J. Membrane Biol. 158, 109–118 (1997)

The Journal of
Membrane
Biology

Ion, Fluid and Charge Transport Across *Necturus* Intestinal Epithelium in Response to Alanine

B. Shachar-Hill, A.E. Hill
Physiological Laboratory, Downing St., Cambridge CB2 3EG, UK

Received: 5 August 1996/Revised: 7 February 1997

Abstract. Fluxes of Na, Cl and volume were followed across *Necturus* small intestine under zero voltage clamp. 20 mM L-alanine doubles the net Na and fluid transfer. Although there is a ouabain-sensitive Na pump present in *Necturus* a major fraction of the net Na flux can be measured for an hour after application of 10^{-3} M ouabain. Collected fluid transferred by the epithelium is quasi-isotonic over a range of luminal osmolarities from 100 to 250 milliosmolar in alanine saline. The net Na fluxes account for the Na found in this transported fluid. Fluid transfer also shows a large ouabain-insensitive fraction after the addition of alanine.

Compartmental analysis of ^{22}Na-loaded epithelium was used to separate cellular and paracellular fluxes. The estimated Na concentration in the cell derived from its Na content is 9–10 mM, in agreement with that determined with microelectrodes. The Na efflux from cell to serosa is stimulated by alanine, but this increase accounts for only a quarter of the simultaneous rises in Na, fluid and current flow across the epithelium.

The increase of Na efflux from the cell induced by alanine is apparently insensitive to ouabain although the cell Na content rises to *circa* 20 mM but no higher even after 20 hr. From the initial rate of rise of Na in the cell on treatment with ouabain the activity of the Na pump can be estimated to be ~92 pM/cm^2 · sec, a value much smaller than the transepithelial net flux.

The results are not consistent with the standard model in which Na-alanine influx stimulates the Na pump and enhances fluid transport by osmotic coupling in the lateral interspace system. A scheme is proposed based upon that for absorption in *Necturus* gallbladder by which alanine stimulates an active paracellular fluid transfer driven by motile elements of the junction.

Correspondence to: A.E. Hill

Key words: *Necturus* intestine — Epithelium — Alanine — Ion transport — Fluid transport

Introduction

The small intestine of *Necturus* shows a net transport of ions and water from the lumen to the serosa which is stimulated by alanine. As part of a study of the bidirectional fluxes of dextran molecules through the paracellular system in response to the presentation of L-alanine, we have measured the fluxes of the major ions and water; this was necessary in order to determine the parameters of absorption which are required to set the dextran fluxes in context. In a comparable system, the gallbladder epithelium of *Necturus*, previous work has shown that it is not possible to incorporate the ion fluxes at the membranes, and therefore through the cells, into a scheme in which the Na pump is the driving force for the coupling of salt and water flow across the epithelium [5, 8]. Subsequent studies using radiolabeled dextrans as paracellular markers showed that virtually all the fluid flow is transjunctional and that isotonic fluid flow does not occur by local osmotic equilibration [6, 16].

In the intestine the situation is more complicated in two respects: first, the fluid and salt flows are modulated by the presence of metabolites in the luminal bath; second, it is generally assumed that there is recirculation of water and salt occurring as well. The bidirectional fluxes cannot simply be assumed to be passive plus active in the direction of the net flux, and only passive in the opposite direction. Nevertheless, the addition of metabolites can be used as a convenient switch to study changes in ion and water fluxes, and the net fluxes have direct relevance for the fluid uptake mechanism, in particular for the interpretation of the flux of molecules which only use the paracellular system, as described in a companion paper [7]. Alanine is very effective in stimulating the water

and salt transport from the intestinal lumen of *Necturus maculosus* and therefore affords a method for modulating fluid transport in comparison with *Necturus* gallbladder epithelium where the fluid transport rate is ''constitutive.''

The aims of this study were twofold. First, to characterize the overall transport of ions and water by the epithelium in response to alanine, to determine whether the absorbate is isotonic, and to measure the sensitivity of the fluxes to ouabain. Second, to measure the rate of Na efflux from the cells, in particular the ouabain-sensitive fraction, by compartmental washout analysis to see whether the rate altered when the tissue was presented with alanine, as would be expected from a cytoplasmic stimulation of the Na pump. A comparison of the cellular and transepithelial Na fluxes should give information about the role of the pump in fluid production. The technique of compartmental analysis has been developed for analyzing ion fluxes in *Necturus* gallbladder epithelium [8] and has been used here for analyzing the cellular Na flux in the intestine.

Materials and Methods

Tissue and Solutions

Necturus maculosus was kept in aerated, filtered tanks at 13–14°C and fed regularly. Sections of small intestine of 5–10 cm were removed from below the bile duct and stripped of the muscle coat under a dissecting microscope. Pieces were mounted into Perspex cassettes where the tissue was held in place by outer O-rings; inside these were circular flat rings whose faces were spaced 30 μm apart, and which sealed the tissue without crushing it. The area of epithelium exposed to the saline baths was 0.283 cm^2. Adjacent pieces were used in parallel chambers to measure forward and back fluxes simultaneously in any experiment.

Necturus salines were buffered at pH 7.2 and contained (mM/l) either Na 100, K 3.6, Ca 1, Mg 1, Cl 104, phosphate 3.6, gassed with pure oxygen, or Na 100, K 2, Ca 1, Mg 1, Cl 84, bicarbonate 20, gassed with 95% O_2:5% CO_2; in experiments where the latter was used it is referred to as ''bicarbonate saline.''

