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Calcium uptake in isolated brush-border vesicles from rat small intestine

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Ca^{2+} uptake in brush-border vesicles isolated from rat duodena was studied by a rapid-filtration technique. Ca^{2+} uptake showed saturation kinetics, was dependent on the pH and ionic strength of the medium and was independent of metabolic energy. Uptake activity was readily inhibited by Ruthenium Red, La^{3+}, tetracaine, EGTA, choline chloride and Na^+ or K^+. The effect of variations in medium osmolarity on Ca^{2+} uptake and the ionophore A23187-induced efflux of the cation from preloaded vesicles indicated that the Ca^{2+}-uptake process involved binding to membrane components, as well as transport into an osmotically active space. Scatchard-plot analyses of the binding data suggested at least two classes of Ca^{2+}-binding sites. The high-affinity sites, $K_a = (2.7 \pm 1.1) \times 10^4 M^{-1}$ (mean ± S.D.) bound 3.2 ± 0.8 nmol of Ca^{2+}/mg of protein, whereas the low-affinity sites ($K_a = 60 \pm 6 M^{-1}$) bound 110 ± 17 nmol of Ca^{2+}/mg of protein. In the presence of 100 mM-NaCl, 1.7 and 53 nmol of Ca^{2+}/mg of protein were bound to the high- and low-affinity sites respectively. Decreased Ca^{2+}-uptake activity was observed in vesicles isolated from vitamin D-deficient as compared with vitamin D-replete animals and intraperitoneal administration of 1,25-dihydroxycholecalciferol to vitamin D-deficient rats 16h before membrane isolation stimulated the initial rate of Ca^{2+} uptake significantly. The data indicated that Ca^{2+} entry and/or binding was passive and may involve a carrier-mediated Ca^{2+}-uptake component that is associated with the brush-border membrane. Altering the electrochemical potential difference across the membrane by using anions of various permeability and selected ionophores appeared to increase primarily binding to the membrane rather than transport into the intravesicular space. Since there is considerable binding of Ca^{2+} to the vesicle interior, a comprehensive analysis of the transport properties of the brush-border membrane remains difficult at present.

In recent years methods have been developed to study transport mechanisms in plasma-membrane vesicles isolated from intestinal and renal cells (Isselbacher, 1974; Mürer & Hopfer, 1974; Kinne, 1976; Sacktor, 1977). The principal advantages of these systems are that they are simpler than whole cells or tissue, permit control over the supply of energy and minimize such complicating factors as compartmentation and intracellular metabolism (Hopfer, 1977).

Previous research with brush-border and basolateral membranes prepared from rat small intestine has been concerned primarily with sugar and amino-acid transport (Hopfer *et al.*, 1973, 1976; Mürer & Hopfer, 1974; Sigrist-Nelson *et al.*, 1975) and relatively few studies of electrolyte translocation (Berner *et al.*, 1976) have appeared in the literature. Gmaj *et al.* (1979) have studied Ca^{2+} transport in brush-border and basolateral plasma-membrane vesicles isolated from rat renal cortex, but Ca^{2+} uptake has not been investigated extensively in vesicles prepared from rat intestinal cells. Freedman *et al.* (1977) reported significant Ca^{2+} influx in vesicles prepared from Golgi, basolateral and microvillus membranes, with a marked decrease in transport evident in vesicles isolated from Golgi and basolateral membranes from vitamin D-deficient animals. Since only limited data are available on the kinetics and mechanisms of Ca^{2+} uptake by isolated brush-border vesicles (Nellans & Kimberg, 1978), the present study was initiated to examine further the physiological role of these membranes in cellular transport.

Abbreviations used: $1,25(OH)_2D_3$, 1,25-dihydroxycholecalciferol; Hepes, 4-(2-hydroxyethyl)-1-piperazine-ethanesulphonic acid; Mes, 4-morpholine-ethanesulphonic acid.

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Materials and methods

Animals and diets

Male Sprague–Dawley rats, purchased from ARS, Madison, WI, U.S.A., were allowed access to demineralized water and diet I, a low-Ca^{2+} (0.06%) and low-phosphorus (0.2%) semisynthetic regimen (Bronner & Freund, 1975) for several days before being killed.

The effects of vitamin D-deficiency and $1,25(OH)_2D_3$ administration on Ca^{2+} uptake were studied essentially as described previously (Miller *et al.*, 1979). Vitamin deficiency was assessed by measuring plasma Ca^{2+} (<6.0mg/dl; Bronner & Freund, 1975) and duodenal Ca^{2+}-binding protein contents (<15nmol of Ca^{2+} bound/g of mucosa; Ueng *et al.*, 1979). The vitamin D-deficient animals were injected intraperitoneally with 0.5μg of $1,25(OH)_2D_3$ 16h before being killed and control animals received injections of water or 0.9% NaCl.

Reagents

$^{45}CaCl_2$ (sp. radioactivity 13.6–26mCi/mg) and D-[U-^{14}C]glucose (sp. radioactivity 4.5Ci/mol) were purchased from New England Nuclear, Boston, MA, U.S.A. Ruthenium Red, D-mannitol, D-cellobiose, valinomycin, phlorizin, carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone and glucose colorimetric kit No. 510-A were obtained from Sigma Chemical Co., St. Louis, MO, U.S.A. The antibiotic Ca^{2+} ionophore A23187 was a gift from Dr. R. Hamill of Eli Lilly and Co., Indianapolis, IN, U.S.A. Other reagents and chemicals used throughout this investigation were of analytical-reagent grade.

Membrane preparation

Animals fed the appropriate diets were killed by cervical dislocation. The duodena were removed and placed in 0.9% NaCl, slit longitudinally, washed free of intestinal contents with saline solution and the mucosa was collected by scraping with a glass slide. The mucosal scrapings were suspended in approx. 75ml of 5mM-EDTA plus 1mM-Hepes/Tris buffer, pH7.5, and homogenzied (12strokes/min) with a motor-driven glass/Teflon homogenizer at a setting of 50–60 on the Powerstat variable transformer. Brush-border membranes and vesicles were prepared from the homogenate essentially as described by Forstner *et al.* (1968*a*) and Hopfer *et al.* (1973). Briefly, purified brush borders were obtained by differential centrifugation and membranes and vesicles were generally isolated after homogenization in 100mM-D-mannitol containing either 1mM- or 10mM-Hepes/Tris buffer, pH7.5. After the final centrifugation (24000*g*) for 30min the vesicles were resuspended in the desired incubation medium with a tuberculin syringe and then used for uptake experiments. All of the above operations were performed at 0–4°C.

Protein concentrations were determined in duplicate by the method of Lowry *et al.* (1951) with crystalline bovine serum albumin as the protein standard.

