D₃-treated chicks were incubated with ⁴⁵Ca either at 0° or at 38°. The uptake of ⁴⁵Ca by the cells is shown in Table 9. Mucosal cells took up much more ⁴⁵Ca at 0° and there was little difference between rachitic and vitamin D₃-treated cells. When cells loaded at 0° were reincubated in calcium-free medium at 38° a considerable amount of ⁴⁵Ca was released. In a total of eight experiments where

Table 4. *Uptake and transport of calcium at 38° by everted sacs of small intestine and exchange of ⁴⁵Ca at 0° and 38°*

Everted sacs of small intestine from rachitic or vitamin D₃-treated chicks were incubated for 30 min. at 30° in Krebs-Henseleit bicarbonate medium containing ⁴⁵CaCl₂ (2mM) and glucose (0.02M). After being rinsed, the sacs were reincubated for 15 min. at 0° or 38° in the same medium but containing non-radioactive CaCl₂. The results are mean values ± s.e.m. for ten sacs (first incubation) or five sacs (for each temperature during second incubation).

	First incubation (15 min. at 38°)				Second incubation (15 min. at 0°)				Second incubation (15 min. at 38°)			
	⁴⁵ Ca accumulated (μg.)		45Ca efflux (μg.)		Percentage exchange of mucosal cellular uptake		45Ca efflux (μg.)		Percentage exchange of mucosal cellular uptake		45Ca efflux (μg.)	
	In mucosal cells	In serosal fluid	In mucosal medium	In serosal fluid	In mucosal medium	In serosal fluid	In mucosal medium	In serosal fluid	In mucosal medium	In serosal fluid	In mucosal medium	In serosal fluid
Rachitic	5.03 ± 1.71	1.70 ± 0.71	0.91 ± 0.27	0.62 ± 0.21	18	12	2.06 ± 0.54	1.03 ± 0.32	41	20	4.08 ± 0.91	25
Vitamin D ₃ -treated	15.5 ± 2.83	9.40 ± 2.34	2.47 ± 0.53	2.43 ± 0.64	16	16	6.35 ± 1.15		41			

mucosal cells were incubated at 38° with ⁴⁵Ca, six experiments showed slightly more ⁴⁵Ca accumulated in the cells from vitamin D₃-treated chicks than in those from rachitic chicks (ratio 1.3–1.5) and in two experiments there was no difference in uptake between the two kinds of cells.

Results obtained with mitochondria were very variable: out of 13 experiments, eight showed a greater uptake by those from vitamin D₃-treated chicks than by those from rachitic chicks (ratio 1.3–1.7) and five showed no difference. No differences were found in the uptake of ⁴⁵Ca by brush borders prepared by the method of Miller & Crane (1961) from rachitic or vitamin D₃-treated chicks.

DISCUSSION

To account for the experimental observations and to locate the site of action of vitamin D₃, various models for transcellular transport, based on the ideas put forward by Parsons (1963), were considered. Essentially a sheet of mucosal cells was considered to have two faces, one bordering the lumen of the intestine and the other, serosal surface, facing the muscle of the gut. Each face was assumed to possess two independent properties: (1) a substrate pump operated by energy obtained from metabolism; (2) a diffusion leak working in the opposite sense to the pump. When considering the active transport of sugars and amino acids, Parsons (1963) considered the pumps to be directed intracellularly; however, when considering a substrate such as Ca²⁺, which is usually present only in low concentrations inside cells, there is no reason why the pumps should not be considered as removing Ca²⁺ from the cell. Vitamin D₃ could be considered to enhance or inhibit either or both of the pumps, or either, or both, of the two leaks. Thus a large number of model systems were considered, but the experimental results obtained allow the elimination of many of these alternatives, and the action of vitamin D₃ can best be accounted for by considering it to inhibit the metabolically driven pump P₁ (Scheme 1). The evidence to support this hypothesis is as follows.

(i) If pump P₁ were inhibited (cf. Scheme 1) there would be a rise in intracellular calcium concentration and this would reach the serosal surface via pump P₂ and diffusion leak K₂. The overall result would be an increased translocation of calcium such as that found with everted sacs from vitamin D₃-treated chicks (Table 3).