Flux Measurements

Experiments were carried out at 18–20°C. Bidirectional fluxes of ^{22}Na and ^{36}Cl were measured across the epithelium by sampling at 15-min intervals and averaged over an hour for each protocol. ^{22}Na was assayed in a gamma counter and ^{36}Cl assayed by liquid scintillation counting. L-Alanine was used at 20 mM and always applied to both sides of the epithelium. Ouabain was also applied to both sides of the epithelium at a concentration of 1.0 mM to ensure rapid inhibition. Solutions were stirred at high speed parallel to the cassette faces by magnetic bars, the rotations being monitored with Hall effect transistor probes. Calculation shows that as the Reynolds number exceeded 8000 the flows were turbulent and consequently unstirred-layer effects in the baths adjacent to the apical cell membrane and the subepithelium were considered to be negligible [16]. Within the subepithelium, which is an unstirrable matrix, USL gradients can be calculated as follows: assuming that the matrix is similar to *Necturus* gallbladder, where the diffusion coefficients of Na and Cl ions are about 20% of free solution values [8], application of the linear diffusion-convection equation with the known values of fluid convection indicates that the concentration difference across the structure is very small, amounting to 2%.

Voltage Clamp

The clamp amplifiers were equipped with series resistance compensation using a continuous automatic bias circuit. These were initialized by balancing the effect of current in chambers with empty cassettes and salines. The efficacy of the compensation was checked before every experiment against an electronic analogue circuit of the tissue in the amplifier. Tissue resistances were monitored with current clamp pulses of 3 mv every 9 min. The output of the clamp circuits was filtered at 20 Hz and the data captured by computer software emulating scrolling recorders.

Fluid Measurements

Volume Flow Rates

Fluid transfer was measured gravimetrically. Stripped epithelium was mounted across the bottom of a cylindrical Perspex chamber (0.66–1.33 cm^2 in area) containing saline and immersed in an aerated bath at room temperature. Weighings were taken every 15 min.

Absorbate Osmolarity and Na Concentration

Stripped epithelium was mounted horizontally in a small chamber in which the mucosal face was perfused with saline from a reservoir. The serosal side faced a closed chamber in which absorbate could be collected. Care was taken to avoid any temperature differences between the tissue and the chamber walls, which can alter the concentration of the fluid by vapor phase distillation of water out of the absorbate. To ensure thermal equilibration the chamber was seated into an aluminium block which was immersed in a constant temperature bath.

At the end of two hours the chamber was opened and fluid removed from the underside of the tissue with a filter paper disc which was immediately transferred to an adjacent vapor phase osmometer (Wescor 5500). Care was taken not to touch the tissue in this procedure. Samples from the mucosal perfusing solution were also assayed. Osmolarities determined in this way are probably upper estimates due to any slight evaporation of the samples. Na in the absorbate was determined by flame photometry.

Cell Efflux Analysis

Technique

The measurement of ion fluxes from the cell and extracellular space by compartmental analysis, and the theory underlying the technique, is described in considerable detail elsewhere for *Necturus* gallbladder epithelium including the estimation of unstirred layers (USL) at both mucosal and serosal surfaces [5]. The same conditions apply to *Necturus* intestine, and the technique is only described here in its essentials.

The stripped epithelium is set up in conditions of steady-state transport between two chambers bathed in bicarbonate saline. The mucosal chamber is filled with ^{22}Na-saline. The serosal chamber is kept

washed with nonradioactive saline by a pump. Previous studies of the diffusion of Na ions in the subepithelium of gallbladder show that the specific activity of Na adjacent to the basal cell membrane must be close to zero i.e., that there is a negligible USL in the subepithelium under these conditions. In the case of stripped intestine, the subepithelium is of similar thickness to that of the gallbladder epithelium and so these conditions apply. The subepithelium thus does not make a contribution to the Na compartments and does not impose any delays. At $t = 0$ the mucosal ^{22}Na saline is replaced with nonradioactive saline and the perfusion of the serosal chamber is directed to a fast fraction collector (15-sec aliquots). The efflux of ^{22}Na into the serosal bath represents the loss from the cell and the extracellular space compartments as they move towards a condition of zero specific activity Each empties as a single exponential with its own time constant.

In experiments to determine the effect of ouabain on the efflux the glycoside, at a concentration of 10^{-3} M, was added to the serosal perfusate at zero time. When ouabain is applied to the serosa it acts quickly. It can be calculated that 98% inhibition of the pump in *Necturus* occurs within 20 sec, taking the diffusion lag into account (*see* Discussion). In some experiments in which the rise of cell Na was measured after inhibition of the Na pump, the tissue was pretreated with ouabain for periods extending up to 40 min at room temperature (20°C) and for one set of treatments kept overnight (20 hr) at 6°C and then warmed to room temperature.

Cell Volume Determination

Stripped *Necturus* intestine was fixed for light microscopy in buffered glutaraldehyde at pH 7.2 with the osmolarity identical to the saline to minimize volume changes. The tissue was then post-fixed in OsO_4, dehydrated and embedded in epoxy resin. 1–2 μm sections were cut and photographed under phase contrast microscopy.

To convert cell contents to concentration the enterocyte volume per unit area was obtained from measurements of cell dimensions from light micrographs of the stripped epithelium. This was 0.0134 cm^3/cm^2.

Results

Analysis of the Cell Efflux

The ^{22}Na contents of the washout samples were fitted to a double exponential (Fig. 1) from which the fast and slow intercepts J_0 and slopes k were extracted. The slow intercept represents the basolateral efflux from the cell and the fast one, the efflux *via* the paracellular system. This has been confirmed in a study of *Necturus* gallbladder, in which the sizes and time constants of the two compartments are very similar to those in intestine, by loading the tissue with radio sucrose as a free space marker. Efflux analysis of the radio sucrose then reveals only one compartment i.e., the efflux curve is a single exponential, with similar time constant to the fast Na compartment of the tissue.

