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FACTORS AFFECTING THE COMPARTMENTALIZATION OF SODIUM ION WITHIN RABBIT ILEUM IN VITRO

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SUMMARY

(1) Net Na^+ loss from rabbit ileum, stripped of its serosal muscle layers, into ice-cold choline chloride is consistent with loss from two separate pools (rate constants 0.102 and 0.011 min^{-1}). Since cell K^+ is lost with a single rate constant, 0.0062 min^{-1}) and inulin, a good extracellular marker, is lost with a single rate constant, 0.082 min^{-1}, it is inferred that the fast rate constant of Na loss characterizes loss from an extracellular pool and the slow constant, loss from an intracellular pool.

(2) The $[Na^+]$ in the inulin space (extracellular) was calculated to be 180 ± 13 (S.D.) mequiv. and the $[Na^+]$ in the intracellular space 30.4 ± 4.1 (S.D.) mequiv., this provides evidence that the paracellular spaces are, at least, 80 mosmol hypertonic to the external Ringer.

(3) There is a saturable galactose-dependent increase in both the intracellular and extracellular $[Na^+]$. Extracellular $[Na^+]$ is increased to 236 ± 22 (S.D.) mequiv. whilst intracellular $[Na^+]$ is increased to 42.6 ± 8.8 (S.D.) mequiv. when Ringer [galactose] is 10 mM. Galactose-dependent increases in total tissue $[Na^+]$ can thus be attributed mainly to the increase in extracellular $[Na^+]$.

(4) Extracellular hypertonicity, both in the presence and absence of galactose, is dependent upon the $[Na^+]$ of the bathing Ringer. 0.1 mM ouabain abolishes the extracellular hypertonicity. This observed extracellular hypertonicity in normally functioning tissue may provide the driving force for transcellular convective flow of salt, water and sugars.

INTRODUCTION

Transcellular flow of water through epithelial cells is presumed to be a response to osmotic pressure acting across the basal-lateral cell borders resulting from deposition of hypertonic NaCl within the lateral spaces by Na^+-pump activity [1].

Good evidence for the existence of such a mechanism would be the finding of extracellular hypertonicity that was dependent on $(Na^+ + K^+)$-ATPase activity. Machen and Diamond have made an indirect measurement of the concentration of NaCl within the lateral spaces of rabbit gall-bladder; they conclude that the lateral space is some 80 mosmol more concentrated than the bathing media [2]. Similarly Zybler et al. [3, 4] have inferred that a high concentration of Na^+ is localized within

the intercellular space of frog skin, however, they attribute this to ion complexation. Wall et al. [5] have sampled intercellular spaces of cockroach rectum by microelectrodes and found them to be around 130 mosmol hypertonic to the bathing media.

Very recently Zeuthen and Monge have reported on the basis of ion sensitive microelectrode studies in rabbit ileum, that intracellular K^+ is present at hypertonic concentrations at the basal pole of the epithelial cells and that the extracellular Cl^- concentration in the region immediately adjacent to the basement membrane is approximately 300 mM [6].

The work described in this paper attempts to measure extra and intra cellular cation concentrations by compartmental analysis. Since sugars are known to stimulate net flux of water and NaCl across intestine [7–9], the effects of a non-metabolized sugar, D-galactose [10] on the tissue distribution and concentrations of Na^+ and K^+ were studied. Also of interest are the effects of theophylline, which reverse the normal absorptive state of intestine with respect to NaCl and water [11] and also ouabain, which inhibits tissue Na^+-pump activity [12].

MATERIALS AND METHODS

(*i*) *Animals.* Male white New Zealand rabbits, fed normally, were killed by intravenous injections of Nembutal, their ileum rapidly excised and washed with ice-cold Ringer's solution. The ileum was stripped of its serosa and external muscle layers as previously described [10].

(*ii*) *Ringer's solutions.* Ringer's solutions contained 140 mM NaCl, 10 mM HCO_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 solutions were gassed continuously with a O_2/CO_2 (95 : 5) gas mixture saturated with water vapour at room temperature. The Na^+ concentration of the Ringer's solution was varied by isosmotic replacement of NaCl with choline chloride. Galactose at concentrations up to 20 mM was added without further adjustment of control solution tonicities. The base, 2,4,6-triaminopyrimidine (Aldrich chemicals) was neutralized to pH 7.4 by addition of HCl prior to use. This procedure altered Ringer $[Cl^-]$ by < 5 mM. Control solutions with respect to triaminopyrimidine contained mannitol to maintain isotonicity. Addition of mannitol had no effect upon tissue ion concentrations and distributions compared to controls minus mannitol. Theophylline was added as the hydrate (B.D.H.). Wash solutions comprised 150 mM choline chloride buffered to pH 7.4 with 5 mM Tris · HCl. Additions of 2,4,6-triaminopyrimidine or theophylline to the wash media were made where appropriate.

(*iii*) *Procedure for wash-out experiments.* Ileum, prepared as above, was opened along the mesenteric border and mounted as a flat sheet on a perspex former covered with half-inch spikes approx. 3 cm apart to maintain an even tension. The former, was then laid in a bath containing the appropriate Ringer at 37 °C for a preincubation period of 1 h. Access of well stirred solution to both epithelial surfaces was ensured by vigorous gassing with O_2/CO_2 (95 : 5). Following preincubation, the former, was transferred to another bath containing the ice-cold stirred wash medium. Measurement of net cation loss was performed discontinuously at time intervals of up to 80 min by estimating $[Na^+]$ and $[K^+]$ in extracts of separate pieces of ileum. The ion and water content of the tissue was also monitored during the preincubation period.

