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ENERGETICS OF COUPLED Na^+ AND Cl^- ENTRY INTO EPITHELIAL CELLS OF BULLFROG SMALL INTESTINE

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Summary

Na^+, K^+ and Cl^- concentrations (c_j^i) and activities (a_j^i), and mucosal membrane potentials (E_m) were measured in epithelial cells of isolated bullfrog (*Rana catesbeiana*) small intestine. Segments of intestine were stripped of their external muscle layers, and bathed (at 25°C and pH 7.2) in oxygenated Ringer solutions containing 105 mM Na^+ and Cl^- and 5.4 mM K^+. Na^+ and K^+ concentrations were determined by atomic absorption spectrometry and Cl^- concentrations by conductometric titration following extraction of the dried tissue with 0.1 M HNO_3. ^{14}C-labelled inulin was used to determine extracellular volume. E_m was measured with conventional open tip microelectrodes, a^i_{Cl} with solid-state Cl^--selective silver microelectrodes and a^i_{Na} and a^i_K with Na^+- and K^+-selective liquid ion-exchanger microelectrodes. The average E_m recorded was —34 mV. c^i_{Na}, c^i_K and c^i_{Cl} were 51, 105 and 52 mM. The corresponding values for a^i_{Na}, a^i_K and a^i_{Cl} were 18, 80 and 33 mM. These results suggest that a large fraction of the cytoplasmic Na^+ is 'bound' or sequestered in an osmotically inactive form, that all, or virtually all the cytoplasmic K^+ behaves as if in free solution, and that there is probably some binding of cytoplasmic Cl^-. a^i_{Cl} significantly exceeds the level corresponding to electrochemical equilibrium across the mucosal and baso-lateral cell membranes. Earlier studies showed that coupled mucosal entry of Na^+ and Cl^- is implicated in intracellular Cl^- accumulation in this tissue. This study permitted estimation of the steady-state transapical Na^+ and Cl^- electrochemical potential differences ($\Delta\bar{\mu}Na$ and $\Delta\bar{\mu}Cl$). $\Delta\bar{\mu}Na$ (—7000 J · mol^{-1}; cell minus mucosal medium) was energetically more than sufficient to account for $\Delta\bar{\mu}Cl$ (1000—2000 J · mol^{-1}).

Introduction

Earlier studies in this laboratory [1] led to the proposal that coupled electroneutral Na^+ and Cl^- influx across the apical membrane of the absorptive cells is a major component of net transepithelial ion transport by isolated bullfrog small intestine. More recently, a similar coupled entry of Na^+ and Cl^- has been identified in mammalian [2] and teleost [3] small intestine, rabbit gallbladder [4] and proximal renal tubules of *Necturus* [5]. Measurements, with Cl^--selective microelectrodes, of intracellular Cl^- activities in the epithelial cells of bullfrog [6] and *Amphiuma* [7] small intestine, and of *Necturus* proximal tubules [5] have shown that, in these tissues, net Cl^- entry from the luminal fluid to the cell interior occurs against an electrochemical gradient. A similar active accumulation of Cl^- by isolated rabbit ileum and gallbladder was suggested on the basis of the electrical potential profile and apparent Cl^- concentrations in the epithelial cells of these tissues [8]. Hence, the proposal [8] that the Na^+ electrochemical potential difference ($\Delta\bar{\mu}Na$) between the luminal fluid and the cell interior could provide the energy required for intracellular Cl^- accumulation in the small intestine, gallbladder, and other epithelial tissues, by a mechanism analogous to that which is now widely accepted for Na^+-dependent intestinal sugar and amino acid transport [9], has aroused much interest. Clearly, the validity of any such proposal depends absolutely on the condition that the energy available from $\Delta\bar{\mu}Na$ be at least sufficient to account for accumulative transfer of the solute (sugar, amino acid, Cl^-, etc.) whose movement from lumen to cell is coupled to that of Na^+. Measurement of the thermodynamic adequacy of $\Delta\bar{\mu}Na$ as an energy source for Na^+-coupled solute transfer [10] is, therefore a necessary pre-requisite for evaluating such hypotheses. In this study K^+, Na^+, and Cl^- activities (a^i_K, a^i_{Na}, a^i_{Cl}) were measured in absorptive cells of isolated bullfrog small intestine immersed in a NaCl medium and the energetic adequacy of $\Delta\bar{\mu}Na$ for Cl^- accumulation by these cells was assessed. In addition, intracellular K^+, Na^+ and Cl^- concentrations were determined under the same conditions.