The contents Q of each compartment are determined by integration of the separate exponentials i.e., for a single compartment

$$J_t = J_0\, exp(-kt) \tag{1}$$

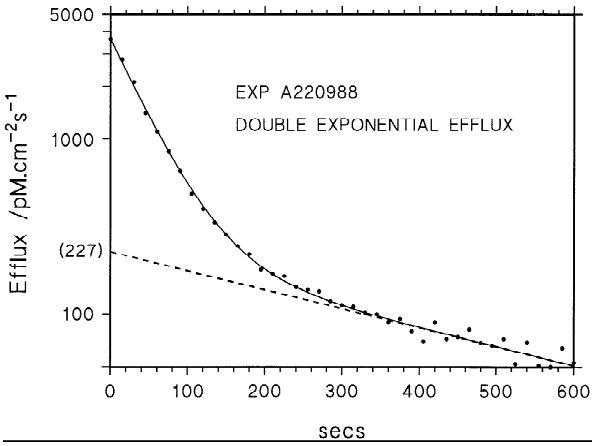

Fig. 1. A representative experiment of efflux into the serosal chamber from ^{22}Na-loaded intestine in bicarbonate saline fitted to a double exponential. The dotted line shows the zero intercept to the slow component. The equation fitted to this data was $y = 3429.8\ exp(-2.19 \times 10^{-2}t) + 226.8\ exp(-2.49 \times 10^{-3}t)$; the intercepts and time constants separate by an order of magnitude.

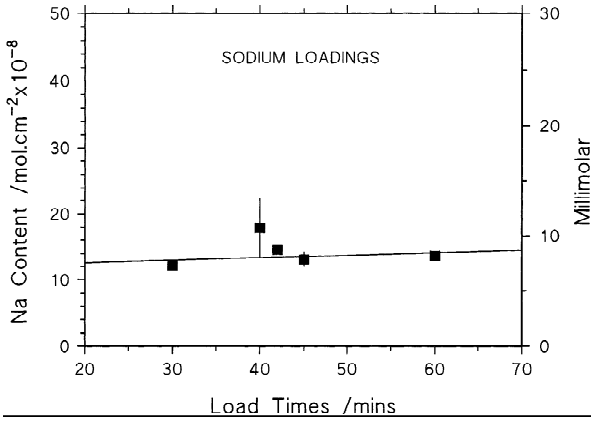

Fig. 2. Contents as determined with Eqs. 1 and 2 after loading for different periods with ^{22}Na in bicarbonate saline. The line is a linear regression with $r^2 = 0.002$ for all experiments.

$$Q = \int_0^\infty J_t dt = J_0/k \tag{2}$$

which does not require that all the radio sodium be washed out, only that the zero time intercept J_0 and the rate constant k be known. To validate that the slow efflux is indeed that from the cell it must be shown that this compartment has the same Na content as the enterocyte. Because the cell is essentially in diffusive equilibrium only with the mucosal bath the specific activity of the cell $s = 1$. The cell Na content Q must be equal to the ^{22}Na content Q^* i.e., $Q = Q^*/s$.

It is very important to ascertain (i) whether the cells

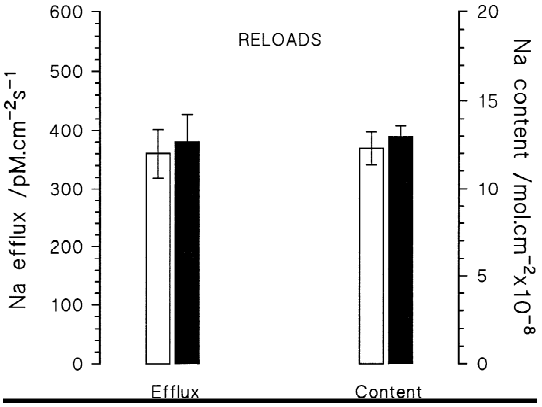

Fig. 3. The effect of reloading on efflux and content of *Necturus* intestine. Tissue was reloaded in bicarbonate saline with ^{22}Na 20 min after the first washout. No significant difference in content or efflux from the cell is observed between the first (open bars) and second (solid bars) washouts.

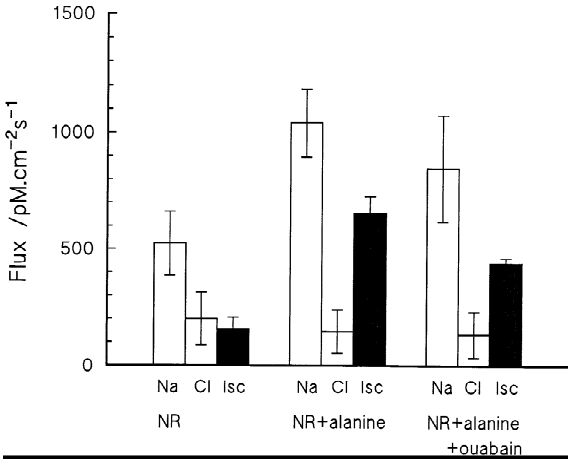

Fig. 4. Charge balance under zero voltage clamp. Currents (Isc) and the net transport of Na and Cl are shown before and after alanine stimulation. Ouabain values are means for the subsequent hour.

are loaded for long enough and (ii) whether the technique is causing the cells to leak; (i) can be calculated if the cellular through-flux of Na is known, but could not be assumed *a* priori. Consequently, cells were loaded for various times (all in excess of calculated values) and the contents *Q* were compared. The results are shown in Fig. 2, where it can be seen that different load times make little difference and that they are already asymptotic; (ii) was investigated by reloading the cells with ^{22}Na 20 min after a washout and repeating the experiment. The results from reloads are shown in Fig. 3 for the slow (cellular) component. It is apparent that the same results are obtained and the slight increase in efflux is not significant. This is probably an indicator of the robustness of *Necturus* tissues in general.

Ion Fluxes and Charge Flow Balance

The net fluxes of sodium and chloride ions across the epithelium under zero voltage clamp are shown in Fig. 4. It is apparent that the clamp current is due to the absorption of sodium and chloride ions in different proportions, the sodium fluxes being much larger than the chloride. The sum of the two is accounted for by the charge flow and there is no special need to invoke a substantial transport of other ions, for which there is no evidence. It would appear that the effect of alanine is to stimulate the net sodium flux without stimulating the net chloride fluxes.