(*iv*) *Estimation of Na^+ and K^+.* Separate pieces of ileum were blotted on Whatman No. 1 filter paper and placed in tared 10 cm^3 conical flasks and weighed. 4 cm^3 of extraction fluid (normally 0.1 N HNO_3, but H_2O was used in experiments involving ^{125}I-labelled albumin) were then added to each flask and extraction was carried out overnight. The [Na^+] and [K^+] of aliquots of extract were determined by flame photometry. Tissues were then dried over a sandbath at 95 °C to determine the tissue dry weight.

(*v*) *Determination of the extracellular space.* The extracellular space was determined from the distribution ratio of ^{14}C-labelled inulin between the bathing solution and the tissue extracellular space. ^{125}I-labelled albumin was also used in some experiments for comparative purposes. Inulin and albumin were added to the preincubation solutions only in tracer quantities.

(*vi*) *Radioisotope counting.* ^{14}C- and ^{125}I-activities were determined from their β-activities using a Packard Tricarb Liquid Scintillation counter. All samples 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 [13]. All activity was corrected for differences in quench between samples of tissue extract and samples of incubation medium by calibrating the external standard against samples of known activity, quenched by varying amounts of chloroform.

RESULTS

(1) *Compartmental analysis of tissue cation content*

(*a*) *Controls.* Figs. 1a and b show the wash-out curves of tissue Na^+ and K^+ for control tissues and tissues preincubated in the presence of 0.1 mM ouabain. Percentage loss is plotted semilogarithmically against time. In control tissues net Na^+ loss is best fitted by a double exponential function of time. Kinetically, therefore, tissue Na^+ exists in two compartments. The $t_{\frac{1}{2}}$ for the fast component is 6.86 ± 1.69 min (S.D.) whilst for the slow component the $t_{\frac{1}{2}}$ is 65.9 ± 29.3 min (S.D.). K^+ loss, in contrast, is best fitted by a single exponential whose $t_{\frac{1}{2}}$ is 96.8 ± 46.8 min (S.D.). This result indicates that K^+ is contained within a single intracellular compartment. Since the $t_{\frac{1}{2}}$ for K^+ loss is similar to the slow component for net Na^+ loss, this component of Na^+ loss can be equated with loss from the intracellular pool. Extrapolation to zero time gives the original amount of intracellular Na^+. The remaining Na^+ is adequately fitted by a single exponential function of time. Since the $t_{\frac{1}{2}}$ of the component is rapid, this Na^+ must be located within a compartment that is relatively open to the bathing solutions. Identification of this compartment as the tissue extracellular space follows from comparison of the $t_{\frac{1}{2}}$ of inulin loss, (8.1 ± 3.6 min (S.D.)) (see section 2 below) to the $t_{\frac{1}{2}}$ of the fast Na^+ exit component (Table I).

(*b*) *Effect of 0.1 mM ouabain.* 0.1 mM ouabain causes a change in the wash-out kinetics for Na^+ and K^+ from the tissue (Fig. 1b). Both net Na^+ and K^+ losses are best fitted by double exponential functions of time. The amount of Na^+ exiting with a long $t_{\frac{1}{2}}$ increases compared to controls ($P < 0.001$) whereas the amount of K^+ exiting with a similar $t_{\frac{1}{2}}$ decreases ($P < 0.001$). This result is consistent with the well known action of ouabain in inhibiting Na^+-pump activity [12], so causing cellular K^+ loss and reciprocal Na^+ gain, and supports the intracellular locations for cations inferred from the wash-out kinetics. That net K^+ loss is best fitted by a double

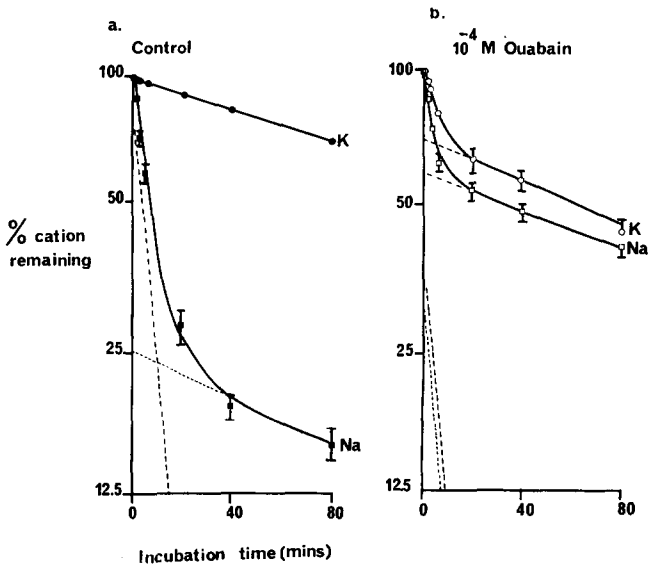

Fig. 1. (a) Wash-out curves for Na^+ (■) and K^+ (●) from stretched rabbit ileum following 1 h preincubation in standard Ringer. Percentage loss is plotted semilogarithmically against time of incubation in the wash media. Error bars denote ±S.E.M. for each point ($n = 27$); these lie within the points where not shown. Dashed lines indicate the least-square regression lines for the two components of net Na^+ loss. Solid lines are drawn by eye. (b) Tissues were preincubated in the presence of 0.1 mM ouabain for 1 h prior to incubation in the wash media. Curves show the wash-outs for Na^+ (□) and K^+ (○). Each point represents the mean of 9 determinations. Error bars denote ±S.E.M. Dashed lines denote the least-square double-exponential fit for the data.

TABLE I

THE $t_{\frac{1}{2}}$ FOR LOSS OF Na^+ FROM THE EXTRACELLULAR TISSUE COMPARTMENT OF RABBIT ILEUM INTO ICE-COLD CHOLINE CHLORIDE BUFFER

Numbers in parentheses represent the number of experiments followed by the number of experimental data points involved in the regression analyses. n.s., not significant.