Uptake methods

The uptake of Ca^{2+} by brush-border vesicles was determined by a Millipore filtration technique. Unless otherwise stated, the incubation medium (total volume 65μl) contained 100mM-D-mannitol plus 10mM-Hepes/Tris buffer (pH7.5), 0.048–1mM-$^{45}CaCl_2$ [(3–5)$\times 10^5$c.p.m.] and membrane protein (30–60μg). The uptake of Ca^{2+} at selected times was terminated by the rapid addition of 3ml of ice-cold 100mM-D-mannitol, 10mM-Hepes/Tris and 150mM-$MgCl_2$, pH7.5. Occasionally, 5mM-EDTA or -EGTA was substituted for $MgCl_2$ in the stop and wash solution with similar results. All diluted samples were immediately filtered through Millipore filters (Type HA, 0.45μm, diameter 25mm) with a model FH 225V filtration unit (Hoefer Scientific Instruments, San Francisco, CA, U.S.A.) and the collected membranes were washed twice with 3ml of the cold stop solution. Presoaking the filters in 10mM-$CaCl_2$ before use or further washing (three to six times) did not significantly decrease the amount of Ca^{2+} retained. The filters were then dissolved in 3–4ml of Bray's scintillation fluid (Bray, 1960) to which 100μl of PCS (Amersham Corp., Arlington Heights, IL, U.S.A.) was added. The radioactivity retained by the membranes was measured in a Nuclear–Chicago liquid-scintillation spectrometer (Unilux II-A) and uptake values were expressed as nmol of Ca^{2+} equivalent/mg of membrane protein. Unless stated otherwise, all uptake assays were conducted in duplicate at 25–26°C with freshly prepared brush-border vesicles. In this study, Ca^{2+} uptake refers to binding plus transport.

Zero-time points were determined by adding 3ml of cold stop solution to 55μl of incubation medium. Membrane protein (10μl; 30–60μg) was then added and the reaction mixtures were immediately filtered, washed and the filters counted for radioactivity as described above. All uptake values were corrected for the amount of radioactivity retained by the filters at zero time, usually 0.01–0.03% of the total radioactivity at 0.48mM- and 1mM-Ca^{2+}. The absolute amounts of Ca^{2+} uptake varied slightly with different membrane preparations and the uptake patterns were highly reproducible.

Preliminary experiments (results not shown) indicated that Ca^{2+} uptake was linear between 10 and 75μg of protein per assay tube and maximum uptake occurred at pH7.5 to 8.0.

The uptake of D-glucose was assayed by a method

similar to that described for Ca^{2+}. The incubation medium contained 100mM-D-mannitol, 1mM-D-[U-^{14}C]glucose (5×10^5c.p.m.), 0.1mM-$MgSO_4$, 100mM-NaCl or 100mM-NaSCN and 1mM- or 10mM-Hepes/Tris (pH 7.5). The stop and wash solution contained 100mM-D-mannitol, 150mM-NaCl, 50mM-$MgCl_2$, 10mM-Hepes/Tris, pH 7.5, and 0.2mM-phlorizin. Values for the non-specific retention of D-[^{14}C]glucose on the filters, usually 0.01–0.03% of the total radioactivity in the incubation medium, were subtracted from the values of the incubated samples.

Enzyme assays

The intestinal disaccharidases sucrase (β-D-fructofuranoside fructohydrolase, EC 3.2.1.26) and lactase (β-D-galactoside galactohydrolase EC 3.2.1.23) were assayed as described by Dahlquist (1974). The Tris/glucose oxidase reagent was prepared from a glucose colorimetric kit (Sigma Chemical Co.). Specific activity was expressed as μmol of glucose formed/min per mg of protein at 37°C and pH 6.0.

Preparation of microvillus membranes for electron microscopy

Brush-border-membrane pellets were fixed in 0.1M-cacodylate buffer (pH 7.3) containing 1.25% glutaraldehyde, post-fixed in 1% OsO_4 and stained with aq. 0.25% uranyl acetate. The membranes were then dehydrated with acetone and embedded in Spurr low-viscosity resin. This sections were cut using an LKB ultramicrotome, double-stained with methanolic uranyl acetate and lead citrate and examined with a Zeiss EM 10 electron microscope operated at 80kV.

Differentiation of binding and transport

Essentially the methods of Hopfer *et al.* (1973) and Kessler *et al.* (1978) were used to evaluate the effect of altering the osmotic pressure on Ca^{2+} uptake by brush-border-membrane vesicles. The vesicles were prepared in 100mM-D-cellobiose and uptake values at equilibrium were determined as described above in the presence of increasing concentrations of D-cellobiose. The vesicles were pre-incubated with D-cellobiose for 10–30min before the initiation of the uptakes by the addition of $^{45}CaCl_2$.

Ionophore A23187-induced Ca^{2+} efflux

Membranes were isolated in 100mM-D-mannitol/10mM-Hepes/Tris buffer, pH 7.5, and equilibrated in a 20-fold excess (v/v) of the same buffer containing 100mM-NaCl for 15min at 37°C. The vesicles were collected by centrifugation, resuspended in the medium containing NaCl and then loaded with either 0.48mM- or 0.96mM-Ca^{2+} for 1–2h at 25°C. When equilibrium had been established the vesicles were diluted 6.5-fold into media containing either 0.1mM-EGTA or 0.1mM-EGTA and 15μM-ionophore A23187. At selected times thereafter, Ca^{2+} release was terminated by dilution with ice-cold 100mM-D-mannitol/10mM-Hepes/Tris/5mM-EGTA, pH 7.5. The diluted samples were then filtered, washed twice more with the same solution and assayed for radioactivity as described previously.

Membrane potential and Ca^{2+} uptake

Membrane potentials across the brush-border membrane were modified as described by Mürer & Hopfer (1974) with anions of various permeability and the ionophore valinomycin, which generates a large K^+ diffusion potential. Carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone was also used to generate an electrochemical potential (inside negative) with vesicles that were loaded with protons by homogenization in 20–50mM-Mes/Tris (pH 5.5) during the initial vesiculation step.

Results

Purification of microvillus membranes

The purification of rat intestinal brush borders and membranes was routinely monitored by light and phase-contrast microscopy and assessed by determining the activity of the membrane-bound disaccharidases sucrase and lactase. An increase in sucrase specific activity of at least 20-fold, compared with that of the homogenate, correlated well with results reported by Forstner *et al.* (1968*a*), Hopfer *et al.* (1973) and Schmitz *et al.* (1973). Sucrase and lactase specific activities in the membranes were 1.5- and 2-fold greater respectively than in the purified brush-border fraction. Electron-microscopic studies further substantiated the purity of the microvillus membranes. Thin sections of several specimens contained a heterogeneous population of membrane vesicles and a few fragments that did not vesiculate. In most cases, the amount of electron-dense material contained within the intravesicular space was minimal and mitochondrial contamination was not apparent.

The transport capability of the brush-border vesicles was assessed by determining D-glucose uptake. The patterns of uptake obtained in the presence of 100mM-NaCl or 100mM-NaSCN were nearly identical with those reported by Mürer & Hopfer (1974). The intravesicular volume estimated from D-glucose distribution varied from 0.9 to 1.4μl/mg of protein with different membrane preparations. Available evidence (Haase *et al.*, 1978) indicates that the brush-border vesicles are oriented predominantly right-side out.