Effect of Alanine and Ouabain on Transepithelial Na Fluxes

The transepithelial Na fluxes are shown in Fig. 5 and the Table where the stimulation due to alanine and its partial

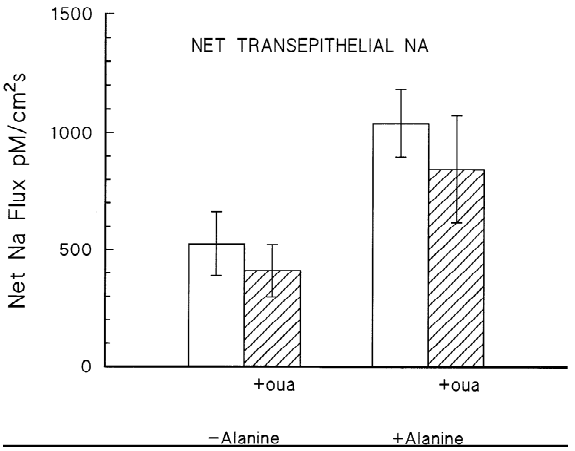

Fig. 5. Transepithelial net Na fluxes before and after alanine stimulation and the effect of ouabain (hatched).

suppression by ouabain can be seen. The fluxes under ouabain are mean values for the first hour after application of the glycoside. There are two points of interest in these results. First, it is clear that the stimulation of sodium transport is due to the absorption of Na ions while the changes in Na backfluxes are not significant. Second, the Na-pump inhibitor ouabain has only a partial (and on a time scale, very slow) effect and the net transport of sodium stimulated by alanine is remarkably resistant to this compound. However, it is important to note that studies on the Na pump in *Necturus* have shown that it is sensitive to ouabain having similar properties to that in other animals (*see* Discussion). In these experiments on *Necturus* intestine the clamp current I_{sc} begins to decline immediately after ouabain is applied in a quasi-exponential fashion with an initial time constant of $3–7 \times 10^{-4}$ sec^{-1} which indicates that the glycoside acts

Table. Transepithelial Na and fluid flows

	NR	NR + ouabain	NR + alanine	NR + alanine + ouabain
Na *ms*	2245 ± 166	2120 ± 242	2724 ± 167	2455 ± 281
Na *sm*	1720 ± 100	1709 ± 159	1684± 109	1609 ± 281
Na net	525 ± 138	411 ± 112	1041 ± 144	846 ± 228
Jv	4.21 ± 0.41		9.59 ± 1.64	3.93 ± 0.76

Na fluxes (pM/cm^2 · sec) measured as isotope flows and fluid flows *Jv* (cm^3/cm^2 · sec × 10^{-6}) followed gravimetrically, ±SE. L-alanine added at 20 mM and ouabain at 1 mM. The flows of both Na and fluid following ouabain addition are means for 1 hr.

upon the Na pump in the intestinal epithelium just as it does in other tissues of the animal.

Fluid Transport

Volume Rates and Sodium Transport

The Table shows the stimulation of fluid transport induced by alanine which is ~230% and falls under ouabain to 93% of the prestimulated level, as averaged over the first hour.

In Fig. 6 the rate of sodium transfer in the fluid based upon its Na content (*see* next section) is compared to the net transepithelial radio-sodium fluxes. The differences are not significant and are consistent with a transfer, before and after alanine stimulation, of isotonic volumes of fluid composed predominantly of sodium saline.

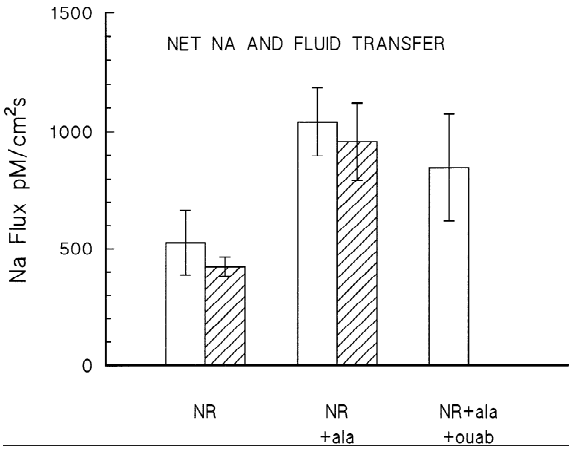

Fig. 6. A comparison of net transepithelial Na fluxes (open bars) with the Na flux calculated from its mean concentration in the transported fluid (hatched).

Tonicity and Na Content of the Absorbed Fluid

The fluid osmolarity is shown in Fig. 7 over a range of luminal values from 100 to 250 mOs. The relationship between absorbate and luminal osmolarity is linear ((correlation)^2 = 0.98) and can be described as quasi-isotonic. The measurements involve a transfer of fluid to an osmometer chamber and there may be a slight rise in tonicity caused by this procedure (*see* Materials and Methods) although this could not be accurately quantified. The measured Na concentration in the absorbate ([Na]/mM) is related to its osmolarity (π/mOs) by the expression [Na] = 0.4π–1.9. The Na salts therefore account for approximately 80% of the osmolarity, a value which may reflect a slight enrichment of the fluid in alanine at the expense of sodium.

Compartmental Analysis of Sodium Transfer

The serosal efflux analysis results are shown in Figs. 8 and 9. In this analysis we consider it an essential test of the method to be able to measure the Na content of the cells. As explained in Materials and Methods, it is possible to do this by calculating the area under the slow exponential i.e., by integrating the cell efflux of labeled Na over time until this compartment is effectively empty (Eq. 2). The value obtained for the cell content in bicarbonate saline is 12.5 × 10^{-8} mol/cm^2 epithelium and using the measured value for the cell volume (0.0134 cm^3/cm^2 epithelium) gives a value for the cell Na concentration [Na$_i$] of 9.3 mM. This may be compared with two values for [Na$_i$] in *Necturus* small intestine determined previously with sodium-selective microelectrodes in solutions without bicarbonate ion: [Na$_i$] = 9.1 mM [14] and a calculated [Na$_i$] of 12.1 mM [9] determined in saline containing 110 mM Na. These values show clearly that the titration of intracellular sodium by ^{22}Na efflux analysis gives correct results and that the measured efflux from the slow phase represents that from the cell.

