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MEASUREMENT OF SODIUM ION CONCENTRATION IN THE UNSTIRRED LAYER OF RAT SMALL INTESTINE BY POLYMER Na^+-SENSITIVE ELECTRODES

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The concentration of sodium ion at the surface of rat small intestine both in vitro and in vivo was measured with plastic polymer sodium ion sensitive electrodes. In vitro the surface sodium ion concentration [Na_s^+] was found to be significantly higher than bulk bathing solutions of 25 mM concentration. This value could be increased by the addition of glucose to the medium and was significantly higher in the jejunum than in the ileum. Ouabain, deoxycholic acid and dithiothreitol all reduced the [Na_s^+] under in vitro conditions. In vivo, very high values for [Na_s^+] were found in the jejunum (approx. 80 mM) when the bulk concentration was 25 mM, indicating a substantial local accumulation of sodium ion at or near the brush border. This could be reduced by omission of glucose from the buffer and further reduced when magnesium was the substituent cation rather than choline. Consequently, in vivo, an appropriately orientated sodium ion gradient persists in the face of adverse tissue to bulk solution concentration gradients and potentially explains why solute absorption occurs under these circumstances. The further reduction of the correctly aligned sodium ion gradient, as required by the gradient hypothesis, by magnesium-substituted buffers indicates that there is no real need to postulate additionally a sodium-independent magnesium-sensitive glucose transport system in vivo.

Introduction

The intestinal absorption of sugars and many other dietary constituents is thought to be mediated by gradients of sodium ion concentration across the luminal membrane of the enterocyte. This sodium gradient hypothesis, based largely on the work of Crane [1] proposed that active transport of sodium ions at the basolateral membrane lowers the intracellular sodium ion concentration sufficiently to provide a chemical gradient at the mucosal membrane. It is clear that this mechanism would depend on a relatively high extracellular sodium ion concentration at the outer face of the mucosal membrane. In the in vitro state, this condition can be easily fulfilled, simply by maintaining a high concentration in the bathing solutions. Under these circumstances, transport dependence on extracellular sodium ion concentration has been demonstrated repeatedly [2,3]. However, in the in vivo state, it is not clear that the luminal sodium ion concentration will always be higher than that intracellularly, especially when low sodium ion containing buffers are perfused through the lumen. In fact, verification of the sodium gradient hypothesis in vivo is difficult: sodium-dependent solute transport can be shown either transiently [4] or not at all [5–10]. Clearly, there is dependence in vivo on the luminal sodium ion content [8] but whether this is in the form proposed by the sodium ion gradient hypothesis is uncertain.

When the lumen is perfused in vivo with sodium ion concentrations at or about the estimated intracellular concentration, sodium-linked solute

transport persists [8]. For this reason, it has been proposed that verification of the sodium gradient hypothesis relies almost exclusively on in vitro data and may not explain solute transport in vivo, indeed that there are other sodium-independent systems [11]. On the other hand, some reconciliation of in vivo data with the model would be provided, if the sodium ion concentration at the enterocyte border differed from the bulk solution concentration and to a large extent, the bulk solution did not reflect actual conditions prevailing at the mucosal side of the proposed gradient. The presence of unstirred layers is well known and it is possible that these may act as a reservoir for sodium ion, after recycling through the tight junctions, as has been proposed in Alvarado's model [12]. In this model, the recycling of sodium ions would make any stoichiometric calculations difficult since ions would recycle and engage with possibly several different solute molecules before exiting from the system. Perhaps more importantly, it would explain why almost no studies have demonstrated 'gradient-like' dependence in vivo.

For this reason, an attempt was made to verify Alvarado's hypothesis. Studies were undertaken with sodium ion sensitive electrodes in an attempt to measure the sodium ion concentration at the brush border membrane. This was carried out with all-plastic surface sodium ion electrodes, both in vitro and in vivo, in the rat small intestine under various experimental circumstances.

