Studies on the Effect of Vitamin D on Calcium Absorption and Transport

BY GEORGE HASHIM* AND IRWIN CLARK

*Institute of Nutrition Sciences and Departments of Biochemistry and Orthopaedic Surgery,
Columbia University, New York, N.Y. 10032, U.S.A.*

(Received 7 October 1968)

1. Mucosal cells of the small intestine obtained from rats deprived of vitamin D or given excessive amounts of the vitamin accumulated significantly more calcium than did cells from control animals. 2. Mucosal cells from vitamin D-deficient rats released less calcium than did cells from normal or hypervitaminotic D animals. 3. Studies *in vivo* showed that the transfer of ^{45}Ca from the intestine to the blood was delayed in vitamin D deficiency, but was accelerated in hypervitaminosis D. 4. The findings support the thesis that vitamin D is involved in the release of calcium rather than in its uptake by mucosal cells. 5. Further evidence is presented suggesting that uptake of calcium by intestinal mucosal cells at 0° is primarily passive, whereas at 38° uptake and release are effected by an active process that depends on energy derived from glycolytic activity.

It is well established that absorption *in vivo* of calcium from the small intestine is dependent on vitamin D (Harris & Innes, 1931; Nicolaysen, 1937; Greenberg, 1945). Studies *in vitro* using the everted-gut-sac technique showed that previous treatment of the animals with vitamin D enhanced calcium transport against concentration gradients from fluids bathing the intestinal mucosa to those bathing the serosa (Schachter & Rosen, 1959; Harrison & Harrison, 1960; Schachter, Kimberg & Schenker, 1961). There is, however, disagreement whether this transport is the result of increased active transport (Schachter *et al.* 1961), increased permeability of the intestine to calcium (Harrison & Harrison, 1960, 1965; Wasserman & Kallfelz, 1962) or increased formation of a protein carrier for calcium (Wasserman & Taylor, 1963, 1966a; Taylor & Wasserman, 1965). In such preparations, the initial step in the transfer of calcium involves the uptake of calcium by mucosal cells, and this process is probably the same *in vivo*. However, the continued transfer of calcium through the other layers of the intestine, i.e. lamina propria, sub-mucosa, muscularis externa and serosa, does not occur in the living animal, where calcium is transferred from mucosal cells into the vascular system. These layers of the intestine form diffusion barriers for calcium transport *in vitro* (Wasserman & Taylor, 1966b; Hurwitz, Harrison & Harrison, 1967) and Holdsworth (1965) showed that calcium binding by

the muscle layers of the small intestine was greatly influenced by vitamin D. Therefore, interpretation of experiments designed to study the role of vitamin D or other substances in calcium absorption by measuring calcium transfer across the full thickness of the intestine may be hampered by effects of vitamin D on tissues underlying the mucosal cells, which are the functional absorbing units of the small intestine (Wilson, 1964).

Previous workers have attempted to isolate the absorptive cells from the remainder of the intestine, and have noted some of the conditions necessary for the survival of such preparations (Dickens & Weil-Malherbe, 1941). The uptake of formate into purines was successfully measured by using such a preparation (Paterson & Zborsky, 1955, 1958), although after continued incubation as much as 95% of the DNA was sometimes lost. Also, villi preparations were used for studying the transport and release of ^{45}Ca (Rasmussen, Waldo, Dziewiatkowski & DeLuca, 1963). More recently attempts were made to prepare epithelial cells free from contaminating mesenchymal cells by enzymic digestion (Harrer, Stern & Reilly, 1964; Ferris, 1966), and Ferris (1966) showed that oxygen consumption was almost linear over a period of 1 hr.

This paper describes the effect of vitamin D and other conditions on cell survival and uptake and release of calcium by mucosal cell suspensions of the small intestine of rats, which mainly contain absorptive cells. Such preparations permitted the

* Present address: Salk Institute for Biological Studies, La Jolla, Calif. 92037, U.S.A.

study of calcium uptake and release, independent of underlying tissues that are present in intestinal slices or sacs. Further, the effects of vitamin D on the transfer of calcium *in vivo* from the intestine to the blood were also investigated.

MATERIALS AND METHODS

Preparation of rats. Male albino rats of the Holtzman strain were used in all experiments. In studies of the effects of vitamin D deficiency, weanling rats weighing 40–50 g. were fed on a vitamin D-free diet (DeLuca, Gran & Steenbock, 1957) until significant differences were obtained between their body weights and those of controls. This diet (DeLuca *et al.* 1957) was prepared in the laboratory and modified to contain 60% of glucose (Cerelease), 24% of protein (vitamin-free casein), 10% of fat (Mazola oil) and 4.14% of salt mixture. The complete diet contained 0.8% of Ca, 0.5% of P, 0.12% of Na, 0.36% of K, 0.05% of Mg, 0.10% of Cl and 0.11% of Fe. The diet also contained trace amounts of Cu, Mn, Zn and I, in addition to all vitamins known to be required by the rat, except vitamin D. Vitamin D₂ (75 i.u.) in 0.1 ml. of sesame oil were given orally, three times per week, to control and pair-weighted rats. In the vitamin D-toxicity studies, rats weighing 200–250 g. were used. Toxic doses of vitamin D (10 000 i.u./100 g. body wt./day) were also given orally to rats in 0.1 ml. of sesame oil. The rats were grouped as follows, so that the mean weight of each group was the same: control, pair-weighted and vitamin D-deficient or hypervitaminotic-D. The pair-weighted group received restricted amounts of food daily to keep its body-weight gain about the same as that of the vitamin D-deprived or hypervitaminotic-D group. All rats were deprived of food overnight (16–20 hr.) before they were decapitated. In the experiment designed to measure the release of calcium *in vitro*, the rats, after being kept without food overnight, received ⁴⁵Ca by stomach intubation and 30 min. later were killed.