In the absence of alanine the fluxes from the cell compartment shown in Fig. 9 (366 ± 32 pM/cm^2 · sec) are of comparable size to the net flux across the epithelium (516 ± 140) although somewhat smaller. But, the efflux is not ouabain sensitive and this lack of inhibition is also seen in the transepithelial fluxes (Fig. 5). It

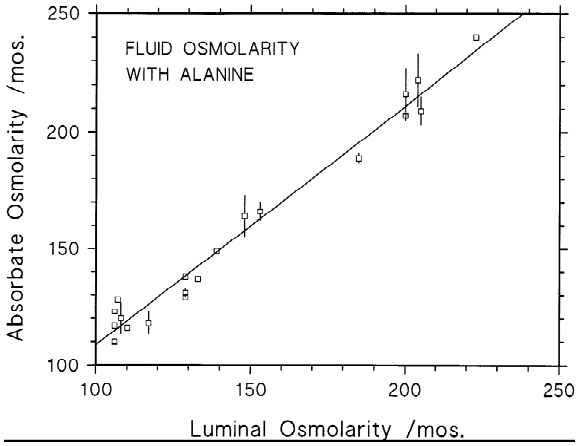

Fig. 7. The measured osmolarity of the serosal transportate plotted against that of the luminal saline. The line represents a linear regression ($r^2 = 0.98$).

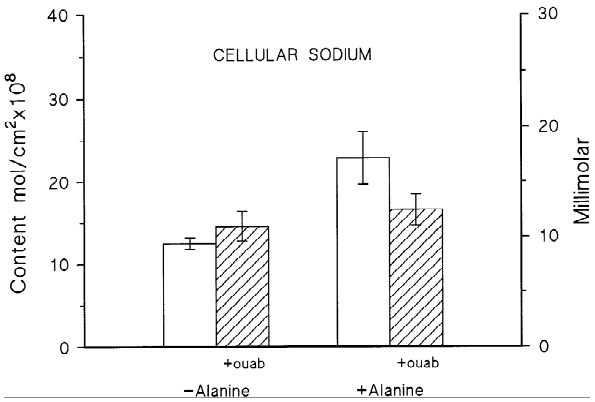

Fig. 8. Content of Na in the cell compartment (Eq. 2) before and after alanine stimulation in bicarbonate saline and the effect of ouabain (hatched). The content can be converted to a concentration using the epithelial volume of 1.34×10^{-2} ml/cm^2 epithelium.

should be remembered that the cell effluxes of Fig. 9 as estimated from the analysis are measured over a period of 10 min, longer than needed to inhibit the pump (20 sec) but over which the flux has not fallen appreciably. However, the transepithelial net Na fluxes are averaged over an hour, during which time the values under ouabain are slowly falling; the values a short time after ouabain (15 min) would be much closer to the pre-ouabain values.

The effects of alanine are also shown in Figs. 8 and 9 where it can be seen that there is a stimulation of both Na content and Na efflux. However, the stimulation of Na efflux from the cell (from 366 to 510 i.e., 144 pM/cm$^2 \cdot$ sec) is small compared to the increase in transepithelial Na flux after alanine (~500 in NR and ~1000 in bicarbonate saline); a comparison of all the flux increases caused by alanine are made below (*see* Discussion and Fig. 11).

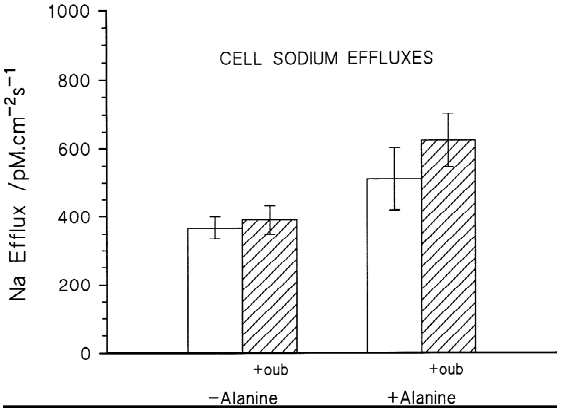

Fig. 9. The Na effluxes from the cellular compartment before and after alanine stimulation in bicarbonate saline with the effect of ouabain (hatched). The efflux is the intercept of the cell component at $t = 0$ (*see* Fig. 1).

Effects of Ouabain on Cell Na Content

In the experiments shown in Fig. 10 the cell Na content was determined by efflux analysis after pretreating the tissue with ouabain for different times. During this time the cell Na would be expected to rise in response to the inhibition of the Na pump and the inwardly directed Na gradient. It is apparent that the cell Na does not approach that of the bath (100 mM) but ''locks'' at about a sixth of this value (~16 mM), assuming an original cell volume of 0.0134 cm^3/cm^2 epithelium. If the tissue has swollen to any degree, the concentration will be even lower. As the content rises this can be used to estimate the rate of pumping: if the pump is inhibited, the immediate rise of cell Na, $(dQ/dt)_{t=0}$ should be equal to the pump rate. If the total cell Na is measured, as in the efflux experiments here, the value of dQ/dt does not depend upon the volume of the cell as it would if cell concentration was measured. This procedure depends upon the pump being inhibited on a time scale which is short compared to the rise of cell Na or the fall of cell potential. Both these criteria are met: the pump is inhibited within 20 sec (*see* Discussion below) and the intracellular Na concentration and potential change very little in that time ([20] and refs. therein). If we fit a third-order polynomial to the first four points of Fig. 10 we obtain an influx at zero time of 92 pM/cm$^2 \cdot$ sec. This value is of the same order as that in *Necturus* gallbladder epithelium [8] (62 pM/cm$^2 \cdot$ sec) and quite small when compared to the cell efflux value (366 pM/cm$^2 \cdot$ sec).