(ii) If pump P₁ were enhanced by vitamin D₃ there would be a greater return of calcium to the lumen and hence a decreased translocation of calcium, which is contrary to the experimental findings.

(iii) If pump P₂ were inhibited there would be a

Table 5. *Effects of iodoacetate on calcium uptake and transport by everted sacs of small intestine*

Everted sacs of small intestine from rachitic or vitamin D₃-treated chicks were incubated for 30 min. at 38° in Krebs-Henseleit bicarbonate medium containing ⁴⁵CaCl₂ (2 mM) and glucose (0.02 M). Some flasks contained iodoacetate (2 mM). The results are mean values ± S.E.M. for eight sacs.

	⁴⁵ Ca translocated (μg./30 min.)			
	No inhibitor		2 mM-Iodoacetate present	
	Rachitic	Vitamin D ₃ -treated	Rachitic	Vitamin D ₃ -treated
Serosal fluid	2.46 ± 0.49	11.68 ± 1.97	2.06 ± 0.38	3.26 ± 0.54
Mucosal cells	2.62 ± 0.61	6.88 ± 1.02	5.58 ± 0.89	7.22 ± 1.27
Mucosal muscle	3.54 ± 0.72	11.74 ± 2.12	5.66 ± 0.94	6.36 ± 1.15

Table 6. *Influence of Ca²⁺ concentration on calcium transport by everted sacs of small intestine*

Everted sacs of small intestine from rachitic or vitamin D₃-treated chicks were incubated for 30 min. at 38° in 0.15 M-NaCl containing glucose (0.02 M) and the stated concentration of Ca²⁺ (with 4 μC of ⁴⁵Ca). The results are mean values ± S.E.M. for four sacs.

Concn. of CaCl ₂ (mM)	⁴⁵ Ca in serosal fluid (μg.)		Ca transported by sacs from vitamin D ₃ -treated chicks/Ca transported by sacs from rachitic chicks ratio (range)
	Rachitic	Vitamin D ₃ -treated	
100*	16.9 ± 2.4	25.4 ± 4.4	1.2-1.8
10	6.5 ± 1.3	15.7 ± 2.2	2.5-3.0
1.0	2.1 ± 0.29	7.9 ± 1.9	3.7-5.6
0.1	0.51 ± 0.27	4.2 ± 2.9	4.2-9.8

* At this concentration some of the mucosal cells were shed into the mucosal fluid.

Table 7. *Transport of calcium by non-everted sacs of small intestine*

Sacs of small intestine from rachitic or vitamin D₃-treated chicks were either everted or non-everted and incubated under the usual conditions (see Table 2). The mean (± S.E.M.) serosal transport of ⁴⁵Ca (of six sacs) was determined.

	Serosal transport of ⁴⁵ Ca (μg./30 min.)	
	Everted sacs	Non-everted sacs
Rachitic	2.15 ± 0.39	1.32 ± 0.61
Vitamin D ₃ -treated	8.20 ± 0.95	1.43 ± 0.49

rise in intracellular calcium concentration but there would be a decrease in the amount of calcium translocated. Since vitamin D₃ increases calcium transport it cannot inhibit pump P₂.

(iv) If pump P₂ were enhanced there would be a decrease in intracellular calcium concentration with an increase in calcium translocation. With everted sacs, vitamin D₃ always caused an increase in intracellular ⁴⁵Ca concentration (Table 3), and therefore it appears that vitamin D₃ does not enhance pump P₂. Further, when everted sacs were preloaded with ⁴⁵Ca at 0° and then incubated at 38° there should have been a noticeable increase in translocation of ⁴⁵Ca if vitamin D₃ was enhancing pump P₂. No such increase could be detected (Table 2).

