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BIDIRECTIONAL SODIUM ION MOVEMENTS VIA THE PARACELLULAR AND TRANSCELLULAR ROUTES ACROSS SHORT-CIRCUITED RABBIT ILEUM*

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SUMMARY

1. It has been confirmed that the agent 2,4,6-triaminopyrimidine decreases Na^+ conductance in the paracellular pathway of rabbit ileum.

2. Triaminopyrimidine has been used as a means of measuring transcellular bidirectional Na^+ flux, and also, of assessing the contribution of the paracellular pathway to transepithelial Na^+ flux.

3. Reduction of Ringer [Na^+] to 25 mM or incubation with 0.1 mM ouabain reduces paracellular Na^+ permeability. This effect may be due to lateral space collapse. Ringer galactose increases serosa to mucosa Na^+ flux by a stimulating reflux through the tight junctions. A proportion of net Na^+ flux in control tissues is due to asymmetry generated in the paracellular pathway. It is likely that this passive asymmetry results from an osmotic pressure gradient across the tight-junction.

4. Measurement of the tissue isotope specific activity ratio together with bidirectional transcellular Na^+ fluxes allows calculation of the four unidirectional fluxes across the mucosal and serosal boundaries. Values obtained for Na^+ entry (J_{12}) and exit (J_{21}) across the mucosal boundary are 7.97 and 7.13 $\mu mol \cdot cm^{-2} \cdot h^{-1}$ respectively. Entry flux (J_{12}) is a saturable function of Ringer [Na^+]. The calculated K_m is 295 mM and the V is 17.6 $\mu mol \cdot cm^{-2} \cdot h^{-1}$. Na^+ entry flux is insensitive to ouabain (0.1 mM). Ouabain results in elevation of exit (J_{21}) flux of Na^+ across the brush border. D-Galactose causes a saturable increase in Na^+ flux (J_{12}) across the mucosal boundary; the K_m for this relationship is 1.2 mM and the V 2.17 $\mu mol \cdot cm^{-2} \cdot h^{-1}$. The stoichiometry between sugar and Na^+ entry is approximately 1 : 1. In contrast to the effect of galactose on entry flux, no change in Na^+ efflux across the mucosal boundary is observed when Ringer [galactose] is raised. This finding is dissonant with the prediction of the Na^+-gradient hypothesis.

The calculated values of exit (J_{23}) and entry (J_{32}) Na^+ fluxes across the serosal border are 16.74 and 15.90 $\mu mol \cdot cm^{-2} \cdot h^{-1}$. 0.1 mM ouabain markedly

* The authors wish to point out the following errors which occurred in a previous paper (BBA 77302, Vol. 433, pp. 597–614). p. 600, Table I: The V for Na^+ activation of 2 mM 3-*O*-methyl glucose, replace $J_{13} = 0.33 \pm 0.54$ by $J_{13} = 0.33 \pm 0.054$ and $J_{12} = 0.33 \pm 0.65$ by $J_{12} = 0.33 \pm 0.065$. The V uptake of β-methyl glucose with Ringer [Na^+] = 140 mM, replace $J_{13} = 0.88 \pm 0.50$ by $J_{13} = 0.88 \pm 0.059$.

reduces both these unidirectional fluxes. This result is consistent with a serosal location of the Na^+-pump. Serosal Na^+ exit flux J_{23} increases as a hyperbolic function of Ringer [galactose]. A small galactose-dependent decrease in entry (J_{32}) is also observed. 0.1 mM ouabain abolishes these galactose-dependent changes.

5. The present findings together with those in the previous paper are discussed in relation to the convective-diffusion model for sugar transport.

INTRODUCTION

Net Na^+ movement across in vitro rabbit ileum results from the asymmetric properties of the mucosal and baso-lateral (serosal) membranes of the epithelial cell layer [1].

Estimates of the unidirectional Na^+ fluxes across the mucosal and basolateral membranes have previously relied upon measurements from three separate experiments performed on separate pieces of tissue [1, 2]. A method for measuring the entry and exit fluxes of sugars simultaneously across the mucosal and serosal borders of rabbit ileum has recently been described [3]. An inherent assumption of this method is that there is no significant route for transepithelial sugar movement other than the transcellular pathway.

There is a considerable body of evidence to show that net transepithelial Na^+ flux occurs via two parallel pathways for Na^+ movement [4–6] Transcellular Na^+ flux is effectively short-circuited by the presence of a high conductance cation-selective shunt, the anatomical location of which is probably the limiting junctions between the epithelial cells together with the lateral intercellular spaces [8, 23]. The shunt pathway comprises 85–95 % of the total tissue conductance [5–7]. Thus the small electrical-potential differences which normally exist across rabbit ileum in open-circuit are an important determinant of the net flow of Na^+ across the tissue. Clamping the transepithelial potential at zero abolishes the potential-sensitive component of flux, but will not affect the diffusional Na^+ flux, via the shunt. Determination of the diffusional flux through the shunt, or its reduction to insignificant levels, is therefore a prerequisite for study of transcellular Na^+ fluxes [9].

Frizzell and Schultz [5] have approached the determination of the shunt permeability to Na^+ by measuring the unidirectional influx of Na^+ as a function of the applied transepithelial potential difference. The shunt permeability to Na^+ at zero potential difference (i.e. the diffusive component via the shunt) may then be determined from the functional relationship between influx and transepithelial potential difference. Errors may arise, not only from within and between experimental variance, but also from current-induced changes in tissue resistance [10] and perm-selectivity [11]. The use of low transepithelial potentials (± 10 mV) [7] does not eliminate this effect because of the high tissue conductance.

Moreno [12, 13] has recently found that 2,4,6-triaminopyrimidine specifically reduces the Na^+ conductance of the shunt pathway in the gall bladder and other low-resistance epithelia e.g. rabbit ileum. It was therefore considered that triaminopyrimidine, by eliminating or significantly reducing the shunt component of transepithelial Na^+ flux, would be useful in the measurement of transcellular Na^+ fluxes in rabbit ileum.

Recently the Na^+-dependence of all four unidirectional fluxes of D-galactose has been examined [14, 15]. Specifically the exit flux across the mucosal boundary was shown to decrease as intracellular [Na^+] was raised. This result contradicts that predicted by the Na^+-gradient hypothesis. Very little information exists concerning the effects of D-galactose on the Na^+ exit flux across the mucosal boundary of sheets of tissue [36]. Furthermore, the finding that extracellular hypertonicity is increased when galactose is added to the Ringer [16] suggests that there is a galactose-dependent stimulation of Na^+-pump activity. This paper describes the effects of D-galactose on all four unidirectional Na^+ fluxes across the mucosal and serosal borders of the cells and the flux of Na^+ via the shunt pathway.

MATERIALS AND METHODS

(*a*) *Flux calculations*

The unidirectional fluxes of Na^+ across the mucosal and serosal borders of rabbit ileum are calculated from three independent measurements, namely the mucosal to serosal flux, J_{13}, the serosal to mucosal flux J_{31} and the ratio R, of the specific activities of radioisotopes coming from the mucosal and serosal solutions within the tissue of compartment 2. $^{24}Na^+$ is used as a label of M-S flux (J_{13}) and $^{22}Na^+$ is used as a label of S-M flux (J_{13}). When the concentrations of Na^+ are equal in the mucosal [1] and serosal compartments [3] then:

$$R = \frac{(\text{cpm})_2^{24}}{(\text{cpm})_2^{22}} \times \frac{(\text{cpm/ml})_3^{22}}{(\text{cpm/ml})_1^{24}}$$

superscripts 24 and 22 refer to the $^{24}Na^+$ and $^{22}Na^+$ tracers respectively. Compartments 1, 2, 3 refer to the mucosal solution, tissue fluid and serosal solution respectively.

Following the method as developed for D-galactose, the unidirectional fluxes are given by the following equations [3]:

$$J_{12} = J_{31} \cdot R + J_{31}$$
$$J_{21} = J_{31}(1+R)$$
$$J_{23} = J_{13}(1+1/R)$$
$$J_{32} = J_{31} + J_{13}/R$$

Three assumptions inherent in the above treatment are relevant to the discussion of Na^+ fluxes across the tissue. (1) There is no significant shunt permeability to Na^+. (2) The tissue behaves kinetically as a single compartment to Na^+ (such that there is no inhomogeneity in the tissue isotope ratio R of $^{24}Na^+/^{22}Na^+$ within the tissue). (3) The estimated variables J_{13}, J_{31} and R are at steady state (i.e. the net flux across the tissue is equal to the net flux across the individual boundaries).

The last assumption is easily verified for Na^+ fluxes, since in tissues showing no conductance change during the total period of flux measurement, 2 consecutive periods of 30 min fluxes, after a 20 min preincubation period, are not significantly different.

Assumptions 1 and 2 require experimental justification. The first assumption, the absence of a significant shunt to Na^+ movement is dependent on the mode of

action of 2,4,6-triaminopyrimidine. The effects of triaminopyrimidine on the Na^+ conductance are described by Moreno [12, 13] and are confirmed of rabbit ileum in Results. The second assumption concerns the state of Na^+ within the tissue. Compartmentalization of Na^+ is evident, since it was found that the extracellular [Na^+] is hypertonic to the [Na^+] of the bathing media [16]. This compartmentalization will not affect the unidirectional flux estimates provided that the tissue ratio R is the same in both intra and extracellular compartments. Localization of one isotope with respect to another will invalidate the method. Analysis of the ratio R within the tissue compartments is therefore of importance to the calculation of unidirectional fluxes as outlined above (see Results, III).

