Effects of Cholecalciferol on the Translocation of Calcium by Non-Everted Chick Ileum *in vitro*

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An apparatus is described that allows perfusion of a non-everted segment of intestine *in vitro* and the study of the accumulation of substances within the mucosal cells. The translocation of Ca^{2+} by rachitic-chick ileum and the effect of pretreatment with cholecalciferol was investigated, with the following conclusions. (1) Entry of Ca^{2+} across the microvilli into mucosal cells is by diffusion; it does not require metabolic energy or the presence of any other inorganic ions. (2) Pretreatment of the chick with cholecalciferol causes increased permeability of the microvillus to Ca^{2+} in both directions (lumen to cell, cell to lumen). The increased transport brought about by cholecalciferol *in vivo* can be partially mimicked by sodium dodecyl sulphate added *in vitro*. (3) The sign and the magnitude of the electrical potential difference prevailing across the ileum does not influence Ca^{2+} transport. (4) Exit of Ca^{2+} from the mucosal cell is temperature-sensitive, requires metabolic energy and Na^+ . (5) Pretreatment with cholecalciferol caused increased movement of Ca^{2+} out of the cell across the basement membranes. This effect of cholecalciferol given *in vivo* could be markedly increased by the presence of dicyclohexylcarbodi-imide in the perfusion fluid. These observations suggested that cholecalciferol increased Ca^{2+} entry (and exit) at the mucosal surface and also caused Ca^{2+} to be more available to the pump at the serosal surface.

There is general agreement that one important effect of cholecalciferol is to increase the translocation of Ca^{2+} from the lumen of the small intestine to plasma (reviewed by DeLuca, 1971). However, there is not a complete understanding of how this increased translocation is brought about. Several barriers to calcium absorption exist in the intestine; first, the mucosal microvilli; second, it is believed that intracellular Ca^{2+} is taken up by organelles, leaving Ca^{2+} at very low concentrations in the cytoplasm (Lehninger, 1970); and third, the exit at the serosal surface across the basement membrane against a concentration gradient. Simple concepts of 'active transport' are therefore unlikely to account for Ca^{2+} translocation, and cholecalciferol might affect any one of these barriers. The literature on this subject is confused because the two main species studied, rats and chickens, differ significantly in their response to cholecalciferol and in the way they absorb Ca^{2+} . Differences are obtained when absorption experiments *in vivo* are compared with techniques *in vitro*. Urban & Schedl (1969, 1970) used perfusion of the small intestine *in situ* of cholecalciferol-deficient or -repleted rats to study Ca^{2+} transport. No effect of cholecalciferol was observed at 0.4 mm- Ca^{2+} , but a significant effect was obtained with 3.4 mm- Ca^{2+} in the perfusing fluid. Flux calculations showed that cholecalciferol had its effect on the serosal surface, causing increased exit of Ca^{2+} from the cell. Wasser-

man & Kallfelz (1962) and Wasserman *et al.* (1966) by using cholecalciferol-deficient or -repleted chicks *in vivo*, found that cholecalciferol increased the permeability of the mucosal surface to Ca^{2+} in both directions, such that at 1.3 mm- Ca^{2+} there was no net absorption of Ca^{2+} . Net absorption was obtained at Ca^{2+} concentrations from 2.7 to 135 mm and cholecalciferol caused an increase of Ca^{2+} translocation. By analysis of the carcasses of chicks that had absorbed Ca^{2+} *in vivo* from the perfused intestine, Holdsworth (1965) found that cholecalciferol caused a tenfold increase in translocation at 1 mm- Ca^{2+} and an eightfold effect at 10 mm. Since the amount of Ca^{2+} found in the mucosal cells at the end of the 30 min perfusion was the same both in cholecalciferol-deficient or in cholecalciferol-repleted intestine, cholecalciferol must have increased entry at the mucosal surface.

Similarly, the work *in vitro* is equivocal. The original investigations on the use of everted sacs of intestine for studying Ca^{2+} transport reported that the active transport of Ca^{2+} required O_2 , and was inhibited by anaerobic conditions and by 2,4-dinitrophenol and CN^- (Schachter & Rosen, 1959; Schachter, 1963). However, Harrison & Harrison (1960), Wasserman (1960) and Sallis & Holdsworth (1962) found that these inhibitors caused only a slight decrease in Ca^{2+} transport by everted sacs. Norman *et al.* (1969), using everted sacs in the apparatus

devised by Helbock *et al.* (1966), found that anaerobic conditions and 1 mM-NaCN increased the transport of Ca^{2+} by cholecalciferol-deficient chick intestine but had no effect on the cholecalciferol-repleted tissue. Everted sacs of rat intestine were used by Wasserman & Taylor (1963) to show that Na^+ ions were not essential for Ca^{2+} transport, but in similar experiments in which K^+ and P_i were omitted from the media, it was shown that Na^+ at a maximum of 40 mM increased Ca^{2+} translocation (Martin & DeLuca, 1969*a,b*).