Methods

Tissue preparation and electrical measurements. Flat sheets of intestinal tissue from North American bullfrogs (*Rana catesbeiana*) were used in this investigation. Animals were killed by a blow on the head and 5 cm lengths of small intestine (immediately distal to the entrance of the common bile duct) were rapidly excised. These were opened along the side of lesser curvature, pinned as flat sheets on a small lucite dissecting board, and stripped of their external muscle layers by blunt dissection [11]. During dissection the intestine was kept moist with Ringer solution *.

Following dissection and stripping, segments were cut from the intestinal sheets and mounted, mucosal side upwards, in a divided chamber of the type described by White and Armstrong [13]. Both the mucosal and serosal aspects

* Histologic examination of sections prepared from segments of stripped intestine showed that the external muscle layers were completely removed, leaving behind an intact epithelial cell layer, basement membrane, lamina propria and portions of the muscularis mucosae. These preparations thus corresponded to the 'partial mucosal strips' of Frizzell et al. [12].

of the tissue were perfused continuously (at 25°C and pH 7.2) with identical oxygenated phosphate Ringer solutions [1]. These contained 105 mM Na^+, 105 mM Cl^- and 5.4 mM K^+. Membrane potentials, and Na^+, K^+, and Cl^- activities were determined by impaling epithelial cells, through their mucosal membranes, with appropriate microelectrodes. These microelectrodes were connected via salt bridges to calomel half-cells which were, in turn, connected to the recording apparatus. The apparatus and circuitry used to measure and record the potentials obtained with these electrodes have been described in detail elsewhere [6,13,14]. A grounded half-cell connected to the mucosal solution via an appropriate salt bridge was used as a reference electrode for all microelectrode potential measurements.

Fabrication of microelectrodes. Solid-state silver microelectrodes of the type described in detail by Armstrong et al. [6] were used to measure a^i_{Cl}. These had slopes in the range 55—60 mV. To determine membrane potentials, a^i_K, and a^i_{Na}, single-barreled micropipettes with tip diameters of about 1.0 μm were drawn in a Kopf 700C vertical puller from 'Kwik-Fil' borosilicate glass capillary tubing (outer diameter 1.2 mm, inner diameter 0.68 mm, W.P. Instruments, New Haven, CT) previously cleaned by boiling in methanol. For membrane potential (E_m) measurements, micropipettes were 'back-filled' (i.e. filled by immersing the open end of the electrode barrel in the electrolyte solution) with 3 M KCl. The tip resistances of these microelectrodes ranged from 10 to 50 MΩ. Liquid ion-exchanger microelectrodes were used to measure a^i_K and a^i_{Na}. These were prepared by the following method. First, the inside surfaces of the micropipettes were made hydrophobic by silanization [15]. This was accomplished by mounting them in a special teflon holder and exposing them for 2 min at 25°C to a stream of dried DDS (dichlorodimethyl silane, Eastman Kodak Company, Rochester, NY) vapor *. Following this they were heated in an oven for 1 h at 110°C. A column of liquid ion-exchanger (200 μm—2 mm long) was then introduced into the tip of each micropipette, either by immersing it, tip downward, in the ion-exchanger solution or by back-filling. Finally, each micropipette was mounted, under 150X magnification, on a microscope stage and filled with an appropriate electrolyte solution (0.5 M KCl for K^+-selective, 150 mM NaCl for Na^+-selective microelectrodes). A fine glass capillary, pulled by hand and connected to a syringe, was used for this purpose. A combination, under microscopic observation, of mechanical agitation with an ultrafine glass fiber mounted in a micromanipulator (Narishige MM 33), and intermittent gentle heating with a microforge [14] was used to dislodge air bubbles and ensure a good contact between the liquid ion-exchanger and the electrolyte solution in the micropipette.

Corning 477317 K^+-selective liquid ion-exchanger was used in K^+-selective microelectrodes. Solutions of the Na^+-selective antibiotic ionophore monensin (10%, w/v, of the free acid) in various organic solvents were used in Na^+-selective microelectrodes.

Measurement of intracellular K^+, Na^+, and Cl^- activities. K^+-, Na^+-, and Cl^--selective microelectrodes were calibrated in 5, 10, 20, 50 and 100 mM KCl and

* The optimal time for this step in silanization can vary considerably on different occasions. The reasons for this are not clear at present. The humidity of the surrounding atmosphere appears to be one factor, but there are probably others, e.g. the precise shape and diameter of the micropipette tip (see ref. 15).

