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The Steady-State Relationship between Sodium and Chloride Transmembrane Electrochemical Potential Differences in *Necturus* Gallbladder

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Summary. Intracellular Cl, K and Na activities (a^i_{Cl}, a^i_K and a^i_{Na}) and transmucosal membrane potential (E_m) in epithelial cells of *Necturus* gallbladder were measured at different external Na concentrations ($[Na]_o$), with liquid ion-exchanger and conventional microelectrodes. Bladders were mounted in a divided chamber at 23 °C between identical HCO_3-free Ringer solutions containing 5 mM K. The pH was 7.2. Tris was substituted for Na. Measurements were made under steady-state conditions as determined by the constancy of the transepithelial potential difference. Both, a^i_{Cl} and a^i_{Na} increased in a saturable fashion with $[Na]_o$. E_m did not change significantly. Average values ($\pm$SEM) under normal conditions ($[Na]_o$ = 100 mM) for a^i_{Cl}, a^i_{Na} and E_m were 16.8 ± 0.8 mM ($n=9$), 9.7 ± 0.6 mM ($n=10$) and -52.6 ± 0.6 mV ($n=26$), respectively. In Na-free media a^i_{Cl} declined to its equilibrium value. a^i_K (96 ± 2 mM; $n=7$) did not change when $[Na]_o$ was varied between 100 and 10 mM but decreased to 80 ± 3 mM ($n=4$) in Na-free media.

Transmembrane electrochemical potential differences, $\Delta\bar{\mu}_j$, for Cl and Na were calculated at four different $[Na]_o$ levels. A highly significant linear relation between $\Delta\bar{\mu}_{Cl}$ and $\Delta\bar{\mu}_{Na}$ was found, indicating that Cl and Na transport are energetically linked. The results support the view that the energy necessary for intracellular Cl accumulation is derived from the simultaneous dissipation of the chemical potential gradient of Na across the apical membrane and that the coupled entry mechanism is electroneutral.

The existence of coupled Na and Cl transport in the gallbladder of a number of animal species (Diamond, 1968) and in other leaky epithelia (Frizzell, Field & Schultz, 1979) has been recognized for some time and is now well-established as an important component of salt and water transfer by these tissues.

There is now compelling evidence that in the gallbladder, and other epithelia, coupled transport of Na and Cl takes place across the luminal membrane of the epithelial cells and that it involves an electroneutral 1:1 coupling between these ions (Frizzell, Dugas & Schultz, 1975; Cremaschi & Henin, 1975; Spring & Kimura, 1978; Frizzell et al., 1979; Reuss & Grady, 1979). Chemical and radiotracer estimates of intracellular Cl (Frizzell et al., 1975) indicated that, in the epithelial cells of rabbit gallbladder, the Cl concentration is above the level corresponding to electrochemical equilibrium across the luminal membrane. This prompted the suggestion that the energy for Cl accumulation is provided by simultaneous dissipation of the transmembrane Na electrochemical potential gradient by a mechanism analogous to that which is now widely accepted for Na-dependent sugar and amino acid transport in the small intestine (Schultz & Curran, 1970). Direct measurement, with Cl-selective microelectrodes, of intracellular Cl activities in a number of leaky epithelia has demonstrated that, under steady-state conditions and in the presence of luminal Na, intracellular Cl accumulation in excess of the requirements for electrochemical equilibrium does in fact occur (Frizzell et al., 1979; Armstrong et al., 1979*a*; Reuss & Grady, 1979; Duffey et al., 1979). Furthermore, measurements of intracellular Na activities in some leaky epithelia that exhibit "uphill" Cl transport across the luminal membrane have shown that the Na electrochemical potential difference across this membrane is, energetically, more than sufficient to account for the steady-state level of Cl accumulation (Armstrong et al., 1979*a*; Reuss & Weinman, 1979). More direct evidence that the transmucosal Na-electrochemical potential difference ($\Delta\bar{\mu}_{Na}$) is the energy source for intracellular Cl^- accumulation has emerged from studies in which Na was completely removed from the mucosal bathing solution. Under these conditions (Spring & Kimura, 1978;

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Reuss & Grady, 1979; Duffey et al., 1978; Frizzell et al., 1979) intracellular Cl falls to the level approximating an equilibrium distribution of Cl across the luminal cell membrane, i.e., the transmembrane Cl electrochemical potential difference ($\Delta\bar{\mu}_{Cl}$) assumes a value close to zero. Although these results strongly support the idea that $\Delta\bar{\mu}_{Na}$ is the direct source of energy for Cl accumulation, they are amenable to alternative explanations. For example, it might be argued that the effect of Na on Cl accumulation is indirect and involves a component of metabolism that depends on the presence of Na. The demonstration of an explicit functional relationship between $\Delta\bar{\mu}_{Na}$ and $\Delta\bar{\mu}_{Cl}$, when $\Delta\bar{\mu}_{Na}$ is systematically varied, would provide additional evidence that $\Delta\bar{\mu}_{Cl}$ depends directly on $\Delta\bar{\mu}_{Na}$. The experiments reported herein are addressed to this problem. In these studies, $\Delta\bar{\mu}_{Na}$ in epithelial cells of *Necturus* gallbladder was varied by altering the external Na concentration, $[Na]_o$. The membrane potential, E_m, together with the intracellular Na, K, and Cl activities (a^i_{Na}, a^i_K, and a^i_{Cl}) were measured under these conditions. The results obtained permit one to define the relationship between $\Delta\bar{\mu}_{Na}$ and $\Delta\bar{\mu}_{Cl}$ in this tissue.

Materials and Methods

Necturus maculosus obtained from Graska Biological Supplies (Oshkosh, Wisc.) were kept in a large aquarium at 4 °C. The animals were killed by a blow on the head and a triple transection of the spinal cord was performed. The abdominal cavity was opened by median incision, the gallbladder was removed, cleaned from adhering liver tissue, emptied, and cut longitudinally. It was then washed free of bile residues in Ringer's solution and mounted as a flat sheet at room temperature (23 ± 1 °C) in a divided chamber (White & Armstrong, 1971). Both the luminal and serosal surfaces of the tissue were independently and continuously perfused by means of a simple gravity device. Both perfusion solutions could be rapidly changed by means of stopcocks located near the appropriate chamber inlets.

