

CALCIUM ABSORPTION BY THE MIDGUT OF THE BLOWFLY, *CALLIPHORA VICINA*

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SUMMARY

The isolated midgut of the adult blowfly, *Calliphora vicina*, can be maintained under internal perfusion for over 6 h, and calcium absorption measured by including ^{45}Ca in the perfusing saline with $[^3\text{H}]\text{inulin}$ as a volume marker. The midgut has a considerable capacity to transport calcium from the lumen (L) to the bathing saline (BS) against its electrochemical gradient and in the absence of an appreciable net water flux across the gut. Calcium absorption (L-BS) shows saturation kinetics, is totally and reversibly inhibited by metabolic poisons and is accompanied by a negligible backflux (BS-L). It is concluded that the midgut of *C. vicina* is capable of active calcium transport and that the entire transepithelial calcium flux occurs *via* a transcellular route. This contrasts with the mammalian duodenum, where absorption occurs *via* a combination of transcellular and paracellular routes.

INTRODUCTION

The mechanisms and regulation of calcium transport across intestinal epithelia have been extensively studied in mammals (Levine, Walling & Coburn, 1982) and, to a lesser extent, in birds (Hurwitz, Bar & Cohen, 1973). However, the histology of vertebrate gut is complex, and to ensure adequate oxygenation of the mucosa *in vitro*, everted sacs have been much used, but the geometry of the villi is then disrupted. Of greater concern is the effect of the disrupted blood supply on removal of absorbed nutrients from the epithelium. *In vitro*, the complex capillary network and lymphatic system of the villi are disrupted and absorbed substances must move passively through the mass of the gut without the benefit of the normal bulk flow of blood.

There have been no comparable studies of calcium absorption in invertebrates. Insects provide particularly amenable subjects since their open circulatory systems can be realistically imitated by the situation *in vitro*, though the tracheal system is disrupted and so the tissue must obtain oxygen from the saline in which it is bathed. However, the midgut is structurally simple: it consists of a single layer of epithelial

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cells surrounded by a loose basement lamina in which muscle fibres and tracheae are embedded (De Priester, 1971), so oxygen access from the saline is unlikely to pose problems. Thus, the internally perfused insect gut mimics the situation *in vivo* at least insofar as the route for ion transport is concerned. In this study, the internally perfused insect midgut has been used to investigate calcium transport by the midgut epithelium.

A preliminary account of part of this work has already been published (Taylor, 1984a).

MATERIALS AND METHODS

Animals

Newly emerged adult blowflies, *Calliphora vicina* R.D. (formerly *C. erythrocephala*), were collected from a laboratory culture and then kept on a diet of sugar and water at room temperature. Female flies between 7 and 14 days after emergence were used for all experiments.

Calcium absorption

The bathing saline had the following composition (mmol l^{-1}): PIPES (piperazine-*N,N'*-bis[2 ethanesulphonic acid]), 5; NaOH, 20.2; NaCl, 152; KCl, 10; CaCl_2 , 2; MgCl_2 , 2; NaH_2PO_4 , 4; malic acid, 2.8; glutamic acid, 2.9; proline, 7; glucose, 10; it was isosmotic with *C. vicina* haemolymph ($358 \text{ mosmol l}^{-1}$). A trace of phenol red was added to provide a continuous check on the pH which was 7.2. The perfusing saline had the same composition as the bathing saline except that the concentration of NaH_2PO_4 was reduced to 1 mmol l^{-1} to avoid precipitation of calcium phosphate at high perfusing calcium concentrations; the concentration of NaOH was reduced to 18 mmol l^{-1} to maintain the pH at 7.2. [^3H]inulin (Amersham, specific activity = $2.48 \text{ Ci mmol}^{-1}$) was included in the perfusing saline at a final activity of about $3200 \text{ c.p.m. } \mu\text{l}^{-1}$ and $^{45}\text{CaCl}_2$ (Amersham, specific activity = $1.6\text{--}2.3 \text{ Ci mmol}^{-1}$) at a final activity of about $350 \text{ c.p.m. } \mu\text{l}^{-1}$ ($^3\text{H}/^{45}\text{Ca}$ ratio of about 9). The CaCl_2 concentration of the perfusing saline was varied between 0.5 and 37 mmol l^{-1} without correction for osmotic activity. The resultant transepithelial osmotic gradient did not appreciably affect the permeability of the midgut to [^3H]inulin (not shown) or to Ca (Fig. 3). In substrate-free salines, glucose, proline, glutamate and malate were all omitted; the pH was corrected with NaOH and the osmolarity maintained by the addition of NaCl. Otherwise they had the same compositions as given above.