Discussion

The results are difficult to fit to any simple scheme whereby the Na pump is the coupling mechanism between Na and fluid transport in the intestine. In many

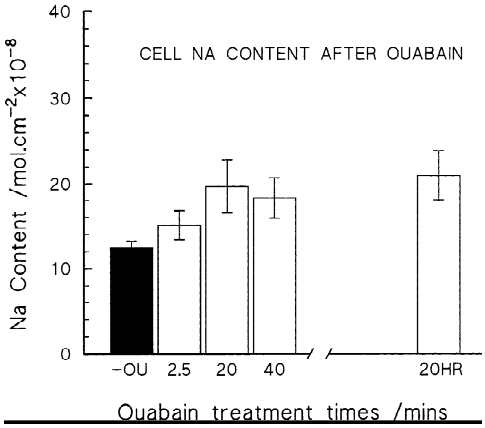

Fig. 10. The rise of Na in the cell in bicarbonate saline determined by washouts at specified times after ouabain treatment. The value at 20 hr was determined after incubation at 6°C.

respects the picture is very similar to that shown for *Necturus* gallbladder. There are two main points to be noted here: the relative insensitivity of the net fluxes to inhibition by ouabain, and the small size of the cell fluxes compared to the transepithelial ones after stimulation by alanine.

Pump Inhibition

Figure 4 shows that ouabain reduces the short-circuit current and net Na transepithelial transport after the addition of alanine, but does not abolish them. Other workers have observed this phenomenon in *Necturus* intestine [4] and claimed that this is due to the insensitivity of *Necturus* as a species. It is essential to draw a clear distinction between the action of ouabain on cellular Na homeostasis and its effect upon transepithelial salt and water transport. There are many instances in the literature where ouabain has only a partial or slow effect upon transepithelial transport, in a manner similar to that described here ([1, 4, 15, 17] for example). However, it has never been demonstrated that ouabain fails to inhibit an epithelial Na-K-ATPase when present in the assay.

Ouabain has a similar effect in *Necturus* to that in other tissues which are sensitive to the glycoside. In the *Necturus* erythrocyte ouabain inhibits $^{82}Rb^+$ ion accumulation with a $K_{0.5}^{-1}$ of 1.6×10^{-6} molar and in the gallbladder epithelium studies with labeled ouabain have shown that the association coefficient of the inhibitor for the pump is 5×10^3 M^{-1} sec^{-1} [8]. This compares well with that determined for mammalian systems [2]. The diffusion time constant measured for ^3H-ouabain in the lateral intercellular spaces, when applied to the serosal bath, is 5.2×10^{-3} sec^{-1}. These two can be combined into a single rate equation representing simultaneous filling of the interspace with ouabain and its binding to the pump sites. The solution of this equation shows that the Na pump is inhibited to 98% in 20 sec [8].

As shown in Results ouabain has an immediate effect as would be expected from its binding constants. The cell Na quickly starts to rise, and if there is a microelectrode in the cell the potential immediately falls. The transepithelial short-circuit current also quickly begins to fall in a quasi-exponential fashion. However, the rate of decline is slow and the current is still 68% of the alanine-stimulated value after an hour. There is a mechanism that can maintain the charge flow through an epithelium for some time: this is the effect of the Na-K gradient between cell and bath running down and driving current through the tissue—the ''replacement current'' effect. The current comprises a Na current entering across the apical membrane and a K current of similar magnitude leaving the basolateral membrane. This phenomenon can easily be demonstrated with tight epithelia, and the time course of clamp current after ouabain has been effectively modelled [11]. This mechanism cannot be invoked here for the reason that the *Necturus* enterocytes do not replace most of their K with Na: the data of Fig. 10 show that the Na content of the cell has only risen from 12 to about 20 ($mol \cdot cm^2 \times 10^{-8}$) over the first 20 min, and remains at this level for long periods, sometimes overnight. The replacement current this Na would transfer is only about 5–6 μA for the first 20 min. This phenomenon of the Na rising to ''lock'' at a value which is still quite low may be due to the inhibition of Na entry by the rise of the cytoplasmic Na concentration. This has been postulated for both ''tight'' epithelia [12, 18, 19] and ''leaky'' ones [3, 10] and may be a general effect.

The net epithelial transport of Na and volume show a similar decline but a substantial fraction of both is also present after an hour (Table and Figs. 5 and 6). It would seem that the remaining net transport of Na and charge, together with a continuation of volume flow, is not directly dependent on the Na-K pump. There is apparently a different coupling process maintained, which declines slowly after ouabain. It has been proposed that the basis of fluid transfer in *Necturus* gallbladder epithelium is an active mechanism resident in the junction [6] and the extension of this idea to *Necturus* intestine in order to explain these results is explored more fully below.

Ouabain has inhibitory effects on most transepithelial flows if left for quite long periods. This may be due to the fact that eventually cellular ion gradients collapse and transepithelial movements, together with other cell transport functions, come to a halt. *Necturus* is very resistant in this respect while most mammalian tissues are very sensitive. It is possible that the Na:K gradients built up by the pump between the cell and exterior contribute energetically to the fluid transport mechanism, and the fact that inhibitors of ATP synthesis often work very slowly would support this idea. The linkage may there-

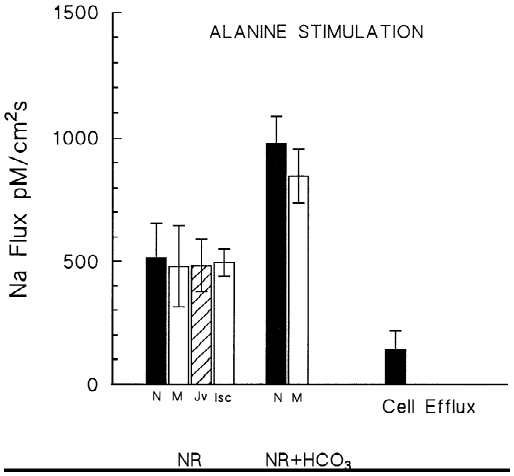

Fig. 11. The stimulations produced by alanine of transepithelial net Na flux (N), mucosa-to-serosa Na flux (M), fluid transfer (J_v) and current (I_{sc}) expressed as equivalent Na fluxes, compared with the cellular Na efflux.

fore be indirect, as opposed to that envisaged in the conventional scheme where the Na pump and fluid movements are simply osmotically coupled.