Epithelial cells are held together at the mucosal surface by desmosomes, tight junctions that are assumed not to be significantly permeable to water-soluble substances. Wasserman *et al.* (1973) have, however, suggested that part of the Ca^{2+} translocated by intestine passes through these junctions rather than across the cell. It seemed possible that the process of turning the intestine 'inside-out' to prepare everted sacs could damage some of these tight junctions. For this reason an apparatus was built that allows the study of Ca^{2+} transport in non-everted intestine. A re-investigation of Ca^{2+} transport *in vitro* was made to try to identify the factors involved during the passage of Ca^{2+} across each of the barriers mentioned above.

Materials and Methods

Management of chickens

White crossbred (Leghorn $\times$ Australorp) cockerels, 1 day old, were obtained from a local poultry farm and were raised under artificial light on a diet deficient in cholecalciferol for a period of 4 weeks as described previously by Sallis & Holdsworth (1962). Only those birds with satisfactory signs of cholecalciferol deficiency (as judged by X-ray examination) were used. To study the effect of cholecalciferol, the chicks were given 400 i.u. of cholecalciferol (Koch-Light Laboratories, Colnbrook, Bucks., U.K.) in 0.1 ml of propylene glycol by intramuscular injection 16 h before the experiment.

Apparatus for perfusion of non-everted intestine *in vitro*

The apparatus was made entirely of Perspex and consisted of a small chamber (D, Fig. 1*a*). The dimensions of the inner portion where perfusion takes place were 1 cm $\times$ 2 cm (cross-section) $\times$ 8 cm (height). When the 5 cm portion of gut with its Perspex support were in position, 5 ml of medium would completely cover the whole length of the gut. The outer chamber of D formed a water jacket and

Fig. 1. Apparatus for the perfusion of non-everted segments of intestine *in vitro*

The details of this Perspex chamber are described in the text. A 5 cm segment of intestine was attached to the removable holder (Fig. 1*b*) and placed in the inner chamber of the water-jacketed Perspex vessel shown in Fig. 1*a*). Medium from vessel C flows through the intestine and was returned to C by a peristaltic pump E. The volume of medium bathing each side of the segment of intestine was 5 ml. Vessels A and B were attached when the electrical potential was measured.

was maintained at 37°C. An 18-gauge hypodermic needle was cemented through the chamber to provide O₂+CO₂ (95:5) to saturate the outer serosal medium.

The perfusion fluid was contained in a vessel (C) of 1 cm cross-section and 6 cm in height. Medium flowed from C via silicone-rubber tubing to the top of the segment of gut, down through the gut and was returned to C by a small peristaltic pump (E; Watson Marlow Ltd., Marlow, Bucks., U.K.). The diameter and length of tubing were kept as short as possible so that with gut and tubing full of medium, a total of 5 ml of fluid ensured that vessel C remained half-full. O₂+CO₂ (95:5) was supplied via an 18-gauge hypodermic needle through the side of C. The height of vessel C was such that the total head of pressure on the gut was less than 4 cm of H₂O. When the electrical potential was to be measured, 'bridges' made with the particular perfusion medium being used were solidified with 1% agarose in polythene tubes, and served to connect vessels A to C and B to D. Platinum electrodes in vessels A and B were connected to a vibrating-reed electrometer (0.1 pA sensitivity; Nuclear-Chicago, Des Plaines, Ill., U.S.A.) and to a recorder. A holder for the segment of gut was made from Perspex as shown in Fig. 1(b). The connecting tube (F) on to which one end of the gut was tied was able to slide in a hole so that the length of gut could be varied by 0.5 cm to keep the segment extended but not under tension (the segment tends to increase in length during perfusion). Short-circuit experiments were done by passing a fine Ag/AgCl wire down through the tube connecting C to the intestine until the electrode was centrally placed within the intestine and the other electrode was placed parallel to it and close to the outside of the intestine. The electrodes were connected via a potentiometer, a 1.5 V Hg cell and a sensitive 100 μ A-meter.

Experiments with non-everted intestine

The portion of chick small intestine between the attachment of the yolk sac to the caecum was washed out with warm 0.15 M-NaCl or 0.15 M-Tris-HCl, pH 7.2, and removed from the bird under ether anaesthesia. The end of the gut near the attachment to the yolk sac was attached to F by tying with linen thread, and a segment 5 cm long was cut and tied to the holder (Fig. 1b). The next 5 cm portion of gut was retained for the determination of the Ca²⁺ and P contents of the gut before perfusion.

The holder with intestine attached was immersed in 5 ml of medium in chamber D and 5 ml of medium was added to C. All the media had been equilibrated with the gas phase and brought to 37°C. Small samples of media were withdrawn from vessels D or C at various time-intervals and the whole of the remaining fluid was usually removed after 30 min.

The holder was withdrawn, rinsed with non-radioactive medium and approx. 100 ml of medium passed through the lumen of the gut. The intestinal segment was then removed, cut longitudinally and carefully blotted with tissue. The mucosal cells were scraped off with a glass slide and the cells and residual muscle were placed in tared vessels and weighed. The tissues were dried at 105°C to constant weight.

Experiments with everted sacs in vitro

Everted sacs were prepared as described by Sallis & Holdsworth (1962) and incubated either at 0°C or 37°C in Krebs-Henseleit bicarbonate Ringer (Dawson & Elliot, 1959) containing 10 mM-glucose and 1 mM-⁴⁵CaCl₂ (0.5–1 μ Ci/ μ mol). After 15 min the sacs were removed and rinsed with non-radioactive media at the appropriate temperature. The serosal fluid was removed, fresh serosal and mucosal medium containing ⁴⁰Ca was applied and the sacs were incubated for a further 15 min at 37°C. Serosal and mucosal fluids and the whole remaining sac were analysed for ⁴⁵Ca.