(*b*) *Experimental*

(*i*) *Ringer's solutions.* Ringer's solutions comprised 140 mM NaCl, 10 mM $KHCO_3$, 0.4 mM K_2PO_4, 2.4 mM K_2HPO_4, 1.2 mM $CaCl_2$, and 1.2 mM $MgCl_2$. All were continuously gassed with a gas mixture O_2/CO_2 (95 : 5) saturated with water vapour at room temperature. The [Na^+] of the Ringer was varied by isosmotic replacement of NaCl by choline chloride. The base 2,4,6-triaminopyrimidinium was neutralized by addition of HCl prior to use. Control solutions contained mannitol in equivalent quantities to maintain isotonicity.

(*ii*) *Animats.* Male white New Zealand rabbits were fed normally and killed by intravenous injection of Nembutal. Terminal ileum was rapidly excised, washed, and stripped of its serosa and muscle layers prior to mounting in the flux chambers.

(*iii*) *Flux chambers and voltage clamp.* The apparatus used in these studies consisted of temperature regulated flux chambers designed to measure bidirectional transepithelial fluxes in 6 adjacent pieces of tissue. The potential differences across the tissues were continuously voltage-clamped (potential difference = 0) with a six channel short-circuit current device giving automatic compensation for solution resistance [17]. Electrical isolation of each set of chambers in the block of 6 was achieved by trimming surplus interconnecting tissue and isolating the remaining tissue by use of oil-containing conduits running between each piece of tissue. Calomel potential-sensing electrodes were connected to the bathing solutions via saturated KCl bridges (1 mm internal diameter) immobilized by a 1 % agar gel. The tips of these bridges were held within 2 mm of the tissue. Current was passed through large area Ag/AgCl electrodes, also connected to the bathing solutions by KCl bridges. The tips of the current passing bridges opened at the back of the flux chambers. The geometry of the flux chambers ensured a homogeneous clamp current over the whole tissue surface.

(*iv*) *Flux measurements.* $^{22}Na^+$ and $^{24}Na^+$, obtained from the Radiochemical Centre Amersham, as aqueous isotonic solutions of NaCl, were added to the serosal and mucosal solutions respectively in quantities sufficient to give increments of at least 2000 cpm in 1 cm^3 of solution within the contralateral chamber during 30 min.

The initial volume of Ringer in each chamber was 7.5 cm^3. 1 cm^3 samples were taken 20, 50 and 80 min following addition of radio-isotopes. Extraction of the isotopes from the tissue at the end of the flux period to determine the isotope ratio R is as previously described [3].

(*v*) *Radio-isotope counting.* $^{24}Na^+$ was counted in a Packard Tricarb liquid scintillation counter by its Cerenkov radiation. All samples counted for $^{24}Na^+$

activity were diluted with 10 cm^3 of distilled water. Background subtraction of activity due to the presence of $^{22}Na^+$ was made after recounting samples following complete decay of all $^{24}Na^+$ activity (2 weeks following the first count). $^{24}Na^+$ activity was corrected for decay during the initial counting period. $^{22}Na^+$ activity was determined, following the decay of all $^{24}Na^+$ activity, from its β-emission using the Tricarb counter. All samples for β-counting were diluted with 10 cm^3 of scintillation cocktail (500 cm^3 toluene, 500 cm^3 Triton X-100, 5.0 g 2,5-diphenyloxazole). All activity was corrected for errors due to quench using an external standard.

(*vi*) *Distribution of the tissue isotope ratio R within the tissue.* Operationally and experimentally, rabbit ileal tissue can be divided into two parts [18]. (a) The epithelial cell layer and underlying tissue to the lamina propria and (b) the remaining submucosa and muscle. Each part has an associated extracellular space which can be freed of inorganic cation by washing in ice-cold isotonic choline chloride [16]. Hence estimates of the tissue isotope ratio R may be made in each of 4 separate tissue compartments for Na^+ by scraping the mucosal from the submucosal layers [18] before and following a wash period in ice-cold choline chloride.

(*vii*) *Characterization of the action of 2,4,6-triaminopyrimidine on rabbit ileum.* The conductance of the paracellular route in rabbit small intestine may be estimated by three methods. (a) Voltage-dependent influx of Na^+. The approach outlined by Frizzell and Schultz [5] was used; unidirectional flux J_{13} was determined as a function of applied potential difference. For rabbit ileum it has been shown that

$$J_{13}^{Na^+} = J_{c13}^{Na^+} + J_{d13}^{Na^+} \xi^{-\frac{1}{2}}$$

where $J_{13}{}^{Na^+}$ is the total transepithelial unidirectional flux from mucosa to serosa. $J_{c13}{}^{Na^+}$ is the unidirectional flux from M-S via the cellular route. $J_{d13}{}^{Na^+}$ is the diffusive component of unidirectional flux from mucosa to serosa via the high conduction pathway. $\xi^{-\frac{1}{2}} = \exp(ZF\Delta\psi_{13}/RT)^{-\frac{1}{2}}$ where, Z, F, R and T have their usual meanings. $\Delta\psi_{13}$ is the transepithelial potential difference. A plot of J_{13} versus $\xi^{-\frac{1}{2}}$ gives a straight line with a slope of J_{d13} and intercept of $J_{c13}{}^{Na^+}$ [5].

(b) Dilution and biionic potentials. Transepithelial potential differences were recorded in open circuit during isosmotic replacement of the mucosal bathing solution (initially 140 mM NaCl Ringer). Isosmotic replacement of NaCl was made with mannitol, choline chloride, KCl, or Na_2SO_4. All solutions contained 0.1 mm ouabain to abolish the spontaneous potential difference across the tissue and thus eliminate possible complications from changes in active transport potentials [5]. Saturated KCl bridges minimise errors due to asymmetric junction potentials. Absolute errors using this approach are approximately 2–3 mV [19] but are constant with respect to potential difference changes in the presence of triaminopyrimidine.

(c) Conductance measurements. Conductance measurements were made under voltage-clamp by measuring the current required to clamp the tissue in response to short (10 s) depolarizing and hyperpolarizing steps (5 mV) of command potential applied to the tissue intermittently.

(*viii*) *Statistical methods.* Errors of grouped results, except where stated, are routinely expressed as the standard error of the mean. Statistical comparisons were made using two tailed Student's t tests for unpaired samples. Significance levels were obtained by comparison of calculated t values to the tabulated distribution of t.

RESULTS

I Effects of 2,4,6-triaminopyrimidine of the shunt conductance

(*a*) *Voltage-dependent mucosa to serosa Na^+ fluxes* (J_{13}). Figs. 1a and b show the variation of $J_{13}^{Na^+}$ when plotted as a scatter diagram against the applied potential difference expressed as the function $\xi^{-\frac{1}{2}}$ (see Materials and Methods) for control and triaminopyrimidine-treated tissues (20 mM). The regression equation gives $J_{c13}^{Na^+}$ as 6.14 μmol · cm^{-2} · h^{-1} and $J_{d13}^{Na^+}$ as 5.44 μmol · cm^{-2} · h^{-1} for controls. These values are similar to those obtained by other workers [5–7]. The presence of triaminopyrimidine causes a significant change in the slope of the regression line ($p < 0.05$). Indeed the slope (J_d) plus triaminopyrimidine is not significantly different from zero ($p > 0.2$). $J_{c13}^{Na^+}$ increases slightly to 9.17 μmol · cm^{-2} · h^{-1} in the presence of triaminopyrimidine. This value is not significantly different ($p > 0.1$) from $J_{c13}^{Na^+}$ for control tissues.

That 20 mM triaminopyrimidine reduces the shunt component of transepithelial ($J_d^{Na^+}$) flux without affecting transcellular movement ($J_{c13}^{Na^+}$) is evident from the observation that flux J_{13} (total) at zero potential difference plus triaminopyrimidine (7.02 μmol · cm^{-2} · h^{-1}) is similar to the value for transcellular Na^+ movement (J_{c13}) derived from the potential sensitive fluxes (above) in control tissue.

(*b*) *Dilution and biionic potentials.* Table I shows the biionic potentials recorded after isosmotic replacement of the Ringer in the mucosal solution by the following solutions: choline chloride, KCl, Na_2SO_4 and mannitol. As found by other workers [5], mannitol replacements gave a large serosa negative (−18 mV) potential. The presence of 20 mM triaminopyrimidine reduced this potential to −9.8 mV. The difference compared to control values is significant ($p < 0.02$). That this reduction is due specifically to a reduction in Na^+ permeability without concurrent changes in the

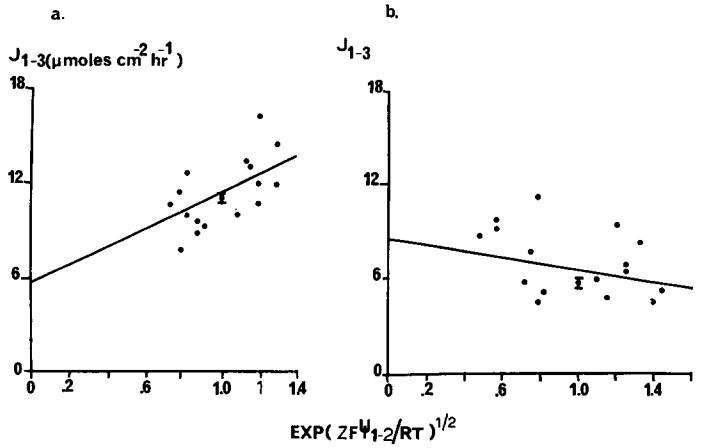

Fig. 1. (a) Relationship between mucosa to serosa flux, J_{13} and the applied potential difference (expressed as the relationship exp $((ZF\Delta\psi_{13}/RT)^{-\frac{1}{2}})$ for control tissues. Data were pooled from four experiments (8 tissues) and normalized about the flux at zero potential difference. The solid line is the least-squared regression line for the data ($J_{13}^{Na} = 6.14 + 5.44\ \xi^{-\frac{1}{2}}\ r = 0.46\ p < 0.05$). (b) Relationship between flux J_{13} and the applied potential difference for tissue incubated in the presence of 20 mM triaminopyrimidine. Data were normalized from four experiments (8 tissues). The least-square line for the data gives ($J_{13} = 9.57 - 2.18\ \xi^{-\frac{1}{2}}\ r = 0.27\ P > 0.05$ n.s.).