	$t_{\frac{1}{2}}$ (min)±S.D.
1. Control (9,108)	6.86±1.69
2. +20 mM 2,4,6-triaminopyrimide (3,36)	5.57±1.68
3. +0.1 mM ouabain (3,36)	2.36±0.56*
4. +10 mM galactose (4,48)	3.51±1.68*
5. +10 mM theophyline (3,36)	1.21±0.64**
6. +10 mM theophyline+20 mM galactose (3,36)	2.96±0.61* (6-4 n.s. $P > 0.8$)

* Student's t test $P < 0.01$ (experimental against control values).
** Student's t test $P < 0.001$ (experimental against control values).

exponential function in the presence of ouabain, suggests that the K^+ remaining within the tissue has equilibrated with the extracellular compartment.

Since the intestine is a complex folded tissue [14] the half-times of ion loss ($t_{\frac{1}{2}}$) from both the extracellular and intracellular spaces are lumped parameters representing loss from many different, but operationally similar, compartments. Small

changes in the $t_{\frac{1}{2}}$ for the extracellular component may indicate a change in the location of the bulk of extracellular Na^+ within the extracellular space. The $t_{\frac{1}{2}}$ for loss of extracellular Na^+ in the presence of ouabain is less than control values (Table I). The difference between these values is significant ($P < 0.01$). This result may indicate a shift in extracellular Na^+ away from regions of active Na^+-pumping in ouabain-treated tissues.

(*c*) *The effects of the tight junction agent 2,4,6-triaminopyrimidine.* The direction of loss of Na^+ from the region of high Na^+ concentration located within the lateral spaces will be determined primarily, by the resistance of the tight junctions. If this resistance is low, Na^+ loss will be rapid, since net loss will occur through the tight junctions rather than by Na^+ traversing the relatively long distance across the submucosal tissue layers. The base 2,4,6-triaminopyrimidine specifically increases the resistance of intestine by reducing the Na^+ conductance of the tight junctions [15]. (That 20 mM 2,4,6-triaminopyrimidine has no effect on Na^+-pump activity can be seen in Table IV which shows that 20 mM 2,4,6-triaminopyrimidine has no significant effect upon intracellular cation concentrations.) 20 mM 2,4,6-triaminopyrimidine has no significant effect upon the rate of net Na^+ loss from the extracellular space ($P > 0.4$) of stretched ileum indicating that the serosal resistance to net Na^+ loss is low compared to the resistance of tight junctions. Incubating ileum without stretching it on the former (see Materials and Methods), causes tissue curling and an increased serosal exit resistance. In this case the fast extracellular component of net Na^+ loss coming from the mucosal surface of control tissues is completely eliminated by 20 mM 2,4,6-triaminopyrimidine; thus demonstrating that the extracellular component of net Na^+ loss in unstretched tissue is located distal to the tight junction.

(*d*) *Effects of galactose and theophylline (10 mM).* Both 10 mM galactose and theophylline significantly increase the rate of loss of Na^+ from the extracellular space of stretched tissue (Table I). The galactose-dependent decrease in $t_{\frac{1}{2}}$ may be associated with an increased tissue permeability due to changes in the widths of the lateral spaces [10, 7] (see also section 2 below). Theophylline is thought to reduce the width of the paracellular pathway through increased cellular cyclic-AMP levels [17]. The observed decrease in the $t_{\frac{1}{2}}$ compared to controls, may be due to partial exclusion of extracellular Na^+ from the epithelial layers. Since theophylline treated tissue with galactose present has a $t_{\frac{1}{2}}$ for extracellular Na^+ loss that is not significantly different from controls with only 10 mM galactose present ($P > 0.8$) (see also sections 3c and 4d below), it can be inferred that galactose reverses this effect of theophylline.

(2) *Extracellular space determinations: choice of an extracellular marker*

The calculation of intracellular and extracellular cation concentrations from the kinetic data depends upon the accuracy of the estimated extracellular space. Schultz et al. [18] have shown that inulin is an adequate marker for the extracellular space of rabbit ileum. This is confirmed in the present study. Fig. 2a shows that equilibration of ^{14}C-labelled inulin activity with the extracellular space is rapid and that no slow entry component is discernable. Inulin loss into the ice-cold wash medium is also shown (Fig. 2b). The percentage activity of ^{14}C-labelled inulin remaining in the tissue is plotted semilogarithmically against the incubation time. Loss is rapid and is adequately fitted by a single exponential function of time. Virtually all activity is lost after 20 min incubation. The $t_{\frac{1}{2}}$ for inulin loss is 8.17 ± 3.6 min (S.D.). This finding

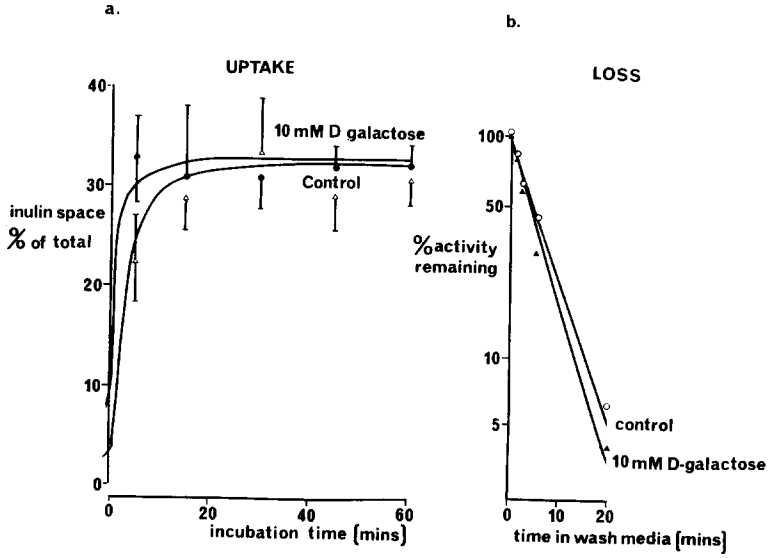

Fig. 2. (a) Equilibration of ^{14}C-labelled inulin with the extracellular space for control Ringer (△) and Ringer containing 10 mM galactose (●). Error bars denote the S.E.M. for each point ($n = 9$). (b) Loss of ^{14}C-labelled inulin from preincubated tissue into ice-cold wash medium. Percentage activity remaining in the tissue is plotted against time of incubation for control tissues (○) and tissues preincubated in Ringer containing 10 mM galactose (△). Solid lines represent the least-square lines for the data. Each point is the mean of 9 determinations.

shows that kinetically, the extracellular space behaves as a single compartment with respect to inulin (see Materials and Methods).