Methods

1. Physiological procedures

(a) In vitro. Sections of freshly exised proximal jejunum or distal ileum from male Wistar rats (250 g) were incubated in both 10 mM glucose-containing or glucose-free Krebs-Henseleit bicarbonate buffer maintained at 37°C and gassed with 95 : 5% (v/v) O_2/CO_2 gas mixture. Proximal jejunum was defined as 10 cm distal to the ligament of Treitz and distal ileum was regarded as being 10 cm proximal to the ileocaecal valve. Fresh tissue taken from rats under sagatal anaesthesia (15 mg/kg) was loosely pinned onto the floor of cork-lined incubation vessels so as to provide stability for measurement without preventing access of the oxygenated Ringer to both mucosal and serosal surface. Each tissue flask was individually and continuously gassed with the mixture. Tissues were incubated either in normal (high sodium) Ringer or 'low sodium' Ringer in which sodium ion was replaced either with choline chloride or magnesium sulphate to give a final sodium ion concentration of 25 mmol/l.

(b) In vivo. Similar areas of proximal jejunum and distal ileum were located in male Wistar rats (250 g) maintained under sagatal (15 mg/kg) anaesthesia. For location of the distal ileum a mid-line abdominal incision was made in the lower abdomen and the ileocaecal valve found. The procedure for proximal jejunum location involved first cannulating the duodenum at the pyloric antrum and perfusing 10 ml of saline distally. It was possible to note the disappearance and subsequent re-emergence of fluid in the first loop distal to the ligament of Treitz which was designated proximal jejunum. In both areas a short section of bowel was exteriorised and ligated between the arcades of the mesenteric vasculature. Proximal to this, the bowel was sectioned. From the cut end, the loop was opened along the anti-mesenteric border and pinned onto the base plate of a perspex frame, analogous to one side of an Ussing chamber. When a short 5 cm length of bowel had been pinned, the proximal end was sectioned. A second plate was clamped onto the base plate (see Fig. 1) allowing a section of bowel to be contained within a chamber (mucosa uppermost) forming its base. In this way, a section of intestinal mucosa was exposed, with intact vasculature, and with bleeding from the sides and ends of the tissue prevented by clamping. With care no blood loss and negligible subsequent leakage of bathing fluid occurred during this procedure. The chamber was held in position by a Prior micromanipulator above the body of the animal and was perfused with the appropriate bathing solution. A constant level in the chamber was achieved by means of an exit pump from the chamber draining off any excess pumped in by an input pump. In this way the mucosa could be conveniently exteriorised with intact blood supply and could be constantly perfused.

(c) Oxygen uptake studies. Oxygen uptake was monitored in some in vitro circumstances with a Clark-type oxygen electrode obtained from Rank

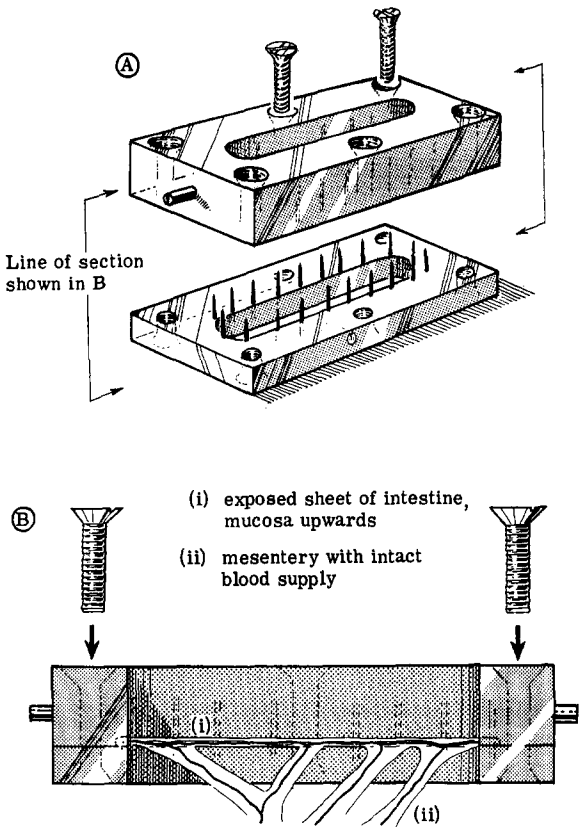

Fig. 1. Diagram of the in vivo perfusion chamber.