TABLE I

MANNITOL DILUTION POTENTIALS AND BIIONIC POTENTIALS ±20 mM TRIAMINOPYRIMIDINE (PRESENT IN BOTH MUCOSAL AND SEROSAL SOLUTION)

All measurements refer to mucosal replacement of 140 mM NaCl Ringer (pH 7.2) and are expressed relative to the mucosal solution. All solutions contained 0.1 mM ouabain. Potentials were recorded after a 20-min preincubation period during which time the spontaneous potential difference had declined to zero. Errors are expressed as the S.E.M. Numbers in parentheses are the number of experiments. p values are for Student's t test for the effect of triaminopyrimidine.

Mucosal dilution	Control	Triaminopyrimidine (20 mM)	p
Mannitol	−18.28±2.6 (6)	−9.88±1.26 (6)	< 0.02
Choline chloride	−21.64±1.45 (12)	−11.9±1.57 (12)	< 0.001
KCl	+ 4.72±0.31 (6)	+4.52±0.26 (6)	> 0.7
Na_2SO_4*	+ 6.04±0.47 (12)	+4.56±0.98 (12)	> 0.2

* Isotonicity to control 140 mM NaCl Ringer was ensured by addition of Mannitol.

Cl^- permeability is evident from the following observations. (1) The choline/Na^+ biionic potential is reduced by a similar amount compared to the mannitol dilution potential. (2) The K^+/Na^+ and the SO_4/Cl biionic potentials are unaffected by triaminopyrimidine ($p > 0.7$ and $p > 0.2$ respectively). The Na^+ permeability for the shunt pathway is 0.038 cm · h^{-1} (this value is derived from J_{d13}, since the permeability may be defined as $P_{ij} = J_{ij}/C_i$ at zero potential difference.

A reduction of 50 % in the observed dilution potential reduces P_{Na^+} to 0.0153 cm · h^{-1} (calculated from the Goldman-Hodgkin-Katz equation assuming P_{K^+} = 0.04 cm · h^{-1} and P_{Cl^-} = 0.019 cm · h^{-1}) [5]. Since the $P_{Na^+} : P_{K^+}$ ratio does not change, it can be concluded that triaminopyrimidine reduces the partial ionic conductance of K^+ in the shunt to the same extent as it affects Na^+ conductance.

Additional experiments (Simmons, N. L., unpublished) have confirmed Moreno's findings [13] that the monocationic form of triaminopyrimidine is the active form and that the K_m for its action is ≈ 2.0 mM. Triaminopyrimidine is used at saturating concentrations (20 mM total added base) in all flux experiments, (see below).

(c) *Conductance measurements.* Table II shows the open-circuit potentials and conductance measurements of tissues incubated in Ringer's solutions containing 140, 75 and 25 mM Na^+ ±triaminopyrimidine. The open-circuit potential changes from positive to negative as the Ringer [Na^+] is reduced from 140 mM to 25 mM. Concurrently the conductance decreases; this result is consistent with the presence of a cation-selective shunt across the intestine, impermeable to choline [9]. Triaminopyrimidine decreases tissue conductance at 140 mM Na^+ ($p < 0.001$). This effect is not significant at 25 mM Na^+ ($p > 0.4$). The triaminopyrimidine-dependent reduction in total tissue conductance (3.03 $m\Omega^{-1} \cdot cm^{-2}$) at140 mM Na^+ is equivalent to a reduction in P_{Na^+} of the shunt to 0.0216 cm · h^{-1}.This observed reduction in Na^+ permeability is similar to the values obtained fromdiffusion/biionic potential measurements.

Sections a, b, and c above show that there is a specific reduction in shunt permeability to Na^+ due to triaminopyrimidine. Since the P_{Na^+} of the shunt is

TABLE II

CONDUCTANCE MEASUREMENTS ±20 mM TRIAMINOPYRIMIDINE

NaCl replacement was made with choline chloride. (Ringer pH 7.2). Errors are expressed as ±S.E.M. Numbers in parentheses represent the number of separate determinations. Determinations of conductance and potential difference were made 5–15 min subsequent to the start of each experiment.

	$[Na^+]$ (mM)	Open circuit potential difference (mV)	Conductance $m\Omega^{-1} \cdot cm^{-2}$
Control	140	1.48 ±0.14 (43)	10.39±0.26 (46)
	75	0.97 ±0.21 (30)	9.52±0.61 (26)
	25	−0.26 ±0.28 (15)	7.14±0.62 (15)
+20 mM triaminopyrimidine	140	1.34 ±0.24 (31)	7.29±0.51 (38)
	75	0.707±0.212 (22)	7.26±0.41 (22)
	25	−0.65 ±0.33 (10)	6.57±0.79 (10)

known in both the presence and absence of triaminopyrimidine, transcellular permeabilities can be derived. Na^+ fluxes measured in the presence of triaminopyrimidine consist mainly of the transcellular component.

II Bidirectional transepithelial flux measurements

(*a*) *Effects of 2,4,6-triaminopyrimidine* In 140 mM Na^+ Ringer, 20 mM triaminopyrimidine reduces both mucosa-serosa flux, J_{13} and serosa-mucosa flux, J_{31} compared to control values (see Table III). The decrease in flux in both directions is highly significant ($p < 0.001$). The triaminopyrimidine-dependent reduction in flux is equivalent to a reduction in the unidirectional M-S Na^+ permeability (P_{13}) from 0.061 to 0.039 cm · h^{-1}. This reduction in permeability by 0.022 cm · h^{-1} is consistent with the observed reduction in the paracellular shunt permeability to Na^+ described in I above.

(*b*) *Effects of replacement of Ringer Na^+ by choline on the bidirectional Na^+ fluxes*

(1) *Control tissues.* In control tissues replacement of Ringer Na^+ with choline causes a reduction in both M-S and S-M Na^+ flux. This decrease in flux is directly proportional to the reduction in Ringer $[Na^+]$, hence no change in the bidirectional transepithelial Na^+ permeability is observed on reducing Ringer $[Na^+]$ from 140–25 mM (see Table III). The observed decrease in net Na^+ absorption is also directly proportional to the reduction in Ringer $[Na^+]$.

(2) *Effects of triaminopyrimidine.* (i) *Triaminopyrimidine-dependent reduction in bidirectional transepithelial Na^+ flux.* As with tissue bathed in Ringer containing 140 mM NaCl, (IIa above) triaminopyrimidine also reduces the bidirectional transepithelial flux of Na^+ at lower concentrations. The extent of this reduction varies with Ringer $[Na^+]$. This is shown in Table IV where it can be seen that the triaminopyrimidine-dependent reduction in both M-S and S-M Na^+ permeability falls as Ringer $[Na^+]$ is reduced from 140–25 mM. With Ringer $[Na^+]$ at 140 or 75 mM, the triaminopyrimidine-dependent reductions in both M-S and S-M transepithelial Na^+ fluxes are highly significant ($p < 0.001$). However, with Ringer $[Na^+] =$ 25 mM, a small non-significant reduction in transepithelial Na^+ flux is obtained in the

TABLE III

MEASURED BIDIRECTIONAL MUCOSA TO SEROSA AND SEROSA TO MUCOSA Na^+ FLUXES (J_{13} AND J_{31})

All errors are ±S.E.M. Bidirectional permeabilities were calculated from the relationship $P_{1j} = J_{1j}/C_1$.

Condition	n	J_{13} (μmol · cm^{-2} · h^{-1})	P_{13} (cm · h^{-1})	J_{31} (μmol · cm^{-2} · h^{-1})	P_{31} (cm · h^{-1})	J_{Net} (μmol · cm^{-2} · h^{-1})
140 mM Na^+	22	8.59±0.47	0.061±0.003	6.76±0.45	0.048±0.003	1.82±0.35
140 mM Na^++20 mM triaminopyrimidine	18	5.46±0.28	0.039±0.002	4.61±0.25	0.032±0.002	0.84±0.23
75 mM Na^+	37	4.86±0.22	0.064±0.002	3.81±0.13	0.050±0.002	1.05±0.16
75 mM Na^++20 mM triaminopyrimidine	29	3.59±0.13	0.047±0.002	3.01±0.13	0.040±0.002	0.57±0.11
25 mM Na^+	11	1.47±0.24	0.059±0.009	0.95±0.08	0.038±0.003	0.52±0.18
25 mM Na^++20 mM triaminopyrimidine	11	1.33±0.10	0.053±0.004	0.97±0.05	0.039±0.002	0.36±0.07
140 mM Na^++0.1 mM ouabain	6	6.02±0.48	0.046±0.003	5.93±0.06	0.042±0.004	0.32±0.31
140 mM Na^++0.1 mM ouabain+20 mM triaminopyrimidine	6	4.82±0.34	0.034±0.002	4.81±0.26	0.034±0.002	0.02±0.14
140 mM Na^+ Ringer+ 20 mM galactose	11	10.16±0.58	0.072±0.004	7.15±0.49	0.051±0.003	3.01±0.33
140 mM Na^+ Ringer+ 20 mM galactose+ 0.1 mM ouabain	5	6.01±0.96	0.043±0.006	5.88±0.81	0.042±0.005	0.24±0.30
140 mM Na^+ Ringer+ 20 mM triaminopyrimidine+ +20 mM galactose	13	7.21±0.47	0.051±0.003	4.12±0.23	0.029±0.001	3.08±0.41
140 mM Na^+ Ringer +20 mM triaminopyrimidine +20 mM galactose +0.1 mM ouabain	5	5.69±0.18	0.041±0.001	5.37±0.20	0.038±0.001	0.32±0.07

TABLE IV

TRIAMINOPYRIMIDINE-DEPENDENT REDUCTIONS IN BIDIRECTIONAL PERMEABILITIES

The number of degrees of freedom is given by the figures in parentheses.