Media

Krebs-Henseleit bicarbonate Ringer with 10 mM-glucose and 1 mM-Ca²⁺ was the standard medium used. In some experiments 1 mM-Sr²⁺ was substituted for Ca²⁺. The gas phase was either O₂+CO₂ (95:5) or N₂+CO₂ (95:5).

To investigate the role of various ions on Ca²⁺ transport, the medium contained 65 mM-Tricine, 8 mM-Tris and 185 mM-mannitol. The pH of this solution was 7.2 and the osmotic pressure was 260 mosmol. When additions were made to this medium these additions were substituted for equiosmolar amounts of mannitol. Cations and anions were added paired with buffer ions that are very poorly absorbed by mucosal cells (Good *et al.*, 1966). Phosphate and chloride solutions were prepared by adjusting 0.1 M solutions of the acids to pH 7.2 with Tris base. Na⁺ solutions were prepared by adjusting 0.5 M-Tricine to pH 7.2 with NaOH and measuring Na⁺ by flame photometry (approx. 25 mequiv./ml). Ca²⁺ solution was prepared by warming 0.74 g of Ca(OH)₂ in 50 ml of water and containing 4.25 g of *N*-tris(hydroxymethyl)methyl-2-aminoethane-sulphonic acid (Tes), and finally diluting to 100 ml to give 0.1 M-Ca²⁺.

Calcium-binding protein

Calcium-binding protein was prepared from rachitic-chick small intestine after treatment of the chicks 72 h before with 400 i.u. of cholecalciferol. It was isolated and purified by the method of Wasserman *et al.* (1968).

Analysis of tissues

Muscle or cells from the intestine were digested with 1 ml of HNO_3 (10%, w/v) and 0.5 ml of HClO_4 (60%, w/w) and evaporated to dryness. The residues were dissolved in 2 ml of 0.5M- HClO_4 and portions taken for radioactive counting of ^{45}Ca and ^{32}P , for the determination of Ca by atomic absorption, and that of P_i by the method of Taussky & Shorr (1953).

Counting of radioactive samples

A Beckman liquid-scintillation counter was used with the scintillation fluid of Bray (1960) and counts were corrected for quenching. To compare results from many experiments the data were recalculated to express the results as if the total radioactivity in c.p.m. in 5 ml of medium were 1000 (^{45}Ca or ^{32}P). Actual total counts varied from 7000000 to 16000000 c.p.m. per 5 ml of medium.

Results

Determination of cell water and the 'leakiness' of the tissue

The Krebs-Ringer medium was used with 1 mg (1 μCi) of polyethylene [1,2- ^{14}C]glycol (New England Nuclear Corp., Boston, Mass., U.S.A.) added to the mucosal fluids. After 30 min perfusion of the ileum, cells and mucosal muscle were separated as described above. The intracellular water was the loss of weight on drying minus the extracellular fluid measured by using the radioactive polyethylene glycol (Jackson *et al.*, 1970), and the results are shown in Table 1. The radioactivity of the perfusion fluid did not change, showing that there was no net transport of water. Only 0.0019% of the [^{14}C]polyethylene glycol was detected in the serosal fluid. Similar segments that had been everted and studied in the same way gave variable results with 0.0023–0.029% of the initial [^{14}C]polyethylene glycol found in the serosal fluid.

Ca^{2+} transport in Krebs-Ringer medium

Krebs-Ringer bicarbonate containing 10 mM-glucose, 1 mM- Ca^{2+} and 1 mM- P_i was used as a standard physiological medium and the transport of Ca^{2+} was calculated from the changes in radioactivity of ^{45}Ca . The change in Ca^{2+} of the perfusion fluids measured by atomic absorption was insignificant, but tissues actually lost Ca^{2+} to the medium during the 30 min perfusion, as shown in Table 2. For this reason the net transport of Ca^{2+} could not be calculated and the results are shown as ^{45}Ca (c.p.m.) translocated.

There was an initial lag of approx. 5 min in the appearance of ^{45}Ca either in the serosal or in the mucosal fluids but thereafter the rate of accumulation was linear with time for at least 50 min. The situation reached after 30 min perfusion is shown in Fig. 2. Treatment of rachitic chicks with 400 i.u. of cholecalciferol 16 h before the perfusion brought about the expected increase in Ca^{2+} translocation in the direction $\text{M} \rightarrow \text{S}$ (from mucosal to serosal fluid). Fig. 2 illustrates that the increased transport brought about by cholecalciferol is due both to increased entry into the cell and increased exit from the cell at the serosal surface. Entry of Ca^{2+} from the serosal surface to the mucosal cell is the same for ilea from both cholecalciferol-deficient and -repleted birds but exit from cells of cholecalciferol-repleted birds to the mucosal fluid is facilitated and leads to a greater movement of Ca^{2+} from serosal to mucosal fluid (Fig. 2). Thus pre-treatment with cholecalciferol seems to increase the permeability to Ca^{2+} of the microvillus of the cell in either direction. The translocation of ^{45}Ca from mucosal to serosal fluids and from serosal to mucosal fluids is almost equal for rachitic chicks, whereas for the cholecalciferol-pretreated chick the flux from mucosal to serosal fluid is twice as great as the flux from serosal to mucosal fluid.