In studies of calcium uptake and release *in vivo*, the animals were killed at timed intervals after the oral administration of ⁴⁵Ca. Serum was collected and analysed for calcium by a fluorometric procedure (Kepner & Hercules, 1963); ⁴⁵Ca was measured by liquid-scintillation counting (counting efficiency 80%) with Bray's (1960) solution, and phosphate by a modification of the method of Fiske & Subbarow (1925).

Preparation of mucosal cell suspensions. Immediately after decapitation, the small intestine (from the pylorus to the ileocaecal valve) was removed, flushed with 25 ml. of cold 0.9% NaCl and cut into 10 cm. segments. A glass rod was then inserted into each segment and a longitudinal incision was made by passing the sharp edge of a scalpel over the intestine. The open strip was then laid flat over a large Petri dish inverted over ice. The mucosa was gently scraped with glass slides and the cell scrapings were suspended in cold 0.9% NaCl, filtered through nylon cloth to remove connective tissues and large masses of extraneous material and centrifuged at 500 g for 5 min. Histological examinations of the intestine before and after scraping showed that mainly the tips of the villi were removed. Examination of the cells after centrifugation revealed that they were mostly absorptive cells, although some small lymphocytes were also present. The sedimented cells were

resuspended in Krebs–Ringer bicarbonate buffer (Krebs & Henseleit, 1932), previously gassed with O₂+CO₂ (95:5) to maintain pH 7.4. For anaerobic conditions the medium was gassed with N₂+CO₂ (95:5). The medium also contained tracer amounts of ⁴⁵Ca, which was used to follow the uptake of calcium by the cells, except in studies where ⁴⁵Ca was administered to the rats and its release from mucosal cells was measured.

Incubation of mucosal cell suspensions. Incubation of cell suspensions was carried out at 38° in a Dubnoff shaker in an atmosphere of O₂+CO₂ (95:5) or N₂+CO₂ (95:5) for the required times. After incubation, a sample of the suspension was centrifuged at 500 g for 3–5 min. and the pellet washed twice with ⁴⁵Ca-free cold buffer. The sedimented mucosal cells were extracted twice with 0.5 M-HClO₄ at 70°. Portions of the extract were used for determination of DNA (Burton, 1956), calcium (DeLuca *et al.* 1957) and ⁴⁵Ca (Bray, 1960). Since there was the possibility of non-specific adsorption of ⁴⁵Ca on cell surfaces as well as its penetration into the cells, cells incubated at 0° were used as controls and the calcium uptake was defined as the difference between the ⁴⁵Ca uptakes by cells at 38° and at 0°. Also, since a variable amount of cells always survived to the end of each incubation period, the uptake was expressed as c.p.m./μg. of DNA of the cells sedimented at 500 g for 5 min., and calcium uptakes were expressed as the difference between the ratios of ⁴⁵Ca to DNA at 38° and at 0° of washed mucosal cells sedimented after incubation. Serum specific radioactivity was calculated by the following formula, which takes into account differences in body weight:

$$\text{Serum specific radioactivity} = \frac{\text{c.p.m./ml. of serum}}{\text{c.p.m./dose of } ^{45}\text{Ca}} \times \frac{1}{\mu\text{g. of Ca/ml.}} \times \frac{\text{Body wt. (g.)}}{100}$$

RESULTS

Stability of cell suspensions. Measurement of cellular DNA of sedimented cells before and after incubation (Table 1) in Krebs–Ringer bicarbonate buffer, pH 7.4, showed that about 75% of the cells disintegrated after 15 min. of incubation at 38° when the medium contained 2.5 mM-calcium. Increasing the calcium concentration to 5.0 mM improved DNA recovery, and at a calcium concentration of 10 mM the recoveries were about 75–80%; in some experiments recoveries were almost 100% (Table 2). At 0°, DNA recovery was practically complete, regardless of the calcium concentration in the medium. At 38° the uptake of calcium (c.p.m./μg. of DNA) rose with decreasing calcium concentration whereas at 0° the uptake of calcium (c.p.m./μg. of DNA) fell (Table 1). Although the uptake of ⁴⁵Ca at 38° rose with decreasing calcium concentration, it was considered that greater cell stability was more desirable and 10 mM-calcium was used in all other experiments. It appeared that, as the concentration of calcium in the medium fell, the amount of calcium entering the cell increased, i.e. net ⁴⁵Ca uptake (Table 1, next to last column). In reality this was not so, since the

