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Intracellular Chloride Activities in Rabbit Gallbladder: Direct Evidence for the Role of the Sodium-Gradient in Energizing "Uphill" Chloride Transport

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Summary. Intracellular chloride activities, $(Cl)_c$, in rabbit gallbladder were determined by using conventional (KCl-filled) microelectrodes and Cl-selective, liquid ion-exchanger, microelectrodes. The results indicate that in the presence of a normal Ringer's solution, $(Cl)_c$ averages 35 mM; this value is 2.3 times that predicted for an equilibrium distribution across the mucosal and baso-lateral membranes. On the other hand, when the tissue is bathed by Na-free solutions, $(Cl)_c$ declines to a value that does not differ significantly from that predicted for an equilibrium distribution.

These results, together with those of Frizzell *et al.* (*J. Gen. Physiol.* **65**:769, 1975) provide, for the first time, compelling evidence that (i) the movement of Cl from the mucosal solution into the cell is directed against an electrochemical potential difference (23 mV); and (ii) this movement is energized by coupling to the entry of Na down a steep electrochemical potential difference.

Finally, our data suggest that (i) Cl exit from the cell across the basolateral membrane may be coupled to the co-transport of a cation or the countertransport of an anion; and (ii) the mechanism responsible for active Na extrusion from the cell across the baso-lateral membrane is rheogenic (electrogenic), and is not the result of a neutral Na-K exchange.

In vitro preparations of fish and rabbit gallbladder, bathed on both surfaces with identical bicarbonate-Ringer's solutions, actively absorb Na and Cl at rapid rates; at the same time the transepithelial electrical potential difference (ψ_{ms}) is very small (less than 1 mV) and most often the mucosal solution is electrically positive with respect to the serosal solution. Further, active transepithelial transport of Na is abolished when Cl is replaced with an inert anion (e.g., SO_4 or isethionate), and active Cl absorption is abolished when Na is replaced with inert cations (e.g., choline, K) (Diamond, 1962; Wheeler, 1963; Diamond, 1964; Diets-

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chy, 1964; Frizzell, Dugas & Schultz, 1975). These findings suggested the presence of an obligatory, neutral transport mechanism which mediates the coupled movements of Na and Cl across one or both of the limiting membranes of the epithelial cell.

Frizzell *et al.* (1975) demonstrated that the unidirectional influxes of Na and Cl across the mucosal membrane of rabbit gallbladder are mediated by an obligatory, neutral, coupled mechanism similar to that described earlier by Nellans, Frizzell and Schultz (1973) for rabbit ileum. In addition, Frizzell *et al.* (1975) found that the intracellular concentration of exchangeable Cl is greater than that predicted by an equilibrium distribution across the mucosal membrane and that it declined toward the equilibrium value when Na in the bathing media was replaced with choline. These observations prompted the suggestion that the entry of Cl into the cell from the mucosal solution is directed against an electrochemical potential difference and that the energy required for this "uphill" movement is derived, at least in part, from coupling to the entry of Na down a steep electrochemical potential difference. If so, Cl exit across the baso-lateral membrane would be a passive process directed down an electrochemical potential difference, and the energy for active transcellular Cl transport would be derived, at least in part, indirectly from the Na pump mechanism at the baso-lateral membrane responsible for the maintenance of a low intracellular Na activity. The findings and conclusions of Frizzell *et al.* (1975) are in close agreement with those of Henin, Cremaschi and their collaborators (1974, 1975, 1976) and Rose and Nahrwold (1976).

However, the notion that the intracellular electrochemical potential of Cl is greater than that in the bathing media was based on measurements of the distribution of ^{36}Cl in the epithelium using the distribution of inulin to correct for the content of Cl in "extracellular spaces". This approach has obvious, and potentially serious, shortcomings. For example, if the distribution of inulin underestimates the "true" extracellular space, intracellular Cl concentration will be overestimated. Further, even if the extracellular space is estimated accurately, the concentration of exchangeable intracellular Cl may not be a valid measure of the thermodynamic activity of cytoplasmic Cl, $(Cl)_c$.

The purpose of the present investigation was to determine the thermodynamic activity of intracellular Cl in rabbit gallbladder directly by using conventional and Cl-sensitive microelectrodes and to evaluate rigorously the Na-gradient hypothesis for transcellular Cl transport by this tissue.