The control Ringer's solution contained, in mM: NaCl, 100; KH_2PO_4, 0.8; K_2HPO_4, 2.3; Ca-gluconate, 1.8; and mannitol, 21. The pH was adjusted to 7.2. NaCl was substituted by Tris (Tris-hydroxymethyl-aminomethane) titrated with HCl to pH 7.2. Since the amounts of Cl and of Tris are different at this pH (Colowick & Kaplan, 1955), Tris Cl was added in an amount sufficient to bring the Cl concentration to 100 mM. The mannitol content was varied to maintain the same osmolarity in all solutions. The measured osmolality was 210 ± 3 mOsm (SD, $n = 7$) (Advanced Osmometer 3W, Advanced Instruments, Needham Heights, Mass.). Both bathing solutions were continuously gassed with O_2.

Electrical Measurements

The transepithelial potential difference, E_{Tr}, was continuously measured by two calomel half cells connected to the mucosal and serosal solutions by means of 3 M KCl-agar bridges. The mucosal half cell was connected to ground and the serosal one to the input of a high impedance preamplifier with input resistance $> 10^{14}\ \Omega$ and unit gain (311J, Analog Devices, Norwood, Mass.). The output of the preamplifier was connected to a millivoltmeter (1350, Data Precision, Wakefield, Mass.) and to one channel of a strip-chart recorder (Gould-Brush Mark 240, Cleveland, Ohio). Microelectrodes were connected by Ag-AgCl wires to another high impedance preamplifier. Initially, connection between the Ag-AgCl wire and the preamplifier was by a coaxial cable with a grounded shield. In later experiments the time response of the recording system was improved (*see* Figs. 2 and 3) by using a guarding system. The potentials registered by the microelectrode with reference to the mucosal half cell were recorded simultaneously on a millivoltmeter (165 Keithley, Cleveland, Ohio), a second channel of the Brush recorder, an oscilloscope (5103 N, Tektronix, Beaverton, Ore.), and an audiomonitor.

Fabrication and Calibration of Microelectrodes

Single-barrelled micropipettes with tip diameters < 1 μm were drawn in a Kopf 700C (David Kopf Inst., Tukunga, Calif.) vertical puller from "Kwik-Fil" borosilicate glass capillary tubing[1] (outer diameter 1.2 mm, inner diameter 0.68 mm, W.P. Inst., New Haven Conn.) previously cleaned by immersion in approximately 1 N HNO_3 solution followed by boiling in methanol[2]. For E_m measurements, micropipettes were "back-filled" with 1 M KCl. The tip resistances of these microelectrodes in normal NaCl-Ringer ranged from 20 to 40 MΩ. Liquid ion-exchanger microelectrodes were used to measure intracellular Cl, K and Na activities (a^i_{Cl}, a^i_K and a^i_{Na}). These were prepared by a modification of the method of Fujimoto and Kubota (1976). The micropipettes were silanized by immersing the tips in a fresh solution (0.0025–0.004%) of silicone polymer (1107, Dow Corning, Midland, Mich.) in acetone for approximately 7 sec. After this, the micropipettes were cured for 20 min on a hot plate. This silanization technique yielded a greater proportion of successful microelectrodes than previous methods in which the inside of the micropipettes were exposed to the vapor of dimethyldichlorosilane or dimethylchlorosilane (Armstrong et al., 1979*a*). A column of liquid ion-exchanger (200 μm–2 mm long) was then introduced into the tip of each micropipette by back filling. Finally, each micropipette was mounted, under 150× magnification, on a microscope stage and filled with appropriate electrolyte solution (0.5 M KCl for K- and Cl-selective microelectrodes, 0.5 M NaCl for Na-selective microelectrodes). A fine glass capillary, pulled by hand and connected to a syringe, was used for this purpose.

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[1] In a few experiments thinner walled capillaries ("Ultra-Tip" Omega Dot capillaries, 1.0 mm OD, Frederick Haer & Co., Brunswick, Me.), drawn in a horizontal Narishige PD-5 puller, were used to measure E_m. When filled with 1 M KCl, the resistances of these microelectrodes were typically 4–8 MΩ. There was no difference between the E_m values measured with these microelectrodes and those recorded with thicker walled microelectrodes. However, when thin-walled glass was used to make ion-selective microelectrodes, the electrode potential, in many instances, was not linearly related to log a at lower activities. This effect is probably due to shunting of the microelectrode potential through a hydrated glass layer (Armstrong & Garcia-Diaz, 1980). Therefore ion-selective microelectrodes made from thin walled glass micropipettes were not used in these experiments.

[2] More recently, we have found that glass capillaries cleaned by boiling in a concentrated detergent solution followed by thorough rinsing in distilled water and oven drying, provide micropipettes that are easier to fill with aqueous solutions or ion-selective resins without trapping air bubbles in the shank of the microelectrode.

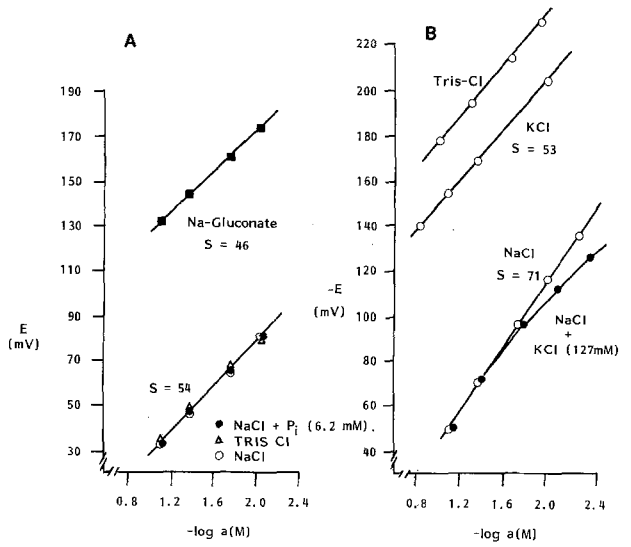

Fig. 1. (*A*): Calibration of a Cl-selective microelectrode. The solutions with P_i in addition to NaCl contained 1.6 mM KH_2PO_4 and 4.6 mM K_2HPO_4. (*B*): Calibration of a Na-selective microelectrode. In both *A* and *B*, *S* is the slope, in mV, of the electrode potential for a decade change in activity.