The midgut was cannulated at the proventriculus (Fig. 1) and the lumen flushed with perfusing saline containing a trace of the dye, azorubin-S, to check for leaks. This saline was then replaced with labelled perfusing saline and the cannula connected to a $100\text{-}\mu\text{l}$ gas-tight syringe (Hamilton). The internal perfusion was driven by a motorized Agla micrometer syringe drive at between 0.92 and $0.96 \mu\text{l min}^{-1}$; the precise rate was measured for each experiment. All guts were perfused for 10 min before collecting any samples to ensure that the entire lumen (volume = $1.92 \pm 0.12 \mu\text{l}$, mean $\pm \text{s.e.}$, $N = 27$) had been flushed at the operational rate before any measurements were made. This interval was also sufficient for

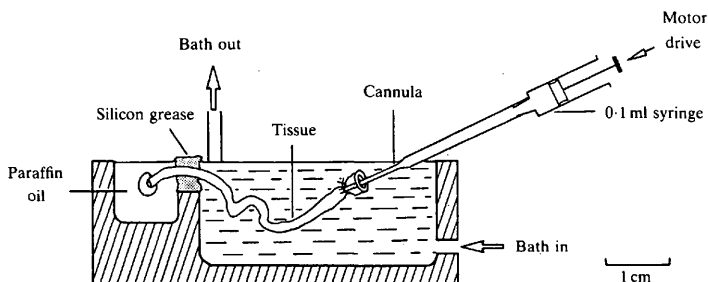


Fig. 1. Internal perfusion system. The bath temperature was monitored with a miniature, thermistor thermometer and maintained at 19°C by a cooling stage mounted beneath the bath. [^3H]inulin and $^{45}\text{CaCl}_2$ were included in the perfusing saline. The bath (volume = 2.2 ml) was perfused with oxygenated bathing saline at about 0.9 ml min^{-1} and the gut lumen perfused with perfusing saline at about $0.94 \mu\text{l min}^{-1}$. Calcium absorption was calculated from the change in the ratio of $^3\text{H}/^{45}\text{Ca}$ between the saline and the droplet collected under liquid paraffin.

calcium absorption to reach a steady state (not shown). The bath had a volume of 2.2 ml and was perfused with oxygenated bathing saline at 19°C at about 0.9 ml min^{-1} ; the exact rate was determined for each experiment.

To measure calcium absorption, the change in the ratio of [^3H]inulin to ^{45}Ca after it passed along the gut was determined. Such a protocol requires that inulin is restricted to the gut lumen and neither enters, nor is adsorbed to, the epithelium. Use of [^3H]inulin as a 'volume marker' can be justified since although small amounts, up to 3.5%, of the ^3H label perfusing the lumen cross the midgut epithelium, this leak is unaffected by factors which influence calcium transport. Nor is the ^3H label appreciably adsorbed to the epithelium. The small amount (about 330 c.p.m.) that is adsorbed reaches equilibrium within 5 min and is not, therefore, a source of error in these experiments, in which the first samples were collected after 10 min. The ^3H activities of aliquots of bathing saline were measured and from these the leakiness of the midgut to ^3H determined. Since 43% of the ^3H label was lost when the bathing saline was dried at room temperature, it seems very likely that the leak of ^3H label is largely attributable to movement of H_2O which had become labelled by exchange of ^3H with [^3H]inulin. An appropriate correction was applied to the ^3H activity of perfusate samples to take account of the small ^3H leak across intact guts. Guts in which more than 3% of the ^3H label crossed into the bathing saline were assumed to be damaged and were rejected.