Equilibration and loss of ^{14}C-labelled inulin activity are facilitated by the presence of 10 mM galactose. This finding correlates with the observation that apparent access of galactose to the serosal membranes of the epithelial cells from the serosal fluid is a function of net galactose flux [10].

The inulin space for controls expressed as a percentage of the total tissue water is 33.21±2.97% (S.E.M.). This value agrees closely with values determined by other workers [18]. The ^{125}I-labelled albumin space is 28.2±2.4% (S.E.M.) of the total tissue water. These two values are not significantly different ($P > 0.2$) (Table II). Equilibration of ^{125}I-labelled albumin with the extracellular space is similar to inulin, in having a single rapid time constant. The difference between the inulin and albumin spaces may be interpreted as being due to partial exclusion of albumin [19], on the basis of its greater molecular weight.

Examination of Table II shows however, that the ^{125}I-labelled albumin space responds less readily than the inulin space to changes in tissue H_2O observed during incubation with galactose. For this reason inulin was routinely used as the extracellular marker and all calculations were based on these measurements.

3. *Factors affecting tissue water*

(*a*) D-*Galactose.* D-Galactose causes tissue swelling as judged by increments in the tissue wet/dry weight ratio at the end of the preincubation period (Tables II and

TABLE II

COMPARISON OF THE ^{14}C-LABELLED INULIN AND ^{125}I-LABELLED ALBUMIN SPACES FOR CONTROL TISSUES ±10 mM D-GALACTOSE

All errors denote ±S.E.M., figures in parentheses are the number of experimental data points.

	Wet weight/dry weight ratio	Extracellular water expressed as a % of the total	Ratio of the weight of extracellular water/tissue dry weight
^{14}C-labelled inulin controls	6.62±0.38 (24)	33.21±2.97 (24)	2.37±0.26 (24)
^{125}I-labelled albumin controls	6.53±0.21 (18)	28.20±2.40 (18)	2.19±0.18 (18)
^{14}C-labelled inulin +10 mM D-galactose	9.62±0.43 (21)	31.34±2.09 (21)	2.83±0.22 (21)
^{125}I-labelled albumin +10 mM D-galactose	9.47±0.50 (14)	25.50±0.95 (14)	2.36±0.18 (14)

TABLE III

EFFECTS OF D-GALACTOSE, Na^+ REPLACEMENT, OUABAIN, 2,4,6-TRIAMINOPYRIMIDINE AND THEOPHYLLINE ON THE EXTRACELLULAR SPACE

All measurements were made after 45–60 min of incubation. Errors are given as the S.E.M. Asterisks denote values for experimental determinations that are significantly different from control values (Student's *t* test unpaired data).

Experimental conditions	*n*	Wet weight to dry weight ratio	Inulin space as a % of total water	Weight of extracellular H_2O/tissue dry weight
Control	24	6.62±0.38	33.21±2.47	2.37±0.25
+10 mM galactose	21	9.62±0.43**	31.34±2.09	2.83±0.22 ($P < 0.1$)
+0.1 mM ouabain	17	6.59±0.46	46.1 ±2.56*	2.98±0.18 ($P > 0.1$)
0.1 mM ouabain +10 mM galactose	19	6.98±0.56	45.60±2.32*	2.89±0.14 ($P > 0.1$)
75 mM Na^+ Ringer	12	7.01±0.46	36.16±4.56	2.39±0.26
75 mM Na^+ Ringer +20 mM galactose	12	9.71±0.46***	35.30±7.40	2.89±0.32 ($P > 0.1$)
25 mM Na^+ Ringer	8	6.52±0.16	29.31±2.89	2.19±0.19
25 mM Na^+ Ringer +20 mM galactose	8	8.25±0.25*	29.80±4.03	2.46±0.26
140 mM Na^+ +10 mM theophylline	18	5.86±0.25 ($P < 0.1$)	25.56±1.80*	1.87±0.14 ($P < 0.1$)
10 mM theophylline +20 mM galactose	6	9.05±0.56**	34.82±6.70	3.15±0.61 ($P < 0.2$)
140 mM triaminopyrimidine	14	7.16±0.34	31.11±2.20	2.14±0.16
20 mM triaminopyrimidine +20 mM galactose	10	10.8±0.96***	32.50±3.80	2.73±0.19 ($P < 0.2$)