Corning 477317 and 477315 liquid ion-exchangers (Corning Medical, Medfield, Mass.) were used in K- and Cl-selective microelectrodes. The liquid ion-exchanger Na-selective microelectrodes were as described in detail elsewhere (O'Doherty, Garcia-Diaz & Armstrong, 1979). The resistance of the ion-selective microelectrodes was of the order of $10^{10}\,\Omega$ (*see* legends to Figs. 2 and 3 for an estimate of the time response).

The ion-selective microelectrodes were calibrated in electrolyte solutions with concentrations covering the physiological range. Ionic activities in these solutions were obtained from the data published by Conway (1952) or from the Debye-Hückel equation (Armstrong, Byrd & Hamang, 1973). Calculated lines of regression between the electrode potential and the logarithm of the primary ion activity always had $r > 0.9995$.

Figure 1*A* shows the calibration of a Cl-selective microelectrode in solutions of 100 to 10 mM NaCl or Tris Cl. The slopes for 33 of these electrodes ranged from 47.9 to 56.3 mV/decade change in chloride activity (mean 51.7). It is evident from Fig. 1*A* that the microelectrode response to Cl ions is the same in NaCl and in TrisCl and is not affected by the addition to NaCl solutions of 6.2 mM P_i, that is, approximately the amount of intracellular P_i found in a variety of tissues (Conway, 1957; Stermann, Wagle & Decker, 1978). That HCO_3 ions did not significantly influence the response of Cl-selective microelectrodes in these experiments may be inferred from the fact that the Ringer's solution used was HCO_3-free and that the selectivity ratio, $k^{-1}_{ClHCO_3}$ measured by the separate solution method, SSM (Method *Ia*, Moody & Thomas, 1971), in 0.1 M NaCl and 0.1 M $NaHCO_3$ solutions ranged from 9.2 to 11 ($n = 5$). To check for interference from other intracellular anions, three experiments were performed where a^i_{Cl} was determined after substitution of external Cl by gluconate. The Cl-selective microelectrodes showed a high selectivity ratio, k^{-1}_{ClGluc}, (*see* Fig. 1*A*). With 6 microelectrodes k^{-1}_{ClGluc} ranged from 72 to 125. It was found that, when gluconate was substituted for external Cl, a^i_{Cl} decreased exponentially with a mean time constant of 40 min. After 80 min, a^i_{Cl} was 3.5 ± 0.9 mM (SEM) and reached a stable value of 1.0 ± 0.3 mM approximately 4 hr after the change. This value of "apparent" intracellular Cl probably reflects the effect of other unspecified cellular anions on the microelectrode potential.

K-selective microelectrodes were calibrated in 200, 100, 50 and 10 mM KCl solutions. In 21 microelectrodes the slopes ranged from 60.6 to 65.8 mV/decade (mean 63). Selectivity ratios, determined by the SSM in 0.1 M salt solutions, were 20.2–49.4 for K over Na and 40.6–75.2 for K over Tris. Fig. 1*B* shows the calibration of a Na-selective microelectrode. In pure NaCl solutions with concentrations ranging from 100 to 5 mM, the slopes of 20 microelectrodes varied between 65.3 and 76.3 mV/decade (mean 69.5). k^{-1}_{NaK} measured by the SSM in 0.1 M salt solution[3] ranged from 18 to 32. Even with these moderate values of k^{-1}_{NaK}, the change in the electrode potential between the 10 and 5 mM NaCl solutions containing 127 mM KCl, is about 10–15 mV. This provides sufficient sensitivity to record changes in a^i_{Na}. The reason for this decrease in k^{-1}_{NaK} compared to previously reported values for these microelectrodes (O'Doherty et al., 1979; Steiner et al., 1979) is not completely clear, but changes in the method of preparing and silanizing the micropipettes can alter the resistance of the hydrated glass layer and produce changes in the overall microelectrode selectivity (Armstrong & Garcia-Diaz, 1980). In 8 microelectrodes k^{-1}_{NaTris} ranged from 40.6 to 75.2.

Measurement of Intracellular Ionic Activities

The use of the Nicolsky equation (Moody & Thomas, 1971) to determine the intracellular activity of an ion i in the presence of an interfering ion j requires a knowledge of k_{ij}. This is usually calculated by the SSM in 0.1 M salt solutions. However, it is often observed with liquid ion-exchanger microelectrodes (Edelman et al., 1978; Armstrong et al., 1979*a*; Armstrong & Garcia-Diaz, 1980) that the slope of the electrode potential *vs.* log a is larger for the primary than for interfering ions.[4] Consequently, the selectivity ratio, k^{-1}_{ij}, is concentration dependent. This introduces an uncertainty in the calculation of ionic activities. This uncertainty is particularly serious in the case of the Na-selective microelectrodes. These are affected not only by K, but also by extracellular Ca (Steiner et al., 1979). Therefore, in the present experiments intracellular ionic activities were calculated by reading directly from the calibration curve the value of the intracellular electrode potential after correction for the mean E_m obtained in the same tissue under the same conditions with a conventional open tip microelectrode. For K- and Cl-selective microelectrodes the readings were taken from the calibration curve in pure univalent salt solutions (KCl and NaCl, respectively). For the Na-selective microelectrodes, the calibration curve in solutions containing variable amounts of NaCl together with 127 mM KCl (Fig. 1*B*) was used for this purpose. This amount of KCl corresponds to a mean a_K of 98 mM. This is the mean value of a^i_K found in *Necturus* gallbladder epithelium when the external Na concentration was varied between 10

[3] k^{-1}_{NaK} values for these microelectrodes measured by the fixed interference method, FIM (method *IIa*, Moody & Thomas, 1971) in solutions containing different amounts of NaCl together with 127 mM KCl agree well with those determined by the SSM. k^{-1}_{NaK} values measured by the FIM ranged from 17.3 to 31.3.