Increased permeability of the mucosal surface does not appear to be the only effect of cholecalciferol. All additional effect causing release of ^{45}Ca from the cell can be seen by comparing the ratio of ^{45}Ca in serosal

Table 1. *Weight of tissue and intracellular water in 5 cm of ileum*

Segments of chick ileum were perfused for 30 min in Krebs-Ringer bicarbonate medium containing 1 mg (1 μCi) of [^{14}C]polyethylene glycol in the mucosal fluid. After washing, the cells and muscle were dried and the loss of weight on drying was corrected for 'external water', which was assessed from the radioactivity of the tissue. The results are mean values $\pm$ S.E.M. for 32 segments for each treatment.

Treatment	Tissue	Wet weight (g)	Calculated intracellular water (g)
Rachitic	Cells	0.2169 ± 0.0176	0.1718 ± 0.0188
	Muscle	0.0796 ± 0.0048	0.0594 ± 0.0042
Cholecalciferol-pretreated	Cells	0.2150 ± 0.0161	0.1634 ± 0.0159
	Muscle	0.0763 ± 0.0041	0.0583 ± 0.0034

Table 2. *Calcium content of ileum before and after perfusion*

A 10cm length of ileum distal to the yolk sac was divided into two portions. One portion was taken immediately for separation of mucosal cells and muscle and the Ca^{2+} content determined. The second 5cm portion was perfused for 30min in Krebs-Ringer bicarbonate medium containing 10mm-glucose, 1mm- Ca^{2+} , and 1mm- P_i . The results are mean values $\pm$ S.E.M. for 80 segments for each treatment.

Treatment	Tissue	Ca^{2+} ($\mu\text{g}/5\text{cm}$ segment)	
		Before perfusion	After perfusion
Rachitic	Cells	17.1 ± 2.3	5.2 ± 0.4
	Muscle	6.1 ± 0.8	2.8 ± 0.2
Cholecalciferol-pretreated	Cells	16.9 ± 2.4	$5.9 \pm 0.4^*$
	Muscle	5.8 ± 0.9	3.3 ± 0.3

* Significantly greater than for rachitic birds; $P = 0.01$.

fluid/(^{45}Ca in mucosal cells). The ratios are 0.40 for rachitic and 1.04 for cholecalciferol-pretreated birds. The combined effects of increased entry and increased exit account for the fivefold increase in ^{45}Ca translocation (mucosa to serosa) brought about by treatment with cholecalciferol. Results almost identical with those shown in Fig. 2 were obtained when 1mm- ^{90}Sr was substituted for ^{45}Ca in the medium.

Effect of various substances on Ca^{2+} transport

Non-everted segments were studied in Krebs-Ringer bicarbonate medium containing 10mm-glucose, 1mm- ^{45}Ca and the various substances added to either the mucosal or serosal fluids. The ^{45}Ca accumulated in mucosal cells and muscle and serosal fluid is shown in Table 3. The results are for ^{45}Ca moving from mucosal to serosal fluid, since the substances had no effect on the movement in the reverse direction.

Anaerobic conditions ($\text{N}_2 + \text{CO}_2$) did not prevent Ca^{2+} entering the cells but had a slight effect on exit from the cell (Table 3). Inhibitors interfering with energy production, iodoacetamide, 2,4-dinitrophenol or dicoumarol (results for these latter substances are not shown, but are similar to those for iodoacetamide), again had no effect on entry of ^{45}Ca into the cell but caused ^{45}Ca to accumulate within the cell, so that less ^{45}Ca reaches the serosal surface. When 1mm-sodium dodecyl sulphate was present in the fluid perfusing the mucosal surface, ^{45}Ca flux from mucosa to serosa was increased in ilea of both rachitic and cholecalciferol-pretreated birds (Table 3). The effect of sodium dodecyl sulphate when present in the serosal surface was only slight and it had no effect on the flux from serosa to mucosa. Thus sodium dodecyl sulphate *in vitro* partially mimics the effect of cholecalciferol given *in vivo*, by increasing entry of to Ca^{2+} the mucosal cell. Inspection shows that the

Fig. 2. *Translocation of Ca^{2+} by chick ileum*

Ilea from rachitic (a) or cholecalciferol-pretreated (b) chicks were perfused for 30min as described in the Materials and Methods section with Krebs-Ringer bicarbonate medium containing 10mm-glucose, 1mm- Ca^{2+} and 1mm- P_i . The ^{45}Ca accumulated by muscle, cells and fluids has been corrected to correspond to a total initial radioactivity of 1000 c.p.m. either in the mucosal (M $\rightarrow$ S) or serosal (S $\rightarrow$ M) fluids. The results are the means of 48 ilea for each direction and each treatment, and the significance of the differences between rachitic and cholecalciferol-pretreated chicks for each membrane crossed is given by numbered superscripts: 1, $P < 0.01$; 2, $P = 0.02$; 3, $P = 0.01$; 4, $P > 0.2$; 5, $0.1 < P < 0.2$; 6, $P = 0.02$.

ratios ^{45}Ca in serosal fluid/ ^{45}Ca in the cell (rachitic 0.43; cholecalciferol-pretreated 0.92) are not significantly different from ratios obtained in the absence of sodium dodecyl sulphate and shows that sodium dodecyl sulphate does not have the property of enhancing release of ^{45}Ca from the cell as does cholecalciferol given *in vivo*.