The open end of the midgut was held under liquid paraffin in the second chamber (Fig. 1) which was separated from the bathing saline by silicon grease. Samples of $1 \mu\text{l}$ of perfusate were collected under liquid paraffin in microcaps (Cam Lab) and transferred to 5 ml 'Biofluor' liquid scintillation cocktail (N.E.N.). Radioactivity was measured in a Packard Tri Carb model 3225 liquid scintillation counter and the ^{45}Ca and ^3H activities of each sample calculated after correction for background activity and overlap of the energy spectra. All samples were equally quenched. Calcium absorption was calculated from the change in the ratio of $^3\text{H}/^{45}\text{Ca}$ between the stock and perfusate after correction for the small ^3H label leak across intact guts.

Calcium backflux

To determine the flux from bathing saline to gut lumen, ^{45}Ca was included in the bathing saline ($3000 \text{ c.p.m. } \mu\text{l}^{-1}$). The lumen was perfused as before, though with neither ^{45}Ca nor $[^3\text{H}]\text{inulin}$ in the perfusing saline. Backflux was monitored by collecting $2\text{-}\mu\text{l}$ samples of perfusate, and the rate at which ^{45}Ca appeared was taken as a measure of calcium backflux. To economize in the use of ^{45}Ca , a pool of 10 ml of bathing saline was recirculated for up to 2 h . Previous experiments had shown that calcium absorption was unaffected by recycling the saline.

Transepithelial potentials

For measurements *in situ*, flies were dissected from the dorsal surface and the abdominal air sacs removed to expose the gut, the animal was held open with glass pins and the entire preparation flooded with bathing saline. Transepithelial potentials (PDs) were measured with low resistance electrodes (about $10 \text{ M}\Omega$) filled with $3 \text{ mol l}^{-1} \text{ KCl}$ and the electrical connection made with chloridized silver wire. The reference electrode was earthed. Potentials were measured with a Keithley model 300B electrometer. Between each impalement, the asymmetry potential was determined with both electrodes in the saline and this subtracted from the measured PD. Otherwise, all procedures were as described by Dow (1981).

For measurements *in vitro*, midguts were set up for internal perfusion and the PD measured with the earthed reference electrode in the bathing saline and the other in the drop of perfusate under liquid paraffin.

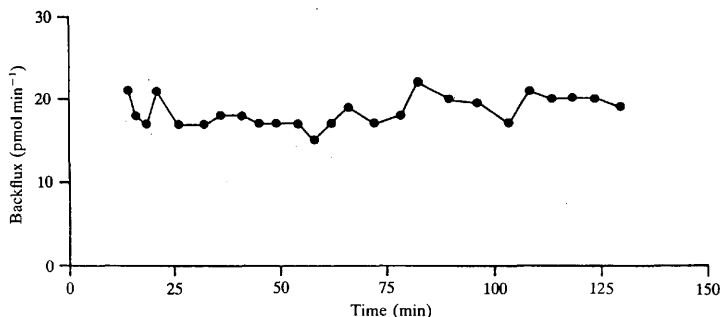


Fig. 2. Calcium backflux across the isolated midgut. Results from a single midgut are shown. The calcium flux from the bathing saline to the lumen was monitored for over 2 h of perfusion. The perfusing saline contained $8 \text{ mmol l}^{-1} \text{ CaCl}_2$, other constituents of the perfusing and bathing salines were as given in the text.