* $P < 0.05$
** $P < 0.01$
*** $P < 0.001$

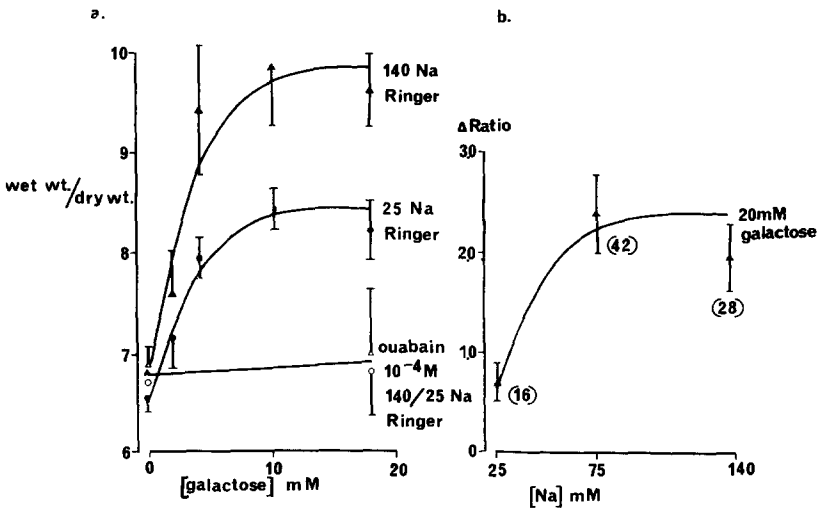

Fig. 3. (a) The effect of D-galactose (0 to 20 mM) on tissue water (the tissue wet weight/dry weight ratio) following preincubation in Ringer solutions containing $[Na^+]$ values of 140 mM (▲) and 25 mM (●). Values for 75 mM Na^+ are coincident with those for 140-mM Na^+ and are omitted for clarity. The effect of 0.1 mM ouabain is also shown at 140 mM Na^+ (△) and 25 mM Na^+ (○) ±20 mM galactose. (b) Increments in the wet/dry weight ratio for paired data ±20 mM D-galactose as a function of the $[Na^+]$ of the bathing media. Figures in parentheses are the number of experimental points. Error bars denote ±S.E.D.

III). This increased tissue water is a saturable function of the galactose concentration of the preincubation medium (Fig. 3a) at Ringer $[Na^+]$ of 140, 75 and 25 mM. The calculated K_m values (galactose concentration giving half maximal swelling) at these Na^+ concentrations are 3.06±0.2 mM (S.E.), 3.1±0.9 mM (S.E.) and 3.2±0.2 mM (S.E.) respectively, which are in agreement with the observed K_m values for galactose influx across the brush-border of the epithelial cells [10, 20]. The amount of tissue swelling at constant galactose concentration of 20 mM is dependent on the Ringer $[Na^+]$; on reducing the $[Na^+]$ below 75 mM, the absolute amount of swelling is reduced (Fig. 3b). The K_m value for this relationship is 45±9 (S.E.) mequiv. This value is similar to that determined for the $[Na^+]$ giving maximal stimulation of both convective and diffusive permeabilities for galactose entry across the mucosal brushborder [10].

Table III shows that galactose-dependent swelling is paralleled by an increased extracellular space. Since no increase in the ratio of extracellular water/total tissue water is observed, it may be concluded that galactose causes concurrent increases in both the cell water, and the extracellular space.

(b) 0.1 mM ouabain. The data of Table III show that the addition of 0.1 mM ouabain to the preincubation medium has no significant effect on tissue water as judged by the wet weight/dry weight ratio ($P > 0.9$). Ouabain also abolished the galactose-dependent increase in wet/dry weight ratio.

With ouabain present the extracellular space increases to 46% of the total wet weight compared to control values of 33.2% ($P < 0.01$). These last two results in conjunction indicate cell shrinkage.

TABLE IV

CALCULATED INTRACELLULAR AND EXTRACELLULAR CATION CONCENTRATIONS BASED UPON THE EXTRACELLULAR SPACE DETERMINATIONS OF TABLE III

Errors are given as the standard deviation. *n*, number of experiments; figures in parentheses are the number of data points involved in the regression analyses. Footnote denotes values for experimental determinations that are significantly different from control values. n.s., not significant.

Experimental conditions	*n*	Intracellular [Na^+] mequiv./l cell H_2O	Extracellular [Na^+] mequiv./l cell H_2O	Intracellular [K^+] mequiv./l cell H_2O
Control 140 mM Na^+	9	30.68± 4.13 (108)	180 ±13.2 (81)	115.6 ± 5.6 (189)
+10 mM D-galactose	4	42.06± 8.40[c] (48)	236.5±22.7[d] (36)	102.1 ± 4.7[c] (84)[c]
0.1 mM ouabain	3	137.10±17.3[d] (36)	146.4±16.2[b] (27)	27.9 ± 5.9[d] (63)
0.1 mM ouabain +20 mM D-galactose	3	143.2±21.6[d] (36)	149.6±18.0[c] (27)	22.4 ± 4.3[d] (63)
75 mM Na^+	2	30.34± 6.4 (24)	115.5±13.6 (18)	109.9 ± 3.2 (42)
75 mM Na^+ +20 mM D-galactose	2	29.5 ± 3.8 (24)	148.8±15.0 (18)	114.1 ± 2.7 (42)
25 mM Na^+ Ringer	2	12.8 ± 3.6 (24)	36.2±12.4 (18)	115.6 ± 4.6 (42)
25 mM Na^+ +20 mM D-galactose	2	14.9 ± 4.1 (18)	40.4± 9.6 (24)	110.4 ± 4.3 (42)
140 mM Na^+ +10 mM theophylline	3	35.9 ± 4.2[e] (27)	192.0±21.6[e] (36)	108.9 ± 7.2[e] (63)
10 mM theophylline +20 mM D-galactose	3	42.1 ± 6.4[b] (27)	226.1±20.4[c] (36)	105.6 ± 4.4[a] (63)
140 mM Na^+ +20 mM triaminopyrimidine	2	37.1 ± 3.3[e] (24)	158 ±12.3[e] (18)	111.6 ±10.3[e] (42)
20 mM triaminopyrimidine +20 mM galactose	2	45.80± 4.6[c] (24)	245.1±11.1[c] (18)	97.67± 7.5[b] (42)

[a] $P < 0.05$, [b] $P < 0.02$, [c] $P < 0.01$, [d] $P < 0.001$, [e] n.s.

Ouabain is known to cause tissue swelling (e.g. in brain and red cells [22, 23]). Also Schultz [18] has shown cell swelling with ouabain in mucosal slices of intestine. The apparent difference of the present results with these findings may result from the necessary stretching of the ileum in the present study.