Condition	P_{13} (control)$-P_{13}$ (triaminopyrimidine) ($cm \cdot h^{-1}$)		P_{31} (control)$-P_{31}$ (triaminopyrimidine) ($cm \cdot h^{-1}$)	
140 mM Na^+	0.022 $\pm$0.002	(38)	0.015$\pm$0.002	(38)
140 mM Na^++0.1 mM ouabain	0.012 $\pm$0.003	(10)	0.012$\pm$0.004	(10)
75 mM Na^+	0.0169$\pm$0.002	(64)	0.011$\pm$0.001	(38)
25 mM Na^+	0.005 $\pm$0.003	(20)	0.007$\pm$0.003	(20)

presence of triaminopyrimidine. The difference between the triaminopyrimidine-dependent reduction in transepithelial Na^+ permeability at Ringer [Na^+] = 140 mM, compared to the lesser triaminopyrimidine-dependent reduction with Ringer [Na^+] = 25 mM is highly significant ($p < 0.001$).

These results indicate that there is a Na^+-dependent variable resistance within the paracellular pathway in series with the tight-junction (see Discussion).

Table IV also shows that the triaminopyrimidine-dependent reductions in M-S and S-M Na^+ fluxes at Ringer [Na^+] concentrations of 140 mM and 75 mM are not equal. The reduction in M-S flux is significantly larger than the reduction in S-M flux ($p < 0.05$, for both cases). When Ringer [Na^+] is reduced to 25 mM, no significant difference in the reduction of M-S and S-M bidirectional permeabilities due to triaminopyrimidine is seen ($p > 0.1$).

The asymmetric action of triaminopyrimidine is also evident in the triaminopyrimidine-dependent reduction in net Na^+ flux (J_{net}) (Table III). A significant reduction of J_{net} due to triaminopyrimidine occurs both at 140 mM and 75 mM Ringer [Na^+] ($p < 0.05$, $p < 0.05$ respectively). No significant decrease in J_{net} at 25 mM Ringer [Na^+] in the presence of triaminopyrimidine is seen ($p > 0.4$).

These results suggest that triaminopyrimidine may block a portion of net flux that is paracellular (see Discussion).

(*ii*) *Effect of variation of Ringer* [Na^+] *on the bidirectional transepithelial* Na^+ *flux in the presence of 20 mM triaminopyrimidine.* On reducing Ringer [Na^+] with 20 mM triaminopyrimidine present, there is a progressive increase in M-S Na^+ permeability (Table III) ($p < 0.001$). This progressive increase in permeability indicates that the triaminopyrimidine insensitive (transcellular) flux is a saturable process whose existance, in control tissue, is obscured by concurrent changes in the permeability of the paracellular pathway on varying Ringer [Na^+]. The K_m for triaminopyrimidine insensitive M-S flux is 357$\pm$43 mM (S.D.) and the V is 20.2$\pm$8.4 (S.D.) $\mu mol \cdot cm^{-2} \cdot h^{-1}$. There is no significant change in S-M Na^+ permeability on reducing Ringer [Na^+] from 140–25 mM.

(*c*) *Effects of 0.1 mM ouabain on the bidirectional transepithelial* Na^+ *flux*

(1) *Control tissue.* 0.1 mM ouabain reduces M-S Na^+ flux compared with untreated tissue ($p < 0.02$). A slight reduction is also seen in the mean S-M Na^+ flux following addition of 0.1 mM ouabain, however the reduction is not significant

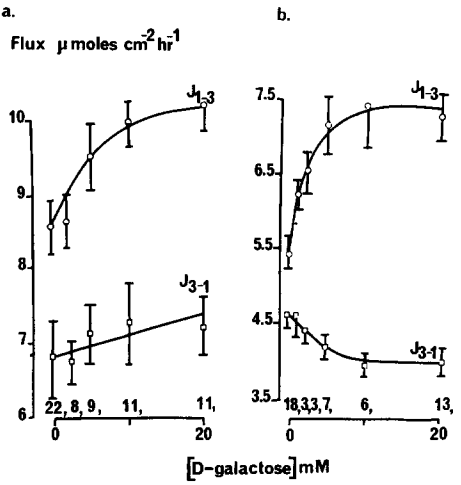

Fig. 2. The effect of D-galactose (0–20 mM) upon the bidirectional fluxes J_{13} and J_{31} under voltage clamp (potential difference = 0) for (a) control tissues (b) tissues incubated in the presence of 20 mM triaminopyrimidine fluxes are averaged from two separate flux periods of half-hour (see Materials and Methods). Error bars, denote the standard error of the mean for each data point. The number of data points for each level of Ringer [galactose] used is shown above the x-axis.

($p > 0.3$). With ouabain present, the net Na^+ absorption does not differ significantly from zero ($p > 0.6$) Table III.

(2) *Triaminopyrimidine treated tissue.* As with control tissue, ouabain abolishes the net flux across tissue in the presence of 20 mM triaminopyrimidine by reducing Na^+ flux J_{13} without significantly affecting Na^+ flux J_{31}. The extent of the triaminopyrimidine-dependent reduction in bidirectional Na^+ flux is significantly less ($p < 0.01$) in the presence of ouabain than in its absence (Table IV). This result indicates that ouabain has a similar effect to that of reducing Ringer [Na^+] on the triaminopyrimidine-sensitive (shunt) conductance.

In the presence of 0.1 mM ouabain there is a symmetrical reduction in bidirectional (M-S and S-M) Na^+ permeability due to triaminopyrimidine. This result contrasts with the asymmetric action of triaminopyrimidine in control tissues (b, ii).

(*d*) *Effects of* D-*galactose on the bidirectional transepithelial* Na^+ *fluxes*

(1) *Control tissues.* It has been shown previously on numerous occasions [20], that addition of galactose to the Ringer solution bathing isolated small intestine, increases M-S Na^+ flux. As can be seen in Fig. 2a this increase in Na^+ flux J_{13} is a saturable function of Ringer [galactose]. The K_m for the galactose-dependent increase in flux is 1.64 ± 0.35 (S.D.) mM and the V is 2.08 ± 0.47 $\mu mol \cdot cm^{-2} \cdot h^{-1}$. Galactose also slightly stimulates S-M Na^+ flux in control tissues (see also Table III). A similar effect of sugar on S-M Na^+ flux in guinea pig intestine [21] has been reported.

(2) *Effects of 0.1 mM ouabain on the galactose-dependent increase in bidirectional* Na^+ *flux.* As was shown in Section (c), ouabain reduces both the M-S and S-M Na^+ fluxes. It can be seen in Table III that ouabain also abolishes the galactose-dependent increments in both M-S and S-M flux.

(3) *Effects of 20 mM triaminopyrimidine.* In the presence of 20 mM triaminopyrimidine, the absolute levels of the bidirectional transepithelial Na^+ fluxes are

reduced, but triaminopyrimidine has no effect on the galactose-dependent increment in M-S Na^+ flux (Fig 2b). This result is consistent with the view that triaminopyrimidine acts exclusively to reduce the Na^+ permeability at the tight-junction.

In contrast to the effect of galactose on S-M Na^+ flux in control tissue, galactose significantly decreases S-M Na^+ flux when triaminopyrimidine is present ($p < 0.01$) (one-way analysis of variance). This result is similar to the sugar-dependent reduction in S-M Na^+ flux noted previously by Quay and Armstrong [22] in bull-frog small-intestine.

0.1 mM ouabain reduces M-S flux with 20 mM triaminopyrimidine present ($p < 0.001$). Since ouabain also abolishes the galactose-dependent decrease in S-M Na^+ flux seen with triaminopyrimidine present, ouabain effectively increases S-M Na^+ flux with galactose present in the Ringer (Table III). Thus with triaminopyrimidine present, the action of ouabain in abolishing net Na^+ flux is due both to a reduction in M-S Na^+ flux and an increase in S-M Na^+ flux. These actions strongly resemble the previously described reciprocal effects of ouabain on bidirectional transepithelial galactose flux [3, 14].

(4) *Effects of replacement of Ringer Na^+ with choline on galactose-dependent bidirectional transepithelial Na^+ fluxes.* Fig. 3 shows the galactose-dependent changes in M-S and S-M Na^+ flux following addition of 20 mM galactose to the Ringers containing 25, 75, and 140 mM Na^+ in both the presence and absence of 20 mM triaminopyrimidine. The galactose-dependent increment in M-S Na^+ flux in both the presence and absence of 20 mM triaminopyrimidine is identical and increases in direct proportion to the Ringer [Na^+]. The small galactose-dependent increase in S-M Na^+ flux is lost on reducing the Ringer Na^+ to 25 mM. With 20 mM triaminopyrimidine present, the galactose-dependent decrease in S-M Na^+ flux is also lost, on reducing Ringer [Na^+] from 140–25 mM.