RESULTS

Calcium transport in vitro

In the salines used for these experiments, the isolated midgut remains motile, and will transport calcium for over 6 h with only a few percent decline in the rate. From Fig. 2, it is clear that a steady-state backflux must be attained within a few minutes (certainly before the first samples were collected), since backflux measurements were stable for over 2 h of perfusion. This is consistent with the suggestion that the pool from which the ^{45}Ca had come equilibrated rapidly with the bathing saline. Therefore, the backflux rate was calculated by assuming that the calcium appearing in the lumen had the same specific activity as that in the bathing saline.

The characteristics of calcium transport are shown in Fig. 3: absorption (lumen to bathing saline, L-BS) and backflux (BS-L). Since calcium activities were not measured in these experiments and the calcium concentration of the perfusing saline must change as it passes along the gut and calcium is absorbed, the results are not amenable to rigorous kinetic analysis. Nevertheless, it is plain that calcium absorption was saturable and had a maximum transport capacity of $2.4 \text{ nmol min}^{-1}$.

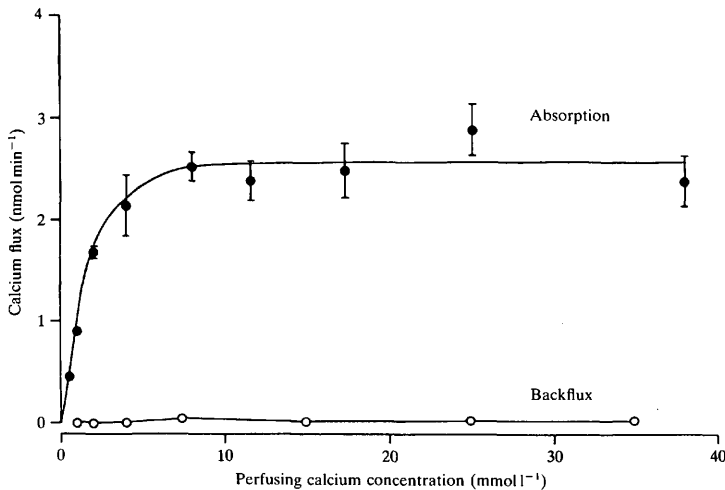


Fig. 3. Calcium fluxes across the entire midgut. After 10 min of internal perfusion, steady state absorptive and back fluxes were attained. Samples of perfusate or bathing saline were then collected at 2–3 min intervals during the next 30 min. These flux measurements were averaged for each gut. All points shown in this figure are the means of between 3 and 9 guts \pm s.e. Calcium absorption is the flux from lumen to bathing saline and the backflux is the flux from bathing saline to the lumen. In all experiments the bathing saline contained $2 \text{ mmol l}^{-1} \text{ CaCl}_2$.