Kinetics of Ca^{2+} uptake

The time course and effect of Ca^{2+} concentration

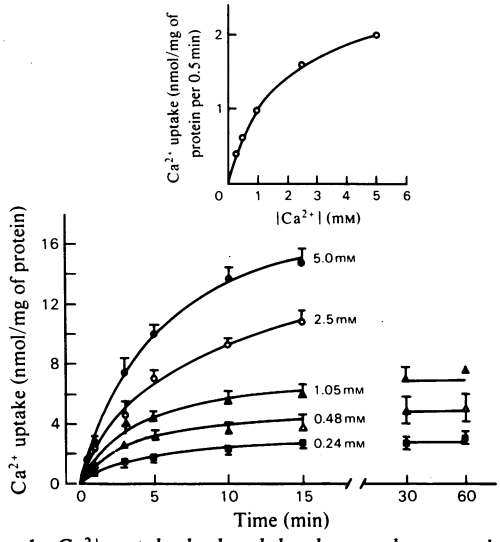

Fig. 1. *Ca^{2+} uptake by brush-border-membrane vesicles at several Ca^{2+} concentrations*
Vesicles were isolated in 100 mM-D-mannitol/10 mM-Hepes/Tris buffer, pH 7.5; the incubation medium contained the same buffer and in addition 100 mM-NaCl.

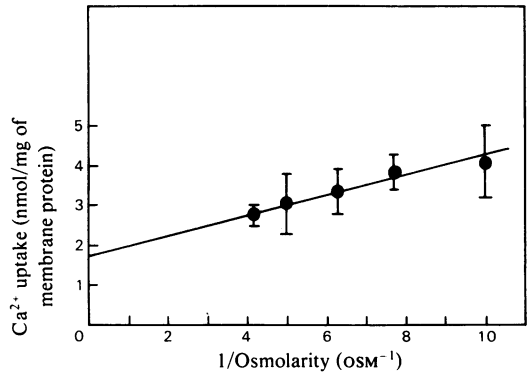

Fig. 2. *Influence of medium osmolarity on Ca^{2+} uptake by brush-border-membrane vesicles*
Vesicles were prepared in 100 mM-D-cellobiose. Uptake values at 10 min were determined in the presence of increasing concentrations of D-cellobiose plus 2.5 mM-$MgCl_2$, 0.48 mM-Ca^{2+} and 10 mM-Hepes/Tris buffer, pH 7.5.

on the uptake process at 25°C are shown in Fig. 1. Ca^{2+} uptake was time-dependent and the initial rate of accumulation, as well as the amount of Ca^{2+} taken up at equilibrium, was a function of the Ca^{2+} concentration. The initial rate of Ca^{2+} uptake, determined at 0.5 min, showed saturation kinetics with half-maximal activity at approx. 1.1 mM (Fig. 1 insert), an observation that agrees reasonably well with results reported previously by Nellans & Kimberg (1978).

Distinction between Ca^{2+} binding and transport

A comparison of the data for D-glucose transport and Ca^{2+} uptake gave the first indication that considerable binding of Ca^{2+} to brush-border membranes occurs. The osmotically active space occupied by 1 mM-D-glucose, which is almost completely transported (Hopfer *et al.*, 1973), was 0.9–1.4 μl/mg of protein, in contrast with a calculated intravesicular volume of 6 μl/mg of protein for 1 mM-Ca^{2+} at equilibrium.

Supportive evidence for Ca^{2+} binding is given in Fig. 2, which illustrates the effect of increasing medium osmolarity (decreased vesicle volume) on Ca^{2+} uptake by brush-border vesicles. Ca^{2+} accumulation decreased as the osmolarity of the incubation medium was increased by the addition of D-cellobiose. Extrapolation to infinite medium osmolarity or zero intravesicular space (ordinate intercept of the plotted data) indicated that approximately half of the Ca^{2+} accumulated by the vesicles was the result of binding to membrane components. The uptake values at 30 and 60 min (results not shown) were also extrapolated essentially to the same intercept on the ordinate.

Inhibitors of Ca^{2+} uptake

The effects of temperature and selected inhibitors on Ca^{2+} uptake by brush-border vesicles are shown in Table 1. When uptake assays were conducted at 0°C, an appreciable decrease, approx. 50%, in Ca^{2+} accumulation was evident and little uptake at 25°C was observed when the incubation medium contained 5 mM-EGTA. A substantial decrease in uptake was also noted when the extravesicular medium contained either $LaCl_3$, choline chloride or Ruthenium Red. Ouabain had no significant effect. Tetracaine (1 mM) decreased Ca^{2+} uptake by about 40%. However, when the vesicles were pre-incubated in the presence of the anaesthetic for 30 min before the initiation of the uptake by the addition of $^{45}CaCl_2$, Ca^{2+} accumulation was decreased by over 60%. The data again strongly support the existence of considerable binding, since tetracaine readily adsorbs to biomembranes and may compete with and displace membrane-bound Ca^{2+} from the fixed negative sites of the membrane (Papahadjopoulos, 1972). Verapamil, which blocks Ca^{2+} penetration across the cardiac sarcolemma (Fleckenstein *et al.*, 1969), but is a weak inhibitor of Ca^{2+} binding and uptake by isolated mitochondria and sarcoplasmic reticulum (Balzer, 1972), inhibited Ca^{2+} uptake by brush-border vesicles by approx. 45% (Table 1).

Effect of ionophore A23187 on Ca^{2+} uptake

A rapid transient accumulation of Ca^{2+} by brush-border vesicles occurred in the presence of the antibiotic Ca^{2+} ionophore A23187 (Fig. 3). Equilibrium values were essentially the same in the presence or absence of the ionophore after 30min incubation at 25°C.

Effects of EGTA and ionophore A23187 on Ca^{2+} release

Fig. 4. shows the effects of EGTA and ionophore A23187 on the release of Ca^{2+} from preloaded vesicles. The vesicles were pre-equilibrated with 100mM-NaCl, loaded with 0.48mM-$^{45}CaCl_2$ and, at the time indicated by the arrow, were diluted 6.5-fold into media containing either 0.1mM-EGTA or 0.1mM-EGTA and 15μM-ionophore A23187. EGTA chelated external free Ca^{2+} as well as that Ca^{2+} which was reversibly bound to the external surfaces of the membrane. The more rapid release of Ca^{2+} on addition of ionophore A23187 indicated the presence of an EGTA-inaccessible Ca^{2+} pool and substantiated the occurrence of a transmembrane process. Ca^{2+} may therefore occur in the free ionized form or may be readily exchangeable within the vesicle interior. Similar results have been reported (Gmaj *et al.*, 1979) for Ca^{2+} uptake into brush-border- and basolateral-membrane vesicles isolated from rat kidney cortex. The small percentage (3.6–12.3%) of Ca^{2+} that remained associated with the vesicles after treatment with the ionophore in repeated experiments was considered to be tightly bound to the inner surface of the membrane. The inner membrane of the vesicle is impermeable to EGTA (Reed & Bygrave, 1974) and the Ca^{2+} discharged by ionophore A23187 would be complexed by EGTA in the extravesicular medium and wash solution. Ionophore A23187-induced Ca^{2+} release was also observed with vesicles that were not pre-equilibrated with 100mM-NaCl.