(*c*) *10 mM theophylline.* Theophylline causes a reduction in the tissue wet/dry weight ratio compared to control values ($P < 0.1$) (Table III). This is accompanied by a reduction in the amount of extracellular water as judged by the inulin space, ($P < 0.05$). These effects are, therefore, in agreement with previous observations [24]. Addition of 20 mM galactose reverses the theophylline dependent reductions in the wet to dry weight ratio and the inulin space. These results are consistent with the view [17] that theophylline-mediated secretion is associated with the reduction of the extracellular spaces. Galactose reverses this effect by stimulation of net water and NaCl absorption.

4. *Calculated intracellular and extracellular cation concentrations*

(*a*) *Control values.* The calculated intracellular and extracellular Na^+ concentrations for controls are 30.7 ± 4.1 (S.D.) and 180.0 ± 13.3 mequiv. l respectively (Table IV). The extracellular Na^+ concentration is significantly greater than the $[Na^+]$ of the Ringer's solution ($P < 0.01$); hence the previously held assumption that the Na^+ concentration of the extracellular space is equivalent to that of the bathing medium [18] is invalid. The use of this assumption will lead to an overestimate of the intracellular $[Na^+]$. The calculated intracellular $[Na^+]$ in this study is approximately 50 % below previous estimates [18, 25]. The intracellular $[K^+]$ concentration is 115.7 ± 5.6 (S.D.) mequiv. l^{-1} cell H_2O, a value which agrees closely with previous data [18, 25]. Estimates of intracellular $[K^+]$ are not subject to error from elevated extracellular concentrations, hence this agreement is to be expected .The hypertonicity of the extracellular space is at least 80 mosmol, a value similar to that deduced by Machen and Diamond [2] for the lateral spaces of rabbit gall bladder.

(*b*) *0.1 mM ouabain.* Ouabain causes a redistribution of both Na^+ and K^+ within the tissue (Table IV). The intracellular $[K^+]$ falls to 27.9 ± 5.9 (S.D.) mequiv. whilst there is a concurrent increase in the cell $[Na^+]$ to 134 ± 17.6 (S.D.) mequiv. Ouabain also abolishes the observed hypertonicity of the extracellular space. The $[Na^+]$ of the extracellular space falls to 146 ± 16.0 mequiv. This is not significantly different from the Na^+ concentration of the bathing fluid ($P < 0.8$) but is significantly below the control values ($P < 0.02$). Intracellular Na^+ and K^+ concentrations were probably not at equilibrium at the end of the preincubation period, since intracellular $[K^+]$ is significantly larger that the concentration contained within the bathing medium ($P < 0.05$).

(*c*) D-*Galactose.* Both intracellular and extracellular Na^+ concentrations are saturable functions of the [D-galactose] (Fig. 4). Intracellular $[Na^+]$ increases from 30.7 ± 4.1 (S.D.) mequiv. to 42.1 ± 8.4 (S.D.) mequiv. at 10 mM galactose. Extracellular $[Na^+]$ increases from 180 ± 13.2 (S.D.) mequiv. to 236.5 ± 22.7 mequiv. with 10 mM galactose present in the Ringer ($P < 0.001$).

It can be seen that the large increases in tissue $[Na^+]$ observed by Koopman and Schultz [25] in the presence of galactose, are not solely due to an increased intracellular Na^+ content, but are due, primarily, to an increased extracellular $[Na^+]$. There is a reciprocal decrease in the intracellular K^+ concentration as intracellular

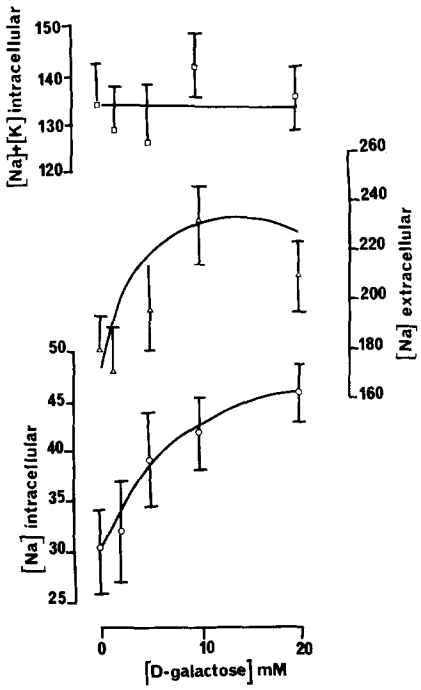

Fig. 4. The effect of D-galactose on the calculated values of intracellular [Na^+], (○); intracellular [Na^+]+[K^+], (□) and extracellular [Na^+], (△). Error bars denote ±S.D. for each point ($n \geqq 42$).

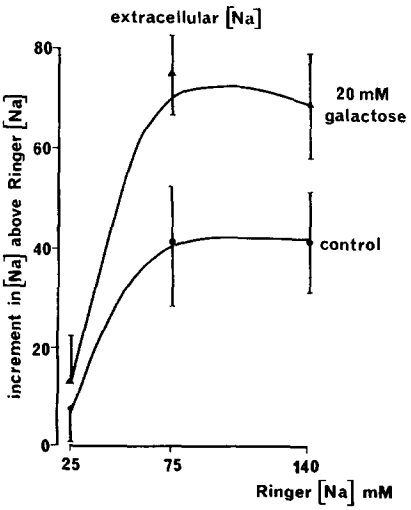

Fig. 5. Effect of variation in the [Na^+] of the preincubation medium on the calculated [Na^+] of the extracellular space expressed as an increment above the preincubation solution [Na^+] for controls (●) and in the presence of 20 mM of galactose (▲). Error bars denote ±S.D. ($n \geqq 23$).