Table 1. *Effect of calcium concentration on DNA recovery and net calcium uptake by mucosal cells in vitro*

Mucosal cells were incubated at pH 7.4 in an atmosphere of O₂ + CO₂ (95:5) in a complete medium that consisted of Krebs-Ringer bicarbonate buffer containing (final concentrations) 10 mM-calcium, a tracer quantity of ⁴⁵Ca, 0.25 M-sucrose and 0.02 M-glucose, and were shaken for 15 min. in a Dubnoff shaker. The medium was diluted with calcium-free medium to obtain the other calcium concentrations (5.0 and 2.5 mM). The percentage of DNA recovered was calculated as 100 × the amount of DNA that remained in the sedimented cells after incubation divided by the amount of DNA in the sedimented cells before incubation. Results are given as the mean ± s.d. of three determinations.

Expt. no.	Concn. of calcium (mM)	Temp.	...	DNA recovered (%)		Radioactivity of ⁴⁵ Ca in cells (c.p.m./μg. of DNA)		Net ⁴⁵ Ca uptake (c.p.m./μg. of DNA)	
				38°	0°	38°	0°		
1	10.0			76 ± 2	84 ± 1	1573 ± 28	1194 ± 13	379	379
	5.0			40 ± 1	102 ± 13	1705 ± 16	722 ± 16	983	491*
	2.5			26 ± 5	101 ± 2	2485 ± 262	594 ± 12	1791	448*
2	10.0			83 ± 3	89 ± 4	475 ± 11	312 ± 1	163	163
	5.0			54 ± 5	96 ± 4	591 ± 25	239 ± 39	352	176*
	2.5			28 ± 6	98 ± 3	947 ± 17	197 ± 7	750	188*

* These are the values for net ⁴⁵Ca uptake corrected for dilution (see the text).

Table 2. *Effect of pH on net calcium uptake by mucosal cells in vitro*

Mucosal cells were incubated in complete medium as in Table 1 for 15 min.; pH 7.4 was attained by gassing the medium for 10 min. with O₂ + CO₂ (95:5), pH 7.9 by further gassing with 100% O₂ for 10 min., and pH 5.0 and pH 6.1 by adjusting the pH of the medium with dil. HCl and then gassing with 100% O₂ for 10 min. Two rats were used in each experiment.

pH	Temp.	...	DNA recovered (μg.)		Radioactivity of ⁴⁵ Ca in cells (c.p.m./μg. of DNA)		Net ⁴⁵ Ca uptake (c.p.m./μg. of DNA)
			38°	0°	38°	0°	
5.0			216	227	17	10	7
5.0			216	224	23	14	9
6.1			170	162	18	7	11
6.1			153	147	12	8	4
7.4			135	134	439	181	258
7.4			132	132	394	175	219
7.9			161	158	211	104	107
7.9			155	165	221	78	143

same amount of radioactive isotope was used in each suspension but the concentrations of unlabelled calcium were different, i.e. the specific radioactivity of the solution containing 2.5 mM unlabelled calcium was four times that of the 10 mM-calcium solution and twice that of the 5.0 mM-calcium solution. Thus if the net uptake at 2.5 mM was divided by 4 and that at 5.0 mM by 2, the uptake of ⁴⁵Ca was about the same in each case when the dilution was taken into account (Table 1, last column). Incubation of cell suspensions for 15 min. at different pH values had essentially no effect on the final pH or on the stability of the cell suspensions as measured by the amount of DNA recovered at 38° and at 0° (Table 2). However, calcium uptake was greatest at pH 7.4; at pH 7.9 it fell considerably and it was negligible at pH 5.0 and 6.1.

As the incubation time increased, the number of cells sedimented decreased, as measured by the DNA content of the extract, and at 120 min. about 80% of the cells had disintegrated (Fig. 1). Further, the stability of the epithelial cell suspensions was unaffected by the vitamin D status of the animals.

Calcium uptake in vitro. Calcium uptake by cells incubated at 0° was considerably less than that by cells incubated at 38° at pH 7.4 (Tables 1 and 2). When glucose was omitted from the medium (Table 3) uptake was decreased by more than 50%. It was not affected when the incubation was done in an atmosphere of nitrogen. In the presence of cyanide (0.085 mM) or arsenate (10 mM), however, uptake was about twice that of the control. Although actinomycin D (10 μg./100 g. body wt., injected intraperitoneally) had no effect on calcium

uptake, it prevented the enhanced uptake usually seen after vitamin D treatment (Table 3). Calcium uptake by mucosal cells from rats treated with

Fig. 1. Percentage recovery of DNA from mucosal cells incubated for different times in complete medium at pH 7.4 (see Table 1). The percentage of DNA recovered was determined by measuring DNA of sedimented cells before and after incubation. ■, Control; ▨, vitamin D-deficient; ▩, hypervitaminotic D. The bars show the s.d. from the means of four determinations.