Attempts to increase ^{45}Ca translocation of rachitic ilea by including the calcium-binding protein in the perfusion fluid were unsuccessful even when ten times the concentration of calcium-binding protein normally found in 5cm of ileum was present. Whatever the role of calcium-binding protein in Ca^{2+} transport, it seems unlikely that its effect is exercised on the outer surface of the mucosal cell.

The two inhibitors of ATPases*, ethacrynic acid and *NN*-dicyclohexylcarbodi-imide, had very different effects on Ca^{2+} transport. Neither prevented

* Abbreviation: ATPase, adenosine triphosphatase.

Table 3. *Flux of ^{45}Ca from mucosal to serosal fluids*

A 5 cm segment of ileum from rachitic (R) or cholecalciferol-pretreated (CC) birds was established in Krebs-Ringer bicarbonate medium as described in the Materials and Methods section. The mucosal fluid contained 1 mM- ^{45}Ca and the serosal fluid 1 mM-Ca (non-radioactive). The results show the total ^{45}Ca radioactivity (c.p.m.) accumulated in 30 min by serosal fluid, mucosal cells or muscle, adjusted to an initial total radioactivity of 1000 c.p.m. in the mucosal fluid (actual c.p.m. varied from 7×10^6 to 16×10^6). Substances were added to either mucosal (M) or serosal (S) fluids. Each substance tested was compared with a similar number of control segments tested on the same day and the significance was tested within this test. The mean value of the control groups shown is the overall mean of all the control segments.

Type of segment	No of ilea	Additions	Fluid to which added	^{45}Ca radioactivity (c.p.m.) in		
				Serosal fluid	Cells	Muscle
R	80	None	—	2.02 ± 0.51	5.04 ± 1.89	0.83 ± 0.43
CC	80	None	—	10.54 ± 2.47	10.15 ± 2.35	3.04 ± 1.01
R	12	$\text{N}_2 + \text{CO}_2$ (95:5)	—	$1.64 \pm 0.47^*$	6.51 ± 1.75	0.86 ± 0.41
CC	12	$\text{N}_2 + \text{CO}_2$ (95:5)	—	$8.47 \pm 1.82^*$	11.13 ± 2.12	2.52 ± 0.96
R	8	Iodoacetamide (2 mM)	M	1.82 ± 0.45	$7.44 \pm 1.68^*$	1.61 ± 0.55
R	8		S	2.02 ± 0.52	6.45 ± 1.80	1.34 ± 0.47
CC	8		M	$4.29 \pm 1.15^\dagger$	$12.61 \pm 2.30^\dagger$	3.83 ± 0.85
CC	8		S	$7.97 \pm 1.57^\dagger$	$11.15 \pm 2.41^*$	3.25 ± 0.90
R	8	Sodium dodecyl sulphate (1 mM)	M	$5.43 \pm 0.95^\dagger$	$12.66 \pm 2.04^\dagger$	$2.14 \pm 0.84^*$
R	8		S	2.84 ± 0.60	6.17 ± 1.65	1.23 ± 0.75
CC	8		M	$15.25 \pm 3.13^\dagger$	$16.64 \pm 2.90^\dagger$	$3.44 \pm 0.96^*$
CC	8		S	$11.66 \pm 2.72^*$	8.92 ± 1.48	2.47 ± 0.78
R	8	Ethacrynic acid (1 mM)	M	$1.52 \pm 0.37^*$	6.84 ± 1.51	0.70 ± 0.36
R	8		S	$1.22 \pm 0.40^\dagger$	$7.26 \pm 1.75^\dagger$	0.76 ± 0.40
CC	8		M	$6.27 \pm 1.14^\dagger$	$13.48 \pm 2.30^\dagger$	2.82 ± 0.77
CC	8		S	$5.93 \pm 1.09^\dagger$	$14.94 \pm 2.85^\dagger$	$2.14 \pm 0.62^*$
R	8	<i>NN</i> -Dicyclohexylcarbodi-imide	M	2.16 ± 0.50	6.28 ± 1.50	1.63 ± 0.45
R	8	(0.1 mM)	S	2.64 ± 0.37	5.36 ± 1.32	1.70 ± 0.53
CC	8		M	$19.18 \pm 3.21^\dagger$	9.45 ± 1.90	3.18 ± 1.10
CC	8		S	$24.34 \pm 3.85^\dagger$	10.55 ± 1.78	3.66 ± 0.96

* Different from the corresponding control ilea without inhibitor, $0.02 < P < 0.05$.

† Different from the corresponding control ilea without inhibitor, $P < 0.02$; all other results not significantly different, $P > 0.2$.

entry of Ca^{2+} into the mucosal cell, but ethacrynic acid prevented the exit of Ca^{2+} , causing an accumulation of Ca^{2+} within the cell for intestine of both cholecalciferol-deficient and -repleted birds. Dicyclohexylcarbodi-imide affected only the gut of cholecalciferol-repleted birds and caused a marked increase of ^{45}Ca translocations without leading to the accumulation of ^{45}Ca within the cell.