[4] We found in these experiments that the slopes of the liquid ion-exchanger microelectrodes in pure solutions of the primary ion are larger for Na-selective than for K-selective microelectrodes, and these are larger than for Cl-selective microelectrodes. The reason for this is not completely clear at present. Since the same mucosal reference electrode and KCl-agar bridge was used for experimental measurements and for calibration, it may be due in part to the development of a junction potential at the tip of this bridge.

Table 1. Intracellular K activities, a_K^i, under variable external Na, $[Na]_o$

	E_m (mv)	a_K^i (mM)	n	P
Control	-51 ± 2	99 ± 3	3	–
$[Na]_o = 10$ mM	-51 ± 3	99 ± 2		
Control	-50 ± 1	95 ± 3	4	< 0.05
$[Na]_o = 0$	-50 ± 2	80 ± 3		

Control and test experiments were done with the same gallbladder. Values shown are mean ± SEM; n = number of gallbladders.

and 100 mM (Table 1). In every calibration, one test solution had the same composition as the external medium (Edelman et al., 1978).

Experimental Procedure

Microelectrodes were advanced perpendicularly to the tissue by means of a micromanipulator (MM-33, Narishige, Japan) until they were in the luminal solution and allowed to reach a steady potential before impaling the cells. Further advance was accomplished under microscopic observation (Stereomicroscope III, Zeiss, New York, N.Y.) by means of a hydraulic micromanipulator (MO-10, Narishige, Japan). After several impalements, the microelectrode was calibrated as already described. Impalements were continued and a second calibration was then performed. If the electrode potential differed by more than 2 mV between the luminal fluid and the test solution of the same composition, the measurements were discarded. If the discrepancy was less than 2 mV, it was taken into account in the calculation of intracellular activities.

After mounting the tissue in the chamber, at least 30 min were allowed for stabilization of the electrical parameters before beginning impalements. Experiments were usually begun with the normal Ringer's solution (containing 100 mM NaCl) bathing both sides of the tissue. After at least 10 acceptable impalements with an open-tip microelectrode and at least 5 with an ion-selective microelectrode were obtained, both the luminal and serosal media were changed to another with a lower Na concentration. Immediately after changing the solutions E_{Tr} became more negative, reaching a pseudo-stationary level in about 1 min and slowly decaying in an exponential fashion during 40–80 min to the initial value which was close to zero. The magnitude of the initial change in E_{Tr} was inversely related to the external Na concentration, $[Na]_o$. When a new steady-state was reached, impalements with microelectrodes were resumed. Student's t-test was used for statistical comparisons.

Results

Representative recordings of acceptable impalements with open-tip and ion-selective microelectrodes are shown in Figs. 2 and 3. The criteria used for accepting impalements were: (i) There was an initial sharp deflection on impalement (in a positive direction with K-selective microelectrodes and in a negative direction with the others). (ii) This was followed by the establishment of a steady potential. (iii) The steady-state potential remained constant within 0.5 mV until withdrawal of the microelectrode; and (iv) following withdrawal, the electrode potential returned rapidly to within 2 mV of its initial value in the luminal solution.[5] In a few cases (Fig. 3) the initial sharp deflection was followed by a relatively slow depolarization and subsequent repolarization, until a steady value, usually higher than the initial peak, was reached. This behavior has been observed previously with open-tip microelectrodes (White & Armstrong, 1971) and has been ascribed to local membrane damage and subsequent spontaneous repair (or sealing of the microelectrode to the membrane) at the site of impalement. In other instances, following the initial deflection,

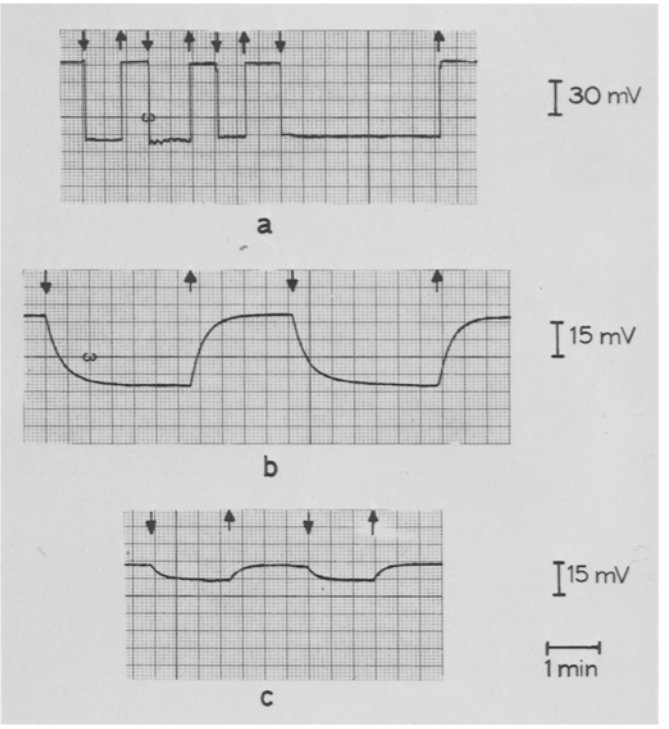

Fig. 2. Some acceptable impalements with conventional (*a*) and Cl-selective microelectrodes (*b* and *c*) in the same gallbladder. ↓ and ↑ indicate time of impalement and withdrawal of the microelectrode. Note stability and reproducibility of the impalements. The second impalement in *a* shows small oscillations occasionally observed in *Necturus* gallbladder. This experiment was started in Na-free Ringer (*c*) and later the solutions were changed to normal Ringer (*b*). In these earlier experiments the microelectrode was connected to the preamplifier by a coaxial cable with a grounded shield, and the time response of the ion-selective microelectrodes was in excess of 1 min (*see b*).

[5] In these experiments no attempt was made to record the fractional resistance of the luminal and basolateral membranes. However, the use of this parameter as an index of acceptable impalements is very limited since it depends on the presence of other stability criteria, such as those described above (*see* Appendix in Graf & Giebisch, 1979).