Na^+, K^+ and P_i had no effect on the release of Ca^{2+} from brush-border vesicles isolated from duodenal mucosal scrapings. Gmaj *et al.* (1979) have reported an Na^+/Ca^{2+}-exchange system in basolateral-membrane vesicles prepared from rat

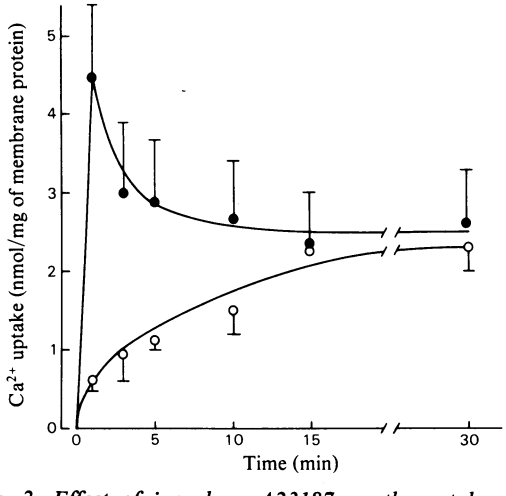

Fig. 3. *Effect of ionophore A23187 on the uptake of Ca^{2+} by brush-border-membrane vesicles*

The vesicles were isolated and incubated as described in the legend to Fig. 1. The stop and wash solution contained 100mM-mannitol, 10mM-Hepes/Tris, 100mM-NaCl and 5mM-EGTA, pH 7.5. Stock solutions of ionophore A23187 were prepared in ethanol. The final ethanol concentration in the incubation medium after the addition of the ionophore was less than 0.5%. ●, 0.96mM-Ca^{2+} + ionophore A23187 (15μM); ○, 0.96mM-Ca^{2+}.

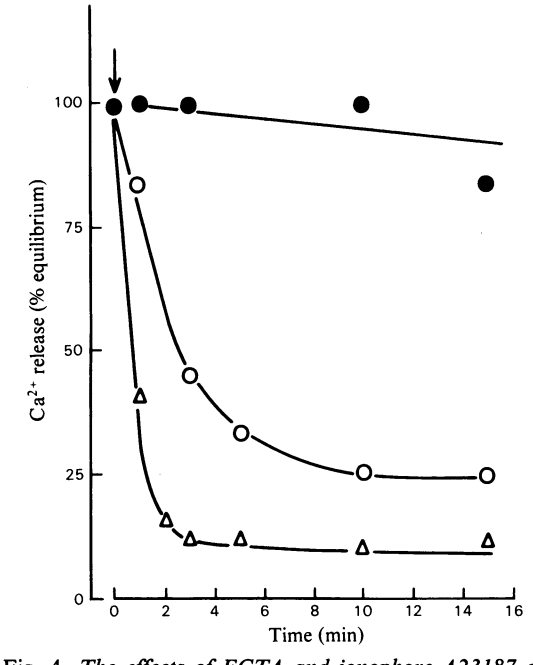

Fig. 4. *The effects of EGTA and ionophore A23187 on the release of Ca^{2+} from brush-border vesicles*

The vesicles (3.7mg of protein/ml), isolated in 100mM-D-mannitol/10mM-Hepes/Tris (pH 7.5), were equilibrated in a 20-fold excess (v/v) of the same buffer containing 100mM-NaCl for 15min at 37°C. The membranes were then cooled on ice and centrifuged at 24000*g* for 15min. The pellet was suspended in 1ml of the above buffer and loaded with 0.48mM-$^{45}CaCl_2$ for 60–90min at 25°C. At $t = 0$, the vesicles were diluted 6.5-fold with buffer (●), 6.5-fold with buffer containing 0.1mM-EGTA (○) and 6.5-fold with buffer containing 0.1mM-EGTA and 15μM-ionophore A23187 (△).

kidney cortex, but Na^+ had no effect on Ca^{2+} efflux from renal brush-border vesicles.

Effect of ionic strength on Ca^{2+} uptake

The imposition of an Na^+ gradient (medium > vesicle), with Cl^- as the accompanying anion, markedly decreased Ca^{2+} accumulation by brush-border vesicles (Fig. 5). Similar results were obtained when the external medium contained 10–100 mM-KCl (Fig. 5) or 100 mM-choline chloride (Table 1). Since Ca^{2+} uptake was decreased by Na^+ and not affected by ouabain (Table 1), significant contamination by basolateral membranes may be excluded.

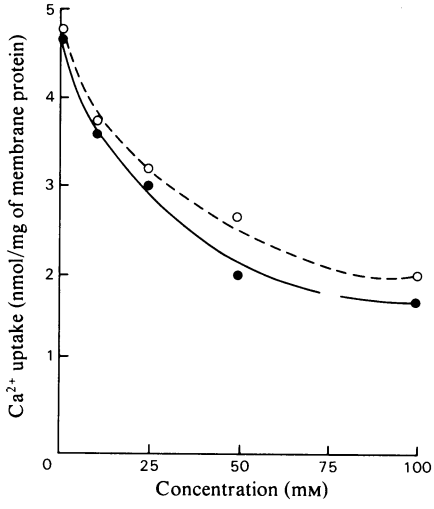

Fig. 5. *Ca^{2+} uptake as a function of extravesicular NaCl (●) and KCl (○) concentrations*
The vesicles were isolated in 100 mM-D-mannitol/10 mM-Hepes/Tris buffer (pH 7.5). The incubation media contained 0.48 mM-$^{45}CaCl_2$ and the uptake values were determined after incubation at 25°C for 15 min.

Table 1. *Conditions affecting Ca^{2+} uptake in brush-border vesicles*

Condition	Ca^{2+} uptake (%)* 0.5 min	15 min
Control	100	100
0°C	52.2	46.3
Ruthenium Red (0.1 mM)	73.3	56.0
Verapamil (1 mM)	—	55.4
EGTA (5 mM)	6.5	5.1
Tetracaine (1 mM)	89.9	60.8
(1 mM)‡	66.6	37.1
Ouabain (0.1 mM)	—	105.5
Choline chloride (100 mM)	73.3	41.8
$LaCl_3$ (0.1 mM)	88.8	42.6
(1 mM)	7.4	13.4

* Values are expressed as percentages of control uptakes determined at 0.5 and 15 min in 100 mM-mannitol/10 mM-Hepes/Tris buffer (pH 7.5).

† Control assays were conducted at 25°C with either 0.48 mM- or 0.96 mM-$CaCl_2$ in the incubation medium.

‡ Vesicles were pre-incubated with tetracaine 30 min before the addition of $^{45}CaCl_2$.