Brothers, Bottisham, Cambridge in accordance with the method of Leese and Bronk [13]. Segments of intestinal tissue were incubated in Krebs-Henseleit bicarbonate buffer at 37°C and the oxygen tension measured over a period of 15 min polarographically, results being displayed on a Leeds Northrup Speedomax paper chart recorder. The rate of change in oxygen tension in the electrode chamber was derived by drawing by hand a best fit to the experimentally derived results. The slope of this line gave the oxygen uptake by the tissue which was then corrected for dry weight of sample after drying the tissues overnight at 100°C to constant weight. Results are expressed as μl/mg dry weight per h as is the convention for Q_{O_2} measurements.

2. Electrode construction

A significantly different modification of the Oehme method [14] of plastic membrane construction allowed rapid manufacture of low impedance electrodes in sufficient quantities. 5 mg of sodium ion ligand [15] were dissolved in 2 ml of tetrahydrofuran solvent along with 60–90 mg of poly(vinyl chloride) (either Lonzavyl or available poly(vinyl chloride) tubing fragments) and 30 mg of plasticiser (dibutyl sebacate). This was cast into a small dish and the tetrahydrofuran was allowed to evaporate. When sufficient tetrahydrofuran had evaporated, leaving the mixture close to the point of congealing, poly(vinyl chloride) tubing of 1.5 mm outer diameter was dipped into the mixture. By capillarity, enough mixture was taken up to form a liquid meniscus in the poly(vinyl chloride) tubing which subsequently bonded with the tubing when all tetrahydrofuran evaporated. As the cast membranes were sufficiently durable, excess poly(vinyl chloride) from the tip could be cut away leaving the sensing membrane exposed, forming a flush surface. No dead space was therefore present in these electrodes, exactly as in the original procedure which involved sealing on pre-formed poly(vinyl chloride) ligand membranes onto poly(vinyl chloride) tubing with tetrahydrofuran. The electrodes were filled with 1000 mM NaCl. These electrodes had an average slope of some 50 mV per decade over the concentration range 25–150 mM NaCl and had an average resistance of about 100 MΩ. Potassium ion electrodes of similar electrical characteristics were obtained by substituting valinomycin for the sodium ligand.

3. Recording details

The sodium ion sensing poly(vinyl chloride) electrodes were coupled to either a Keithley 610 C electrometer or a Pye-Unican 9409 voltmeter, both of sufficiently high input impedance, via chlorided silver wire. The low side consisted of an inert (3% agar: 3 M KCl) agar bridge electrode connected to a silver chloride half-cell. Output from the electrometer was recorded on a Leeds and Northrup Speedomax paper chart recorder. In both in vivo and in vitro experimental protocols, electrodes were calibrated in 25 mM and in 150 mM [Na^+] containing solutions to check the adequacy of the electrode slope factor. Both the sodium and the inert electrodes were then placed in the buffer bulk solutions maintained at 37°C by incubation in vitro and by heat exchange prior to luminal perfu-

sion in vivo. After recording the bulk solution signal, the sodium ion electrode was placed onto the surface of the mucosa under direct visual control with the aid of a Prior micromanipulator. There was an immediate change in signal which was recorded until a steady state was achieved, usually within 2 min. Such a change was not solely due to contact artefact since positioning electrodes onto cotton swabs immersed in buffer gave no such change when the electrodes made contact. The electrodes were then taken off the surface and the bulk solution sodium ion concentration was once again measured.

4. Analysis of results

For the in vitro experiments, normally, an experiment consisted of paired tissues from the same rat in high and low sodium, with and without glucose in the buffer. This meant that differences between estimates were the variables used for statistical evaluation. Similarly, with in vivo experiments, the experimental protocol involved flushing with various solutions each animal acting as its own control and again allowing paired sample comparisons. When this was not possible, e.g. comparison of jejunal loops with ileal loops, unpaired statistical procedures were adopted. Both paired and unpaired data were tested for significance by Student's *t*-test.

5. Solutions and chemicals used

Krebs-Henseleit bicarbonate buffer in standard form with modifications was used throughout and gassed with the appropriate mixture. Sodium ion substitution was achieved with choline chloride to maintain ionic strength and osmolarity. Where the effect of high concentrations of magnesium ion were required, sodium chloride was substituted by magnesium sulphate which maintained osmolarity but not ionic strength. However, the increase in ionic strength from approx. 310 to 545 mmol/l changes the activity coefficient from 0.71 to 0.68 introducing an error of the order of 4% in the electrode measurements.