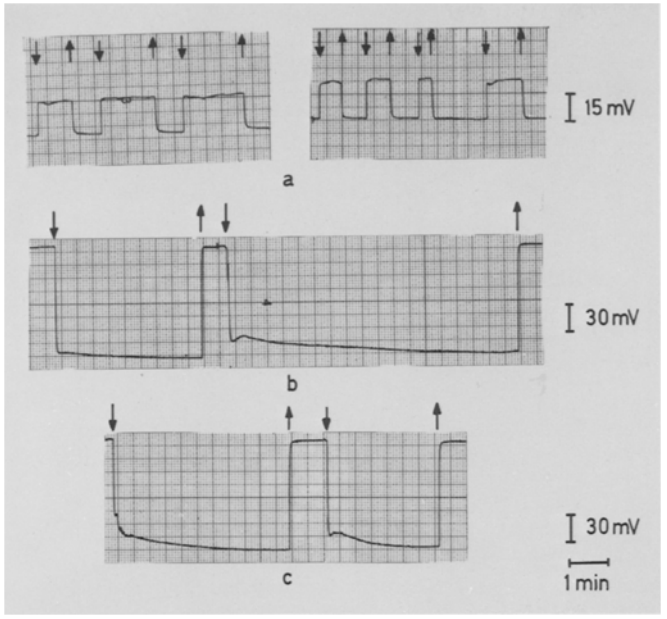

Fig. 3. Recording of some acceptable impalements with K-selective (*a*) and Na-selective microelectrodes (*b* and *c*) in different gallbladders. Arrows are as in Fig. 2. In these experiments the input of the preamplifier was guarded, and the time response of the ion-selective microelectrodes was much shorter than in the experiments illustrated in Fig. 2.

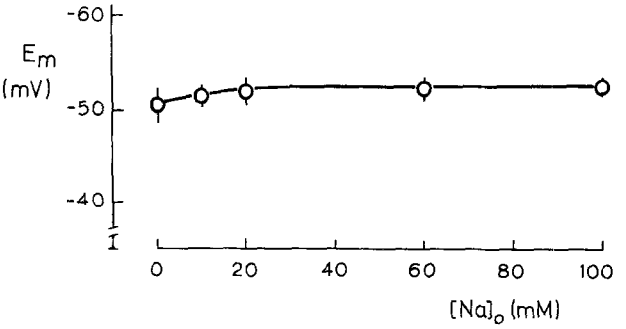

Fig. 4. Membrane potential, E_m, as a function of the external Na concentration, $[Na]_o$. Values shown are mean ± SEM. Number of gallbladders: 26 (100 mM), 7 (20 mM), 11 (10 mM), and 8 (0 mM).

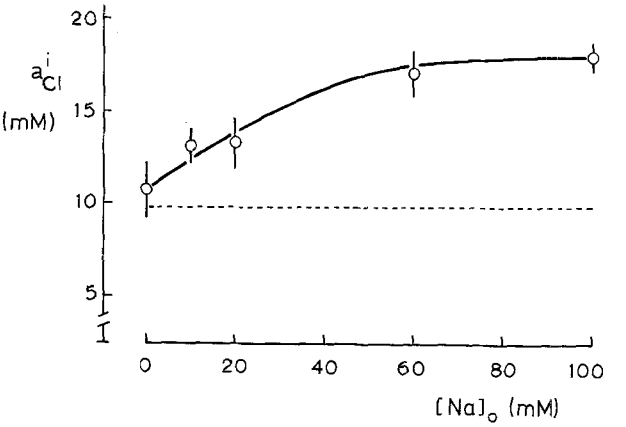

Fig. 5. Intracellular Cl activity, a^i_{Cl}, as a function of the external Na concentration, $[Na]_o$. Values shown are mean ± SEM. Number of gallbladders: 9 (100 mM), 5 (60 mM), 3 (20 mM), 4 (10 mM), and 4 (0 mM). The dotted line represents the electrochemical equilibrium value of a^i_{Cl} for the mean E_m of −52 mV.

the electrode potential depolarized continuously without reaching a stable value. This kind of impalement probably reflects permanent damage of the membrane and associated leakage across it. Such results were discarded.

In normal Ringer's solution E_m was -52.6 ± 0.6 mV ($n=26$). Since E_{Tr} was not significantly different from zero in these experiments (-0.3 ± 0.06 mV, $n=42$, $p>0.4$), virtually the same potential difference exists across the basolateral membrane. When the external K concentration of 5.4 mM used in the present experiments and the high P_K of the *Necturus* gallbladder epithelial cells (*see* Fig. 3 of Reuss & Finn, 1975*a*) are taken into account, this value of E_m agrees well with those previously reported by others (Frömter & Diamond, 1972; Reuss & Finn, 1975*a*; Van Os & Slegers, 1975). As observed by others (Reuss & Weinman, 1979), much of the scatter in the intracellular potentials observed in these experiments resulted from animal-to-animal variation. Individual impalements in the same epithelium, either with open-tip or ion-selective microelectrodes, did not differ by more than 5 mV (Figs. 2 and 3).

Figure 4 shows that, when Na was replaced by Tris, there was no significant difference in the steady-state E_m at different $[Na]_o$ levels. A similar result was obtained by Reuss and Grady (1979) when N-methyl-D-glucamine was used to replace Na. When Na was replaced by choline, these authors observed a depolarization of the membrane. A depolarization by choline has also been observed in rabbit gallbladder (Duffey et al., 1978), flounder intestine (Duffey et al., 1979), and in tubular kidney cells (Kikuta & Hoshi, 1979). Therefore, the use of Tris as a substitute for Na in the present experiments permitted the transmembrane chemical potential difference for Na to be changed without altering the electrical component of the Na electrochemical gradient. A further advantage of using Tris was that, as reported by Reuss and Grady (1979), K-sensitive electrodes were found to be about 200 times more selective to choline than to K. On the other hand, the selectivity of K and Na electrodes for Tris was sufficiently low to permit any effect of intracellular Tris on the electrode response to be disregarded (Materials and Methods and Fig. 1*B*).