NaCl solutions. a_K, a_{Na}, and a_{Cl} in these solutions were calculated from the Debye-Huckel equation [10]. As reported elsewhere [6], the Cl^--selective microelectrodes used in these experiments were substantially free from interference by other anions present in significant amounts in the experimental system. Therefore intracellular Cl^- activity (a^i_{Cl}) was estimated directly from the equation

$$a^0_{Cl}/a^i_{Cl} = 10^{\Delta E/S} \quad (1)$$

In Eqn. 1, a^0_{Cl} is the calculated Cl^- activity of the (mucosal) bathing medium (81 mM, Table I), S is the slope of the electrode potential as a function of $\log a_{Cl}$ in the calibrating solution, and ΔE is the change (corrected for the mucosal membrane potential) in the potential recorded when the Cl^--selective microelectrode is moved from the mucosal medium to the interior of an absorptive cell. The mean mucosal membrane potential observed in these experiments (—34 mV) was used in each situation where correction of observed microelectrode potentials for this parameter was required.

The K^+-selective microelectrodes used in this study had slopes ranging from 55.7 to 64.5 mV/decade change in a_K. Their average K^+/Na^+ selectivity was 25 (range 15—62) and their resistances were about 10^9 Ω. Some of these selectivities are considerably lower than the K^+/Na^+ selectivity (80/1) of Corning macroelectrodes containing the same exchanger. A decrease in selectivity with decreasing tip diameter is a frequently observed property of cation-selective liquid ion-exchanger microelectrodes [15,16]. Nevertheless, the K^+-selectivities obtained in this study were adequate to measure intracellular K^+ activity (a^i_K) without significant interference from Na^+ or other cellular cations. However, because of the high a_{Na}/a_K ratio in the bathing medium (81/4, Table I), a^i_K (following correction of the intracellular electrode potential for E_m) was estimated from the calibration curve for the microelectrode in pure KCl solutions [16] rather than from an equation analogous to Eqn. 1 *.

Initially, in making Na^+-selective microelectrodes, nitrobenzene was used as the solvent for monensin. Kraig and Nicholson [18] reported that such microelectrodes had Na^+/K^+ selectivities of about 15/1. In our hands, the Na^+/K^+

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* The rationale for this procedure is as follows: The potential registered by a K^+-selective microelectrode immersed in the bathing medium is given by

$$E = E_0 + S \log(a^0_K + k_{KNa}a^0_{Na}) \quad (2)$$

where E_0 is the electrode potential in a solution for which the term in parentheses is unity and k_{KNa} is a selectivity coefficient which is, essentially, the reciprocal of the K^+/Na^+ selectivity ratio of the electrode [17]. In the cell interior, a^i_{Na} is relatively low (Table I). Therefore, in intracellular recordings, the product $k_{KNa}a^i_{Na}$ can be neglected and the electrode potential (corrected for E_m) can be represented with sufficient accuracy by the equation

$$E = E_0 + S \log a^i_K \quad (2a)$$

From eqns. 2 and 2a one can derive the expression

$$(a^0_K + k_{KNa}a^0_{Na})/a^i_K = 10^{\Delta E/S} \quad (3)$$

as the analog of Eqn. 1. While it is entirely feasible to 'correct' Eqn. 3 for the term $k_{KNa}a^0_{Na}$, using the calculated value of a^0_{Na} and the measured value of k_{KNa} for any given microelectrode, it is apparent (Table I) that, under the conditions of the present experiments, when $k_{KNa} > 0.05$ (i.e. K^+/Na^+ selectivity <20/1), $k_{KNa}a^0_{Na} > a^0_K$. Thus, even a small error in the estimate of k_{KNa} could, under these conditions, significantly affect the final value of a^i_K obtained. The procedure outlined in the text above avoids this source of error.

selectivities obtained (1/1—6.5/1) were substantially lower than this. Similar low selectivities have been observed by others (White, J.F., personal communication) and may reflect the fact that, as noted above for K^+-selective microelectrodes, the selectivity of monensin microelectrodes decreases as the tip diameter is reduced. In an attempt to improve the Na^+ selectivity of these electrodes, we tried a number of other solvents. These included hexanol, 3-nitro-*O*-xylene and the Corning 477317 liquid ion-exchanger used to make K^+-selective microelectrodes *. No significant improvement in Na^+/K^+ selectivity was noted, but microelectrodes containing Corning 477317 liquid ion-exchanger had much lower time constants than those containing the other solvent systems used in this investigation. The slopes and resistances, in NaCl solutions, of these Na^+-selective microelectrodes (58.5—73.2 mV/decade change in a_{Na} and approx. $10^9\ \Omega$) did not differ appreciably when different organic solvents were used and, although most measurements were made with microelectrodes containing Corning 477317 liquid ion-exchanger, a^i_{Na} measurements obtained with different solvent systems were pooled (Table I).