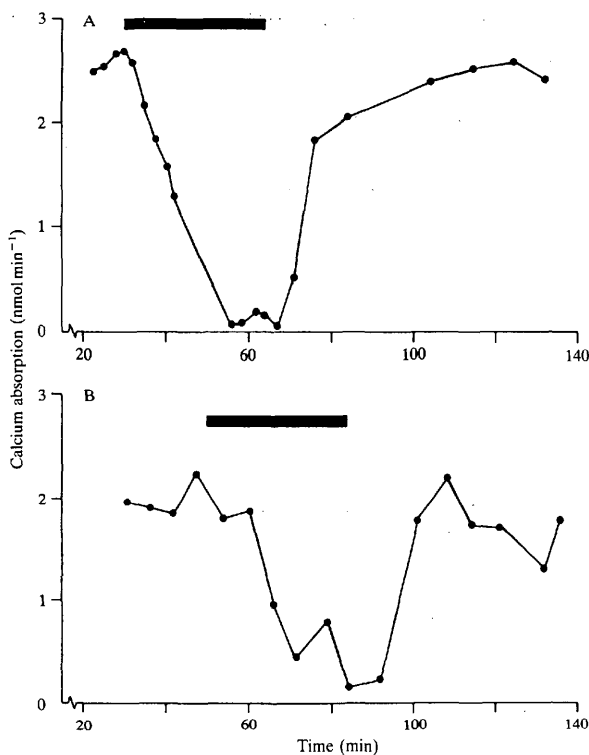


Fig. 4. Effects of 2,4-dinitrophenol (DNP) on calcium absorption. The results from a single midgut are shown. 0.1 mmol l^{-1} DNP was supplied in the bathing saline for the times shown by solid bars. (A) 4 mmol l^{-1} perfusing calcium. (B) 37 mmol l^{-1} perfusing calcium. In both experiments, the bathing saline contained $2 \text{ mmol l}^{-1} \text{ CaCl}_2$. At both perfusing calcium concentrations, DNP totally and reversibly inhibited calcium absorption.

Calcium absorption was essentially complete at perfusing calcium concentrations less than 2 mmol l^{-1} , even though the luminal perfusion rates were much faster than would occur in an intact fly. At each of the perfusing calcium concentrations tested, the backflux of calcium was negligible and never exceeded 2% of absorbed calcium.

Addition of 0.1 mmol l^{-1} 2,4-dinitrophenol (DNP) to the bathing saline wholly and reversibly inhibited calcium absorption both at saturating ($37 \text{ mmol l}^{-1} \text{ Ca}$, Fig. 4B) and sub-saturating concentrations ($4 \text{ mmol l}^{-1} \text{ Ca}$, Fig. 4A) of perfusing calcium. Other metabolic inhibitors, NaCN (2 mmol l^{-1}) and sodium arsenate

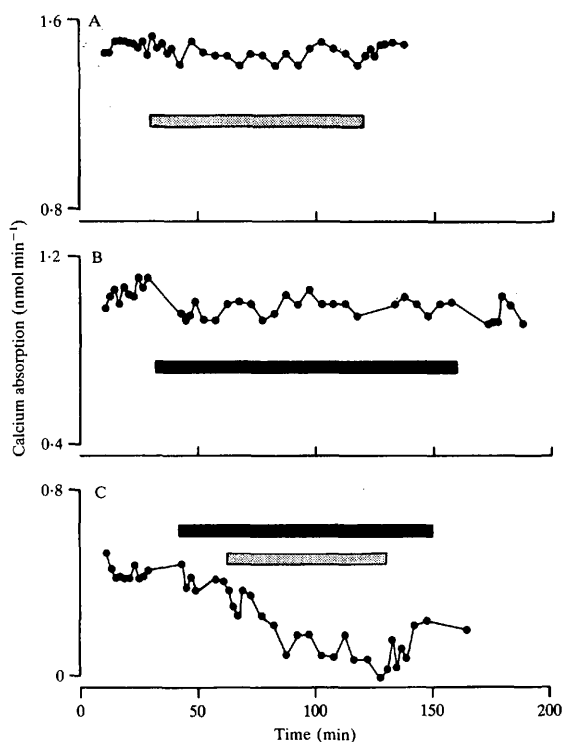


Fig. 5. Effects of substrate removal on calcium absorption. Each figure shows the results from a single gut which was either bathed in substrate-free bathing saline (shaded bars) or perfused with substrate-free perfusing saline (solid bars). In all experiments, the CaCl_2 concentrations of the perfusing and bathing salines were 4 mmol l^{-1} and 2 mmol l^{-1} respectively. Calcium absorption is inhibited when substrates are absent from both salines (C) but not when they are omitted from only the bathing (A) or perfusing (B) saline.

(10 mmol l^{-1}), in the bathing saline also reversibly inhibited calcium absorption (not shown).

Removal of metabolic substrates from either the perfusing or bathing salines did not affect calcium absorption; but when they were omitted from both salines, absorption was inhibited (Fig. 5).