Table 3. *Effect of varied incubating conditions on net ^{45}Ca uptake by mucosal cells*

Mucosal cell suspensions were incubated in complete medium at pH 7.4 (Table 1). Actinomycin D was given intraperitoneally (10 $\mu\text{g.}/100\text{g. body wt.}$) and vitamin D₂ orally (5000 i.u. in 0.1 ml. of sesame oil) 20 hr. before mucosal cell suspensions were prepared. The control value was arbitrarily set at 100.

Incubation conditions	Net ^{45}Ca uptake (% of control)
Complete medium (control)	100
No glucose	41
Atmosphere of N ₂	88
Potassium cyanide (0.085 mM)	215
Sodium arsenate (10 mM)	178
Actinomycin D (10 $\mu\text{g.}/100\text{g.}$)	98
Actinomycin D (10 $\mu\text{g.}/100\text{g.}$), vitamin D ₂ (5000 i.u.)	110

large oral doses of vitamin D was higher than that of control cells after 15 min. incubation (Table 4), and rose with increasing doses of the vitamin until a maximum was reached with a dose of 5000 units. With larger doses (up to 25000 units), calcium uptake, although greater than that by control cells, was lower than that by cells from rats given 5000 units. Fig. 2 shows that at 30 min. more calcium was taken up by cells from vitamin D-treated rats as compared with control cells, but at 60 min. and particularly at 120 min. cells from the vitamin D-treated rats took up significantly less calcium than did control cells.

Calcium uptake by mucosal cells from vitamin D-deficient rats was also higher than that of control cells, whereas uptake by cells from rats given restricted amounts of food daily (pair-weighted control) was the same as that of controls (Table 5). As the incubation was continued (Fig. 3), cells from vitamin D-deficient rats accumulated more calcium than did those of controls, up to 2 hr.

Calcium release in vitro. Table 6 shows the results of experiments designed to study calcium release from cells prelabelled *in vivo* with ^{45}Ca . Mucosal cells from vitamin D-deprived rats lost essentially no calcium, whereas those from hypervitaminotic D animals tended to lose more calcium than did cells from control or pair-weighted animals.

Calcium uptake and release in vivo. Table 7 shows the effects of vitamin D deprivation and toxicity on gains in body weight and on serum concentrations of calcium and phosphorus. Both deprivation and excess of vitamin D markedly decreased weight gains. Vitamin D deficiency lowered the serum calcium concentration and raised the serum phosphorus concentration, whereas hypervitaminosis D raised the serum calcium concentration significantly. After a single oral dose of ^{45}Ca , the specific radioactivity of the mucosal cells of the rats given large amounts of vitamin D was significantly less than that of control cells at 15 min.; from 30 min. on there was no difference between the

Table 4. *Effect of oral vitamin D on net calcium uptake by mucosal cells in vitro*

Control rats were given their last regular dose (75 i.u.) of vitamin D₂ orally 16 hr. before the mucosal cell suspensions were prepared in complete medium at pH 7.4 (Table 1). The calcium uptake of the control is set arbitrarily at 100%. The radioactivities are given as means $\pm$ s.d. for four determinations.

Vitamin D ₂ (i.u.)	Temp.	Radioactivity (c.p.m./ $\mu\text{g.}$ of DNA)		Net ^{45}Ca uptake	
		38°	0°	(c.p.m./ $\mu\text{g.}$ of DNA)	(% of control)
Control		426 $\pm$ 87	103 $\pm$ 17	323	100
2500		831 $\pm$ 31	145 $\pm$ 18	688	213
5000		1379 $\pm$ 68	188 $\pm$ 10	1191	369
10000		975 $\pm$ 82	186 $\pm$ 4	793	246
15000		605 $\pm$ 8	115 $\pm$ 1	490	152
25000		669 $\pm$ 6	154 $\pm$ 18	515	159

specific radioactivities for treated and control rats (Fig. 4). On the other hand, the serum specific radioactivity of the treated rats was the same as that of the controls at 15 min., but was significantly greater at 30 min.; there were no differences after this time (Fig. 5). In contrast, specific radioactivity in mucosal cells of vitamin D-deficient rats was significantly higher than that of controls (Fig. 6) at 15, 30 and 60 min., whereas the ^{45}Ca specific radioactivity of the serum of vitamin D-deficient rats was significantly lower than that of controls at all times (Fig. 7).

DISCUSSION

Although uptake and release of calcium by isolated mucosal cells may not necessarily reflect calcium absorption as it occurs *in vivo* any more accurately than does calcium uptake by the everted gut sac, the isolated mucosal cell system does obviate some of the difficulties inherent in the use of everted gut sacs and intestinal rings, such as passage of ions through tissues not normally involved in calcium absorption (submucosal tissue layers and serosal membranes). Also, one can clearly separate the processes of uptake by the cell

Fig. 2. Effect of vitamin D treatment on net ^{45}Ca uptake by mucosal cells incubated *in vitro* for different times. Groups of control rats were given the following amounts of vitamin D_2 16 hr. before mucosal cells were isolated: ○, none (control); ■, 2500 i.u.; ▲, 5000 i.u. Mucosal-cell suspensions were incubated in complete medium at pH 7.4 (see Table 1). The bars show the s.d. from the means of three or four determinations.