Ca^{2+} transport in Tricine-Tris-mannitol media

For these experiments the intestine was washed out *in vivo* with 0.15 M-Tris-HCl, pH 7.2, and the non-everted 5 cm of ileum was preincubated for 10 min in the Tricine-Tris-mannitol medium. This treatment ensured that all extracellular Na^+ was removed, since at the end of a 30 min perfusion less than 1 mequiv. of Na^+ /litre could be detected in the medium.

In the absence of any other permeable ion, ^{45}Ca was transported by the ileum in the Tricine-Tris-mannitol medium almost as well as in the Krebs-

Ringer medium. The results are shown in Fig. 3. It should be pointed out that a permeable anion is continuously produced in the form of lactate, e.g. in 30 min $4.6 \pm 0.4 \mu\text{mol}$ appears in the serosal fluid and $6.5 \pm 0.6 \mu\text{mol}$ in the mucosal fluid. Addition of 10 mM- Cl^- or 1 mM- P_i had no effect on ^{45}Ca transport. In the absence of Ca^{2+} , the translocation of P_i was not affected by cholecalciferol pretreatment, but in the presence of Ca^{2+} there was always less P_i transported and the ratio of P_i transported by cholecalciferol-pretreated to that transported by rachitic birds was 1.5:1.

Influence of Na^+ and of electrical potential on ^{45}Ca transport

Addition of Na^+ (as the sodium salt of Tricine) to the Tricine-Tris-mannitol medium caused increased ^{45}Ca transport to a maximum at 25 mequiv. of Na^+ /litre as shown in Fig. 4. At an Na^+ concentration of 25 mequiv./litre, ^{45}Ca translocation was greater than

Fig. 3. Effect of Na^+ on the translocation of Ca^{2+} by chick ileum in Tricine-Tris-mannitol medium

Fig. 4. Effect of Na^+ on electrical potential and Ca^{2+} transport in chick ileum

Ilea of cholecalciferol-pretreated chicks were perfused with Tricine-Tris-mannitol medium containing 10 mM-glucose, 1 mM- ^{45}Ca , and increasing amounts of the sodium salt of Tricine. The potential difference in mV (—) was measured 10 min after the start of perfusion. The ^{45}Ca accumulated in the serosal fluid after 30 min (----) is shown on the left-hand ordinate as mean values $\pm$ S.E.M. The results, for eight ilea, were corrected to a total initial ^{45}Ca radioactivity in the mucosal fluid of 1000 c.p.m.

Ilea from rachitic (a) or cholecalciferol-pretreated (b) chicks were perfused for 30 min as described in the Materials and Methods section by using Tricine-Tris-mannitol medium containing 10 mM-glucose and 1 mM- ^{45}Ca . The ^{45}Ca accumulated by muscle, cells and fluids is shown and has been adjusted to correspond to a total initial radioactivity of 1000 c.p.m. in either the mucosal (M $\rightarrow$ S) or serosal (S $\rightarrow$ M) fluids. Two results are shown for each tissue; those in parentheses were obtained when 25 mequiv. of Na^+ /litre was present in the medium. The significance of the effect of Na^+ on each of the processes (by superscripts numbered 1–12) is given below for 16 ilea for each direction, M $\rightarrow$ S or S $\rightarrow$ M. The effect of cholecalciferol on ^{45}Ca translocation is similar to that shown in Fig. 2, but significance values are not shown. Significance of the effect of Na^+ on ^{45}Ca transport: rachitic: 1, $P < 0.01$; 2, $P = 0.05$; 3, $P = 0.01$; 4, $P = 0.02$; 5, $P > 0.1$; 6, $P = 0.05$; cholecalciferol-pretreated: 7, $P < 0.01$; 8, $P = 0.01$; 9, $P = 0.01$; 10, $P = 0.02$; 11, $P = 0.02$; 12, $P = 0.02$.

in Krebs-Ringer medium, but at 75 mequiv. of Na^+ /litre transport of ^{45}Ca by the two media was similar.

In Krebs-Ringer bicarbonate medium with 10 mM-glucose the resistance measured in the apparatus was 260 Ω without, and 280 Ω with, the ileum in position (measured in both directions). The short-circuit current was 34 μA and the potential difference was 24 mV (serosal positive). No attempt was made to

measure ^{45}Ca transport under short-circuit conditions, since it is likely that Ag^+ released from the electrodes may affect the intestine (Clarkson & Toole, 1964). ^{45}Ca transport was measured instead in the Tricine-Tris-mannitol medium to which increasing amounts of Na^+ were added. Electrical potentials (measured 10 min after commencing the experiment) were -46 mV at zero Na^+ concentration, rose rapidly to pass through zero at 5 mequiv. of Na^+ /litre, then slowly increased to a value of +36 mV at 75 mequiv. of Na^+ /litre. Fig. 4 shows that ^{45}Ca transport by ilea from cholecalciferol-pretreated birds rose to a maximum at 25 mequiv. of Na^+ /litre, then declined, but there is no correlation between the potential difference of the ileum with ^{45}Ca transport. At 25 mequiv. of Na^+ /litre the ions Ca^{2+} , Mg^{2+} , K^+ , Cl^- and PO_4^{3-} made only slight contributions to the potential difference, in accord with previous statements that Na^+ is the main source of potential difference (Clarkson *et al.*, 1961).