From the change in concentration of $[^3\text{H}]$ inulin as it passed along the midgut, net water absorption was estimated to be less than 1 % of the total saline flow along the midgut.

By first measuring calcium absorption by the entire midgut, and then with only

the anterior half left in the bathing saline, the relative transport capacities of the two halves of the midgut were estimated. Normally the posterior midgut will see a reduced perfusing calcium concentration since some calcium will be absorbed by the anterior half. However, when perfused with saline containing 8 mmol l^{-1} calcium, the transport mechanism was saturated along the entire length of the midgut. Under these conditions, $44 \pm 5\%$ (mean \pm s.e., $N = 4$) of calcium absorbed was accounted for by the anterior half, suggesting that both halves of the midgut are about equally capable of calcium absorption.

Transepithelial potentials

The lumen of the midgut has a negative potential when recorded either *in vivo* or *in vitro*. *In vivo*, the PDs were as follows, [mean \pm s.e., (N); all expressed as the potential in the haemolymph or bath relative to the lumen]: thoracic ventriculus $+21 \pm 5 \text{ mV}$ (5), abdominal ventriculus $+26 \pm 5 \text{ mV}$ (4), mid-helicoid midgut $+21 \pm 5 \text{ mV}$ (6) and post-helicoid midgut $+17 \pm 3 \text{ mV}$ (6) (terminology of Graham-Smith, 1934). *In vitro*, a PD of $+41 \pm 4 \text{ mV}$ (4) was recorded when the lumen was perfused with saline containing 2 mmol l^{-1} calcium and when the perfusing calcium concentration was 20 mmol l^{-1} , the PD was $+39 \pm 3 \text{ mV}$ (4). The PD was abolished when 0.1 mmol l^{-1} DNP was added to the bathing saline and fully recovered after removal of the inhibitor.

The positive PD will oppose net movement of calcium from the lumen to the bathing saline. From the Nernst equation, if the calcium distribution across the midgut were wholly passive, the luminal calcium activity must exceed that in the bathing saline by a factor of about 24 for a net flux from lumen to bathing saline to occur. In none of the experiments reported was the ratio of calcium activities sufficient to lead to passive net calcium absorption. Since there is negligible net water absorption, any effects of solvent drag are insignificant. It therefore seems reasonable to conclude that active calcium absorption occurs.

DISCUSSION

The isolated midgut of *C. vicina* can actively absorb calcium at a maximal rate of $2.4 \text{ nmol min}^{-1}$, an enormous transport capacity when compared to the calcium content of a recently emerged adult (220 nmol) or to a mature batch of eggs (87 nmol). Active calcium absorption has also been reported to occur across the isolated midgut of the Lepidopteran, *Hyalophora cecropia*. In this animal, however, active transport merely aids the diffusion of calcium down its electrochemical gradient (Wood & Harvey, 1976).

In mammals, net calcium absorption by the small intestine is usually the difference between two relatively large unidirectional fluxes: absorption and secretion (Walling & Rothman, 1969). Calcium absorption by the duodenum is best described by curvilinear kinetics (Pansu, Bellaton & Bronner, 1981; Walling & Rothman, 1969). However, the two components cannot be directly equated with the transcellular and paracellular routes for calcium absorption. Undoubtedly, the saturable component of the transepithelial flux corresponds to a saturable entry mechanism to enterocytes (Miller & Bronner, 1981; Nellans & Kimberg, 1978), but

in addition, a fraction of the transcellular flux may be accounted for by a non-saturable entry mechanism (O'Donnell & Smith, 1973; Papworth & Patrick, 1970). The extrusion mechanism is presumably never normally saturated. The paracellular flux probably occurs through leaky tight junctions. Although the small intestine is a 'leaky' epithelium (Frizzell & Schultz, 1972), it is not uniformly leaky. The complexity, numbers and depth of tight junctions between epithelial cells increase as they migrate from the crypts to the tips of the villi (Madara, Trier & Neutra, 1980). Furthermore, lanthanum and barium ions penetrate the simple tight junctions found between many of the goblet cells of the villus, but do not penetrate the more complex junctions found between absorptive cells (Madara & Trier, 1982). These results suggest that within the intestinal epithelium there may be foci of high ionic permeability. It is not known whether these foci provide the main route for paracellular calcium movement.