Deoxycholate was purchased from Koch-Light Ltd, U.K., dithiothreitol, (Cleland's reagent), ouabain and valinomycin from Sigma, U.K. Inorganic chemicals, analar grade were obtained from B.D.H. Ltd, Poole, U.K. Pure poly(vinyl chloride) powder (S-704) was obtained from Lonzavyl, Switzerland. Sodium ion sensitive ligand [15] was a generous gift from Professor Simon, Organic Chemistry Laboratorium, E.T.H., Zurich, Switzerland.

Results

Prior to any biological measurements, the sodium ion-sensitive electrodes were calibrated with respect to their response and to any factors which might cause artefactual changes in the recorded signal. When an electrode was placed on the surface of the intestine in vitro, an immediate change in signal occurred (a typical in vitro trace is shown in Fig. 2). A change of the order of 20–40 mV was typically seen, requiring about 90 s to achieve a steady-state value which was taken as the final signal from which concentration was calculated. Given the stability of the electrode because of its low impedance relative to other more typical ion-sensitive electrodes, the final concentration could be estimated to within 1 or 2 mV, although this did represent margins of error of the order of 10 mM in absolute terms. However, the form of measurement was always comparative between bulk and surface solution and consequently, on conversion, the measured concentrations were remarkably consistent. The relatively slow response time compared with the electrode response in aqueous solutions is probably because measurements are made in a mucus layer. On occasions,

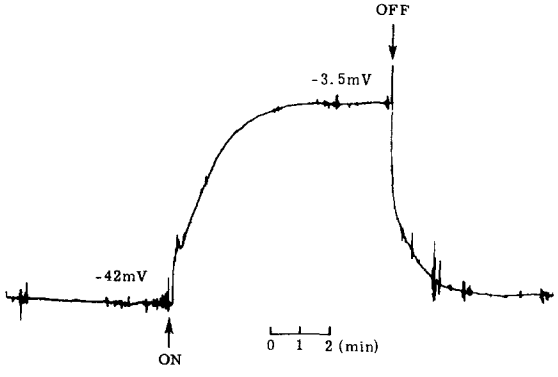

Fig. 2. Typical trace showing the change in potential difference when the sodium ion-sensitive electrode was placed onto the surface of the intestine in vitro (on) and subsequently taken off the surface (off).

when the electrode was taken off the surface, the re-estimated bulk solution sodium ion concentration was slightly higher than before. However, on removing any adherent mucus, the electrode then measured the same signal for the bulk phase as it had done previously.

Measurements of the surface sodium ion concentration [Na_s^+] were carried out in the proximal jejunum in high (150 mM) and low (25 mM) Na^+ containing solutions in the presence and absence of added glucose (Table IA). In the high sodium bulk solutions, [Na_s^+] was not significantly different from the bulk solution values. The surface concentrations were somewhat lower, especially in the presence of glucose, as might be expected but the ability to demonstrate a significant difference was probably outside the capability of the particular electrode type. In contrast, when low sodium buffers were used as the incubation medium, the surface concentration was conspicuously higher at 59 mM ($P < 0.002$) than the bulk phase value in the presence of 10 mM glucose substrate. When glucose was absent [Na_s^+] was lower at 42 mM but still higher ($P < 0.01$) than the bulk phase value.

When ouabain, the ($Na^+ + K^+$)-ATPase inhibitor, was included in the medium in vitro there was a significant reduction ($P < 0.002$) in the surface concentration compared with the control solutions incubated in the low sodium bulk phase buffers (Table IA). Subsequent removal of glucose from the bathing medium did not reduce the surface concentration further. However, both in the presence and absence of ouabain, [Na_s^+] was still significantly ($P < 0.01$) above the bulk phase concentration. Much the same results were obtained when the unconjugated form of bile salt, deoxycholic acid, was included in the incubation medium. Both in the presence and absence of added glucose, there was a significant reduction ($P < 0.01$) of surface sodium ion to values below the control value, although still somewhat above the bulk values. The presence of dithiothreitol (Cleland's reagent) which is a substance known to dissolve mucus caused an even greater fall in the surface concentration in the presence of glucose. Parallel studies on identical tissue from paired samples showed that the presence of dithiothreitol had no effect on the ability of the tissue to support metabolism, as judged by the lack of effect (Q_{O_2} of 16.5 ± 2.1 μl/mg dry wt. per h [15]) on the tissue oxygen uptake, compared with control values (13.4 ± 1.8 [13]). The effect of dithiothreitol is clearly related to its mucolytic properties. In contrast to the proximal jejunum, distal ileum showed a much lower [Na_s^+] of the order of 33 mM when the bulk solution value was 25 mM. However, this was still significantly ($P < 0.01$) above the bulk concentration. Lack of glucose produced a marginally lower surface concentration but this was not significant. The ileal [Na_s^+] was very significantly ($P < 0.05$) lower than that for the jejunum, both in the presence and absence of added glucose.