Materials and Methods

Tissue and Solutions

Gallbladders were obtained from white rabbits (2–3 kg) of either sex which were sacrificed with intravenous pentobarbital. The sac was opened along its hepatic border, yielding a flat sheet, and rinsed free of bile with a standard buffered electrolyte solution (composition given below). The muscle layer was stripped from the epithelium with glass microscope slides (Frizzell *et al.*, 1975). This "mucosal strip" preparation was mounted vertically between two halves of a plexiglass chamber, with an aperture of 0.6 cm^2 (Fig. 1). The serosal side of the mucosal strip preparation was supported by nylon mesh. Both chamber halves were filled with approximately 30 ml of a buffered electrolyte solution which was maintained at 37 °C by water jackets (Fig. 1) and gassed with a mixture of 95% O_2 and 5% CO_2. The volume of the solution bathing the luminal side of the epithelium was somewhat larger than that bathing the serosal side; this resulted in a hydrostatic pressure difference of 3–4 mm H_2O which gently pressed the tissue against the underlying nylon mesh and minimized floating movements.

No attempt was made to short circuit the tissue, because the spontaneous transmural potential difference, ψ_{ms}, was always less than 1.0 mV.

The composition of the standard electrolyte solution was (mM): 140 Na, 124 Cl, 21 HCO_3, 5.4 K, 2.4 HPO_4, 0.6 H_2PO_4, 1.2 Mg, 1.2 Ca, and 10 glucose; the pH was 7.4. Sodium-free solutions were prepared by isotonic replacement of Na with choline.

Microelectrode Fabrication

Conventional microelectrodes were drawn from thin-walled, fiber-filled, borosilicate capillary glass having a 0.86-mm inside diameter and a 1.5-mm outside diameter (WP Instruments). The glass was cleaned by immersion in nitric acid and repeated rinsing with distilled water, and the electrodes were pulled on a horizontal puller (Narishige Scientific, Ltd., Model PN-3). The resulting tip had a resistance of $7 \times 10^6\ \Omega$, when filled with 3 M

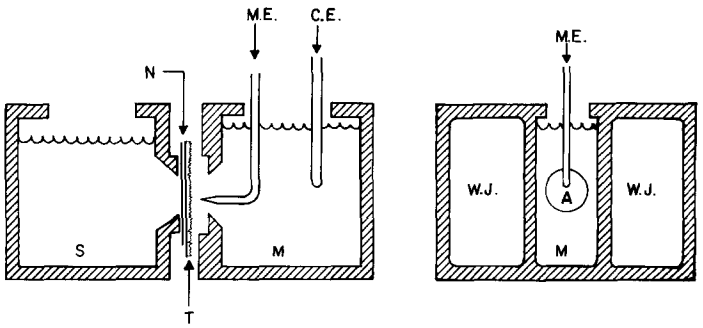

Fig. 1. Side view (left) and end-view (right) of chamber. *M* and *S* are the mucosal and serosal solutions: *T* is the tissue; and *N* is the nylon support. *M.E.* and *C.E.* are the microelectrode and calomel reference electrode, respectively. *W.J.* are the water-jackets and *A* is the aperture (0.6 cm^2). The tissue is clamped between the two half-chambers in a manner that minimizes edge damage

KCl.[1] The electrodes were heated approximately 15 mm from the tip and bent 90° (Fig. 1). The electrode tip was then filled by first immersing the untapered, open end in 3 M KCl and then submerging the entire electrode tip down. Electrodes were stored in 3 M KCl for no more than 5 hr before use.

Chloride-selective electrodes were pulled in the same manner. The tips were submerged for 15 sec in a filtered 1% solution of Q-8-5479 octadecyl functional silane (Dow Corning Corp) in *n*-chloronaphthaline, the electrode was dried for 1 hr at 200 °F, and a 90° bend was then made in the body. Either Corning 477315 (Dow Corning Corp) or Orion 92-17-02 (Orion Research, Inc.) chloride ion exchanger was drawn into the tip to a length of approximately 250 μm by applying a vacuum to the open end. The electrode body was then back-filled with 1 M KCl using a fine syringe needle. Air bubbles were removed from the salt solution by applying a vacuum to the untapered, open end while keeping the tip submerged in the ion exchanger. The resulting electrodes had a tip resistance of approximately $10^{10}\,\Omega$. Chloride electrodes were stored in 1 M KCl and were generally used within 5 hr.