Vitamin D status and Ca^{2+} uptake

Littermates were reared from weaning, with or without access to vitamin D_2, and brush-border membranes were isolated from the duodena when vitamin D deficiency was established. Ca^{2+} uptake by the respective membrane preparations, expressed as a function of the external Ca^{2+} concentration, is shown in Fig. 6(*a*). At concentrations between

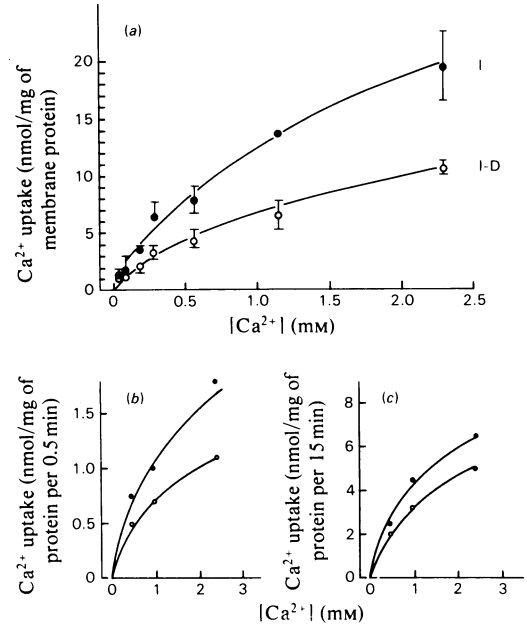

Fig. 6. *Ca^{2+} uptake by brush-border vesicles prepared from vitamin D-replete (●) and vitamin D-deficient animals (○) without (a) and with (b and c) intraperitoneal injection of 0.5 μg of $1,25(OH)_2D_3$ 16 h before killing*
(*a*) Each value, the mean of duplicate uptakes, is shown with associated error bars. The incubation medium contained 100 mM-D-mannitol, 10 mM-Hepes/Tris, 100 mM-NaCl, pH 7.5, and membrane protein (I; 29 μg/assay tube; I-D, 34 μg/assay tube). The animals had been raised from weaning on low Ca^{2+} diets with (I) or without (I-D) vitamin D. Before being killed the plasma Ca^{2+} concentrations of the replete and deficient animals were 9.5 (±0.1) mg/dl and 6.3 (±0.1) mg/dl respectively. (*b* and *c*) The incubation medium contained 100 mM-D-mannitol, 10 mM-Hepes/Tris and 100 mM-NaCl, pH 7.5. Treatment raised the plasma Ca^{2+} concentration from, 5.63 ± 0.14 to 7.68 ± 0.41 mg/dl (means ± S.E.M.).

0.048 mM and 2.3 mM, Ca^{2+} accumulation was significantly greater in membranes from vitamin D-replete animals. The observed effect was not due to variations in vesicle size or extent of vesiculation, since the intravesicular volumes calculated from D-glucose uptakes for the vitamin D-replete and vitamin D-deficient preparations were $1.31 \pm 0.2\,\mu l$/mg of protein and $1.40 \pm 0.3\,\mu l$/mg of protein respectively. The data correlate reasonably well with those reported by McLaughlin *et al.* (1980).

In subsequent experiments, Ca^{2+} uptake was also increased in vesicles isolated from vitamin D-deficient rats that had been injected intraperitoneally with $1,25(OH)_2D_3$, as compared with membrane preparations from untreated vitamin D-deficient animals (Fig. 6*b*). The difference in initial rates (0.5 min) at 2.4 mM-Ca^{2+} was approx. 33% and the vesicle volumes for the treated and untreated preparations were essentially identical (0.9 μl/mg of protein). The time course of 0.48 mM-Ca^{2+} accumulation by vesicles isolated from vitamin D-deficient and $1,25(OH)_2D_3$-treated rats is shown in Fig. 7. After 60 min of incubation at 25°C, the uptake in vesicles from vitamin D-deficient animals was approximately the same as that observed in vesicles from $1,25(OH)_2D_3$-treated animals. If Ca^{2+} uptake were passive, an effect of vitamin D-deficiency would be demonstrable as a change in the rate of uptake and not as a difference in the equilibrium value. The data therefore suggest that a major physiological effect of $1,25(OH)_2D_3$ is to increase the passive binding and/or entry of Ca^{2+}. The electrochemical potential of Ca^{2+} in intestinal cells suggests that Ca^{2+} influx is passive, whereas extrusion must be active (Mircheff *et al.*, 1977).

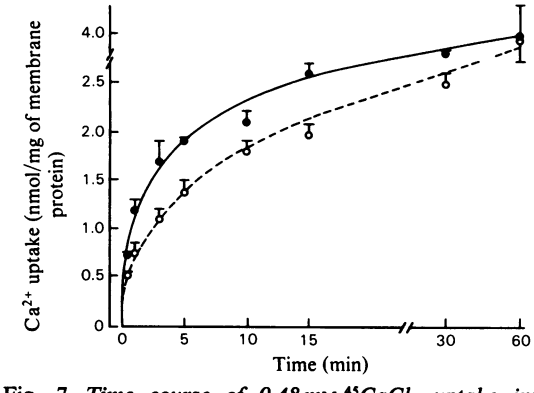

Fig. 7. *Time course of 0.48 mM-$^{45}CaCl_2$ uptake into vesicles from vitamin D-deficient animals* (○) *and vitamin D-deficient animals treated wtih $1,25(OH)_2D_3$* (●), *as described in the legend to Fig. 6(b)*

The incubation medium was the same as given in the legend to Fig. 6(*a*).

Hildmann *et al.* (1980) recently identified an ATP-dependent Ca^{2+}-pump mechanism, as well as a Ca^{2+}/Na^+-exchange system in basolateral membranes isolated from rat enterocytes. These findings are similar to those found for renal membrane preparations (Gmaj *et al.*, 1979).

Scatchard analyses of Ca^{2+} binding

A typical Scatchard plot of Ca^{2+} binding to brush-border membranes is shown in Fig. 8. The biphasic nature of the curve indicated the presence of at least two types of binding sites. The Ca^{2+}-binding data were fitted to a two-term Mass-Action equation. A least-squares computational method (Li & Katz, 1977) was employed that simultaneously averaged to the best values of n_1, n_2 and the respective association constants (K_a). The high-affinity sites, $K_a = 2.7 \pm 1.1 \times 10^4\,M^{-1}$ (mean ± S.D.) bound 3.2 ± 0.8 nmol of Ca^{2+}/mg of protein, whereas the low-affinity sites ($K_a = 60 \pm 6\,M^{-1}$) accommodated 110 ± 17 nmol of Ca^{2+}/mg of protein. The number of low-affinity sites available for Ca^{2+} binding decreased markedly in the presence of 100 mM-NaCl (Fig. 8), 0.1 mM-$LaCl_3$, 0.1 mM-Ruthenium Red, and at pH 6.6 (A. Miller & F. Bronner, unpublished work).