In vivo experiments (Table IB) gave results

TABLE I

(A) The effect of 0.6 mM ouabain, 1 mM deoxycholic acid and 1 mM dithiothreitol (Cleland's reagent) on surface [Na^+] in the presence and absence of 10 mM glucose in rat small intestine in vitro. All tissues incubated in 25 mM Na^+ (choline substituted) Krebs-bicarbonate buffer gassed with 95:5% (v/v) O_2/CO_2 mixture. Results expressed as mean + S.E., with number of animals in parentheses. n.d., not determined.

	In vitro surface [Na^+] (mM)	
	− glucose	+ glucose
Normal Krebs	147 ± 15(7)	138 ± 8(7)
25 mM Na^+-Krebs control jejunum	42 ± 5(7)	59 ± 4(7)
Ouabain	44 ± 4(4)	45 ± 3(4)
Deoxycholate	42 ± 4(4)	45 ± 1(4)
Dithiothreitol	–	37 ± 2(6)
25 mM Na^+-Krebs control ileum	31 ± 1(5)	33 ± 2(5)

(B) Surface [Na^+] and [K^+] in rat proximal jejunum in vivo incubated in the above buffer (25 mM Na^+) and in magnesium-substituted low [Na^+] (25 mM) buffer.

	Surface [Na^+] (mM)	Surface [K^+] (mM)
Choline-substituted		
with 10 mM glucose	79 ± 3 (6)	5.4 ± 0.9(6)
without glucose	58 ± 4 (6)	n.d.
Magnesium-substituted		
25 mM Na^+ with 10 mM glucose	42 ± 3(10)	3.4 ± 0.7(6)
150 mM Na^+ normal Krebs control	(see above)	17.5 ± 2.1(6)

broadly in agreement with the in vitro experiments. The $[Na_s^+]$ for in vivo jejunum in the presence of glucose was approx. 80 mM and was significantly higher than the comparable ($P < 0.05$) in vitro results and very much higher ($P < 0.001$) than the bulk perfusing fluid value of 25 mM, being on average about 3-times higher. In the absence of glucose $[Na_s^+]$ was lower than in the presence of glucose ($P < 0.01$) at 58 mM, although again very much higher than the bulk solution value. Samples of tissue taken from loops exposed to low sodium ion containing buffers did not show any significant reduction in their rate of metabolism (Q_{O_2}, 15.0 ± 2.0 (5) μl/mg dry wt. per h) showing that perfusion of the low sodium-ion containing buffers did not damage the mucosa. In these experiments, sodium ion was replaced with choline to balance the total osmotic and ionic strength. When the sodium ion was compensated for by including magnesium sulphate in the perfusion fluid, there was a further significant fall ($P < 0.02$) in $[Na_s^+]$ in the absence of glucose, compared with the choline substituted buffers. However, again, even this very low $[Na_s^+]$ of approx. 42 mM was still significantly ($P < 0.01$) above that of the bulk solution concentration. As a check on the possible influence of surface $[K^+]$ on measurements, in vivo experiments were similarly carried out using valinomycin electrodes. The low levels of $[K^+]$ recorded (Table IB) combined with the selectivity of the sodium ligand were not such to significantly infere with the $[Na^+]$ estimations. However they did interestingly demonstrate a higher $[K^+]$ of 17.5 mM at the surface which was reduced to 5 mM on perfusing with low $[Na^+]$ buffer. This low $[K_s^+]$ also fell significantly when Mg^{2+}-substituted buffer was used by a further 1.5 mM to approx. 3.4 mM mirroring the similar fall in $[Na_s^+]$.