Cell Effluxes of Na

The cellular effluxes shown in Fig. 9 are not reduced by ouabain, which is in conformity with the behavior of the net transepithelial fluxes shown in Fig. 5. While the two are comparable in magnitude in the absence of alanine (about 400–500 pM/cm^2 · sec) it is clear that the increase in cell efflux caused by alanine, which on the conventional model is due to the stimulation of the sodium pump, is far too small to account for the rise in transepithelial net flux. In Fig. 11 the two are compared.

It is interesting to compare this result with the situation revealed by previous work. In *Necturus* intestine the sodium transport is stimulated by galactose, but the rise in cellular sodium concentration is not sustained and falls back to its prestimulated level in less than 10 min. The net Na transport rate, however, as measured by short-circuit current, increases by 400% to a steady level [9]. Similar effects are seen in the stimulation of net Na transport in rabbit gallbladder epithelium by bicarbonate [13]. The conclusions drawn in both papers are that (i) there cannot be a kinetic stimulation of the basolateral Na pumping consistent with the known properties of the Na-K pump (*see* Discussion in [9]) and therefore this simple scheme must be called into question, and (ii) the only possible mechanism must be an involvement of more pump: the effective V_{max} of the overall Na transport is increased as opposed to its K_m. Either ''inactive'' pumps in the membrane are being activated, other than by a rise in $[Na]_c$, or new pump is being recruited into the membrane. It is not apparent what the signal or mechanism for this process would be.

It is clear from Fig. 11 that such a mechanism of pump recruitment cannot be operating because the increase in Na efflux from the cell is far too small to account for the rise in net transepithelial Na flux. Furthermore, the cell Na efflux is ouabain insensitive (Fig. 9) which is not consistent with the operation of a Na-pump; insensitivity of transepithelial charge flow to ouabain has also been noted by others [4] but as we have seen, *Necturus* possesses Na pumps of normal ouabain-sensitivity. The intestinal short-circuit current begins to decline immediately after the application of ouabain (*see* Results).

When this technique of efflux analysis is applied to *Necturus* gallbladder epithelium it reveals a ouabain-sensitive fraction amounting to 62 pM/cm^2 · sec, but there is a large insensitive fraction [8]. Here the effluxes seem to be insensitive, but we obtain a comparable pumping rate of 92 pM/cm^2 · sec from the initial rise of cell Na after ouabain, as calculated above.

Na and Charge Flow Across the Epithelium

A central feature of the stimulations caused by alanine, shown in Fig. 11, is that the clamp current is much larger than the rise in cell efflux, at least by 350%. If the actual pump rate is even smaller than the cell effluxes i.e., only 92 pM/cm^2 · sec, the discrepancy becomes much greater. This is again an indication that the increase in charge flow across the epithelium cannot be explained simply by an increase in Na pumping, although it is certainly due to an increase in overall Na transport. If the increase is due to a junctional mechanism similar to that which may operate in gallbladder, it must transfer charge independently of the pump, which would account for the discrepancy. It is possible that the clamp current is a measure of cell Na pumping only in ''tight'' epithelia which do not transport isotonically.

The insensitive fraction, although it cannot be a leak [8], represents a loss of radio-sodium from the cytoplasmic compartment. We propose in Fig. 12 a mechanism to account for the overall transport features of the epithelium, before and after stimulation by alanine. This scheme is consistent with that proposed for *Necturus* gallbladder [6, 8] and that which emerges from a study of the intestine using paracellular probes [7]. Essentially, the driving element is a compartment of the junction which uses active contractile elements to transport Na and water in isotonic proportions. As the net Cl fluxes are always much smaller than the Na net fluxes, the junctional compartment must discriminate against Cl ions; this could be on the basis of size, but is more likely to be because it is negatively charged. In this case the junctional mechanism is contributing to the clamp cur-

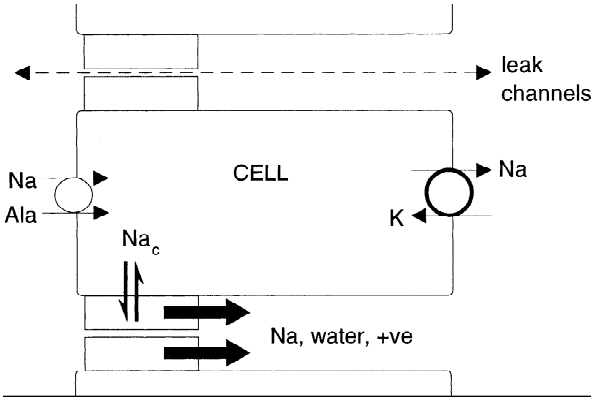

Fig. 12. A proposed scheme of junctional transfer, initially as isotonic fluid flow through active junctional elements. The junction is also traversed by passive bidirectional leak channels. In passing through the junctional compartment Na can exchange with Na in the cytoplasmic pool.

rent under short-circuit conditions in a manner that is indistinguishable from the Na pump, and the persistent current after ouabain may originate from this source.