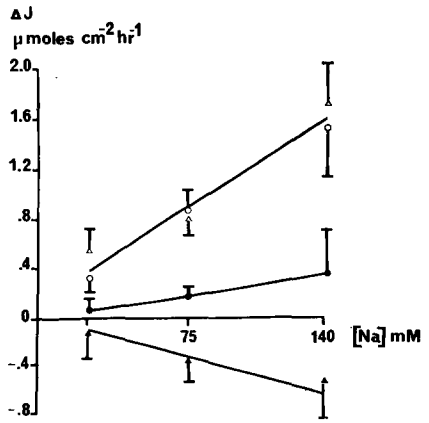

Fig. 3. The effect of Ringer [Na^+] variation upon the galactose-dependent change in bidirectional fluxes in the presence (△, ▲) and absence (○, ●) of 20 mM triaminopyrimidine. Error bars denote the standard error of the difference between Na^+ flux in control tissues and in tissues with 20 mM triaminopyrimidine present. Filled symbols denote S-M flux, open symbols M-S flux.

III The tissue isotope specific activity ratio of $^{24}Na : {}^{22}Na^{+}$ (R)

The tissue isotope specific activity ratio of $^{24}Na^{+} : {}^{22}Na^{+}$ (R) is 0.669±0.057 (Table V) for control tissues. Hence the steady-state distribution of Na^{+} within the tissue is characterized by a relatively greater proportion of Na^{+} originating from the serosal solution. Partial replacement of Ringer Na^{+} by choline has no significant effect upon the ratio R.

Triaminopyrimidine (20 mM) reduces the amount of Na^{+} originating from the mucosal solution compared to control values at all Na^{+} concentrations tested ($p < 0.05$, $p < 0.001$, $p < 0.001$ for 140, 75 and 25 mM Na^{+} respectively). Since triaminopyrimidine is known to reduce M-S flux by reducing the Na^{+} permeability of the paracellular pathway (I and II), the reduction in the tissue isotope ratio may be associated with this effect.

0.1 mM ouabain increases the ratio R towards unity for all experimental conditions tested. In control Ringer (140 mM Na^{+}) the ratio R increases from 0.669 to

TABLE V

THE TISSUE RATIO OF $^{24}Na^{+} : {}^{22}Na^{+}$

Errors are expressed as ±S.E.M. Figures in parentheses are the number of separate determinations.

Conditions	Control	Galactose (20 mM)	Ouabain (10^{-4} M)
140 mM Na^{+}	0.669±0.057 (22)	0.880±0.100 (13)	0.980±0.013 (6)
+20 mM triaminopyrimidine	0.540±0.041 (18)	0.735±0.030 (11)	0.792±0.160 (6)
75 mM Na^{+}	0.770±0.048 (37)	0.950±0.060 (29)	0.890±0.06 (4)
+20 mM triaminopyrimidine	0.574±0.042 (29)	0.650±0.040 (16)	0.910±0.050 (4)
25 mM Na^{+}	0.668±0.061 (11)	0.652±0.091 (20)	1.140±0.070 (3)
+20 mM triaminopyrimidine	0.588±0.043 (11)	0.633±0.029 (20)	0.69 ±0.04 (3)

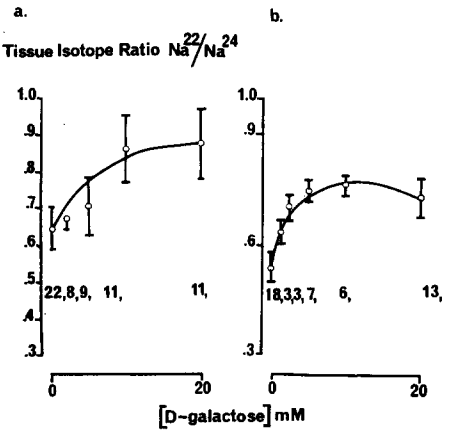

Fig. 4. The effect of galactose (0–20 mM) upon the isotope specific activity ratio ($^{24}Na^{+} : {}^{22}Na^{+}$) within the tissue fluid following incubation to determine bidirectional fluxes for (a) control tissues (b) tissues incubated in the presence of triaminopyrimidine. The number of data points for each level of Ringer galactose is shown above the x-axis.

0.980 following addition of ouabain. The difference between these two values is statistically significant ($p < 0.02$). In the presence of triaminopyrimidine a significant increase in R following addition of ouabain is also observed ($p < 0.05$).

D-Galactose increases the proportion of Na^+ originating from the mucosal solution within the tissue both in the presence and absence of triaminopyrimidine. This increase is a saturable function of Ringer [galactose] (Figs. 4a and b). Replacement of Ringer Na^+ by choline reduces the galactose-dependent increment in the tissue isotope ratio (Table V).

IV The distribution of the tissue isotope ratio within the extracellular and intracellular tissue compartments

The measurements of the tissue isotope specific activity ratio (R) are made from whole tissue determinations. This section is designed to show that these measurements (and hence the unidirectional flux calculations) are not subject to error due to inhomogeneity of the distribution of $^{24}Na^+$ to $^{22}Na^+$ within the tissue. Experimentally three tissue compartments for Na^+ can be identified (see Materials and Methods).

Table VI shows the results of the analysis of control tissues, tissues incubated in the presence of ouabain and tissues incubated in the presence of 20 mM galactose. With control tissue, no effect of washing on the ratio R is observed in either whole tissue ($p > 0.5$), submucosal layers ($p > 0.9$) or epithelial scrapings ($p > 0.6$). Similarly, no significant difference exists between the R ratio in whole tissue and submucosal layers ($p > 0.2$) or whole tissue and epithelial scrapings ($p > 0.2$), or between epithelial scrapings and submucosal layers ($p > 0.6$). Identical effects of washing or scraping are seen in galactose and ouabain-treated tissues.

These results support the validity of the method of determining unidirectional flux in the case of Na^+, since it is evident that this preparation of rabbit ileum may be treated kinetically as a single compartment.

TABLE VI

THE TISSUE DISTRIBUTION OF $^{24}Na^+ : {}^{22}Na^+$ WITHIN THE EXTRACELLULAR AND CELLULAR COMPARTMENTS OF RABBIT ILEAL TISSUE

Results are all expressed as the mean of 9 determinations from three experiments.

	Unwashed	Half-hour choline wash
Whole tissue		
Control	0.802 ±0.04	0.851 ±0.076
0.1 mM ouabain	0.95 ±0.08	0.913 ±0.090
20 mM galactose	0.905 ±0.09	0.892 ±0.097
Submucosal layers		
Control	0.892 ±0.065	0.900 ±0.075
0.1 mM ouabain	1.06 ±0.15	0.880 ±0.060
20 mM galactose	1.05 ±0.102	0.791 ±0.048
Mucosal scrapings		
Control	0.866 ±0.029	0.898 ±0.08
0.1 mM ouabain	1.13 ±0.140	1.04 ±0.134
20 mM galactose	0.815 ±0.08	0.915 ±0.096

DISCUSSION

(A) Bidirectional Na^+ flux through the paracellular pathway

(1) *The effects of triaminopyrimidine on tissue bathed in Ringer.* The shunt permeability to Na^+ calculated from the functional relationship between unidirectional transepithelial Na^+ flux and the applied transepithelial potential difference is 0.038 cm · h^{-1}, this is similar to the values obtained by others [5–7]. It is evident that 20 mM triaminopyrimidine reduces the permeability of the paracellular route, since it abolishes the potential difference-dependent Na^+ flux, reduces the Na^+ diffusion potential, the Na^+-choline biionic potential and the total tissue electrical conductance (Section I, Results). However, even with 20 mM triaminopyrimidine present, a small residual shunt permeability to Na^+ remains; by application of the Goldman-Hodgkin-Katz equation to the Na^+-choline biionic potentials this residual permeability is calculated to be 0.015 cm · h^{-1}.

These effects of triaminopyrimidine are all consistent with the view proposed by Moreno [12, 13] that triaminopyrimidine acts by blocking the cation selective sites within the tight junction between the mucosal borders of the epithelial cells.

When the [Na^+] in the Ringer is reduced by replacement with choline or when 0.1 mM ouabain is added to the Ringer, the triaminopyrimidine-dependent reductions in both P_{13} and P_{31} are decreased (see Table IV). This suggests that the overall permeability, P_t of the shunt pathway is the resultant of two resistances in series, the tight junction, tj and the lateral space, ls hence: $1/P_t = 1/P_{tj} + 1/P_{ls}$ [7]. If the lateral space, by virtue of its capacity to change shape is considered as a variable resistor, then when the lateral space is wide open, (low resistance), a large increase in the resistance of the tight junction following addition of triaminopyrimidine will cause a greater reduction in the total permeability of the shunt pathway than when the lateral space is closed (high resistance). Huss and Marsh [24] and Sackin and Boulpaep [25] have recently described a modified version of Diamond and Bossert's standing-gradient hypothesis [26] which is consistant with the above views. Sackin and Boulpaep modify the standing-gradient hypothesis to take into account the open-ended tight junction, the homogenous distribution of Na^+-pump sites along the lateral intercellular space and the unstirred layer effect in the submucosal space. Huss and Marsh's modification makes the assumption that the baso-lateral membrane forms a distensible compartment which responds to the hydraulic pressure of fluid within the space. This pressure is generated by fluid entry into the space caused by the osmotic pressure gradients across the baso-lateral border and tight junction.

Reducing the Na^+-pump activity at the lateral-basal border by decreasing Ringer [Na^+] or by ouabain addition, will reduce the hypertonicity of Na^+ sequestered in the space (as was demonstrated in the previous paper [16]), reduce the distension of the lateral-basal membranes and thus reduce the permeability of the lateral space. This mechanism therefore explains the reduced effect of triaminopyrimidine on transepithelial conductance in low [Na^+] Ringer and in ouabain-poisoned tissue.