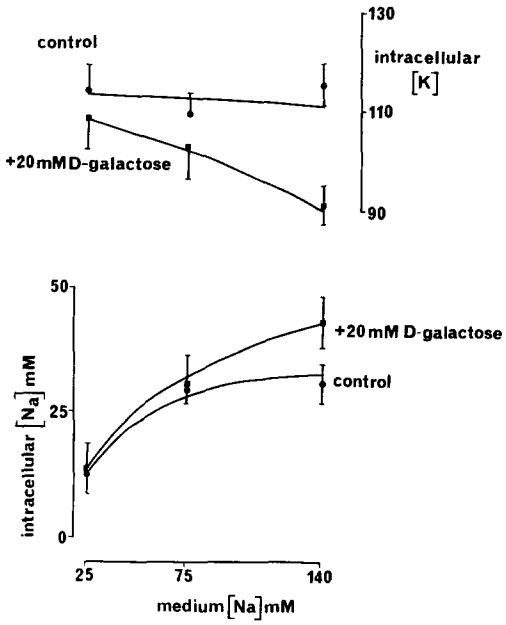

Fig. 6. Effect of variation in the $[Na^+]$ of the preincubation medium on the calculated intracellular cation concentrations $\pm$20 mM D-galactose. Error bars denote $\pm$S.D. ($n \geqq 23$).

$[Na^+]$ increases. This result agrees with the observations of Brown and Parsons [26] and Koopman and Schultz [25]. The sum of intracellular $[Na^+]$ and $[K^+]$ is invariant with galactose concentration (Fig. 5). Galactose-dependent tissue swelling can, therefore be inferred to be due to an increased solute content of both the extracellular and cell fluid.

Variation in the Ringer $[Na^+]$ in the range 0–140 mequiv. causes saturable increases in the extracellular hypertonicity in the presence and absence of galactose (Fig. 5). The hypertonicity of the extracellular fluid is virtually abolished by reduction of Ringer $[Na^+]$ to 25 mequiv. This result confirms those already obtained with 0.1 mM ouabain (i.e. that extracellular hypertonicity is dependent on the action of the Na^+-pump). Galactose probably stimulates the Na^+-pump by increasing influx of Na^+ into the cell.

Fig. 6. shows the variation of intracellular Na^+ and K^+ concentrations as a function of the preincubation Ringer $[Na^+]$. 20 mM galactose has no significant effects upon intracellular Na^+ or K^+ until Ringer $[Na^+]$ is raised above 75 mequiv.

In Ringer containing 140 mequiv. Na^+ and 20 mM galactose the intracellular $[Na^+]$ is raised to 46 ± 8.4 mM (S.D.) whilst intracellular $[K^+]$ falls to 102 ± 4.6 mequiv. (S.D.). This result shows that in 140 mequiv. Na^+ Ringer, the passive galactose-stimulated net Na^+ entry starts to saturate the capacity of Na^+ extrusion mechanism.

(*d*) *The effects of 10 mM theophylline.* The calculated intracellular Na^+ and K^+ concentrations of tissues in the presence of 10 mM theophylline (Table IV) are not significantly different from control values ($P > 0.2$ for both Na^+ and K^+).

Extracellular [Na^+] is unaffected by theophylline ($P > 0.4$). Thus, reduction in the volume of the extracellular space does not alter extracellular hypertonicity. 20 mM galactose in the presence of theophyllline increases both intracellular and extracellular Na^+ concentrations by similar amounts to those observed in control tissues (Table IV). Intracellular [K^+] also falls slightly, following addition of galactose.

DISCUSSION

This study has shown that the extracellular space of rabbit ileum contains NaCl at a concentration which is at least 80 mosmol hypertonic to the external Ringer. This observation directly corroborates the model of fluid absorption proposed by Diamond and Bossert [1]. Previously, only indirect support for this hypothesis has been obtained from experiments with mammalian epithelia [2] although direct estimates of the osmolarity of the intercellular space of cockroach rectum has yielded evidence of a solution which is 130 mosmol hypertonic. However, uncertainty as to the composition of this fluid remains.

The estimates of extracellular fluid hypertonicity in this study rely on the accuracy of the extracellular space determination. If the inulin space underestimates the true extracellular space by 30 % in Ringer, or by 70 % in Ringer + 20 mM galactose, the [Na^+] in the space could be isotonic. However, errors of this magnitude would mean that the monovalent cation concentration of the cell fluid would be 170 and 230 mequiv. respectively, which are improbably large. The calculated tonicities of the intracellular cations on the basis of the measured space are consistent with the mean cell solute concentration being isotonic with the external Ringer. Further confirmation of the reliability of the results is provided by the observation that, either addition of ouabain, or reduction of Ringer [Na^+] to 25 mequiv. abolishes the hypertonicity of the extracellular fluid compartment. These results indicate that the extracellular hypertonicity is produced by the action of the Na^+-pump which Diamond and Bossert [1] predicted would deposit hypertonic NaCl into the lateral intercellular space.

Rather than an overestimate of the extracellular fluid hypertonicity, the data reported here is probably an underestimate of the hypertonicity present in the lateral intracellular spaces of actively transporting epithelia. Small intestinal transport activity is thought to be localized to the apical part of the intestinal villus, consequently fluid coming from inactive regions will tend to dilute the hypertonic reabsorbant towards isotonicity [27]. Hence the lumped estimate of the extracellular fluid concentration will underestimate the [Na^+] in the actively transporting region of the tissue and overestimate the concentration in parts distant to the active pump sites.