Na^+-selective microelectrodes containing monensin have been shown to perform satisfactorily in extracellular fluids [18]. However, their use for measuring intracellular Na^+ activities does not yet appear to have been reported elsewhere. Therefore, a detailed account of our calibration procedure may be of interest. It should be emphasized that, despite their low Na^+/K^+ selectivity ratios, these electrodes can be successfully used to measure intracellular ionic activities. Indeed, liquid ion-exchanger microelectrodes with very similar characteristics have been employed for this purpose [19]. In solutions containing mixtures of Na^+ and K^+, the potential registered by these electrodes will, in general, be influenced by both ionic species and may, by analogy with Eqn. 2, be written

$$E = E_0 + S \log(a_{Na} + k_{NaK} a_K) \quad (4)$$

Like Eqn. 2, Eqn. 4 assumes that, aside from K^+, there are no other interfering ions in the system. Also, in Eqn. 4, k_{NaK} corresponds to k_{KNa} in Eqn. 2.

In extracellular fluid, the Na^+/K^+ activity ratio is relatively high. Hence, the term $k_{NaK} a_K$ can usually be neglected in extracellular recording [18]. In the cell interior, because of the low Na^+/K^+ selectivity ratios of these microelectrodes, the potential due to this term will not only contribute significantly to the total electrode potential, but, in view of the relatively low Na^+/K^+ activity ratio found in most cells [16], may be expected, in many cases, to exceed the potential arising from a_{Na}. Thus, accurate evaluation of both a_K and k_{NaK} is an essential pre-requisite to the measurement of a_{Na} with these microelectrodes. To determine a^i_{Na} in epithelial cells of bullfrog small intestine we used the following procedure (Fig. 1A). Each microelectrode was first calibrated in solutions of pure NaCl and pure KCl, and k_{NaK} was calculated from the data obtained [14]. This value of k_{NaK}, together with the average a^i_K value found in the present study (80 mM, Table I) was inserted into Eqn. 4 and the curve relating the electrode potential, E, to $\log a_{Na}$ was calculated (bottom curve, Fig. 1A). The correspondence between the actual behavior of the microelectrode and this

* This is a solution of potassium tetrachlorophenylborate in 3-nitro-*O*-xylene.

calculated curve was then tested by calibration in solutions containing 10, 25, 50 and 100 mM NaCl, together with 104 mM KCl ($a_K \cong 80$ mM). Electrodes were accepted for use only if their experimental potentials fell within ±2 mV of the corresponding values calculated from Eqn. 4 (Fig. 1A). With monensin microelectrodes, it frequently happened that, in pure KCl solutions, the electrode potential was not a linear function of $\log a_K$. An example of such behavior (which has not previously been reported for monensin microelectrodes) is shown in Fig. 1B. Under these conditions, which probably reflect interference with the electrode response to K^+ by another cation, e.g. H^+ [17], the behavior of the microelectrode in solutions containing Na^+ and K^+ cannot be described by Eqn. 4, since k_{NaK} is not constant. As shown in Fig. 1B, such microelectrodes can be calibrated empirically for use in cells for which a_K^i is known. They were not, however, used in this investigation.

K^+, Na^+, Cl^- and water content of epithelial cells. Mucosal cell water, K^+, Na^+ and Cl^- were measured as follows: Segments of stripped intestine were immersed for 3 h, with continuous oxygenation, in the medium described above. Inulin (0.2%, w/v), labelled with [*carboxy*-^{14}C]inulin (New England Nuclear), was incorporated as an extracellular marker. Following immersion, the segments were gently blotted, weighed while wet, dried to constant weight at 105°C, and extracted for 72 h in 0.1 M HNO_3 as described elsewhere [20]. Tissue Na^+, K^+, and Cl^-, together with the ^{14}C activity in the tissue (relative to that in the bathing medium) were determined, in aliquots of this extract, by atomic absorption spectrophotometry (Perkin-Elmer model 303) and liquid scintillation spectrometry (Packer Tri-Carb model 3375), respectively. The