(2) *Effects of* D-*galactose on the shunt permeability to Na^+.* In tissue bathed in Ringer, galactose increases both M-S and S-M Na^+ flux. When triaminopyrimidine is added to the Ringer, no reduction in the galactose-dependent increments in M-S Na^+ flux is observed, however there is a small, though significant, galactose-dependent decrease in S-M flux. Similar reductions in S-M Na^+ flux in response to sugars (but in

the absence of triaminopyrimidine) have been noted in bull-frog intestine [22]. It may be concluded that the galactose-dependent increments in M-S Na^+ flux are due to increased transcellular Na^+ movements which is insensitive to the action of triaminopyrimidine. The galactose-dependent increase in S-M Na^+ flux in the absence of triaminopyrimidine may result from increased Na^+ flux via the paracellular shunt pathway following enlargement of the intercellular spaces resulting from increases in the extracellular hypertonicity, as previously observed [16]. This conclusion is supported by the observation that the galactose-dependent increases in S-M Na^+ flux are abolished by ouabain.

Similar increases in Na^+ flux have been obtained in Necturus kidney tubules [30] and rat small intestine [31] following saline loading of the animals. Saline loading causes an increased shunt conductance [30], an increased inulin permeability [31], and a decreased net absorption of Na^+ due to increased S-M Na^+ flux [31, 30]. Lateral space distension is thought to give rise to increased tight-junction permeability [31].

Binder et al. [21] have shown that glucose, galactose and 3-*O*-methyl glucose all increase S-M Na^+ flux in isolated guinea-pig intestine. However they found no sugar or amino acid-dependent increase in S-M Na^+ flux in rabbit ileum [20]. The observed galactose-dependent increments in S-M Na^+ permeability, reported in this paper, reflect similar and concurrent increases in S-M galactose [3], 3-*O*-methyl glucose and β-methyl-glucoside permeability seen at high concentrations of these sugars [32].

(3) *Evidence for asymmetric passive Na^+ movements via the shunt pathway.* In Table IV it may be seen that the decrease in M-S Na^+ flux following addition of 20 mM triaminopyrimidine to Ringer is significantly greater than the decrease in S-M Na^+ flux. Thus triaminopyrimidine causes a significant reduction in net Na^+ absorption. Since it was shown previously that triaminopyrimidine is without effect on (a) the distribution of Na^+ and K^+ between the cells and the external solution [16] and (b) the galactose-dependent increase in transcellular Na^+ movement and also (c) the transport and accumulation of galactose (unpublished results), it may be inferred that triaminopyrimidine only affects the passive permeability of the shunt to Na^+, without affecting cell metabolism or cell membrane permeability.

However, if triaminopyrimidine affects only passive Na^+ permeability within the tight junction, why does it reduce M-S Na^+ flux more than S-M Na^+ flux in actively transporting tissue? A possible explanation for these findings is that, in actively transporting tissue, the osmotic pressure gradient existing across the tight junction, due to hypertonic saline contained within the lateral intercellular space, causes both Na^+ and water to be dragged from the mucosal solution via the tight junction. Additionally fluid is drawn from the cell across the lateral-basal border. Triaminopyrimidine reduces Na^+ mobility within the tight junction, hence will reduce the osmotically induced net flow of Na^+ across the tight junction.

In support of this explanation, of the passive asymmetric flux of Na^+ across the tight junction, it can be seen that the triaminopyrimidine-sensitive reduction in M-S and S-M Na^+ flux is symmetrical when 0.1 mM ouabain is present in the Ringer or when Ringer $[Na^+]$ is reduced to 25 mM. In both these conditions it was previously shown that the fluid within the lateral intercellular space is approximately isotonic [16], hence no osmotically induced drag of Na^+ is expected.

The magnitude of the osmotically-induced passive net flux of Na^+ across the

tight junction is $\cong 1\ \mu mol \cdot cm^{-2} \cdot h^{-1}$. This comprises the greater part of the total net flux of Na^+ across the tissue in control conditions.

A model describing the generation of passive net fluxes of Na^+ has been described by Schafer et al. [27] and used to explain the asymmetric flux of solutes across the Necturus proximal tubule. The driving force for the net flow of Na^+ is considered to be the transtubular concentration differences of Cl^- and HCO_3^- coupled to the existence of different reflexion coefficients for HCO_3^- and Cl^- at the tight junction. Sackin and Boulpaep [25] predict that passive net Na^+ uptake across the tight-junction may occur due to solvent-drug and electrical-potential effects.

The results shown in Fig. 2b indicate that in the presence of triaminopyrimidine, the S-M Na^+ flux progressively decreases as Ringer [galactose] is raised. Since this interaction is apparent only when triaminopyrimidine is present, the process retarding Na^+ flux must occur at some site other than the tight junction.

(B) Transcellular Na^+ flux

As triaminopyrimidine markedly reduces the paracellular component of transepithelial Na^+ flux, bidirectional transepithelial Na^+ flux determinations in the presence of triaminopyrimidine provide a basis for the estimation of the transcellular flux components. However, even with triaminopyrimidine present, a small residual passive permeability to Na^+ remains ($= 0.015\ cm \cdot h^{-1}$) which may lead to overestimation of transcellular flux. This residual Na^+ permeability may represent a leakage pathway in which ionic permeabilities follow their free-solution mobilities. Such a situation exists in the in vitro gall-bladder [13] preparation.

Unidirectional Na^+ fluxes across the mucosal boundary

Controls. The calculated influx and efflux J_{12} and J_{21} of Na^+ across the mucosal border of tissue bathed in Ringer are 7.97 and 7.13 $\mu mol \cdot cm^{-2} \cdot h^{-1}$ respectively (Table VII). These values agree reasonably well with those obtained by direct estimation of Na^+ influx across the mucosal border of rabbit ileum after correction for a shunt component of Na^+ influx [2].

TABLE VII

CALCULATED UNIDIRECTIONAL Na^+ FLUXES ACROSS THE MUCOSAL AND SEROSAL BORDERS IN THE PRESENCE OF 20 mM TRIAMINOPYRIMIDINE

All errors are expressed as the S.E.M. Values given are $\mu mol \cdot cm^{-2} \cdot h^{-1}$

Condition	n	J_{12}	J_{21}	J_{23}	J_{32}
140 mM Na^+ Ringer	18	7.97±0.42	7.13±0.46	15.46±1.44	14.60±1.33
140 Na^+ Ringer +0.1 mM ouabain	5	8.62±0.52	8.61±0.45	10.90±1.26	10.80±1.19
140 Na^+ Ringer +20 mM D-galactose	13	10.15±0.53	7.07±0.32	17.29±1.87	14.02±0.63
140 Na^+ Ringer +20 mM D-galactose +0.1 mM ouabain	5	8.49±0.50	8.62±0.89	11.42±0.43	11.55±0.62
75 mM Na^+ Ringer	29	5.31±0.21	4.74±0.22	10.76±0.69	10.19±0.67
25 mM Na^+ Ringer	11	1.91±0.13	1.55±0.10	3.74±0.38	3.38±0.31

(*b*) *Effects of replacing Ringer* Na^+ *by choline and* 0.1 *mM ouabain on the unidirectional* Na^+ *fluxes across the mucosal border.* The effects of reducing Ringer [Na^+] and of 0.1 mM ouabain on the calculated unidirectional Na^+ fluxes across the mucosal border are shown in Table VII. As expected both influx J_{12} and efflux J_{21} of Na^+ are reduced as Ringer [Na^+] is reduced from 140–25 mM. After correction of the transepithelial fluxes for the residual passive component remaining with 20 mM triaminopyrimidine present, a hyperbolic relationship between Ringer [Na^+] and Na^+ influx across the brush-border emerges. The apparent K_m of Na^+ for this transport process is 295 mM whilst the V is 17.6 mol · cm^{-2} · h^{-1}. Curran et al. [28] previously found a linear relationship between Na^+ influx and Na^+ concentrations. The present data are, however, similar to those of Nellans et al. [29]. It is observed that with 0.1 mM ouabain present, there is no significant reduction in Na^+ influx ($p > 0.5$), but the steady-state efflux of Na^+ from the tissue into the mucosal solution is raised. These results coincide with those obtained in several previous studies: e.g., Chez et al. [33], measured Na^+ influx directly without correcting for the shunt permeability, and found no effect of ouabain on Na^+ influx either. Since with ouabain present, the intracellular [Na^+] rises, these results showing increased Na^+ efflux are as expected.

(*c*) *Effects of* D-*galactose.* The effects of galactose on the unidirectional Na^+ fluxes across the mucosal border are shown in Fig. 5a. Influx J_{12} increases as a hyperbolic function of Ringer [galactose]. The K_m is 1.2±0.32 (S.D.) mM and the V is 2.71±0.69 μmol · cm^{-2} · h^{-1}. This result is substantially the same as observed previously by Goldner et al. [34] who measured Na^+ influx across the mucosal border directly.

The unidirectional efflux of Na^+, J_{21}, in the presence of 20 mM triaminopyrimidine is not raised by galactose. Indeed a small (but non-significant) decrease is observed in Na^+ efflux when Ringer [galactose] is raised from 0–20 mM. Since

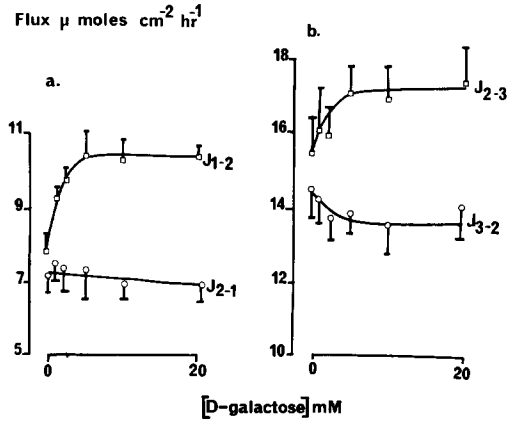

Fig. 5. The effects of galactose (0–20 mM) upon the calculated unidirectional Na^+ fluxes across (a) the mucosal boundary and (b) the serosal boundary. All data shown are for triaminopyrimidine-treated tissues. Error bars denote the standard error of the mean in each instance.

galactose increases intracellular [Na^+] [16] and reduces the electrical potential difference across the mucosal border by $\approx$ 10 mV [35], Na^+ exit should be increased independently of any possible additional acceleration due to a galactose-dependent Na^+ efflux component predicted by the Na^+-gradient hypothesis [36]. Solvent drag, resulting from net fluid flow across the brush border, as described by Koefoed-Johnson and Ussing [38], could account for the discrepancy between the observed efflux and that predicted. (See Appendix).