Estimates of small-intestinal intracellular [Na^+] based on the assumption that the extracellular fluid is isotonic to the external bathing medium are invalidated by the findings reported in this paper. The high cell [Na^+] reported by Koopman and Schultz and the large galactose-dependent increase in tissue [Na^+], can now be ascribed to an increase in NaCl deposition, within the extracellular fluid, rather than to an increase in intracellular [Na^+] induced by inhibition of the Na^+-pump resulting from reduced cell [ATP], as they suggest. It is of interest that the low value of intracellular [Na^+] found here accords with direct determinations of intracellular Na^+ activity made with Na^+-sensitive microelectrodes in bullfrog small intestine [28].

The intracellular activity coefficient of Na^+ in this study was low (≈ 0.5); however, this low activity coefficient may be in error, since the overall intracellular Na^+ from which the activity coefficient was determined, was obtained by assuming that the extracellular fluid was isotonic with the bathing medium.

The large increment in $[Na^+]$ in the extracellular space following addition of 20 mM galactose to the preincubation Ringer may provide an explanation for the observed stimulation of net water and salt absorption by sugars [7–9]. An increase in the osmotic gradient across the basal-lateral border will increase net water flow across this border from the cell fluid. The cell water will be replaced in turn by increased flow across the brush-border as a consequence of the increase in intracellular solute concentration. Barry et al. [8] have made a similar suggestion.

A possible reason for the galactose-dependent increase in Na^+ accumulation in the extracellular space may be that there is an increase in passive net Na^+ influx across the brush-border as a result of sugar linked coupled transport [29]. The increase in intracellular $[Na^+]$ will increase the Na^+-pump activity at the lateral-basal border of the cell, provided that the pump is not already saturated [30].The double-membrane model described by Curran and McIntosh [31] and used as a basis for the quantitative description of isotonic reabsorption of fluid across gall-bladder epithelium by Diamond and Bossert [1] has been adapted to the description of asymmetric sugar flux across the brush-border of small intestine and to accumulation of the sugar within the tissue fluid [19, 32]. The observed concentration polarization of K^+ at the inner surface of the basal membrane, the hypertonic concentration of Cl^- in the extracellular fluid found by Zeuthan and Monge [6] and the hypertonic extracellular Na^+ found in this study are completely in accord with this model.

Some of the results in this paper have been communicated to the Physiological Society [33].

REFERENCES

1 Diamond, J. M. and Bossert, W. H. (1967) J. Gen. Physiol. 50, 2061–2083
2 Machen, T. E. and Diamond, J. M. (1969) J. Membrane Biol. 1, 194–213
3 Zylber, E. A., Rotunno, C. A. and Cereijido, M. (1973) J. Membrane Biol. 13, 199–216
4 Rotunno, C. A., Zylber, E. A. and Cereijido, M. (1973) J. Membrane Biol. 13, 217–232
5 Wall, B. J., Oschman, J. L. and Schmidt-Nielson, B. (1970) Science 167, 1497–1498
6 Zeuthen, T. and Monge, L. (1975) Phil. Trans. R. Soc. Lond. B. 71, 277–281
7 Esposito, G., Faelli, A. and Capraro, V. (1969) Arch. Sci. Biol. 48, 341–356
8 Barry, R. J. C., Smyth, D. H. and Wright, E. M. (1965) J. Physiol. 181, 410–431
9 Fullerton, P. M. and Parsons, D. S. (1956) Quart J. Exptl. Physiol. 41, 387–397
10 Naftalin, R. J. and Curran, P. F. (1974) J. Membrane Biol. 16, 257–278
11 Field, M. (1971) Am. J. Physiol. 221, 992–997
12 Glynn, I. M. (1964) Pharmacol. Rev. 16, 381–407
13 Fox, B. W. (1968) J. Appl. Radiat. Isot. 19, 717–732
14 Trier, J. S. (1968) in Handbook of Physiology, (Code, C. F., ed.), Section 6, Vol. III, pp. 1125–1175, American Physiological Society
15 Moreno, J. M. (1975) J. Gen. Physiol. 66, 97–115
16 Bindslev, N., Tormey, J. McD. and Wright, E. M. (1974) J. Membrane Biol. 19, 357–380
17 Di Bona, D. R., Chen, L. C. and Sharp, G. W. C. (1974) J. Clin. Invest. 53, 1300–1307
18 Schultz, S. G., Fuisz, R. E. and Curran, P. F. (1966) J. Gen. Physiol. 49, 849–865
19 Maizels, M. and Remington, M. (1959) J. Physiol. 145, 658–666

20 Goldner, A. M., Schultz, S. G. and Curran, P. F. (1969) J. Gen. Physiol. 53, 362–383
21 Naftalin, R. J. and Holman, G. D. (1974) Biochim. Biophys. Acta 373, 453–470
22 Towfighi, J. and Gonatas, N. K. (1973) Lab. Invest. 38, 170–184
23 Poznansky, M. and Soloman, A. K. (1972) J. Membrane Biol. 10, 259–266
24 Holman, G. D. and Naftalin, R. J. (1975) Biochim. Biophys. Acta 406, 386–401
25 Koopman, W. and Schultz, S. G. (1968) Biochim. Biophys. Acta 173, 338–340
26 Brown, M. M. and Parsons, D. S. (1969) Biochim. Biophys. Acta 59, 249–252
27 McElligot, T. F., Beck, I. T., Dinda, P. K. and Thompson, S. (1975) Can. J. Physiol. Pharmacol. 53, 439–450
28 Lee, C. O. and Armstrong, W. McD. (1972) Science 175, 1261–1264
29 Goldner, A. M., Schultz, S. G. and Curran, P. F. (1969) J. Gen. Physiol. 53, 362–383
30 Glynn, I. M. (1968) Br. Med. Bull. 24, 165–169
31 Curran, P. F. and MacIntosh, J. R. (1962) Nature 193, 347–348
32 Holman, G. D. and Naftalin, R. J. (1975) Biochim. Biophys. Acta 373, 453–470
33 Naftalin, R. J. and Simmons, N. L. (1975) J. Physiol. 252, 87–88 (P)