Intracellular Cl activities, a^i_{Cl}, at different $[Na]_o$ values are shown in Fig. 5. These values are not corrected for the "apparent" a^i_{Cl} of 1 mM found when

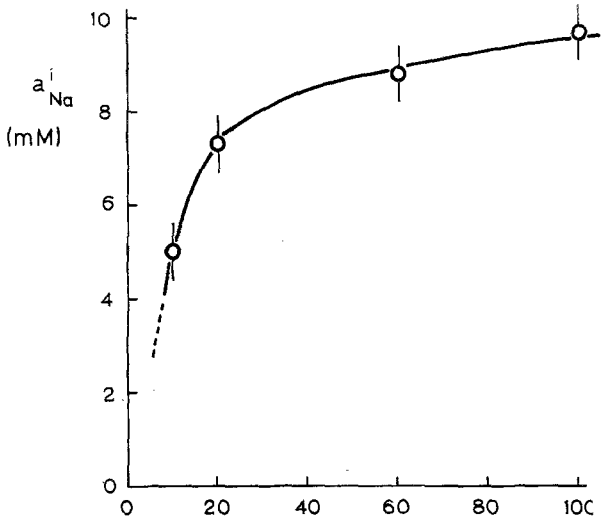

Fig. 6. Intracellular Na activity, a^i_{Na}, as a function of the external Na concentration, $[Na]_o$. Values shown are mean ± SEM. Number of gallbladders: 10 (100 mM), 3 (60 mM), 4 (20 mM), and 4 (10 mM).

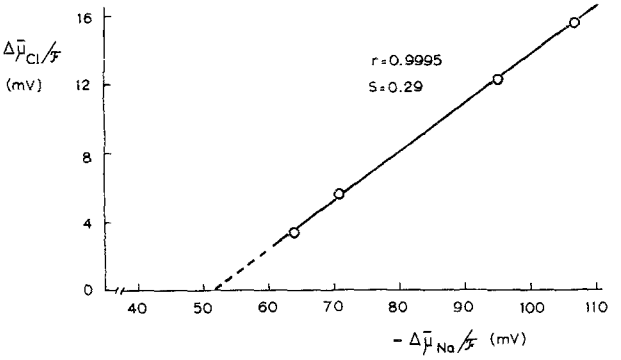

Fig. 7. Relationship between Cl and Na electrochemical potential differences across the cell membrane. Values calculated from the mean values in Figs. 4, 5 and 6 at four different $[Na]_o$ levels (10, 20, 60 and 100 mM). The correlation coefficient, r, was highly significant ($P < 0.001$).

all the external Cl was substituted by gluconate (*see* Materials and Methods). As reported for this and other leaky epithelia (Duffey et al., 1978; Frizzell et al., 1979; Armstrong et al., 1979*a*; Reuss & Grady, 1979), a^i_{Cl} was above the electrochemical equilibrium value under control conditions and decreased to the equilibrium level when all the external Na was removed. Under control conditions the "corrected" a^i_{Cl} was 16.8 ± 0.8 mM ($n=9$), whereas in a Na-free medium it was 9.8 ± 1.5 mM ($n=4$). Some of these experiments were begun in Na-free solutions, and an increase of a^i_{Cl} was observed following a change to a higher $[Na]_o$ (Fig. 2). This indicates the reversibility of the effect of $[Na]_o$ on a^i_{Cl}. The behavior of a^i_{Cl} over a range of $[Na]_o$ has not previously been reported. Figure 5 shows that a^i_{Cl} increases in nonlinear fashion with $[Na]_o$, reaching saturation at a $[Na]_o$ of approximately 60 mM. Although the scatter of the data due to animal-to-animal variation prevents the accurate calculation of a K_t for the effect of $[Na]_o$ on a^i_{Cl}, this parameter appears to lie between 10 and 20 mM.

In order to determine a^i_{Na} accurately, it is necessary, as already explained, to have a previous estimate of a^i_K. Table 1 shows that, in these experiments, a^i_K did not change when the external Na was decreased from 100 to 10 mM. A mean value of 98 mM for a^i_K was, therefore, used in calibrating Na-selective microelectrodes. When the epithelium was bathed in a Na-free medium a^i_K decreased to 80 mM (Table 1). Intracellular Na activity, a^i_{Na}, was measured at $[Na]_o$ levels of 100, 60, 20 and 10 mM. In 10 gallbladders under control conditions, a^i_{Na} was 9.7 ± 0.6 mM. As shown in Fig. 6, it increased in a saturable fashion with $[Na]_o$.

The transmembrane electrochemical potential difference, $\Delta\bar{\mu}_j$, for Na and Cl can be calculated from the experimental data reported herein by the equation:

$$\frac{\Delta\bar{\mu}_j}{F} = \frac{RT}{F} \ln \frac{a^i_j}{a^o_j} + z_j E_m \qquad (1)$$

where a^i_j and a^o_j are the activities of the ion j in the cell and the bathing medium, respectively, E_m is the membrane potential and z_j, R, T and F have their usual meaning. When $\Delta\bar{\mu}_{Na}$ and $\Delta\bar{\mu}_{Cl}$ were calculated from the mean experimental data obtained at four different $[Na]_o$ levels it was found that these parameters were linearly related ($P < 0.001$) as shown in Fig. 7. The value of $\Delta\bar{\mu}_{Na}/F$ for which $\Delta\bar{\mu}_{Cl}$ is zero (Fig. 7), -51 ± 0.8 mV (SD), did not differ significantly from the mean value of E_m (-52 mV) found in the present experiments.

Discussion

Electrical Potentials

The zero value of E_{Tr} found in these experiments under control conditions makes this preparation symmetrical with respect to its electrical potential profile. E_{Tr} in the gallbladder of *Necturus* (Frömter, 1972; Reuss, 1979; Reuss & Grady, 1979; Reuss & Weinman, 1979) and other species (Diamond, 1968; Frizzell et al., 1979) is usually small. This has been attributed to the existence of an electroneutral NaCl transport

mechanism in the gallbladder (Wheeler, 1963; Dietschy, 1964; Diamond, 1968) and to a very low electrical resistance of the paracellular shunt pathway relative to that of the transcellular pathway (Frömter, 1972; Frömter & Diamond, 1972; Van Os & Slegers, 1975; Reuss & Finn, 1975*a, b*). In our experiments, E_{Tr} underwent a transient hyperpolarization to more negative values when both bathing solutions were changed to one with a lower $[Na]_o$. Since the subserosal tissue retards equilibration between the fluid in the intercellular space and the serosal bathing solution, diffusion of Na and Tris takes place across the tight junction after a change in solution. The negative transient hyperpolarization of E_{Tr} indicates that $P_{Na} > P_{Tris}$ across the junctions. Similar results were obtained by Van Os and Slegers (1975) with choline and by Reuss and Finn (1975*a*) when N-methyl-D-glucamine or tetraethylammonium was substituted for luminal Na.