Membrane potential and Ca^{2+} uptake

Since Ca^{2+} is an electrolyte, one would expect its uptake to be influenced by the electrical potential

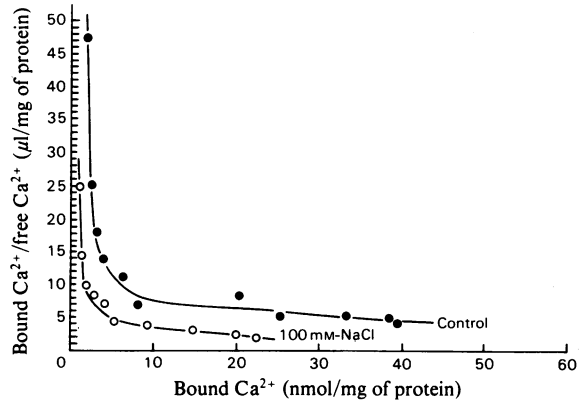

Fig. 8. *Scatchard plots of concentration-dependent Ca^{2+}-binding in brush-border vesicles*

Membranes were isolated in 100 mM-D-mannitol/10 mM-Hepes-Tris buffer (pH 7.5). Each assay tube contained the same buffer, with or without 100 mM-NaCl, 50 μg of membrane protein and the indicated concentrations of Ca^{2+}. When Ca^{2+} uptakes reached equilibrium, each assay tube was diluted (50-fold) with ice-cold water, filtered immediately, washed twice more with buffer containing 150 mM-$MgCl_2$ and the radiolabel retained by the membranes was assessed.

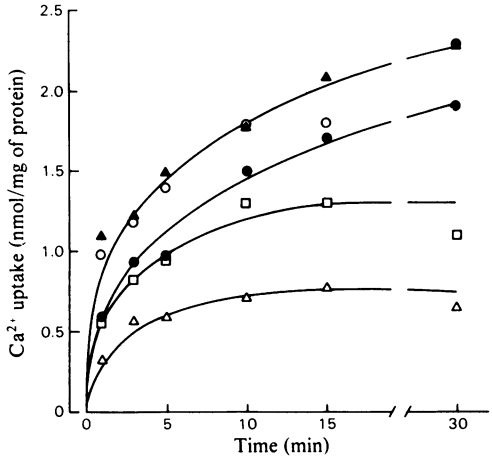

Fig. 9. *The effect of anion replacement on Ca^{2+} uptake by brush-border vesicles*

The vesicles were isolated in 100mM-mannitol/10mM-Hepes/Tris buffer (pH 7.5) and the uptakes of 0.48mM-Ca^{2+} were conducted at 25°C under gradient conditions (medium > vesicle). ●, 100mM-NaCl; ○, 100mM-NaSCN, ▲, 100mM-$NaNO_3$; △, 100mM-Na_2SO_4; □, 100mM-sodium gluconate.

difference across the membrane. The effect of anion replacement on Ca^{2+} uptake by brush-border vesicles is shown in Fig. 9. Replacement of Cl^- by the more permeant anions, SCN^- and NO_3^-, increased Ca^{2+} accumulation appreciably. Decreased uptakes were observed in the presence of the less permeant anions, SO_4^{2-} and gluconate.

In another experimental approach, valinomycin was used to induce an electrogenic movement of K^+ down its concentration gradient, establishing an electrochemical potential (inside negative) across the membrane (Henderson *et al.*, 1969). Vesicles were preloaded with 50mM-K_2SO_4 by homogenization during the initial vesiculation. As shown in Table 2, the valinomycin-induced K^+-diffusion potential had a slight but significant ($P \leq 0.05$) stimulatory effect on Ca^{2+} uptake. Since the experiment was conducted with vesicles that were not pre-equilibrated with Na^+ or K^+, the uptake values were higher than those previously observed. In the presence of an H^+ gradient (vesicles > medium) with carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone, Ca^{2+} uptake differed significantly ($P < 0.05$) from the respective control value (Table 2). Control experiments with D-glucose were conducted simultaneously and, as expected, the addition of valinomycin or carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone produced a characteristic overshoot in D-glucose uptake, whereas the equilibrium values were unaffected by the antibiotic or the proton ionophore (Table 2).

Table 2. *Effect of ionophores on D-glucose and Ca^{2+} uptakes*

The ionophore experiments with D-glucose and Ca^{2+} were conducted essentially as described by Mürer & Hopfer (1974) and as described in the Materials and methods section. Ethanolic solutions of valinomycin (10μg/mg of protein) and carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone (40–80μM) were used and the final ethanol concentration in the medium was 0.38%. $pH_{in} = 5.5$; $pH_{out} = 7.5$. When $pH_{in} = pH_{out}$, the H^+ gradient was dissipated with carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone before Ca^{2+} uptake. Results are means ± S.E.M.

Conditions	D-Glucose uptake (nmol/mg of protein) 0.5 min	15 min
D-Glucose ($Na^+_{out} > Na^+_{in}$)	0.5 ± 0.1	0.85 ± 0.1
D-Glucose + valinomycin ($K^+_{in} > K^+_{out}$)	2.1 ± 0.2	0.90 ± 0.1
D-Glucose + carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone ($pH_{in} < pH_{out}$)	2.0 ± 0.2	0.80 ± 0.1

	Ca^{2+} uptake (nmol/mg of protein) 0.5 min	15 min
Ca^{2+} ($K^+_{in} > K^+_{out}$)	1.1 ± 0.04	6.26 ± 0.21
Ca^{2+} + valinomycin ($K^+_{in} > K^+_{out}$)	1.38 ± 0.36	8.33* + 0.35
Ca^{2+} ($pH_{in} < pH_{out}$)	0.55 ± 0.02	2.4 ± 0.2
Ca^{2+} ($pH_{in} = pH_{out}$)	0.88 ± 0.1	2.6 ± 0.2
Ca^{2+} ($pH_{in} < pH_{out}$) + carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone	1.2* ± 0.1	3.8* ± 0.3

* Differs significantly ($P < 0.05$) from respective control value.

Discussion

Ca^{2+} uptake by brush-border vesicles, prepared from rat duodenal mucosa, consisted of two components: binding and/or incorporation into membrane components, as well as transport into an osmotically reactive space. Ca^{2+} uptake exhibited saturation kinetics, had a pH optimum between pH 7.5 and 8.0, was independent of metabolic energy and was sensitive to the ionic composition of the assay medium. Uptake activity was readily inhibited by Na^+ or K^+ (Fig. 5). If Na^+ were competing with Ca^{2+} for transport one would expect the greatest effect of Na^+ on rates and not on equilibrium values of uptake. The addition of 100mM-NaCl did not appear to have an osmotic effect on the intravesicular space and the equilibrium values differed substantially even after 120min of incubation at 25°C. Therefore, the data suggest that Na^+ decreases Ca^{2+} binding rather than translocation. Ca^{2+}

uptake by plasma-membrane vesicles from rat kidney was also decreased significantly by Na^+ (Moore *et al.*, 1974), but no inhibition in binding was observed with rabbit renal membranes (Palmer & Posey, 1970), rat liver plasma membranes (Schlatz & Marinetti, 1972) and guinea-pig cardiac sarcolemma (Williamson *et al.*, 1975). Katz & Repke (1967) observed an increase in the rate of Ca^{2+} uptake by cardiac microsomal fraction in the presence of low concentrations of Na^+ or K^+, whereas concentrations above 1 M inhibited Ca^{2+} uptake.