It is important to include in this scheme a mechanism for the exchange of Na ions between junctional compartment and the cytoplasm (a ''window'') to explain two observations. (i) In a washout experiment radio-Na can be stripped from the cell into the serosal bath after the pump is inhibited with ouabain, as shown in Fig. 9. This requires another efflux mechanism because the basolateral membrane is virtually Na impermeable; an exchange of Na ions would bring about this radio-Na efflux as observed. (ii) The fluid transferred is quasi-isotonic. If the Na pump were operating independently of the junction then the fluid transfer would be hypertonic because the total Na transfer across the epithelium J_s would be the sum of junctional and basolateral fluxes i.e., $J_s = J_v C_o + P$ where J_v is the fluid transfer rate at tonicity C_o, and P is the pump rate. If a fraction a of the Na entering the cell comes from the junctional compartment then this expression becomes

$$J_s = J_v C_o - aP + P \tag{3}$$

and the tonicity of the absorbate, C_e would then be given by

$$C_e = J_s/J_v = C_o + P(1 - a)/J_v \tag{4}$$

If $a = 0$ (pump and junction were quite independent) then a basal saline concentration of $C_o = 0.1$, a pumping rate P of 92 pM/cm^2 · sec, and a fluid transfer rate of 4.21 × 10^{-6} cm^3/cm^2 epithelium · sec, would lead to a predicted value of C_e of 0.123. This represents a hypertonicity of 23% which is not observed. Therefore, the entry of Na into the cell from a junctional compartment enables the overall transported fluid to approach isotonicity. The magnitude of a is unknown, but in this model it must be a substantial fraction to achieve quasi-isotonicity, and this would lead directly to the behavior seen in Fig. 9. This parameter would function to control the tonicity of the transportate. At present there is no precise value for the amount of Na entering the cell by transporters on the apical membrane, although many studies assume this to be 100%.

The role of the Na-alanine transporter in controlling the rate of Na and fluid transfer needs to be further clarified. Obviously, on the results presented in this paper, it cannot be the simple one of stimulating the pump by raising the internal Na concentration. So much has been concluded in a previous study for the Na-galactose transporter in *Necturus* [9].

This work was supported by the Wellcome Trust.

References

1. Bentzel, C.J. 1974. Expanding drop analysis of Na and H_2O flux across *Necturus* proximal tubule. *Am. J. Physiol.* **226:**118–126
2. Erdmann, E., Schoner, W. 1973. Ouabain-receptor interactions in (Na + K) ATPase preparations from different tissues and species. *Biochim. Biophys. Acta* **307:**386–393
3. Gullans, S.R., Avison, M.J., Ogino, T., Giebisch, G., Schulman, R.G. 1985. NMR measurements of intracellular sodium in the rabbit proximal tubule. *Am. J. Physiol.* **249:**F160–168
4. Gunter-Smith, P.J., Grasset, E., Schultz, S.G. 1982. Sodium-coupled amino acid and sugar transport by *Necturus* small intestine. *J. Membrane Biol.* **66:**25–39
5. Hill, A.E., Hill, B.S. 1987. Steady-state analysis of ion fluxes in Necturus gall-bladder epithelial cells. *J. Physiol.* **382:**15–34
6. Hill, A.E., Shachar-Hill, B. 1993. A mechanism for isotonic fluid flow through the tight junctions of *Necturus* gallbladder epithelium. *J. Membrane Biol.* **136:**253–262
7. Hill, A.E., Shachar-Hill, B. 1997. Fluid recirculation in Necturus intestine and the effect of alanine. *J. Membrane Biol.* **158:**119–121
8. Hill, B.S., Hill, A.E. 1987. Transcellular sodium fluxes and pump activity in *Necturus* gall-bladder epithelial cells. *J. Physiol.* **382:**35–49
9. Hudson, R.L., Schultz, S.G. 1984. Sodium-coupled sugar transport: effects on intracellular sodium activities and sodium-pump activity. *Science* **224:**1237–1239
10. Jensen, P.K., Fisher, R.S., Spring, K.R. 1984. Feedback inhibition of NaCl entry in *Necturus* gall-bladder epithelial cells. *J. Membrane Biol.* **82:**95–104
11. Lew, V.L., Ferreira, H.G., Moura, T. 1979. The behaviour of transporting epithelial cells. I. Computer analysis of a basic model. *Proc. R. Soc. Lond. B.* **206:**53–83
12. Lewis, S.A., Wills, N.K., Eaton, D.C. 1978. Basolateral membrane potential of a tight epithelium: ionic diffusion and electrogenic pumps. *J. Membrane Biol.* **41:**117–148
13. Moran, W.M., Hudson, R.L., Schultz, S.G. 1986. Transcellular

sodium transport and intracellular sodium activities in rabbit gallbladder. *Am. J. Physiol.* **251:**G155–159

14. O'Doherty, J., Garcia-Diaz, J.F., Armstrong, W.McD. 1979. Sodium selective liquid ion-exchange microelectrodes for intracellular measurements. *Science* **203:**1349–1351
15. Schatzmann, H.J., Windhager, E.E., Solomon, A.K. 1958. Single proximal tubules of the *Necturus* kidney. II. Effect of 2,4-dinitrophenol and ouabain on water reabsorption. *Am. J. Physiol.* **195:**570–574
16. Shachar-Hill, B., Hill, A.E. 1993. Convective fluid flow through the paracellular system of *Necturus* gall-bladder epithelium as revealed by dextran probes. *J. Physiol. Lond.* **468:**463–486
17. Spector, D., Hayslett, J.P., Kashgarian, M. 1974. Na-K-ATPase-mediated seasonal variation of sodium transport in *Necturus* kidney. *Am. J. Physiol.* **227:**873–877
18. Weinsten, F.G., Rosowski, J.J., Peterson, K., Delalic, Z., Civan, M.M. 1980. Relationship of transient electrical properties to active sodium transport by toad urinary bladder. *J. Membrane Biol.* **52:**25–35
19. Wills, N.K., Lewis, S.A. 1980. Intracellular Na^+ activity as a function of Na^+ transport rate across a tight epithelium. *Biophys. J.* **30:**181–186
20. Zeuthen, T. 1981. On the effects of Amphotericin-B and ouabain on the electrical potentials of *Necturus* gallbladder. *J. Membrane Biol.* **60:**167–169