Effect of temperature on ^{45}Ca transport

Attempts to use the perfusion apparatus in the cold-room at 2°C gave erratic results and therefore experiments were done with everted sacs kept in ice as described in the Materials and Methods section. Control flasks kept at 30°C were used for comparison. Table 4 shows that at 0°C 50-fold less ^{45}Ca is transported to the serosal fluid by ilea from either rachitic or cholecalciferol-pretreated chickens than at 30°C.

Table 4. *Uptake and transport of ^{45}Ca by everted sacs at 0°C and 30°C*

Everted sacs of the ilea of rachitic (R) or cholecalciferol-pretreated (CC) chicks were given a first incubation of 15 min duration in 5 ml of Krebs-Ringer bicarbonate medium containing 1 mM- ^{45}Ca (5 μCi) at either 0°C or 30°C. After being rinsed on the mucosal surface with 0.154 M-NaCl the sacs were given a second incubation of 15 min at 30°C in the same medium but containing non-radioactive Ca. The ^{45}Ca accumulated by the medium and the ^{45}Ca remaining in the tissue were determined. The results are mean values $\pm$ S.E.M. for six sacs.

Treatment of chick	Total ^{45}Ca radioactivity accumulated (c.p.m.)			
	First incubation: Serosal fluid (15 min at 0°C)	Second incubation		Ileum after 30 min
		Serosal fluid (15 min at 30°C)	Mucosal fluid (15 min at 30°C)	
R	815 $\pm$ 436	20901 $\pm$ 4987	66964 $\pm$ 6739*	240000 $\pm$ 34700*
CC	1247 $\pm$ 504	26655 $\pm$ 5372	94338 $\pm$ 7697*	330200 $\pm$ 45800*
	(15 min at 30°C)	(15 min at 30°C)	(15 min at 30°C)	
R	27299 $\pm$ 6550*	34740 $\pm$ 7334*	57408 $\pm$ 3820*	216000 $\pm$ 24600*
CC	66839 $\pm$ 8492*	77615 $\pm$ 8190*	95597 $\pm$ 10134*	287400 $\pm$ 31000*

* Difference between rachitic and cholecalciferol-pretreated sacs is significant at $P < 0.02$.

This was not due to the inability of ^{45}Ca to enter mucosal cells, since these same sacs on transferring to 30°C transported to the serosal fluid the ^{45}Ca that had been accumulated within the mucosal cells during the previous incubation at 0°C. That ^{45}Ca had entered the cells at 0°C can also be seen from the ^{45}Ca remaining in the tissue after 30 min and also in the ^{45}Ca appearing in the mucosal and serosal fluids of the second incubation.

Discussion

The object of the present study is to understand the effect of the physiologically active form of cholecalciferol on the process of Ca^{2+} translocation across the chick small intestine. The apparatus described allows transport *in vitro* to be studied with the minimum manipulation of the intestine. The potentially damaging technique of everting the intestine is unnecessary, since the effects on Ca^{2+} transport obtained by giving 400 i.u. of cholecalciferol 16 h previously were equal to or greater than results obtained previously by using everted sacs (Norman *et al.*, 1969; Schachter, 1963; Harrison & Harrison, 1960; Wasserman, 1963; Urban & Schedl, 1969). With small and equal volumes on both sides of the transporting membrane it was possible to study movement in either direction, mucosa to serosa or serosa to mucosa, and by measuring Ca^{2+} accumulated in the cells and muscle, to locate where cholecalciferol was influencing Ca^{2+} transport.

The movement of ^{45}Ca across the microvilli both into and out of the cell was increased in cholecalciferol-pretreated chicks (Figs. 2 and 3). These direct measurements confirm the observations by Wasserman & Kallfelz (1962) and indicate that the mem-

branes at this surface have become more permeable to Ca^{2+} . Identical results were obtained when Sr^{2+} was substituted for Ca^{2+} and suggest that the phenomenon was not due to the exchange of ^{45}Ca in the medium with tissue Ca^{2+} .

Calcium-binding protein, whose synthesis is induced by cholecalciferol, may be located at the microvillus surface (Taylor & Wasserman, 1970) and may act as a barrier of Ca^{2+} across these membranes (Wasserman *et al.*, 1968). The suggestion (DeLuca, 1971) that calcium-binding protein secreted by goblet cells into the intestinal lumen causes increased Ca^{2+} entry to the cells, is unlikely, since a large excess of calcium-binding protein in the fluid perfusing through the lumen did not increase Ca^{2+} translocation. Calcium-binding protein may, however, increase Ca^{2+} uptake by embryonic chick intestine in culture (Corradino & Wasserman, 1971). The process of transport across the microvilli seems to be by diffusion, since it was only slightly decreased at 0–2°C (Table 4) and was not affected by inhibitors of glycolysis or oxidative phosphorylation, or by anaerobic conditions. Thus it is unlikely that entry of Ca^{2+} is mediated by a Ca^{2+} -activated ATPase, as suggested by Melancon & DeLuca (1970). The observations of Webbing & Holdsworth (1965, 1966) on the increased Ca^{2+} transport brought about *in vivo* by bile salts, bile alcohol sulphates or sodium dodecyl sulphate have been extended to show that the main effect of sodium dodecyl sulphate *in vitro* was an increased entry of Ca^{2+} across the mucosal surface (Table 3). There was no effect of sodium dodecyl sulphate on the exit of Ca^{2+} across the serosal surface other than that due to increased entry from the lumen. Thus although sodium dodecyl sulphate *in vitro* can mimic the effect of cholecalciferol given

in vivo at the microvilli surface, it cannot mimic the effect of cholecalciferol of releasing Ca^{2+} from sites within the cell.