The substantial decrease in Ca^{2+} uptake by brush-border vesicles in the presence of the hexavalent cation, Ruthenium Red, may have been due to an accumulation of positive charges on the external surface of the membrane, or to a specific interaction with Ca^{2+} binding or transporting sites (Caroni *et al.*, 1977). In rat liver mitochondria, Ruthenium Red reacts specifically with mucopolysaccharides, mucoproteins or muco- or glyco-lipids (Moore & Korey, 1971). In comparison with other plasma membranes in the rat, the polar lipids of the microvillus membrane are rich in glycolipid (Forstner *et al.*, 1968*b*). Ruthenium Red and La^{3+} also both probably bind to phospholipids (Reed & Bygrave, 1974).

Scatchard-plot analyses of the binding data indicated the presence of at least two types of binding sites, high- and low-affinity. The presence of a small number of very-high-affinity binding sites, however, cannot be excluded since Ca^{2+} binding was not examined below 10 μM. Similar cation-binding characteristics have been reported for rat liver plasma membranes (Schlatz & Marinetti, 1972), cardiac sarcolemma (Williamson *et al.*, 1975) and sarcoplasmic reticulum (Chevallier & Butow, 1971).

Although it is not possible at present to identify the nature of the anionic groups in the membrane reacting with Ca^{2+}, the phosphate groups of phospholipids and carboxy groups of proteins are likely prospects. It is generally accepted that acidic phospholipids bind bivalent cations, particularly Ca^{2+}, more strongly than univalent cations. Since approx. 30% of the total lipid in the intestinal microvillus membrane is phospholipid, of which more than 50% is phosphatidylethanolamine and phosphatidylserine (Forstner *et al.*, 1968*b*), the phospholipids may account for sites with low binding affinity. The inhibition of Ca^{2+} binding by Na^+, K^+ and tetracaine, as well as by La^{3+} and Ruthenium Red, is consistent with this observation.

The effect of the membrane potential on Ca^{2+} uptake was investigated under conditions that inhibited binding markedly but not completely. Vesicles were either pre-equilibrated in 100 mM-NaCl before uptake, or uptakes were measured in the presence of Na^+ or K^+ gradients. The lipophilic anions, SCN^- and NO_3^-, rapidly penetrate the membrane in the charged form at pH 7.5 (Gamble & Lehninger, 1973) and facilitate the development of an electrochemical potential (inside negative). As indicated in Fig. 9, Ca^{2+} accumulation increased appreciably when the SCN^- and NO_3^- gradients were directed from the medium to the vesicle interior. The effects of valinomycin and carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone on Ca^{2+} uptake (Table 2) were consistent with the observed effects of the above anions, conditions that enhanced intravesicular negativity stimulated uptake. Since the uptake values obtained in the presence of the ionophores and anion gradients differed significantly from the respective control values even after 90 min of incubation at 25°C, and EGTA in the stop and wash solution would be expected to remove Ca^{2+} that was reversibly bound to the outer membrane surface, the enhanced Ca^{2+} uptakes appear to be due primarily to an increased binding of the cation to the vesicle interior. Although the data indicate that Ca^{2+} uptake is potential-sensitive, an analysis of ion fluxes, whether electrogenic or electroneutral, is difficult at present because of the substantial internal binding of the cation.

Berner *et al.* (1976) have demonstrated an electroneutral Na^+/P_i-co-transport system in brush-border vesicles isolated from rat small intestine that is unaffected by Ca^{2+}. An Na^+/H^+ antiport system has been reported in brush-border-membrane vesicles isolated from rat small intestine and kidney (Murer *et al.*, 1976) and Freedman *et al.* (1978) have presented evidence for an H^+/Ca^{2+} and Mg^{2+}/Ca^{2+} counter-transport in vitamin/D-dependent Ca^{2+} uptake by Golgi-membrane vesicles. Under our experimental conditions, neither Na^+, K^+ nor H^+ appeared to stimulate Ca^{2+} translocation.

The $1,25(OH)_2D_3$-mediated stimulation of Ca^{2+} uptake by brush-border vesicles (Figs. 6*b* and 6*c*) was similar to that reported by Rasmussen *et al.* (1979) for brush-border-membrane vesicles isolated from vitamin D-deficient chicks treated with 1α-hydroxycholecalciferol. Recently, Fuchs & Peterlik (1980) extended these observations to Na^+-dependent P_i transport. They reported that brush-border vesicles from vitamin D-deficient chicks treated with $1,25(OH)_2D_3$ exhibited a 2-fold increase in maximal velocity, with no apparent effect on carrier affinity, as compared with vesicle preparations from untreated vitamin D-deficient animals. Hildmann *et al.* (1980) have shown that $1,25(OH)_2D_3$ treatment of male rabbits stimulated and treatment with disodium 1-hydroxyethane-1,1-bisphosphonate decreased Ca^{2+} uptake by duodenal brush-border-membrane vesicles. The data indicated that Ca^{2+} permeability of the brush-border membrane is maintained by $1,25(OH)_2D_3$ and

decreased when there is interference with the endogenous production of the vitamin D metabolite.

These observations indicate that an early effect of vitamin D is to stimulate the initial rates of Ca^{2+} and P_i transport in the vitamin D-deficient animal. The increased Ca^{2+} uptakes in vitamin D-replete animals may result from an increase in the number of available binding and/or carrier sites (A. Miller, III & F. Bronner, unpublished work). A dependence on dietary vitamin D has been well established for Ca^{2+} absorption *in vivo* (Nicolaysen *et al.*, 1953), for the active transport of Ca^{2+} by gut sacs *in vitro* (Schachter & Rosen, 1959; Dowdle *et al.*, 1960) and for Ca^{2+} uptake by isolated cells *in vitro* (Freund & Bronner, 1975).

Since Ca^{2+} uptake involves binding to cellular components, as well as transport into the intravesicular space, cation movement may be associated with a specific carrier or Ca^{2+}-binding membrane component. Nellans & Kimberg (1978) suggested that a carrier-mediated Ca^{2+}-uptake component exists at the small-intestinal microvillus membrane. The presence of such a component is indicated by the demonstration of saturation kinetics, the presence of a small number of Ca^{2+}-specific high-affinity binding sites with fairly high affinity and the inhibition of the influx component by alkaline-earth cations demonstrated by Nellans & Kimberg (1978).

A vitamin D-inducible Ca^{2+}-binding protein ('$CaBP_x$') has recently been isolated from the 100000*g* supernatant prepared from purified brush borders of rat duodenal mucosa (Miller *et al.*, 1979). This protein differs from the cytosolic vitamin D-dependent Ca^{2+}-binding protein (Wasserman & Feher, 1977) in electrophoretic mobility, tissue location and apparent molecular weight. Since this Ca^{2+}-binding protein is found in the intestinal brush border, is induced by $1,25(OH)_2D_3$ and induction appears to parallel an increase in Ca^{2+} uptake in vitamin D-deficient membrane preparations, it may play a role in Ca^{2+} translocation across the intestinal brush border. In an energy-independent process, Ca^{2+} may be bound, transported and subsequently released to a large number of internal binding sites. However, it must be recognized that the Ca^{2+}-binding protein could also act as a receptor or recognition site, and that Ca^{2+} permeability may be dependent on membrane lipid structure rather than changes in membrane protein content, as suggested by Max *et al.* (1978) and Rasmussen *et al.* (1979). Therefore, the exact role of the Ca^{2+}-binding protein in mediating Ca^{2+} movement across the brush-border membrane remains to be established.