Fig. 3. Effect of vitamin D deprivation on the net ^{45}Ca uptake by mucosal cells incubated *in vitro* for different times. Vitamin D-deficient rats were given the vitamin D-free diet for 70 days. Mucosal-cell suspensions from vitamin D-deficient (●) and control (○) rats were incubated in complete medium at pH 7.4 (see Table 1). The bars show the s.d. from the means of four determinations.

Table 5. Effect of vitamin D deficiency on net calcium uptake by mucosal cells in vitro

Cell suspensions were prepared in complete medium at pH 7.4 (Table 1). The radioactivities are given as means $\pm$ s.d. for four determinations.

Expt. no.	Rats ... Control			Vitamin D-deficient			Pair-weighted		
	Radioactivity (c.p.m./μg. of DNA)		Net ⁴⁵ Ca uptake (c.p.m./μg. of DNA)	Radioactivity (c.p.m./μg. of DNA)		Net ⁴⁵ Ca uptake (c.p.m./μg. of DNA)	Radioactivity (c.p.m./μg. of DNA)		Net ⁴⁵ Ca uptake (c.p.m./μg. of DNA)
	38°	0°		38°	0°		38°	0°	
1	1402 ± 182	701 ± 79	701	2473 ± 129	1029 ± 35	1444	1672 ± 102	948 ± 49	724
2	3254 ± 125	1145 ± 14	2109	4259 ± 156	1009 ± 209	3250	—	—	—
3	2119 ± 92	321 ± 20	1798	3706 ± 117	269 ± 37	3437	1882 ± 86	220 ± 8	1662
4	720 ± 6	345 ± 10	375	1042 ± 167	423 ± 23	619	—	—	—

and release by the cell, at least within short periods of time. Use of hyaluronidase to liberate the absorptive cells from underlying tissue, as reported by Perris (1966) and Harrer *et al.* (1964), may result in less contamination by non-absorptive cells. Histological study of the cells in the present study, however, showed no gross contamination by underlying structures, but did reveal the presence of lymphocytes. However, most of the cells appeared to be epithelial. Thus it was considered that this preparation, which mainly contained absorptive cells, could serve as a useful model for measuring uptake and release of ^{45}Ca as affected by various treatments.

The effect of temperature on ^{45}Ca uptake in these studies is in contrast with that described by Rasmussen *et al.* (1963) and Holdsworth (1965). They reported that mucosal cells took up more ^{45}Ca at 0° than at 38° . This discrepancy can be accounted for by the method of expression of the results. In the studies of Rasmussen *et al.* (1963) the ^{45}Ca liberated into the medium was measured and used as an index of calcium release. However,

in the period for which they incubated their preparations at 33° , most of the cells would have ruptured (Paterson & Zborsky, 1958; and Fig. 1)

Fig. 4. Effect of hypervitaminosis D on the specific radioactivity of calcium in mucosal cells. Mucosal cells of hypervitaminosis D (●) and control (○) rats were isolated at the times indicated after a single oral dose of $^{45}\text{CaCl}_2$. Other details are described in the Materials and Methods section. The bars show the s.d. from the means of three determinations.

Table 6. Percentage loss of ^{45}Ca from mucosal cells previously labelled in vivo

The ^{45}Ca content of cell suspensions at zero time was arbitrarily set at 100%. The results are the percentages of ^{45}Ca lost from the cells after 5 min. incubation in ^{45}Ca -free complete medium at pH 7.4 (Table 1). The results are the means of determinations on two animals.

Expt. no.	Loss of ^{45}Ca (%)			
	Controls	Pair-weighted controls	Vitamin D-deficient	Hypervitaminosis D
1	26	25	3	30
2	12	14	4	26
3	10	—	0	13
	16	—	3	20
4	10	—	0	26

Fig. 5. Serum specific radioactivity from hypervitaminotic D (●) and control (○) rats at the times indicated after a single oral dose of $^{45}\text{CaCl}_2$. Serum specific radioactivity was calculated by the formula given in the Materials and Methods section. The bars show the s.d. from the means of three determinations.

Table 7. Effect of vitamin D deficiency, hypervitaminosis D and food restriction on body weight, serum calcium concentration and serum phosphorus concentration

Experimental details are given under 'Preparation of rats' in the Materials and Methods section. The results are given as means $\pm$ s.d. for 10–15 animals in each group.

	Control	Pair-weighted control	Vitamin D-deficient	Hypervitaminosis D
Body wt. (g.)	292 $\pm$ 18	154 $\pm$ 9	160 $\pm$ 30	182 $\pm$ 19
Concn. of serum calcium (mg./100 ml.)	10.9 $\pm$ 0.3	10.2 $\pm$ 0.4	5.8 $\pm$ 0.2	16.0 $\pm$ 1.3
Concn. of serum phosphorus (mg./100 ml.)	8.7 $\pm$ 0.9	7.4 $\pm$ 0.7	10.4 $\pm$ 2.2	8.5 $\pm$ 0.5

Fig. 6. Effect of vitamin D deficiency on specific radioactivity of calcium in mucosal cells. Mucosal cells from vitamin D-deficient (●) and control (○) rats were isolated at different times after a single oral dose of $^{45}\text{CaCl}_2$. Other details are described in the Materials and Methods section. The bars show the S.D. from the means of three determinations.