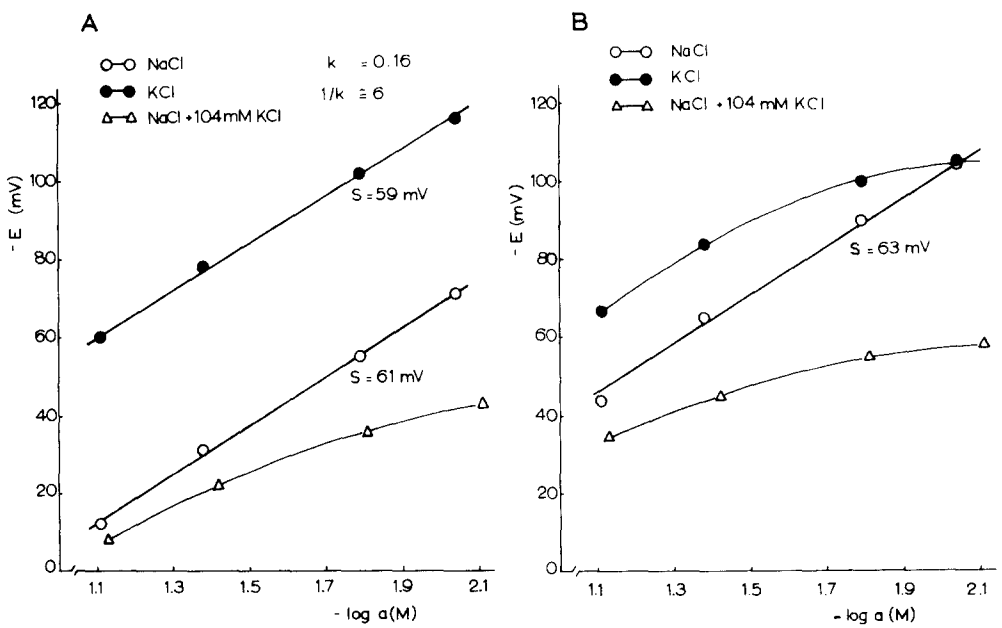

Fig. 1. (A, B) Calibration of two Na^+-selective liquid ion-exchanger microelectrodes containing monensin in pure NaCl, pure KCl and mixed solutions containing a constant amount of KCl ($a_K \cong 80$ mM). $1/k$ (A) is the Na^+/K^+ selectivity ratio.

inulin space, calculated from the tissue/medium ^{14}C activity, was equated to the extracellular water volume [20].

Reagent grade chemicals and on-line distilled water (further purified by two passes through a mixed ion-exchanger) were used to prepare all solutions used in these experiments. Student's *t*-test was used for statistical comparisons. Statements of statistical significance are made at the 0.05 confidence level unless otherwise indicated.

In this study, the criteria used to determine the acceptability of membrane potential recordings and impalements with Cl^--selective microelectrodes were similar, in general, to those established by White and Armstrong [13] and by Armstrong et al. [6] except that constancy (within 1 mV) of the final steady-state electrode potential for a minimum of 30 s, rather than 1 min or more, was considered a sufficient index of stability. Extensive experience in this laboratory has shown that, unless a microelectrode spontaneously falls out of a cell, an electrode potential which has remained constant for 30 s usually remains steady until the microelectrode is withdrawn.

Results and Discussion

Representative recordings of intracellular potentials with a Na^+-selective and a K^+-selective microelectrode are shown in Figs. 2A and 2B. Recordings with these electrodes were considered acceptable if (a) there was an initial sharp deflection on impalement (in a positive direction with K^+-selective and a negative direction with Na^+-selective microelectrodes), (b) this was followed within 10—20 s by the establishment of a steady potential (c) this steady-state potential remained constant within 1 mV until withdrawal of the microelectrode, and (d) following withdrawal, the electrode potential returned rapidly to within 1—2 mV of its initial value in solution. It is of interest that, as was found previously with closed-tip solid-state Cl^--selective microelectrodes [6], the relatively slow depolarization and subsequent repolarization (2—4 min) so frequently observed following impalement of an epithelial cell with an open-tip microelectrode [13] was rarely seen with K^+- or Na^+-selective liquid ion-exchanger microelectrodes. Instead, the time required to establish a steady-

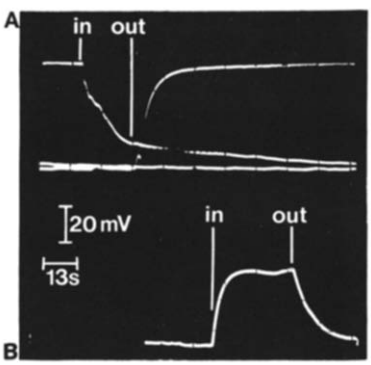

Fig. 2. Oscilloscope tracings of cell impalements with (A) a Na^+-selective and (B) a K^+-selective microelectrode.

state potential following impalement of a cell with these high-resistance microelectrodes appeared to depend mainly on the time constant of the electrode response to the change in a_K or a_{Na} (Fig. 2). A similar type of behavior was observed in previous studies with closed-tip glass microelectrodes [14]. Transient depolarization of the cell membrane following impalement of an epithelial cell with an open-tip microelectrode has been attributed to local membrane damage and subsequent spontaneous repair at the site of impalement [13]. It seems possible that, as a result of silanization, the open tips of liquid ion-exchanger microelectrodes may be less damaging to the cell membrane than those of conventional open-tip microelectrodes.

All ion-selective microelectrodes used in this study were calibrated immediately before and after their use in an experiment. When these procedures revealed a significant change in the characteristics of a microelectrode, results obtained with it were discarded.