Once Ca^{2+} has entered the cell it is subject to the effects of the organelles where mitochondria may decrease the intracellular concentration of Ca^{2+} to $1\ \mu\text{M}$ (Lehninger, 1970). Thus to be transported out of the cell, Ca^{2+} must be pumped across the serosal surface against a concentration gradient, since the extracellular fluid is approx. 2 mM-Ca^{2+} . There must also be some mechanism for the release of Ca^{2+} from the organelles. It was noted in Fig. 2 that cholecalciferol has an effect on the exit of Ca^{2+} from the cell, and the following observations suggest that the site of action is on the intracellular events. Neville & Holdsworth (1968) found that when chicks had been absorbing Ca^{2+} for over 1 h *in vivo* the mucosal cells of cholecalciferol-deficient birds contained twice the amount of Ca^{2+} in the cells of cholecalciferol-repleted birds and suggested that in cholecalciferol deficiency there is a defect in the release of Ca^{2+} . When Ca^{2+} transport is studied in cholecalciferol-deficient chicks either *in vitro* as in Table 3 or *in vivo* (Holdsworth, 1965), the addition of sodium dodecyl sulphate to the intestine of cholecalciferol-deficient birds increased the overall movement of Ca^{2+} . Large amounts of Ca^{2+} accumulated within the intestinal cells, but the serosal pump seemed to be able to pump large amounts of Ca^{2+} even in the absence of cholecalciferol. Therefore it is likely that the effect of cholecalciferol is on the intracellular release of Ca^{2+} to this pump and not on the pump itself.

The study of Ca^{2+} release from organelles and its passage across the serosal membrane cannot be distinguished by the use of intestinal transport experiments *in vitro*, since there are no known specific inhibitors of the two processes. Lack of oxygen or inhibition by iodoacetamide, 2,4-dinitrophenol or dicoumarol causes more Ca^{2+} to be retained within the cell. This indicates that energy is required either for Ca^{2+} release from mitochondria or for the pump in the membrane. Ethacrynic acid was shown by Birge *et al.* (1972) to inhibit a $\text{Na}^+ + \text{Ca}^{2+}$ -dependent ATPase present in the basement membrane of the intestine. This inhibitor caused an accumulation of Ca^{2+} within the cell, thus decreasing overall transport (Table 3). The evidence that this effect is on the serosal membrane is not clearcut, however, since Epstein (1972) has evidence that ethacrynic acid decreases the amount of ATP available for active transport.

The substance *NN*-dicyclohexylcarbodi-imide has been found to inhibit ($\text{Na}^+ + \text{K}^+$)-dependent ATPase (Schoner & Schmidt, 1969). When tested for its effect on Ca^{2+} translocation, *NN*-dicyclohexylcarbodi-imide at 0.1 mM has no effect on ilea of rachitic birds but increased the translocation by ilea of cholecalciferol-pretreated birds twofold. The effect seems to be

on the exit of Ca^{2+} from the cell, since Ca^{2+} concentration within the cell was the same as the control value (Table 3). By whatever process cholecalciferol pretreatment increases the exit of Ca^{2+} from mucosal cells, then *NN*-dicyclohexylcarbodi-imide enhances this process. Beechey *et al.* (1967) proposed a scheme to account for the effects of *NN*-dicyclohexylcarbodi-imide on oxidative phosphorylation in mitochondria, where *NN*-dicyclohexylcarbodi-imide prevented the hydrolysis and phosphorylation of a 'high-energy' intermediate $\text{X}\sim\text{I}$, the energy of which became available for other processes. Thus in ilea transporting Ca^{2+} , *NN*-dicyclohexylcarbodi-imide may make extra energy available for removal of Ca^{2+} from the cell. Again this inhibitor could be affecting either the organelles or the serosal surface. *NN*-Dicyclohexylcarbodi-imide is remarkable in that it is the only inhibitor reported to enhance the effect of cholecalciferol. When Na^+ was absent from the perfusion media, Ca^{2+} accumulated within the cell (Fig. 3). This extends the observations of Harrison & Harrison (1969) and Martin & DeLuca (1969*a,b*) and suggests that for maximum efficiency the pump at the serosal surface requires Na^+ . Measurements of electrical potential across the preparation *in vitro* showed that the sign and magnitude of the potential make little difference to Ca^{2+} translocation (Fig. 4).

Thus it has been established that cholecalciferol increases the permeability of the microvillus surface to Ca^{2+} , but cholecalciferol has a second function in controlling the release of Ca^{2+} from its intracellular location.

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