Microelectrode Calibration

Chloride electrodes were calibrated before each experiment by using standard KCl solutions of 150, 60, 30 mM and the normal Ringer's solution. The circuit consisted of the microelectrode, an Ag/AgCl junction, a high impedance electrometer ($10^{15}\,\Omega$) with a driven probe (WP Instruments, Inc., Model F23-B), and a saturated KCl filled calomel reference electrode. The total potential (E_t) measured in this system can be represented by the expression (Walker, 1971):

$$E_t = E_0 + S \ln(a_i + \sum_j k_{ij} a_j^{z_i/z_j}) \tag{1}$$

where E_o is a constant which lumps miscellaneous circuit potentials (e.g., junction and tip potentials), a_i is the activity of the "primary" ion, a_j is the activity of interfering ion(s), k_{ij} is a selectivity constant of the primary ion (i) over an interfering ion (j), and z is the ionic valence. When no interfering ions are present, a plot of E_t *vs.* $\ln a_i$ yields a straight line with a slope S. This behavior was observed in every instance and the combined calibration data for 23 electrodes are given in Fig. 2. Ion activities were calculated from published activity coefficients (Robinson & Stokes, 1970). The mean value of $S \pm$ SEM was -24.6 ± 0.2 mV at 22 °C, which compares favorably with the ideal value $(RT/z\mathcal{F}) = -25.4$ mV; the correlation coefficient was 0.9997.

Intracellular inorganic anions that may interfere with the determination of intracellular Cl activities include sulfate, phosphate, and bicarbonate. With the exception of

1 This tip resistance is lower than that generally employed in electrophysiologic studies of epithelial tissues. The reason for routinely drawing microelectrodes with tip diameters that correspond to this resistance is that the preparation of Cl-sensitive microelectrodes with much smaller tip diameters proved to be difficult because the tips were frequently clogged during the silanizing process. We chose to employ microelectrodes having the same tip diameters for measurements of the electrical potential difference across the mucosal membrane (ψ_{mc}) and cell Cl activities (ΔE_t) for two reasons. First, as shown by Eq. (5), if ψ_{mc} is influenced by the tip diameter, the use of the same microelectrodes for measurement of both ψ_{mc} and ΔE_t will not affect the calculated value of $(Cl)_c$. Second, the values of ψ_{mc} obtained using 7 MΩ microelectrodes is in excellent agreement with those determined by us and others (*see* below) using microelectrodes with much higher tip resistances.

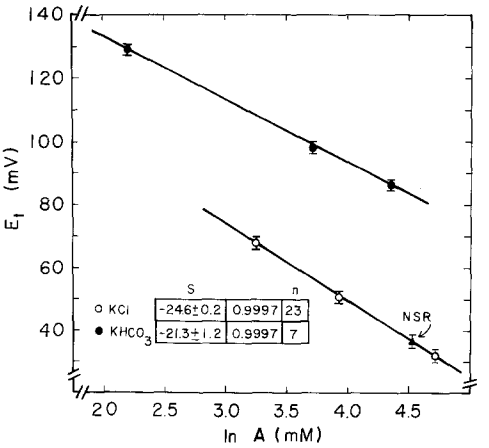

Fig. 2. Calibration data of the Cl-sensitive microelectrodes in solutions of KCl (○) and $KHCO_3$ (●); *NSR* (▲) designates the normal Ringer's solution

bicarbonate, the intracellular activities of these interfering ions are small, resulting in negligibly small $k_{ij}a_j$ terms (Saunders & Brown , 1977). Figure 2 also shows the responses of these microelectrodes to solutions containing 10, 50, and 100 mM $KHCO_3$. k_{ij} for these electrodes was calculated by comparing the electrode potential at equal Cl and HCO_3 activities. This method can be illustrated by writing Eq. (1) for the electrode response in Cl solutions

$$E_{t\mathrm{Cl}} = E_o + S\ln(a_{\mathrm{Cl}}) \tag{2}$$

and in HCO_3 solutions

$$E_{t\mathrm{HCO_3}} = E_o + S\ln[k_{\mathrm{Cl/HCO_3}}\, a_{\mathrm{HCO_3}}] \tag{3}$$

When $a_{\mathrm{Cl}} = a_{\mathrm{HCO_3}}$, Eqs. (3) and (4) yield

$$k_{\mathrm{Cl/HCO_3}} = \exp[(E_{t\mathrm{HCO_3}} - E_{t\mathrm{Cl}})/S]. \tag{4}$$