The present data indicating a lack of any stimulation of Na^+ exit by galactose apparently contradicts studies [37, 36] which demonstrate an increased Na^+ efflux contingent upon cell to mucosal solution gradients of alanine and 3-*O*-methyl glucose. Two important experimental differences between the previous and the present results are, the present use of triaminopyrimidine, which eliminates the possibility that Na^+ efflux across the mucosal boundary is via the tight junctions and the absence of ouabain in the present studies.

(*d*) *Stoichiometry of* Na^+ : *galactose flux within the brush-border.* Goldner et al. [34] calculated the stoichiometry of Na^+ and 3-*O*-methylglucose interaction within

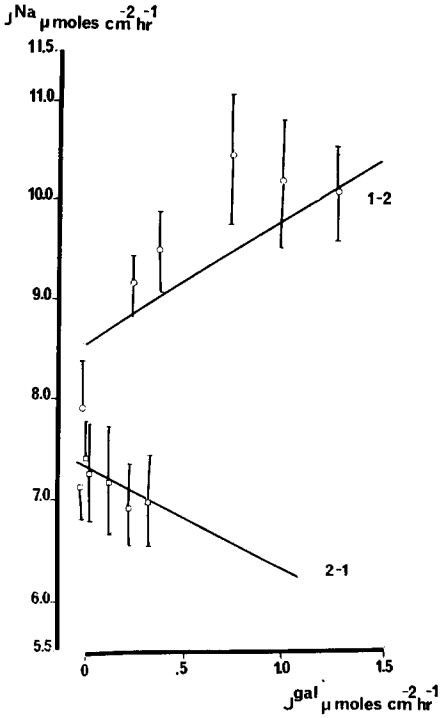

Fig. 6. The relationship between the mucosa to cell flux J_{12} (○) of Na^+ and galactose (and for cell to mucosa flux, J_{21} □). Data for galactose fluxes were taken from ref. 3 corrected for a diffusional component of 0.015 cm · h^{-1}. The regression line for influx (J_{12} Na^+ vs. J_{12} galactose) gives $J_{Na^+} = 8.55 + 1.55\,J$ (galactose) $r = 0.88$). The regression line for cell to mucosa flux (J_{21} Na^+ vs. J_{21} galactoses) gives $J_{Na^+} = 7.33 - 1.03\,J$ (galactose $r = 0.79$). Error bars denote the standard error of the Na^+ fluxes.

the brush-border of rabbit ileum by simultaneously measuring labelled sugar and Na^+ uptake. Whilst it is feasible using the flux method of this present study to measure unidirectional Na^+ and sugar fluxes with quadruple label counting procedures, it was considered that the counting errors thereby introduced would outweigh any advantage gained from simultaneous flux measurement. An alternative approach has been adopted here. Fig. 6 shows the relationship between Na^+ influx (present data)and sugar influx obtained by interpolation of the Michaelis-Menten kinetic parameters of galactose influx in Ringer obtained previously [3]. Although the galactose flux measurements were obtained in open-circuit conditions using the same methods, no effects of short-circuiting upon galactose fluxes have been noted.

The regression line between the increment in Na^+ influx on raising Ringer [galactose] from 0–20 mM and the increment in galactose influx over the same range has a slope of 1.55 ± 0.56 (S.E.). Thus the stoichiometry of Na^+ : galactose interaction for influx across rabbit ileum is not significantly different from that obtained by Goldner et al. for Na^+ : 3-*O*-methylglucose interaction [34].

On plotting the change of Na^+ exit flux across the mucosal border obtained on raising Ringer [galactose] from 0–20 mM against the galactose exit flux J_{21} (gal) on changing Ringer [galactose] over the same range, a slope of -1.03 ± 0.59 (S.E.) is obtained for the regression line of this function. This differs significantly from the slope obtained for the influx stoichiometry ($p < 0.025$) and is contrary to the predictions of the Na^+ gradient hypothesis which implies that the sugar-Na^+ interaction should be similar for both entry and exit across the brush-border [36].

Unidirectional Na^+ fluxes across the serosal border J_{23} and J_{32}

(*a*) *Controls.* The calculated exit, J_{23} and entry flux J_{32} of Na^+ across the serosal border are 16.74 and 15.90 $\mu mol \cdot cm^{-2} \cdot h^{-1}$, respectively. These fluxes greatly exceed those obtained by Schultz et al. [2] who estimated J_{23} and J_{32} to be 4 and 1 $\mu mol \cdot cm^{-2} \cdot h^{-1}$, from data corrected for the shunt fluxes of Na^+. The difference between the results obtained here and those of Schultz et al. [2] is too large to be discounted on the basis of a residual Na^+ shunt permeability in the presence of triaminopyrimidine. Three independently obtained measurements indicate the existance of a large entry flux of Na^+ into the cell across the serosal membranes. These are: (a) the tissue isotope specific activity ratio R, for Na^+ is less than unity, indicating that proportionally more isotope within the tissue fluid originates from the serosal fluid than the mucosal fluid; ouabain increases the isotope ratio R; (b) the permeability of the paracellular route ($0.038 \text{ cm} \cdot h^{-1}$) is significantly less than the total S-M Na^+ permeability in control tissue ($0.048 \text{ cm} \cdot h^{-1}$); (c) triaminopyrimidine reduces S-M Na^+ permeability only to $0.032 \text{ cm} \cdot h^{-1}$, this value is incompatible with S-M Na^+ movement being entirely mediated by the paracellular route, as suggested by Frizzell and Schultz [5]. However, these findings do support the conclusion of Desjeux et al. [7], based on studies of potential difference-dependent S-M Na^+ flux measurements, that there is a significant S-M transcellular flux.

(*b*) *Effects of NaCl replacement by choline chloride and of 0.1 mM ouabain added to Ringer on the unidirectional Na^+ fluxes across the serosal border.* Reduction of Ringer [Na^+] to 25 mM reduces the calculated exit and entry fluxes of Na^+ across the serosal border to 3.74 and 3.38 $\mu mol \text{ cm}^{-2} \cdot h^{-1}$ respectively (Table VII). Ouabain reduces both entry and exit flux of Na^+ across the serosal border at all levels of [Na^+]

tested in the range 25–140 mM. These marked effects of ouabain on serosal Na^+ flux contrast with the small effect of ouabain in the absence of galactose, on the unidirectional Na^+ fluxes across the mucosal border. These effects of ouabain are entirely consistent with the view [9] that the Na^+-pump is situated at the serosal border. It can be observed in Table VII, that as well as a ouabain-sensitive active Na^+ transport process situated at the serosal border, there are also both ouabain-sensitive and ouabain-insensitive-exchange components to unidirectional Na^+ flux across the serosal border. Since ouabain reduces the hypertonicity of the fluid within the lateral intercellular space and hence, the distension of the space and its accessibility to the serosal bathing solution, it is possible that the ouabain-sensitive Na^+-exchange flux simply results from an unfolding of the basal-lateral membranes when the tissue Na^+-pump is activated. This explanation has been previously proposed to account for the ouabain-sensitive component of galactose exchange flux across the serosal membrane [3]. However, further investigation of this process is required in order to define the flux components more exactly.

(*c*) *Effects of galactose on the Na^+ fluxes across the serosal membrane.* Raising Ringer [galactose] increases the exit flux of Na^+ from cells across the lateral-basal border as a hyperbolic function of Ringer [galactose] (Fig. 5b). This result provides support for the view that the tissue Na^+-pump activity is stimulated by Ringer galactose. The activation of the Na^+-pump probably results from the increased entry of Na^+ across the mucosal border with subsequent rises in the intracellular [Na^+]. Consistent with this view, is the finding that ouabain abolishes the galactose-dependent stimulation of Na^+ exit flux, J_{23} across the serosal border (Table VII).

As Ringer [galactose] is raised, there is a slight decrease in the Na^+ entry flux across the serosal border (Fig. 5b). A possible factor contributing to this apparent decrease in Na^+ entry from the serosal fluid is the hypertonic [Na^+] concentration within the extracellular space which may compete with Na^+ coming from the serosal bathing solution for transport sites in the serosal border. Other factors such as an increase in the unstirred layer effects or solvent drag effects within the lateral intercellular space may also play a part in the retardation of Na^+ entry across the serosal border.

Findings consistent with the convective-diffusion model of sugar transport by the small intestine

In previous papers [14, 15, 32] a model has been described for sugar transport in the small intestine which is consistent with the observed unidirectional sugar transport processes across both the mucosal and serosal membranes. It was proposed that the asymmetric transport of sugar across the brush-border arises because of convective-diffusion of sugar via aqueous pores in the brush-border. The force causing the convective flow of fluid across the brush-border and serosal borders is sustained by the osmotic pressure gradient across the lateral-basal border of the epithelial cells. Sugar accumulation within the intestinal epithelial cells is caused by reflection of the sugar at their basal-lateral surfaces.