Intracellular K^+, Na^+ and Cl^- activities and concentrations

The average E_m value recorded in this study was −34.0 ± 1.5 (S.E.) mV (11 animals, 49 impalements). The mean cell water content found in the intestinal mucosa from 8 animals was 3.44 ± 0.12 g/g dry weight. Average values (± S.E.) obtained for K^+, Na^+, and Cl^- concentrations, activities, and related parameters are summarized in Table I, which also shows that measured Na^+ and Cl^- activities in the bathing medium were in satisfactory agreement with those calculated from the Debye-Huckel equation [10].

The mean intracellular K^+ activity found in these experiments (80 ± 4 mM) did not differ significantly from the average value (85 ± 1 mM) previously reported for this parameter in the same tissue bathed with an isotonic chloride-free sodium sulfate Ringer solution [21]. A similar agreement between intracellular K^+ activities in epithelial cells of *Amphiuma* small intestine bathed with NaCl and sodium sulfate media, respectively, was reported by White [16]. These results may be interpreted as indicating that there is no significant

TABLE I

K^+, Na^+, and Cl^- concentrations (c) and activities (a) in the external medium and in epithelial cells of isolated bullfrog small intestine; apparent intracellular activity coefficients (γ^i) and electrochemical potential differences (cell minus mucosal medium) across the mucosal membrane of the epithelial cells ($\Delta\bar{\mu}j$). c and a in mM, $\Delta\bar{\mu}j$ in J · mol^{-1}. Mean values ± S.E. shown for measured parameters. Numbers in parentheses are: number of observations (a^0), number of animals (c^i), or number of animals followed by total number of impalements (a^i).

Ionic species	Medium			Cell interior			
	c^0	* a^0	** a^0	c^i	a^i	γ^i	$\Delta\bar{\mu}j$
K^+	5.4	4.2	—	105 ± 3 (8)	80 ± 4 (8 : 25)	0.76	4020
Na^+	105	81	82 ± 5 (8)	51 ± 3 (8)	18 ± 2 (5 : 20)	0.35	−7000
Cl^-	105	81	81 ± 4 (10)	52 ± 1 (12)	33 ± 2 (13 : 26)	0.63	1050 (1990) ***

* Calculated, assuming a mean ionic activity coefficient of 0.77 [6].

** Measured with ion-selective microelectrodes used to determine corresponding a^i values.

*** Calculated from the data of Armstrong et al. [6].

coupling between transcellular K^+ and Cl^- transport in amphibian small intestine.

The ratio 0.76 for a_K^i/c_K^i (γ_K^i, Table I) found in this study is virtually identical to the value (0.77) calculated on the assumption that all the intracellular K^+ is in free solution in the cytoplasm. This agrees with the data reported for the great majority of cell species for which a_K^i has been directly measured [17,22] and is usually interpreted to mean that there is little if any 'binding' or sequestration of cytoplasmic K^+. As pointed out elsewhere [14,17], this interpretation, in the absence of confirmatory evidence obtained by independent methods is, of necessity, somewhat tentative, since it is based on the assumption that all the cell water behaves as a solvent for K^+, an assumption which is open to serious question.

These results are in sharp contrast to those reported by White [16] for epithelial cells of *Amphiuma* small intestine. This author used K^+-selective microelectrodes similar to those described herein, and found an average a_K^i of 41 mM for tissues bathed in a NaCl medium which contained 90 mM Na^+ and a 2.5 mM K^+. Chemical analysis gave an average c_K^i of 146 mM and a corresponding γ_K^i of 0.28 under these conditions. The reasons for this difference are not readily apparent. It may be, as White [16] has suggested, that intracellular K^+ distribution in bullfrog and *Amphiuma* small intestine is basically different. This, if true, would be somewhat unexpected in view of the fact that K^+ distribution in such widely divergent cell types as mammalian cardiac muscle fibers and neuronal cells of *Aplysia* appears to follow a closely similar pattern [17,22]. In addition, recent experiments in this laboratory [23] indicate that a_K^i in the intestinal absorptive cells of *Necturus*, another amphibian species, is about 108 mM. Further studies are needed to resolve this question.

The mean Na^+ activity observed in this study (18 ± 2 mM) does not differ significantly from the average value (14.4 ± 1 mM) previously found by Lee and Armstrong [21] in a sodium sulfate medium. Because of coupled Na^+ and Cl^- entry, one would suppose that total Na^+ influx across the mucosal membrane of the epithelial cells would be considerably larger in a medium containing Cl^- than in one containing sulfate. The similarity of the a_{Na}^i values found under both sets of conditions therefore suggests a more rapid removal of Na^+ from these cells, i.e. an enhanced rate of Na^+ pumping across the basolateral cell membrane, in chloride than in sulfate media. This is consistent with the finding of Quay and Armstrong [1] that the net mucosal to serosal Na^+ flux in NaCl media greatly exceeds that observed in sodium sulfate media.