Fig. 7. Serum specific radioactivity from vitamin D-deficient (●) and control (○) rats at the times indicated after a single oral dose of $^{45}\text{CaCl}_2$. Serum specific radioactivity was calculated by the formula given in the Materials and Methods section. The bars show the S.D. from the means of three determinations.

and released their ^{45}Ca into the medium. This would account for the rise in the concentration of ^{45}Ca in the medium as the incubation was continued. Further, at the concentration of calcium

in their buffer (0.1 mM) very few cells would survive after incubation at 33° for 15 min. and the radioactive isotope would be released into the medium. As shown in Table 1, after 15 min. of incubation at 38° 75% of the cells incubated in 2.5 mM-calcium disintegrated whereas at 0° they remained intact.

In the studies by Holdsworth (1965), the ^{45}Ca in the supernatant was measured after centrifugation of incubated mucosal cells at 30000g, and the results were expressed as $\mu\text{g. of Ca/mg. of protein}$ in the original incubation mixture. Hence, there is no way to determine the extent of cell breakage, since the protein in the supernatant was not determined after incubation. The present study (Table 1) indicates that there was a greater amount of cell rupture at 38° than at 0° , even at a calcium concentration of 10 mM. Further, it was observed that in the absence of 0.25M-sucrose there was a significant increase in cell breakage during incubation at 38° even at a calcium concentration of 10 mM (G. Hashim & I. Clark, unpublished work). It is therefore possible that the greater amount of radioactive isotope found in the supernatant of mucosal cells at 38° in the studies of Holdsworth (1965) resulted from increased fragility of cells as a consequence of the temperature and the lack of sucrose in the medium. In the present work 0.25M-sucrose was used in all experiments and the ^{45}Ca and DNA of only the cells that sedimented at 500g were analysed, thus avoiding the complications arising from cell breakage. In every experiment, the uptake of $^{45}\text{Ca}/\mu\text{g. of DNA}$ was significantly greater at 38° than at 0° .

Although most of the cells did not survive an incubation for 2 hr. at 38° , as measured by DNA recovery (Fig. 1), those that did survive were viable as measured by the difference in ^{45}Ca uptake at 38° and at 0° (Figs. 2 and 3). However, cell stability alone, as measured by DNA recovery, cannot be considered to be an adequate criterion for measuring active ^{45}Ca uptake since, at low pH (Table 2), ^{45}Ca uptake was negligible and yet DNA recovery was about 100% after 15 min. incubation. At 0° , when metabolic reactions proceed at a minimal rate, the entry of ^{45}Ca into the cell is mostly by passive diffusion. Further evidence for passive diffusion is shown in Table 2, where the ^{45}Ca uptake by the cells at 0° increased with increasing concentration of calcium despite a significant decrease in the specific radioactivity of the incubation medium. It is well established that the rate of diffusion of ions varies with the concentration. Further evidence for passive diffusion comes from the studies with metabolic inhibitors. In the presence of nitrogen, no effect was observed on the uptake of calcium. The large differences between the concentration of $^{45}\text{Ca}/\mu\text{g. of DNA}$ at 0° and at 38° clearly show that entry of the radioactive isotope at 38° is not the

result of simple diffusion but rather the result of some active process. Also, studies of the effect of pH (Table 2) showed that cell stability is maintained for at least 15 min., whereas ^{45}Ca uptake may be altered, indicating that active transfer occurred at pH 7.4, whereas at acid pH only passive transfer occurred. When cyanide or arsenate was present ^{45}Ca uptake was approximately doubled, and in the absence of glucose it was significantly decreased (Table 3). These findings suggest that calcium uptake and release are associated with normal glycolytic activities. In the absence of sufficient oxidizable substrate calcium entry was impaired, and in the presence of respiratory inhibitors such as cyanide release from within the cells was inhibited. By using the everted-gut-sac technique it was demonstrated that, when oxidative phosphorylation was uncoupled with 2,4-dinitrophenol, there was a marked decrease in the transfer of ^{47}Ca from the mucosa to the underlying intestinal coats, although its entry into the mucosa was increased (Schachter, Kowarski, Finkelstein & Ma, 1966). These findings are in accord with the present study, which also shows an accumulation of ^{45}Ca in epithelial cells treated with respiratory poisons (Table 3).

The biphasic nature of the uptake curves shown in Fig. 2 and 3 can perhaps be accounted for by the time necessary for the transition from passive to active uptake. The preparation of cells was at 0° . The slow uptake for the first 30 min. may be accounted for by the time necessary for the cells to reach 38° . At 0° transport would be mainly passive, but as the temperature rose active transport would begin, and very probably at temperatures below 38° there would be a combination of active and passive transport, and at 38° transport would be mainly active.