The values of E_m measured in our experiments agree reasonably well with previous experiments in *Necturus* gallbladder (Frömter, 1972; Van Os & Slegers, 1975; Reuss & Finn, 1975*a*; Graf & Giebisch, 1979). The constancy of E_m at different $[Na]_o$ levels deserves some comment. First, when Na is removed from the medium, the small decrease in a^i_K (Table 1) will depolarize E_m by no more than 3 mV. On the other hand, because the small P^m_{Na} of the luminal membrane (Reuss & Finn, 1975*b*), Na removal will lead to a small hyperpolarization of E_m, opposing the first effect. Under these circumstances it is difficult to recognize possible changes in E_m due to a rheogenic basolateral Na pump that responds to changes in a^i_{Na}, unless the basolateral pump is strongly rheogenic.

Intracellular Ionic Activities

Chloride. As mentioned before, a^i_{Cl} exceeds the equilibrium value corresponding to E_m when *Necturus* gallbladder is incubated in normal Ringer's solution. After Na removal a^i_{Cl} decreases to its equilibrium value. Under control conditions, the ratio of a^i_{Cl} to its equilibrium value, a^i_{Cl}/a^{eq}_{Cl}, was 1.8. Reuss and associates (Reuss & Weinman, 1979; Reuss & Grady, 1979) found a ratio of 4.3 in *Necturus* gallbladder. The principal difference between their experiments and those reported herein is that, in the present experiments, a HCO_3-free medium was used. Interference by HCO_3 ions with the response of the Cl-selective microelectrode in the experiments of Reuss and his associates is not sufficient to explain this difference in a^i_{Cl}/a^{eq}_{Cl}, since in their experiments $[HCO_3]_o$ was only 2.4 mM and $k^{-1}_{ClHCO_3}$ was about 20. It is possible that HCO_3 plays a stimulatory role in NaCl coupled entry in gallbladder, as it does in salt and water movement in this tissue (Diamond, 1964) and in Cl transport by several leaky epithelia (Schultz, Frizzell & Nellans, 1974; Field et al., 1978; Youmans, 1979). The exact nature of the mechanism by which HCO_3 enhances Cl accumulation in the gallbladder is not known. One possibility is that there is a Cl-HCO_3 exchange mechanism in parallel to the NaCl coupled entry and independent of it. Another is that the coupled NaCl entry mechanism is composed of two ion-exchange processes (Na-H and Cl-HCO_3) as proposed by Turnberg (1978) for the ileum. In either of these events, the low P_{CO_2} under the present experimental conditions could be a limiting factor for the formation of intracellular HCO_3 to exchange with external Cl. However, the small amount of HCO_3 employed by Reuss and co-workers and the fact that they used air to oxygenate their solutions might provide enough P_{CO_2} in the cell to enhance HCO_3 formation (mediated by carbonic anhydrase) and sustain a substantial level of Cl-HCO_3 exchange. In the trout urinary bladder, an epithelium with coupled NaCl entry, Fossat and Lahlou (1979) have rejected the possibility of two exchange processes on the basis of the insensitivity of NaCl cotransport to the external pH. Further studies are necessary to elucidate the exact mechanism of the stimulation by HCO_3 of epithelial Cl transport.

The saturable behavior shown by a^i_{Cl} in response to $(Na)_o$ (Fig. 5) could reflect Na dependent carrier mediated Cl entry at the apical side, but any interpretation of this entry mechanism based on the observed behavior of a^i_{Cl} should be regarded with caution since the exact nature of Cl exit from the cell is not known (Cremaschi & Henin, 1975; Reuss & Grady, 1979). If, however, the apical fluxes are the limiting factor for ion transfer (Fossat & Lahlou, 1979; Graf & Giebisch, 1979) the behavior of a^i_{Cl} (and by analogy, that of a^i_{Na}) will reflect the kinetic mechanism of the apical entry. In this context it is interesting that the K_t obtained for salt and water reabsorption in fish gallbladder was about 20 mM (Dietschy, 1964). For the apical influxes and net transepithelial transport of Na and Cl in the trout bladder, a K_t of 8 mM has been found (Fossat & Lahlou, 1979).

Potassium. The mean value of a^i_K found in these experiments under control conditions (96 ± 2 mM, $n=7$) agrees reasonably well with that reported for *Necturus* small intestine (108 ± 4 mM, $n=6$; Garcia-Diaz, O'Doherty & Armstrong, 1978) under the same conditions. Reuss and Weinman (1979) found a value of 87 ± 3 mM, $n=17$, in *Necturus gallbladder*, but they used a lower $[K]_o$ and consequently E_m was higher in their experiments. A more meaningful basis of comparison is the ratio of a^i_K to its equilibrium value,

a^i_K/a^{eq}_K. This ratio was 3.0 in the present experiments and 3.5 in those of Reuss and Weinman. This small discrepancy could be due to the experimental error associated with the low sensitivity of the K-selective microelectrode at high values of a_K (*see* below) and to the different methods used to calculate a^i_K from the electrode potential.