The a_{Na}^i/c_{Na}^i ratio (γ_{Na}^i) observed in the present experiments (0.31, Table I) is far less than the value (0.77) predicted on the assumption that all the cytoplasmic Na^+ is in 'free' solution. Even if one allows for considerable uncertainty about the fraction of cell water that can be considered to act as a solvent for Na^+, this finding strongly suggests that much of the intracellular Na^+, under the conditions of the present experiments, is 'bound' or compartmentalized in one or more regions of cell which are not accessible to Na^+-selective microelectrodes. This is in conformity with the results reported previously for bullfrog small intestine maintained in sodium sulfate media [20] and with the data reported for other species [17,22].

The intracellular Cl^- activity and concentration found in this investigation

gave an a^i_{Cl}/c^i_{Cl} ratio of 0.63 (Table I). This, again, is considerably lower than the value (0.77) predicted on the basis of a free solution model for cytoplasmic Cl^- and could be interpreted as suggestive of some intracellular binding or compartmentation of Cl^-. The uncertainties inherent in the measurement of c^i_{Cl} [17] and those, already mentioned, concerning the status of solvent water in the cell make such a conclusion extremely tentative at this time. White's data [7] for epithelial cells of *Amphiuma* small intestine appear to be less equivocal in this respect. This author found a mean a^i_{Cl} of 17 mM, c^i_{Cl} = 53 mM and γ^i_{Cl} = 0.32. Radioisotope studies indicate that a small fraction (about 10%) of intracellular Cl^- in rabbit ileum is inexchangeable [24]. Thus, on balance, the evidence available at this time favors the view that some fraction of the Cl^- content of epithelial cells of the small intestine is 'bound' or sequestered. This conclusion, together with the results reported above for K^+ and Na^+, agrees with the results reported by Pietrzyk and Heinz [25] on the basis of cell fractionation studies.

The average a^i_{Cl} found in this investigation (33 ± 2 mM) differs markedly from the value (71 ± 5 mM) previously reported by Armstrong et al. [6] under similar conditions. The mean E_m recorded earlier (—24.0 ± 1 mV; ref. 6) also differs significantly from the value (—34 ± 1.5 mV) obtained in the present study. However, this difference in E_m would not alone account for the divergent values obtained for a^i_{Cl}. For example, if an E_m of —24 mV is used to calculate a^i_{Cl} from the Cl^- electrode potentials recorded in the present investigation, one obtains a mean value of 50 ± 3 (S.E.) mM. This is also significantly smaller than the value previously reported [6]. The origin of these discrepancies in E_m and a^i_{Cl} may lie in seasonal variation. In two sets of experiments performed under identical conditions with *Amphiuma* small intestine, White [7] found a marked difference between the average a^i_{Cl} values (28 mM in proximal, 36.5 mM in distal intestinal segments) for animals captured during the summer and the corresponding value (18 mM) in fall and winter animals. In the same experiments the mean E_m increased from 33 mV (proximal) or 24 mV (distal) in summer to 42 mV in fall and winter animals.

Despite the discrepancy between the average a^i_{Cl} value recorded in the present study and that reported previously [6], the intracellular Cl^- electrochemical potential, in both investigations, clearly exceeded the value corresponding to electrochemical equilibrium across the mucosal membrane of the epithelial cells. Thus, both investigations support the conclusion that net Cl^- transport from the luminal medium to the cell interior is an energy-requiring process. Conversely, taking into account the extremely low transepithelial potential difference (0—1 mV) recorded under the conditions of these experiments [1,26], it is evident that net Cl^- exit from the cell interior to the serosal medium takes place down an electrochemical potential gradient.

The Na^+ electrochemical potential gradient and Cl^- accumulation

Table I shows the transmucosal electrochemical potential differences ($\Delta\bar{\mu}j$) for K^+, Na^+ and Cl^- calculated from the electrophysiological measurements performed in the course of this investigation, together with $\Delta\bar{\mu}Cl$ calculated from the data obtained in a previous study [6]. In Table I, $\Delta\bar{\mu}j$ is expressed as the reversible work, in Joules, required to transfer one equivalent of the ion in

question from the mucosal medium to the interior of an absorptive cell and is calculated from the equation