The present studies also show that vitamin D deficiency does not interfere with the entry *in vivo* of calcium from the incubation medium into isolated epithelial cells (Table 5). In fact, mucosal cells from the vitamin D-deficient rats contained more ^{45}Ca than did those of control rats after 15 min. of incubation *in vitro* (Table 5 and Fig. 3) and also after 15, 30 and 60 min. *in vivo* (Fig. 6). The increased amount of ^{45}Ca in the cells from vitamin D-deficient rats may be the result of more rapid uptake, slower release or a combination of both processes. From studies of the release of ^{45}Ca from intestinal cells prelabelled *in vivo* (Table 6), the results clearly show that after 5 min. of incubation, significantly less radioactive isotope was released from cells of the vitamin D-deficient animals. Further evidence, albeit indirect, to support this thesis comes from the study *in vivo*, where comparison of the serum specific radioactivities (Fig. 7) with those of the mucosal cells (Fig. 6) shows that the radioactivity is greater in the vitamin D-

deficient cells than in the control cells, but the radioactivity in the serum of the controls is higher than that in the serum of the vitamin D-deficient animals. This suggests delay in the transfer of ^{45}Ca from the intestine to the vascular system. Thus it appears that normal amounts of vitamin D are not essential for calcium uptake, but are involved in its release from mucosal cells. These findings and those of others (DeLuca & Engstrom, 1961; Engstrom & DeLuca, 1962, 1964) argue against the necessity for vitamin D in the initial steps of calcium absorption. Also, Schachter *et al.* (1966) showed that uptake from the mucosal medium to the mucosa was almost the same in the 'deficient' animals as in those given large amounts (20 000 i.u.) of vitamin D. However, large amounts of vitamin D increased uptake of calcium by the cells (Table 4). The decrease in uptake as the dosage of vitamin D rose from 5000 i.u. to 25 000 i.u. may be the result of toxicity to the cells of this large dose of vitamin D, or of an enhancement of calcium release. Removal of calcium from the mucosal cells is enhanced by excessive doses of the vitamin, since radioactivity in mucosal cells of hypervitaminotic D rats was significantly lower than that in control cells after 15 min. (Fig. 4), whereas serum specific radioactivity of the vitamin D-treated rats at 15 min. was equal to, and at 30 min. was higher than, that of controls (Fig. 5) despite the 40–50% increase in the serum concentration of calcium in the hypervitaminotic D rats (Table 6). If the same amount of ^{45}Ca or less had been transferred, the serum specific radioactivity would have been less. These findings indicate more rapid exit, as well as of entry, of calcium from mucosal cells as a result of excessive amounts of vitamin D. In addition, cells of animals given large amounts of vitamin D (Fig. 2), when incubated for times longer than 30 min., contained significantly less ^{45}Ca than did cells from control rats despite the greater initial uptake by cells from hypervitaminotic D rats at 15 or 30 min. This also indicates more rapid removal from the cells. Further, cells from hypervitaminotic D rats tended to release more ^{45}Ca after 5 min. of incubation than did control cells (Table 5). This rapid removal of calcium from the cells is also in accord with the data of Schachter *et al.* (1966), who observed a significantly greater transfer of ^{45}Ca from the mucosa to the underlying layers of the intestine after the administration of a large amount of vitamin D.

The exact site of accumulation of ^{45}Ca within the epithelial cells is not apparent from these studies. Work with subcellular particles has shown that vitamin D influences the binding and release of calcium by mitochondria and microsomes. Vitamin D *in vitro* or *in vivo* enhances the release of calcium by mitochondria (DeLuca & Engstrom, 1961;

Engstrom & DeLuca, 1962, 1964). Also, cyanide increased the uptake of calcium by cells (Table 3), and a similar event has been shown to occur with mitochondria (Rasmussen, Chance & Ogata, 1965). Thus it is possible that the site of accumulation may be the mitochondria, since vitamin D and cyanide affect release and uptake by the whole cell in the same way that they affect them in mitochondria. Microsomal preparations from chick intestinal mucosa bound calcium *in vitro* (Snellgrove & DeLuca, 1965), and the binding was depressed by the addition of vitamin D. The apparently depressed binding could also have been a consequence of increased release. Recently a protein was isolated from the duodena of vitamin D-treated chicks (Wasserman & Taylor, 1963; Taylor & Wasserman, 1965) and rats (Kallfelz, Taylor & Wasserman, 1967), that depressed calcium binding to cellular debris. It is possible that the influence of vitamin D on calcium transfer may be the result of increased synthesis of such a carrier protein, which would prevent the accumulation of calcium in cells perhaps by rapidly removing it from the cells. It has been shown that actinomycin D suppresses the effect of vitamin D on calcium absorption *in vivo* (Norman, 1965, 1966a,b; Bull, Czarnowski-Misztal & DeLuca, 1965, 1966), and in the present study the antibiotic also prevented the enhancement of calcium uptake *in vitro* (Table 3). Since actinomycin inhibits protein synthesis, these findings suggest that the vitamin may be involved in the formation of a carrier protein, which could possibly prevent intracellular accumulation of calcium by enhancing its transport. However, increased permeability of cellular membranes as a consequence of excess of vitamin D cannot be ruled out in these studies.