When $[Na]_o$ was decreased from 100 to 10mM, no change in a^i_K was observed (Table 1). However, a small decrease was found after complete removal of external Na. This decrease in a^i_K indicates an inhibition of the basolateral Na-K pump following the fall in a^i_{Na}. If, as shown in this (Graf & Giebisch, 1979) and other tissues (Thomas, 1969; Glitsch, 1979), the pump responds to a^i_{Na}, one would predict a reduction by nearly one half in the pump rate when $[Na]_o$ is reduced to 10 mM and a^i_{Na} falls to 5 mM. The observed constancy of a^i_K under these circumstances could have two explanations. First, the mechanism of K exit from the cell is not known. Even if most of the K efflux is diffusive, down its electrochemical potential gradient, part of the K exit could be associated with a neutral KCl efflux (Reuss, 1979). If that is true, the decrease in a^i_{Cl} after $[Na]_o$ reduction (Fig. 5) could lead to a decrease in KCl exit. This might offset the tendency of a^i_K to decrease because of a reduced rate of K pumping into the cell. Second, the sensitivity of the K-selective electrode to a_K at values around 100 mM is very low. For example, the change in electrode potential when a_K varies between 100 and 90 mM is not more than 2 mV. That lies within the range of experimental error for intracellular recording. It is, then, entirely possible that, under control conditions, changes as large as ± 10 mM in a^i_K might remain undetected with the experimental approach used in the present experiments.

Sodium. The mean a^i_{Na} found in these experiments under control conditions (9.7 ± 0.6 mM, $n=10$) is in agreement with previous estimates in *Necturus* small intestine using monensin-based Na microelectrodes (6 ± 1, $n=2$, Garcia-Diaz et al., 1978) and Na-selective microelectrodes similar to those employed in the present studies (9.1 ± 0.9, $n=6$, O'Doherty et al., 1979). It also agrees with the values obtained by Graf and Giebisch (1979) in *Necturus* gallbladder (9.2 ± 3.8, $n=11$) with highly Na-selective glass microelectrodes, if only the more stable values of E_m reported by these authors are chosen to calculate a^i_{Na}. Reuss and Weinman (1979) found an a^i_{Na} of 22 mM in *Necturus* gallbladder. However, as these authors recognized, the low k^{-1}_{NaK} of their Na-selective microelectrodes (about 2.6) and the method they employed for calculating a^i_{Na} from the Nicolsky equation, assuming a constant value of k_{NaK}, severely limits the accuracy of their estimate of a^i_{Na}. The same criticism can be applied to the data of Kimura and Spring (1979) who found a mean a^i_{Na} of 29.7 mM in *Necturus* proximal tubule.

As found with a^i_{Cl}, a^i_{Na} increased in a saturable fashion with $[Na]_o$ (Fig. 6). Again this behavior could reflect the kinetic mechanism of apical NaCl coupled entry if this entry is the rate-limiting step for transcellular Na transport. This seems to be the case since the evidence available at present (Graf & Giebisch, 1979; Glitsch, 1979) indicates that the basolateral Na pump is not saturated under normal physiological conditions. It is interesting in this context that in the sheep Purkinje fiber, where no coupled transport of Na and Cl has been reported, Ellis and Deitmer (1978) found a linear relation between a^i_{Na} and $[Na]_o$.

Relationship Between $\Delta\bar{\mu}_{Cl}$ and $\Delta\bar{\mu}_{Na}$

A corollary of the hypothesis that the uphill entry of Cl into the cells is energized by the simultaneous dissipation of $\Delta\bar{\mu}_{Na}$ across the mucosal membrane is that the level of intracellular Cl accumulation (as represented by its associated energy: $\Delta\bar{\mu}_{Cl}$) should depend on $\Delta\bar{\mu}_{Na}$. Therefore, if $\Delta\bar{\mu}_{Na}$ is varied over a certain range, $\Delta\bar{\mu}_{Cl}$ should change in a directly related way. That there is in fact such a direct relation is shown in Fig. 7. The results shown in this figure thus provide strong support for the view that the simultaneous Na entry into the cell is directly responsible for uphill Cl transport.

Several points about the linear relationship between $\Delta\bar{\mu}_{Cl}$ and $\Delta\bar{\mu}_{Na}$ shown in Fig. 7 deserve further comment. If, as indicated by earlier studies (Frizzell et al., 1979) the NaCl cotransport consists of an electroneutral 1:1 coupling between these ions, one can expect that Cl ions will be at equilibrium ($\Delta\bar{\mu}_{Cl}=0$) when $a^i_{Na}=a^o_{Na}$ ($\Delta\mu_{Na}=0$), and the membrane potential will not contribute to the accumulation of Cl ions. That this is the case is seen from the extrapolation of the line in Fig. 7, where the value of $\Delta\bar{\mu}_{Na}/F$ for which $\Delta\bar{\mu}_{Cl}$ is zero coincides with E_m, indicating that the electrical component of $\Delta\bar{\mu}_{Na}$ does not constitute a driving force for Cl accumulation. This result strongly supports the notion that the coupled mechanism is electroneutral. It should be emphasized that the linear relationship between $\Delta\bar{\mu}_{Cl}$ and $\Delta\bar{\mu}_{Na}$ shown in Fig. 7 was obtained because E_m remained virtually constant when $[Na]_o$ was altered between 10 and 100 mM. If E_m changed when $[Na]_o$ was altered (as occurs, e.g., if choline is substituted for Na) the relationship between $\Delta\bar{\mu}_{Cl}$ and $\Delta\bar{\mu}_{Na}$ would no longer be linear.

The slope of 0.29 indicates that, in order to increase $\Delta\bar{\mu}_{Cl}$ by a given amount, $\Delta\mu_{Na}$ must be in-

creased by almost 3.5 times this amount. A factor that could contribute to the low "apparent coupling efficiency" between Na and Cl transport is Cl exit from the cell. Any exit of Cl from the cell will decrease $\Delta\bar{\mu}_{Cl}$ below the maximum value attainable (Armstrong et al., 1979*b*). Some of this Cl efflux could be a diffusive movement across the basolateral membrane, but the exact nature of Cl exit across this membrane is not known (Reuss, 1979).

In conclusion, we have shown that the activities of both Cl and Na increase in a saturable fashion with $[Na]_o$, whereas E_m is independent of $[Na]_o$. The relationship between $\Delta\bar{\mu}_{Na}$ and $\Delta\bar{\mu}_{Cl}$ shown in Fig. 7 indicates that the transmembrane Na chemical potential difference $\Delta\mu_{Na}$ (and not the electrical component of $\Delta\bar{\mu}_{Na}$) provides the energy for intracellular Cl accumulation.

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