Discussion

Many studies have consistently demonstrated the interdependence of sodium transport and the movement of solutes, particularly the absorption of actively transported sugars. These observations have been incorporated into a familiar paradigm whereby the electrochemical gradient for sodium ion across the enterocyte luminal membrane provides the energy for solute entry into cells [1]. Other explanations of the dependence on sodium ion have been offered [16,17], arising from the apparent inability to completely inhibit glucose absorption when adverse sodium ion gradients are applied in vivo [5–10,18] and less frequently in vitro [19,20]. In contrast, in vivo demonstrations of this dependence have either been of a very transient nature [4] or in experimentally analogous preparations such as the vascularly perfused loop [21]. Clearly a consistent feature has been the difficulty of in vivo verification of the gradient hypothesis. One explanation proposed on the basis of in vitro kinetic experiments [12] is the existence of local accumulation of sodium ion either within the brush border or in an unstirred layer. Under these circumstances, a sufficiently high localised gradient across the brush border membrane would provide the motive force for absorption in the face of apparently disadvantageous luminal solution to cell interior gradients: a proposal apparently irreconcilable [8] with modelling calculations of the maximum possible unstirred layer concentrations. The present experiments demonstrate both in vitro and in vivo that considerable amounts of sodium ion are present close to the surface of the transporting cells, even when the bulk solution approximates the assumed intracellular sodium ion concentration. Whilst no claim is made that these are absolute values, the results indicate that the surface $[Na^+]$ could be between 2- and 3-times greater than the bulk solution value.

What the mechanism for this accumulation can be remains speculative, presumably in vivo a source of sodium ion from the plasma and in vitro recycling of sodium ion through the paracellular pathways, possibly with some retention of ion by the unstirred layer. Both in vivo and in vitro, glucose elevated $[Na_s^+]$ indicating that the increased sodium ion transport caused by glucose manifested itself as a higher surface concentration. Prevention of sodium pumping activity by ouabain or deoxycholate [23,24] led to a reduction of surface concentration. These results support the suggestion made by Alvarado that recycling of sodium ion occurs either round the transporting cells or in a manner analogous to the fluid circuit hypothesis. A buffer zone of sodium ions would account for glucose entry into segments when all sodium was removed from the lumen and could also explain

the partial refractoriness of glucose transport to ouabain and other forms of metabolic inhibition [26,27,28]. What the nature of such a buffer zone consists of, remains at the moment conjectural.

The effect of dithiothreitol on the surface concentration provides an indication of the extracellular structure associated with sodium ion accumulation; this area is often proposed to be an unstirred water layer where forced convection is without effect but where free diffusion as in aqueous solutions occurs [26]. If this were the case, then dithiothreitol, a mucolytic agent with no metabolic effects, should have had little effect. However, if mucus facilitated a high surface concentration either by maintaining the depth of the unstirred layer or by reducing the diffusion coefficient of ions [22], then dithiothreitol would dissolve the extracellular feature responsible for maintaining the high ion concentrations. A second extracellular feature might be the electrostatic attraction provided by surface charge. In normal Krebs, surface $[K^+]$ is significantly higher than the bulk solution value. When bulk sodium is lowered, there was a loss of potassium and sodium from the surface. It may be that the reduction or even reversal of transmural potential difference which occurs under these circumstances allows ions to diffuse away from the surface. The small but highly significant further fall in both ions that occurs when magnesium-substituted buffer is perfused may relate to the shielding of fixed negative charges. Whatever the mechanism, these observations emphasise that the accumulation of sodium ion at the surface is unlikely to be a simple unstirred water layer effect.

In summary, the present data demonstrate a local accumulation of sodium ion near the brush border both in vitro and in vivo when tissues are incubated in buffers containing low bulk sodium ion concentrations. Consequently, the alignment of the electrochemical gradient is still such to allow solute entry as envisioned by the gradient hypothesis. The need to propose separate transport systems that are sodium ion-independent seems unnecessary given these correctly aligned gradients in vivo. These gradients would be refurbished by sodium ion from the vasculature [29] and correctly aligned even after perfusion of solutions with a high magnesium content [8,11] if estimations of intracellular sodium ion content are sufficiently reliable [30]. These observations do not allow a choice to be made between the gradient and other hypotheses but strongly support the former since the apparent contrary evidence in vivo is because the imposed luminal gradient does not reflect the critical brush border gradient.

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