In contrast to the situation in mammals, net calcium absorption by *C. vicina* is determined almost exclusively by the lumen to bathing saline flux (L-BS). There is no significant backflux (BS-L). In further contrast to mammals, the kinetics of calcium absorption are fully saturable and the flux is completely inhibited by metabolic poisons. Even at very high perfusing calcium concentrations (37 mmol l^{-1}), when any passive diffusion would be expected to be at its greatest, the L-BS flux is totally abolished by DNP. This implies that all the calcium that crosses the epithelium does so in an energy-dependent manner. The only tenable explanation of these findings is that calcium absorption by *C. vicina* midgut occurs *via* a wholly transcellular route. Calcium must be pumped out of the cell against the electrochemical gradient and transport is, therefore, presumably ultimately saturable. However, it has not been established by these experiments whether calcium extrusion from the cell or entry at the luminal surface is the rate-limiting step, it is not therefore clear whether calcium entry is a saturable process.

Both *C. vicina* midgut and mammalian duodenum have the capacity to transport considerable amounts of calcium through the cells of their epithelia, and must simultaneously regulate the cytoplasmic calcium activity of the transporting cells. The intracellular calcium activity of duodenum or insect midgut engaged in calcium transport has not been measured though circumstantial evidence suggests that it is unlikely to be much greater than $10^{-6} \text{ mol l}^{-1}$. In all cells which have been studied, the resting cytoplasmic calcium activity is less than $10^{-6} \text{ mol l}^{-1}$; for example, in unstimulated blowfly salivary glands the cytoplasmic calcium activity is less than $10^{-7} \text{ mol l}^{-1}$ (Berridge, 1980). Deleterious effects result if it rises much above this. Several glycolytic enzymes are inhibited if the calcium activity exceeds ($10^{-4} \text{ mol l}^{-1}$) (Kretsinger, 1976). The intestinal microvillus cytoskeleton is dis-aggregated at greater than $10^{-6} \text{ mol l}^{-1}$ calcium (Glenney, Bretscher & Weber, 1980). At concentrations above $10^{-6} \text{ mol l}^{-1}$, mitochondria accumulate calcium which not only reduces ATP production, but if prolonged, will also overload them (Nicholls, 1978). Calmodulin, the ubiquitous mediator of many of the second-messenger effects of calcium, binds calcium in the micromolar range (Cheung, 1980), and could not therefore be an effective signal if the intracellular calcium concentration were to increase beyond this. Despite the lack of direct evidence, it seems reasonable to suppose that the intracellular free calcium concentration in

duodenal and midgut epithelial cells is unlikely to exceed $10^{-6} \text{ mol l}^{-1}$ during calcium transport.

Assuming that the entire weight of the midgut can be equated with the cytosol of the cells involved in calcium transport and that the cytosolic calcium concentration is $10^{-6} \text{ mol l}^{-1}$, then during calcium transport, the hourly flux of calcium exceeds the cytoplasmic pool through which it is transported by a factor of some 150 000. How the epithelial cells maintain their own cellular calcium homeostasis in the face of this massive transcellular flux is a subject of speculation for both invertebrate and vertebrate physiologists (Levine *et al.* 1982). This aspect of transcellular calcium transport by *C. vicina* midgut is discussed in another paper (Taylor, 1984b) in which it is suggested that calcium entry at the apical membranes is regulated by cytoplasmic calcium so that calcium entry never exceeds the capacity of the extrusion mechanisms to pump it out of the cell.

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