(v) If vitamin D₃ increased the diffusion leak K₁ (or K₂ or both) this would lead to increased translocation of calcium. It would be expected that this increased permeability due to vitamin D₃ would also be evident at 0°. The experimental results show no difference in ⁴⁵Ca transport between everted sacs from rachitic and vitamin D₃-treated chicks at 0° (Tables 2 and 4). No effect of vitamin D₃ was found on the movement of ⁴⁵Ca from the serosal to the mucosal compartments (Table 7). This again suggests that the vitamin does not alter diffusion leaks K₂ and K₁, thus increasing the permeability of the cell to calcium.

(vi) If pumps P₁ and P₂ were both inhibited there would be an increase in intracellular calcium concentration but transport across the cell would be limited and dependent solely on diffusion via diffusion leaks K₁ and K₂.

Cooling to 0° should inhibit the metabolically driven pumps, and under these conditions everted sacs from rachitic and vitamin D₃-treated chicks were found to take up and transport the same amounts of ⁴⁵Ca (Table 2). It appears to be necessary to have energy produced from metabolism to be able to show an effect of vitamin D₃.

Isolated mucosal cells took up ⁴⁵Ca at 0° and again no effect of vitamin D₃ was apparent. When these cells that had taken up ⁴⁵Ca at 0° were reincubated at 38°, the ⁴⁵Ca was lost from the cells (Table 9). Further, isolated cells took up more

Table 8. *Distribution of calcium in the mucosa of the small intestine*

Three segments of small intestine from rachitic or vitamin D₃-treated chicks were loaded with ⁴⁵Ca either by perfusion for 30 min. (as in Table 1) or by incubation of the everted sacs (as in Table 2). The medium used was 0.15M-NaCl containing glucose (0.02M) and CaCl₂ (10mM; 10 μC of ⁴⁵C in 10ml.). The segments were rinsed with ice-cold 0.15M-NaCl and the cells and muscle separated. The cells were homogenized with 0.25M-sucrose and the homogenate was separated by differential centrifugation. Cells and muscle for three segments were combined (to produce sufficient material) but the results are expressed as for one segment.

Fraction	⁴⁵ Ca content (μg. accumulated/30 min.)				
	Perfused intestine			Everted sacs	
	Rachitic	Vitamin D ₃ -treated	Sodium lauryl sulphate	Rachitic	Vitamin D ₃ -treated
Debris (600g for 10 min.)	1.6	0.9	8.2	2.3	4.1
Centrifuged at 2000g for 10 min. (refractationed in 20% sucrose to give fraction 1000-3000g for 10 min.)	8.9	7.5	28.1	6.9	11.3
'Mitochondria' (20 000g for 20 min.)	2.0	2.0	2.7	2.0	3.3
'Microsomes' (104 000g for 60 min.)	0.8	0.8	1.4	0.8	0.7
Supernatant (104 000g for 60 min.)	0.9	0.6	1.0	0.8	0.9
Muscle	13.8	18.2	17.3	21.8	31.7
Total Ca	28.0	30.0	58.7	34.6	52.0

Table 9. *Uptake and release of calcium by isolated mucosal cells of the small intestine*

Mucosal cells were scraped from the distal third of the small intestine of rachitic or vitamin D₃-treated chicks. Suspensions of the cells in Krebs-Henseleit bicarbonate medium containing glucose (0.02M) contained 50mg. of protein/ml. The cells in 2.5ml. were made 10mM with respect to ⁴⁵CaCl₂ and incubated for 15 min. at 0° or 38°. Uptake of ⁴⁵Ca was obtained by counting the residual ⁴⁵Ca in the supernatant after centrifugation. Cells were washed twice with ice-cold buffer. The cells that had accumulated ⁴⁵Ca at 0° were then incubated in the above medium for 10 min. at 38°. Results are the means of duplicate determinations.

	⁴⁵ Ca content (μg./125 mg. of protein)			
	Rachitic		Vitamin D ₃ -treated	
	0°	38°	0°	38°
Uptake by cells	80.1	40.0	90.7	41.5
First washing	1.2	23.5	1.3	26.4
Second washing	0.1	10.0	0.3	9.4
Release on reincubation at 38°	54.6		61.0

Scheme 1. Translocation of calcium by the mucosal cells of the small intestine. K₁ and K₂ are diffusion leaks, and P₁ and P₂ are pumps operated by energy from metabolism. Ca_i, Intracellular calcium.