Thus it is proposed that Na^+ interacts with the sugar transport system at two stages, within the brush-border as a modifier of the membrane pore structure thus increasing sugar diffusivity, and at the serosal border, as both activator of the Na^+-pump and as the hypertonic, electrolyte within the lateral-intercellular space. The

new data supplied in this and the previous paper consistent with these views are as follows: (a) Na^+ is present at hypertonic concentrations in the extracellular space of rabbit ileum and the presence of galactose in the Ringer increases the concentration of Na^+ within the space; (b) ouabain prevents Na^+ accumulation to hypertonic levels within the extracellular space in tissue bathed in Ringer either without, or containing galactose; (c) following addition of galactose to the Ringer, Na^+ influx across the brush-border into the cell is increased; the ratio between this increase in Na^+ entry and of galactose entry is approximately 1 : 1; (d) Na^+ efflux across the lateral-basal border is also stimulated when galactose is added to the Ringer as a result of activation of the Na^+-pump activity following the increase in intracellular $[Na^+]$; (e) both the galactose-dependent increases in steady-state Na^+ influx across the brush-border and efflux across the serosal border are abolished by ouabain; (f) in the presence of triaminopyrimidine, Na^+ efflux across the brush-border from the cell, decreases, following addition of galactose to the Ringer. Since it has been shown that the intracellular $[Na^+]$ increases following addition of galactose to the Ringer [16] and that the membrane potential across the brush-border becomes depolarized by actively transported sugar [35], it is expected on the basis of the Na^+-gradient hypothesis and also on application of basic electrochemical principles, that Na^+ efflux should increase, (by approximately 2-fold, see Appendix) when 20 mM galactose is added to the Ringer. The finding that Na^+ efflux decreases is consistent with the view that there is an additional vectorial force within the brush-border retarding Na^+ exit. Since exit of D-galactose, 3-*O*-methyl glucose and of β-methyl glucoside [14, 32] across the brush-border are also retarded following activation of the tissue Na^+-pump, it is likely that the force retarding the exit of these sugars is the same as that which is retarding Na^+ exit, i.e. the viscous flow of water, Na^+ and sugar through the brush-border pores.

A hypothesis consistent with experimental results in both this and previous papers in the series [14, 32] and results with isolated vesicles from brush-borders from small intestine and renal tubules [43, 44] in which there is apparently no convective flow, (but see ref. 32) and in which asymmetric sugar transport is seen in response to net Na^+ movement down its electrochemical gradient, is that both convective and passive Na^+ movements contribute towards the sugar permeability asymmetry across the brush-border.

However, we consider that passive Na^+ movement by itself can only have a minor role in producing sugar permeability asymmetry in whole tissue, since the observed sugar permeability asymmetry and accumulation within the tissue is consistent with convective-diffusion being the sole driving force, [14, 15, 32] whereas the Na^+ gradient is quantitatively insufficient to account for the asymmetric permeability of some sugars across the brush-border and whole epithelium, when intracellular $[Na^+]$ is higher than the $[Na^+]$ in the mucosal solution [42].

APPENDIX

Schlögl [39] has derived an integral solution to an extended form of the Nernst-Planck equation taking account of the effect of a pressure-induced convective flow together with electrically-induced flow of ions across a porous membrane with fixed charges lining the pore.

The Nernst-Planck equation is

$$J_i = C_i V_i - D_i dC_i/dx - D_i z C_i F/(RT) \cdot d\psi/dx \quad (1)$$

where C_i is the concentration of the mobile counterion within the membrane; V_i, is the convective velocity of i; D, the diffusion coefficient; x, the membrane thickness; z, the ion valence; F, the Faraday constant; R, the gas constant; T, the absolute temperature and ψ, the membrane potential.

Schlögl has defined

$$\varepsilon = \exp\,(F\Delta\psi/RT - V_i x/D_i) \quad (2)$$

or as defined here

$$\varepsilon = \exp\,(F\Delta\psi/RT - \mathrm{Pe}) \quad (2a)$$

where Pe is the Peclet number, the ratio of convective: diffusive velocity of univalent ion within the membrane [14].

$\mathrm{Pe} = V_i/P_i$ where $P_i = D_i/x$

also

$$L = \frac{\varepsilon - 1}{\ln \varepsilon} \quad \text{(ref. 39)} \quad (3)$$

By adopting the Goldman constant-field approximation [40] that the potential gradient across the membrane is constant, Schlogl [39] showed that:

$$J_i = \frac{P(C_1 - C_2\,\varepsilon)}{L} \quad (4)$$

Now since

$$\frac{x}{e_x - 1} \simeq e^{-x/2}$$

for small values of x, ref. 5 it follows that the unidirectional fluxes of ions across the membrane are:

$$J_{12} = P_i C_1 \frac{-\varepsilon}{2} \quad \text{and} \quad J_{21} = P_i C_2 \frac{\varepsilon}{2} \quad (5)$$

Where C_1 and C_2 are the concentrations of mobile ion at the proximal and distal side of the membrane, (it is assumed that when convective flow is positive, the direction is 1–2 and Ψ is taken with reference to a zero potential at the proximal membrane surface).

With reference to the data in this paper, it is assumed that convective flow of Na^+ across the brush-border is negligible when Ringer galactose is zero, since net transcellular Na^+ movement is very small, but rises on addition of galactose, as a result of increased hypertonicity within the lateral intercellular space. The passive permeability of the brush-border is unaffected by galactose; (Table VII as there is no change in the bidirectional Na^+ fluxes across the brush-border following addition of galactose in the presence of 0.1 mM ouabain).

$$\frac{J_{12\,Na^+}(20\text{ mM galactose})}{J_{12\,Na^+}(\text{control})} = \frac{10.15\ \mu\text{mol}\cdot\text{cm}^{-2}\cdot\text{h}^{-1}}{7.97\ \mu\text{mol}\cdot\text{cm}^{-2}\cdot\text{h}^{-1}} = 1.27$$

Rose and Schultz [35] have observed that saturating concentrations of actively transported sugars and amino acids depolarize the potential difference across the brush-border from −40 to −30 mV. Thus by applying Eqn. 5

$$\frac{J_{12\,Na^+}(20\text{ mM galactose})}{J_{12}Na^+(\text{control})} = 1.27 = \frac{-\varepsilon/2}{e^{(0.04F/2RT)}}$$

therefore $-\varepsilon/2 = 2.68$. Since the membrane potential with saturating concentrations of sugar in the mucosal bathing medium is −30 mV

$$e^{-(-0.03F/2RT)} = 1.75$$

Now since $\varepsilon = \exp(F\Delta\psi/RT - \text{Pe})$; then $-\varepsilon/2 = \exp(-F\Delta\psi/2RT) + \exp(Pe/2)$. Therefore, $\exp(\text{Pe}/2) = 2.68 - 1.75 = 0.93$ and the Peclet number for Na^+ movement across the brush-border is 0.62. If the convective force on Na^+ within the brush-border is ignored, then the predicted ratio of Na^+ exit flux in the presence of 20 mM galactose: Na^+ exit flux with zero galactose present in the Ringer, is as follows:

$$\frac{J_{21\,Na^+}^{(\text{galactose})}}{J_{21\,Na^+}^{(\text{control})}} = \frac{C_{2Na^+}^{(gal)}\cdot\exp(F\Delta\psi/2RT^{(gal)})}{C_{2Na^+}^{(con)}\cdot\exp(F\Delta\psi/2RT^{(con)})} = \frac{45\times0.5703}{30\times0.4729} = 1.81$$

The values of $C_{2Na^+}^{(gal)} = 45$ mM and $C_{2Na^+}^{(con)} = 30$ mM were obtained from the results of the previous paper [16]. However, the observed flux ratio of Na^+ exit Table VII is:

$$\frac{J_{21Na^+}^{(gal)}}{J_{21Na^+}^{(con)}} = \frac{7.07\ \mu\text{mol}\cdot\text{cm}^{-2}\cdot\text{h}^{-1}}{7.13\ \mu\text{mol}\cdot\text{cm}^{-2}\cdot\text{h}^{-1}} = 0.99$$

If the extended Nernst-Planck equation is applied to the predicted ratio of Na^+ exit fluxes, with the Peclet number derived from Na^+ entry flux included, then:

$$\frac{J_{21Na^+}^{(gal)}}{J_{21Na^+}^{(con)}} = \frac{C_2^{(gal)}\cdot\varepsilon/2^{(gal)}}{C_2^{(con)}\cdot\exp(F\Delta\psi/2RT)^{(con)}} = \frac{45\times0.3724}{30\times0.4729} = 1.18$$

Hence it can be seen that the extended Nernst-Planck treatment of Na^+ flux across the brush-border, where it is assumed that, in the presence of galactose, Na^+ is driven across the brush-border by both the electrical and hydraulic pressure potential gradients, gives solutions which are quantitatively consistent with both the observed rise in Na^+ entry flux and the lack of change in Na^+ exit flux, following addition of 20 mM galactose to the Ringer. Since the passive permeability of Na^+ across the brush-border is 0.06 cm · h^{-1} then $V_i = \text{Pe}\cdot P_i = 0.036$ cm · h^{-1}.

This result indicates that the convective velocity of Na^+ is approximately the same as that of galactose when galactose is present in the Ringer at 20 mM [14, 32]. Since the convective velocity of galactose decreases as [galactose] is increased [14], whereas the convective velocity of Na^+ rises towards that of galactose as [galactose] is increased; this may imply that galactose displaces Na^+ from sites in the brush-border, unaffected by the convective stream, into a more exposed position, perhaps closer to the pore axis.

Some of the results in this paper have been communicated to the Physiological Society [41].

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