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References

Balzer, H. (1972) *Naunyn-Schmiedebergs Arch. Pharmakol.* **274**, 256–272

Berner, W., Kinne, R. & Mürer, H. (1976) *Biochem. J.* **160**, 467–474

Bray, G. A. (1960) *Anal. Biochem.* **1**, 279–285

Bronner, F. & Freund, T. (1975) *Am. J. Physiol.* **229**, 689–694

Caroni, P., Gazzotti, P., Vuilleumier, P., Simon, W. & Carafoli, E. (1977) *Biochim. Biophys. Acta* **470**, 437–445

Chevallier, J. & Butow, R. A. (1971) *Biochemistry* **10**, 2733–2737

Dahlquist, A. (1974) *Anal. Biochem.* **7**, 18–25

Dowdle, E. B., Schachter, D. & Schenker, H. (1960) *Am. J. Physiol.* **198**, 269–274

Fleckenstein, A., Tritthart, H., Fleckenstein, B., Herbst, A. & Grün, G. (1969) *Pflueger's Arch.* **307**, R25

Forstner, G. G., Sabesin, S. M. & Isselbacher, K. J. (1968*a*) *Biochem. J.* **106**, 381–390

Forstner, G. G., Tanaka, K. & Isselbacher, K. H. (1968*b*) *Biochem. J.* **109**, 51–59

Freedman, R. A., MacLaughlin, J. & Weiser, M. M. (1978) *Gastroenterology* **74**, 1123

Freedman, R. A., Weiser, M. & Isselbacher, K. J. (1977) *Proc. Natl. Acad. Sci. U.S.A.* **74**, 3612–3616

Freund, T. & Bonner, F. (1975) *Science* **190**, 1300–1302

Fuchs, R. & Peterlik, M. (1980) *Biochem. Biophys. Res. Commun.* **93**, 87–92

Gamble, J. G. & Lehninger, A. L. (1973) *J. Biol. Chem.* **248**, 610–618

Gmaj, P., Mürer, H. & Kinne, R. (1979) *Biochem. J.* **178**, 549–557

Hasse, W., Schäfer, A., Mürer, H. & Kinne, R. (1978) *Biochem. J.* **172**, 57–62

Henderson, P. J. F., McGivan, J. D. & Chappell, J. B. (1969) *Biochem. J.* **111**, 521–535

Hildmann, B., Schmidt, A., Storelli, C. & Mürer, H. (1980) *Proc. Int. Union Physiol. Sci.* **14**, 472

Hopfer, U. (1977) *Am. J. Physiol.* **233**, E445–E449

Hopfer, U., Nelson, K., Perrotto, J. & Isselbacher, K. J. (1973) *J. Biol. Chem.* **248**, 25–32

Hopfer, U., Sigrist-Nelson, K., Ammann, E. & Mürer, H. (1976) *J. Cell Physiol.* **89**, 805–810

Isselbacher, K. J. (1974) *Harvey Lect.* **69**, 197–221

Katz, A. M. & Repke, D. I. (1967) *Circ. Res.* **21**, 767–775

Kessler, M., Acuta, O., Storelli, C., Mürer, H., Muller, M. & Semenza, G. (1978) *Biochim. Biophys. Acta* **506**, 136–154

Kinne, R. (1976) *Curr. Top. Membr. Transp.* **8**, 209–267

Li, S.-T. & Katz, E. P. (1977) *Calcif. Tissue Res.* **22**, 275–284

Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951) *J. Biol. Chem.* **193**, 265–275

Max, E. E., Goodman, D. B. P. & Rasmussen, H. (1978) *Biochim. Biophys. Acta* **511**, 224–239

McLaughlin, J. A., Weiser, M. M. & Freedman, R. A. (1980) *Gastroenterology* **78**, 325–332
Miller, A., III, Ueng, T.-H. & Bronner, F. (1979) *FEBS Lett.* **103**, 319–322
Mircheff, A. K., Walling, M. N., van Os, C. H. & Wright, E. M. (1977) in *Vitamin D: Biochemical, Chemical and Clinical Aspects Related to Calcium Metabolism* (Norman, A. W., Schaefer, K., Coburn, J. W., DeLuca, H. F., Fraser, D., Grigoleit, H. G. & Herrata, V., eds.), pp. 281–283, De Gruyter, Berlin and New York
Moore, C. L. & Korey, S. R. (1971) *Biochem. Biophys. Res. Commun.* **42**, 298–305
Moore, L., Fitzpatrick, D. F., Chen, T. S. & Landon, E. J. (1974) *Biochim. Biophys. Acta* **345**, 405–418
Mürer, H. & Hopfer, U. (1974) *Proc. Natl. Acad. Sci. U.S.A.* **71**, 484–488
Mürer, H., Hopfer, U. & Kinne, R. (1976) *Biochem. J.* **154**, 597–604
Nellans, H. N. & Kimberg, D. V. (1978) *Gastroenterology* **74**, 1138
Nicolaysen, R., Eeg-Larsen, N. & Malm, O. J. (1953) *Physiol. Rev.* **33**, 424–444
Palmer, R. F. & Posey, V. A. (1970) *J. Gen. Physiol.* **55**, 89–103
Papahadjopoulos, D. (1972) *Biochim. Biophys. Acta* **265**, 169–186
Rasmussen, H., Fontaine, O., Max, E. E. & Goodman, B. P. (1979) *J. Biol. Chem.* **254**, 2993–2999
Reed, K. C. & Bygrave, F. L. (1974) *Biochem. J.* **140**, 143–155
Sacktor, B. (1977) *Curr. Top. Bioenerg.* **6**, 39–83
Schachter, D. & Rosen, S. M. (1959) *Am. J. Physiol.* **196**, 357–362
Schlatz, L. & Marinetti, G. V. (1972) *Biochim. Biophys. Acta* **290**, 70–83
Schmitz, J., Preiser, H., Maestracci, D., Ghost, B. K., Cerda, J. J. & Crane, R. K. (1973) *Biochim. Biophys. Acta* **323**, 98–112
Sigrist-Nelson, K., Mürer, H. & Hopfer, U. (1975) *J. Biol. Chem.* **250**, 5674–5680
Ueng, T.-H., Golub, E. E. & Bronner, F. (1979) *Arch. Biochem. Biophys.* **196**, 624–630
Wasserman, R. H. & Feher, J. J. (1977) in *Calcium Binding Proteins and Calcium Function* (Wasserman, R. H., Corradino, R. A., Carafoli, E., Kretsinger, R. H. MacLennan, D. H. & Singer, F. L., eds.), pp. 293–302, Elsevier/North-Holland, New York
Williamson, J. R., Woodrow, M. L. & Scarpa, A. (1975) *Recent. Adv. Stud. Card. Struct. Metab.* **5**, 61–71