⁴⁵Ca at 0° than at 38° and this finding supports the idea that calcium is pumped out of the cells by metabolically activated pumps. Similar observations were made with rat mucosal cells by Rasmussen, Waldorf, Dziewiatkowski & De Luca (1963).

Sallis & Holdsworth (1962) showed that the energy for calcium transport can come from glycolysis and that iodoacetate would inhibit this process. It seems likely that iodoacetate would inhibit pumps P₁ and P₂. Iodoacetate caused the intracellular ⁴⁵Ca concentration of everted sacs from rachitic chicks to rise, but prevented the transport of this ⁴⁵Ca to the serosal fluid (Table 5). This was the finding predicted above. When sacs from vitamin D₃-treated chicks were studied (where pump P₁ was considered to be already inhibited by vitamin D₃) the further inhibition by iodoacetate did not lead to a significant increase in intracellular ⁴⁵Ca concentration but, by inhibiting pump P₂, did prevent this ⁴⁵Ca reaching the serosal fluid. This difference in response of mucosal cells from rachitic and vitamin D₃-treated chicks to iodoacetate is further evidence for locating the site of action of vitamin D₃ at pump P₁. If vitamin D₃ produced its effect by increasing diffusion leak

K_1 , then inhibition of pumps P_1 and P_2 by iodoacetate would be expected to lead to a rise in intracellular ^{45}Ca concentration in sacs from both rachitic and vitamin D_3 -treated chicks, but this was not observed (Table 5).

(vii) Increasing the concentration of calcium in the mucosal fluid would lead to a greater diffusion of calcium into the cell via diffusion leak K_1 and subsequently to the serosal fluid via diffusion leak K_2 and pump P_2 . At high concentration of calcium the capacity of pump P_1 might be overwhelmed and in these circumstances the difference between the amounts of calcium translocated by gut from rachitic and vitamin D_3 -treated chicks would be less marked. That this is so is shown in Table 6, and this is in agreement with similar findings by Schachter & Rosen (1959), who worked with rat intestine. There is no hint even at 0.1M that translocation of calcium is reaching saturation, and this is consistent with entry by diffusion, whereas diffusion facilitated by 'carrier' molecules would be expected to become saturated.

The above arguments show that the hypothesis that vitamin D_3 inhibits pump P_1 can account for the experimental observations reported in the present paper. Webbing & Holdsworth (1965) have shown that bile and sodium lauryl sulphate will increase calcium translocation in a manner that resembles that of vitamin D_3 . Observations in the present paper show that sodium lauryl sulphate differs in some respects from vitamin D_3 in its effect on calcium transport. Table 1 shows that this detergent increases ^{45}Ca transport *in vivo* but at the same time causes a greater accumulation of ^{45}Ca in the mucosal cells than does vitamin D_3 (Table 1). A more marked accumulation of ^{45}Ca in mucosal cells was seen when sodium lauryl sulphate was used with everted sacs (Table 3). It could be considered that sodium lauryl sulphate was acting as an inhibitor of pumps P_1 and P_2 in a similar manner to iodoacetate. However, the intracellular ^{45}Ca obtained with sodium lauryl sulphate was much greater than that obtained with iodoacetate. Also, it is difficult to see why sodium lauryl sulphate is so effective in the translocation of ^{45}Ca *in vivo* if it is inhibiting pump P_2 , although experiments with everted sacs did show that less ^{45}Ca appeared in the serosal fluid of sacs from vitamin D_3 -treated chicks when sodium lauryl sulphate was present (Table 3). An alternative suggestion (Webbing & Holdsworth, 1965) is that sodium lauryl sulphate (or bile) converts the calcium into a lipid-soluble complex that is more readily taken up by the lipophilic cell membranes. For significant translocation of calcium from lumen to serosal fluid to take place the calcium in the membranes must be released to the serosal fluid. With the small volume of fluid within everted sacs

conditions would be unfavourable for calcium translocation (Table 3), whereas *in vivo*, where plasma is continually flowing past the cells, there is a large volume into which the calcium can be distributed and this could account for the marked translocation of calcium brought about by sodium lauryl sulphate *in vivo* (Table 1).