This work was in partial fulfilment of the requirements for the degree of Ph.D. of Columbia University by G.H. A preliminary report has appeared (Hashim & Clark, 1967). The work was supported by U.S. Public Health Service Grants TIAM-5168-09 and AM04071 and by Atomic Energy Contract no. AT(30-1)2530. I.C. is the recipient of a Career Development Award.

REFERENCES

- Bray, G. A. (1960). *Analyt. Biochem.* **1**, 279.
- Bull, J. E., Czarnowski-Misztal, E. & DeLuca, H. F. (1965). *Science*, **149**, 182.
- Bull, J. E., Czarnowski-Misztal, E. & DeLuca, H. F. (1966). *Proc. nat. Acad. Sci., Wash.*, **55**, 177.
- Burton, K. (1956). *Biochem. J.* **62**, 315.
- DeLuca, H. F. & Engstrom, G. W. (1961). *Proc. nat. Acad. Sci., Wash.*, **47**, 1744.
- DeLuca, H. F., Gran, F. C. & Steenbock, H. (1957). *J. biol. Chem.* **224**, 201.
- Dickens, F. & Weil-Malherbe, H. (1941). *Biochem. J.* **35**, 7.
- Engstrom, G. W. & DeLuca, H. F. (1962). *J. biol. Chem.* **237**, 974.
- Engstrom, G. W. & DeLuca, H. F. (1964). *Biochemistry*, **3**, 203.
- Fiske, G. H. & Subbarow, Y. (1925). *J. biol. Chem.* **66**, 375.
- Greenberg, D. M. (1945). *J. biol. Chem.* **157**, 99.
- Harrer, D. S., Stern, B. K. & Reilly, R. W. (1964). *Nature, Lond.*, **203**, 319.
- Harris, L. J. & Innes, J. R. M. (1931). *Biochem. J.* **25**, 367.
- Harrison, H. E. & Harrison, H. C. (1960). *Amer. J. Physiol.* **199**, 265.
- Harrison, H. E. & Harrison, H. C. (1965). *Amer. J. Physiol.* **208**, 370.
- Hashim, G. & Clark, I. (1967). *Fed. Proc.* **26**, 697.
- Holdsworth, E. S. (1965). *Biochem. J.* **96**, 475.
- Hurwitz, S. H., Harrison, H. C. & Harrison, H. E. (1967). *J. Nutr.* **91**, 319.
- Kallfelz, F. A., Taylor, A. N. & Wasserman, R. H. (1967). *Proc. Soc. exp. Biol., N. Y.*, **125**, 54.
- Kepner, B. L. & Hercules, D. M. (1963). *Analyt. Chem.* **35**, 1238.
- Krebs, H. A. & Henseleit, K. (1932). *Hoppe-Seyl. Z.* **210**, 33.
- Nicolaysen, R. (1937). *Biochem. J.* **31**, 122.
- Norman, A. W. (1965). *Science*, **149**, 184.
- Norman, A. W. (1966a). *Biochem. biophys. Res. Commun.* **23**, 335.
- Norman, A. W. (1966b). *Amer. J. Physiol.* **211**, 829.
- Paterson, A. R. P. & Zborsky, S. H. (1955). *Biochim. biophys. Acta*, **18**, 441.
- Paterson, A. R. P. & Zborsky, S. H. (1958). *Canad. J. Biochem. Physiol.* **36**, 755.
- Perris, A. D. (1966). *Canad. J. Biochem.* **44**, 691.
- Rasmussen, H., Chance, B. & Ogata, E. (1965). *Proc. nat. Acad. Sci., Wash.*, **53**, 1069.
- Rasmussen, H., Waldo, A., Dziwiatkowski, D. D. & DeLuca, H. F. (1963). *Biochim. biophys. Acta*, **75**, 250.
- Schachter, D., Kimberg, D. V. & Schenker, H. (1961). *Amer. J. Physiol.* **200**, 1263.
- Schachter, D., Kowarski, S., Finkelstein, J. D. & Ma, R.-I. W. (1966). *Amer. J. Physiol.* **211**, 1131.
- Schachter, D. & Rosen, S. M. (1959). *Amer. J. Physiol.* **196**, 357.
- Snellgrove, A. & DeLuca, H. F. (1965). *Arch. Biochem. Biophys.* **111**, 23.
- Taylor, A. N. & Wasserman, R. H. (1965). *Nature, Lond.*, **205**, 248.
- Wasserman, R. H. & Kallfelz, F. A. (1962). *Amer. J. Physiol.* **203**, 221.
- Wasserman, R. H. & Taylor, A. N. (1963). *Nature, Lond.*, **198**, 230.
- Wasserman, R. H. & Taylor, A. N. (1966a). *Science*, **152**, 791.
- Wasserman, R. H. & Taylor, A. N. (1966b). *Amer. J. Physiol.* **211**, 419.
- Wilson, T. H. (1964). In *Cellular Functions of Membrane Transport*, p. 215. Ed. by Hoffman, J. F. Englewood Cliffs, N.J.: Prentice-Hall Inc.