For $a_{\mathrm{Cl}} = a_{\mathrm{HCO_3}} = 30$ mM, the calculated $k_{\mathrm{Cl/HCO_3}} = 0.189$ corresponding to a Cl/HCO_3 selectivity of 5.3:1. This value is somewhat less than those reported by Fujimoto and Kubota (1976) and Saunders and Brown (1977) and probably represents a minimal estimate. In any event, since ΔE_t [*see* Eq. (5)] is the change in potential when the tip passes from Ringer's solution into the cell and since the intracellular bicarbonate activity is not likely to exceed that in the surrounding medium, it can be shown that the error in determination of intracellular Cl activity resulting from bicarbonate interference is not likely to exceed 10%. As shown in Fig. 2., the Cl activity of the normal Ringer's solution was 93.7 mM, which is in excellent agreement with the value predicted for a 124-mM solution of NaCl (95.2 mM).

The temperature sensitivity of the Orion and Corning Cl electrodes was determined by comparing calibrations of 5 electrodes at 22 and 37 °C. The results showed only a 3.7% change in slope and a 2.4% difference in absolute magnitude at an activity of 50 mM; the change in slope does not differ from that predicted for an ideal electrode where $S = (RT/\mathcal{F})$. Thus, for convenience calibrations were carried out at 22 °C while the tissues were impaled at 37 °C.

The response time of these electrodes was fast, with an average rise time (to 90% of final value) of 5 sec.

Intracellular Recordings

The chamber containing the tissue was enclosed in a well-shielded Faraday cage, and the microelectrodes were advanced into cells with a remote hydraulic micro-drive (Narashige, Ltd., Model MO-8) outside the cage.

The electrical potential difference across the mucosal membrane ψ_{mc}, with reference to a calomel cell in the mucosal solution (Fig. 1) was monitored by a high impedance ($10^{15}\,\Omega$) electrometer (WP Instruments, Inc., Model F23-B) and recorded on a Brush chart recorder (Model 2400). The criteria for a successful impalement were: (i) an abrupt negative deflection reaching a plateau value; (ii) maintenance of the plateau value with no more than a 10% variation for at least 30 sec; (iii) a return of the deflection to the original baseline when the tip is retracted or spontaneously dislodged.

Chloride selective electrodes were calibrated immediately before use. The microelectrode and the same calomel electrode used for calibration were immersed in the mucosal solution; electrodes were rejected if the value of E_t in the mucosal solution differed from the value obtained for Ringer's solution during calibration. The potential difference between the Cl selective microelectrode and the calomel electrode in the mucosal solution (E_t) was monitored by the same high impedance electrometer and recorded on the Brush recorder. Successful punctures had to conform to the same criteria outlined above; the return to the same value of E_t after retraction from the cell indicated no significant change in tip potential or Cl sensitivity.

The intracellular Cl activity, $(Cl)_c$, was calculated from the deflection in $E_t(\Delta E_t)$ during a successful impalement from the relation

$$\Delta E_t = \psi_{mc} + S \ln[(Cl)_c/(Cl)_m]. \tag{5}$$

Equation (5) assumes that (i) there is no significant interference from other intracellular anions and (ii) the difference between E_o [Eq. (1)] when the tip is in the mucosal solution and when the tip is in the cell interior is simply ψ_{mc}; the results described above and below appear to justify these assumptions.

All results are expressed as the mean $\pm$ the standard error of the mean (SEM) based on the number of tissues studied. A value of $P<0.05$, determined using a paired or unpaired t-test, was accepted as the criterion for a statistically significant difference between means.

Results

Intracellular Chloride Activity in the Presence of Sodium

Typical examples of successful impalements with both conventional and Cl-selective microelectrodes are shown in Fig. 3. As noted previously (Frizzell *et al.*, 1975), values of ψ_{mc} were remarkably consistent for a given tissue and did not change significantly over a period of 1–5 hr; values of ΔE_t also displayed relatively little scatter within a given tissue and were sustained for more than 4 hr. Histograms of the results of all successful impalements are shown in Fig. 4. Both ψ_{mc} and ΔE_t are distributed about a single maximum ($\psi_{mc} \cong -50\,\text{mV}$; $\Delta E_t \cong -20\,mV$) and there is no evidence for more than a single population of impaled cells.