A theory to explain the increased transport of calcium brought about by sugars such as lactose or sucrose has been put forward by Wasserman (1964). He has correlated the effect of these substances on transport with their degree of inhibition of oxidative phosphorylation. No detailed model for transport across the cell was proposed, but the suggestion that lactose is inhibiting the energy supply is compatible with the model proposed in the present paper.

The model in Scheme 1 shows that the diffusion leak and the metabolically operated pump are located at different sites on the cell membrane. However, a leak and a pump can be visualized as occurring at the same site, i.e. diffusion could take place via the pump if this were not completely efficient. Thus substances that alter calcium transport would produce their effects by increasing or decreasing the efficiency of pumps P_1 and P_2 .

The dangers of using the system *in vitro* as a model for what happens during calcium transport *in vivo* were pointed out by Sallis & Holdsworth (1962). However, vitamin D_3 does appear to have effects when using the isolated everted intestine qualitatively similar to those obtained *in vivo*. The important differences noticed when transport of calcium was compared in similar segments of intestine by perfusion *in vivo* and everted sac *in vitro* were: (i) the greater efficiency of the gut from vitamin D_3 -treated chicks *in vivo*, e.g. $38\mu\text{g}$. of ^{45}Ca transported in 30 min. compared with $8\mu\text{g}$. *in vitro* (at 1mm); (ii) after 30 min. the amount of calcium found in the mucosa was always greater *in vitro* than *in vivo*. This latter finding was due to the large amount of ^{45}Ca found in the muscle layer of the everted sacs. With everted sacs where the blood supply has been interrupted and the vessels collapsed, it may be difficult for the ^{45}Ca to pass to the serosal compartment. The ^{45}Ca found in the muscle layer may be calcium on its way from the mucosal cells to the serosal surface. The accumulation of calcium in the muscle layer limits the usefulness of the everted-sac technique for investigating the effect of various factors on calcium transport. An instance of the difficulty of interpreting results can be given when considering Table 5. The ^{45}Ca concentration in the muscle layer of sacs from rachitic chicks increased and that of the sacs from vitamin D_3 -treated chicks decreased as compared with the non-inhibited controls. The elevation of intracellular ^{45}Ca concentration of

cells from rachitic chicks by iodoacetate should increase the amount of ⁴⁵Ca escaping via the reversible diffusion leak K₂ (Scheme 1) to the serosal fluid. It was this ⁴⁵Ca that probably caused the rise in muscle ⁴⁵Ca concentration and only a small proportion found its way to the serosal fluid. Similarly, since less ⁴⁵Ca was leaving the iodoacetate-inhibited cells from vitamin D₃-treated chicks than the corresponding control cells, there was less ⁴⁵Ca in the muscle layer of inhibited sacs.

The location of ⁴⁵Ca in mucosal cells loaded either *in vivo* or *in vitro* was the same and it was mainly present in cell fragments that were centrifuged down between 1000 and 3000g in 20% sucrose. What this fraction represents is not known precisely, but it is most likely to be cell membranes and brush borders. This would be consistent with the idea of pumps located at the cell surface, but the results do not support the suggestion (De Luca, Engstrom & Rasmussen, 1962) that the mitochondria are the main site of vitamin D₃ action.

Implicit in the model proposed is the idea that vitamin D₃ in some way inhibits the metabolic processes that drive pump P₁. Evidence for an inhibition of metabolism by vitamin D₃ has been put forward in a series of papers (De Luca, Gran & Steenbock, 1957; De Luca, Gran, Steenbock & Reiser, 1957; Norman & De Luca, 1964). Both in kidney and in bone a block in metabolism between citrate and α -oxoglutarate was obtained with vitamin D₃-treated rats. It is not known whether vitamin D₃ inhibits citrate metabolism in the intestine of the chick, but it is difficult to believe this inhibition to be the only site of action of vitamin D₃, since the vitamin enhances calcium transport *in vitro* under anaerobic conditions where presumably citrate metabolism is at a standstill.

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