$$\Delta\bar{\mu}j = RT \ln a_j^i/a_j^0 + zE_mF \quad (5)$$

where R, T, z and F have their usual meanings. $\Delta\bar{\mu}$K is included in Table I since it was readily obtained from the available data and may prove to be of interest in relation to the energetics of the baso-lateral Na^+ pump in epithelial cells of the small intestine, and the role of baso-lateral Na^+-K^+ exchange in the maintenance of steady-state Na^+ and K^+ concentrations. As yet, the mechanisms by which these cells regulate their K^+ content has not been studied so intensively as the question of transepithelial Na^+ transport. At this time, the evidence available strongly supports the concept that active Na^+ extrusion across the baso-lateral cell membrane is electrogenic [27]. This is, of course, entirely consistent with the existence of a Na^+-K^+ exchange pump in this membrane providing that its Na^+/K^+ coupling ratio exceeds unity [28]. There is, in fact, considerable evidence that a mechanism of this type is involved in the maintenance of intracellular Na^+ and K^+ concentrations. However, its role, if any, in the regulation of active transepithelial Na^+ transport remains unclear [28].

Table I also shows, for the first time, the quantitative relationship between the energy available from $\Delta\bar{\mu}$Na across the mucosal membrane of epithelial cells of the small intestine and the energy required to maintain the steady-state intracellular Cl^- concentration. It is clear from Table I that, for 1/1 coupled mucosal entry of Na^+ and Cl^-, the energy available from $\Delta\bar{\mu}$Na is more than adequate to account for intracellular Cl^- accumulation. Indeed, of the total reversible energy available from $\Delta\bar{\mu}$Na, as little as 15—30% could, in theory, suffice for this purpose.

In conclusion, two questions which are relevant to the significance of the calculated $\Delta\bar{\mu}j$ values shown in Table I may be briefly considered. First, with isolated *Amphiuma* small intestine, White [7,16] observed apparent increases in Cl^- and K^+ activity when an appropriate ion-selective microelectrode was advanced from the interior of the mucosal solution towards the surface of a villous absorptive cell. Zeuthen and Monge [29] reported a similar apparent increase in a_K for rabbit ileum in vivo. White [7,16] further observed that these local increases in a_K and a_{Cl} were diminished, though not completely abolished, by increased mucosal perfusion rates and suggested that they might be due to insufficient removal, from the immediately vicinity of the absorptive cells, of K^+ and Cl^- which leak passively across the mucosal membranes of these cells or are secreted by other types of cell in the small intestine. In this investigation we found similar apparent increases in a_K, a_{Cl}, and a_{Na} close to the mucosal surface of the tissue. While these were not systematically studied, some observations were made which suggest that they are, at least in part, artifactual. First, it was noticed that, at the time when the extracellular ionic activity apparently increased, a 'dimpling' of the tissue under the microelectrode was visible under the microscope. This suggests that, as described by Suzuki and Frömter [30] for isolated *Necturus* gallbladder, the mucosal cell membrane is pushed inward by the microelectrode before penetration occurs. Second, in some experiments where attempts were made to impale cells with relatively large Na^+-selective

microelectrodes (tip diameters >1.5 μm) changes up to 20 mV in the electrode potential were observed just before penetration occurred and these changes were virtually unaffected by increasing the mucosal perfusion rate from 2 to 200 ml · min^{-1}. These observations do not, of course, exclude the possibility that real variations in ionic activities, due to the presence of unstirred layers, exist close to the surface of the epithelial cells of the small intestine but the existence of such local activity gradients (and especially the occurrence of increased Na^+ and Cl^- activities in this region) in no way challenges the validity of the general conclusion that $\Delta\bar{\mu}Na$ is energetically adequate to account for $\Delta\bar{\mu}Cl$ under the conditions of the present experiments (Table I).

Second, in calculating $\Delta\bar{\mu}j$ (Table I), the assumption was made that a single value could be assigned to a_j^i throughout the cytoplasmic compartment of the cell since, in these experiments, no attempt was made to ascertain the position, with respect to the mucosal membrane, of the microelectrode after penetration. This assumption has been challenged by Zeuthen who proposed [29,31] that gradients of electric and ionic electrochemical potentials exist across the interior of the epithelial cells of gallbladder and small intestine. Such inhomogeneities in cytoplasmic Na^+, K^+ and Cl^- distribution could seriously compromise estimates of $\Delta\bar{\mu}j$, such as those given in Table I, which are based on 'randomized' measurements of intracellular ionic activities. However, the existence of intracellular ionic and electrical gradients such as those proposed by Zeuthen [29,31] has been strongly contested by Suzuki and Frömter [30] who presented evidence that the former worker's results could have arisen from electrode artifacts, in particular, artifacts associated with defective impalements of the mucosal cell membrane and consequent ionic leaks across this membrane. Further, Zeuthen [31] has himself conceded that the existence of such artifacts in his measurements of intracellular potentials cannot be ruled out. For these reasons, the assumption of a uniform a_j^i in calculating the $\Delta\bar{\mu}j$ values shown in Table I appears to be justified at this time.

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