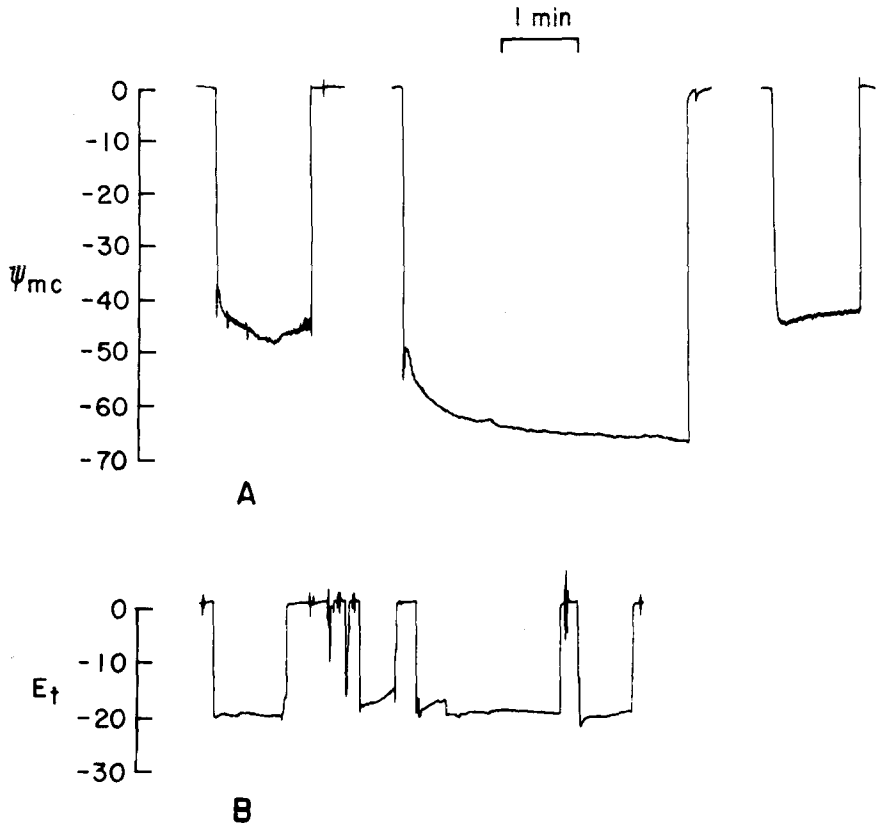

Fig. 3. Typical examples of acceptable impalements with conventional microelectrodes (*A*) and Cl-sensitive microelectrodes (*B*). The recordings of E_t represent four successive impalements on the same tissue with the position of the electrode changed slightly after each impalement and illustrates the uniformity of the response

The data obtained on 11 tissues are tabulated in Table 1. With one exception, the average ψ_{mc} for each tissue fell in the range -40 to -60 mV in agreement with earlier studies (Cremaschi, Henin & Ferroni, 1974; Frizzell *et al.*, 1975; van Os & Slegers, 1975; Henin *et al.*, 1976; Rose & Nahrwold, 1976; Henin *et al.*, 1977). The average value of ψ_{mc} for *all tissues* was -49 ± 2 mV; this value does not differ significantly from the mean of *all impalements* (-50 mV) indicating that it is not influenced by the fact that the number of successful impalements differed among tissues.

With two exceptions, the mean values of ΔE_t for each tissue fell in the range -20 to -30 mV and the overall mean for the 11 tissues was -23 ± 2 mV; this value also does not differ from the mean of all successful

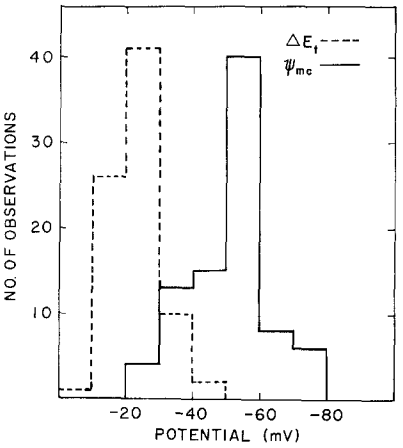

Fig. 4. Histograms of all successful impalements with conventional (ψ_{mc}) and Cl-sensitive (ΔE_t) electrodes

Table 1. Intracellular chloride activities in the presence of a normal Ringer's solution ([Na] = 140 mM)

Tissue	ψ_{mc} (mV)	ΔE_t (mV)	$(Cl)_c$ (mM)	$(Cl)_c^e$ (mM)	$[(Cl)_c/(Cl)_c^e]$
1	−54±4 (2)	−40±3 (3)	53	13	4.1
2	−43±3 (2)	−19±1 (4)	35	19	1.8
3	−50±0 (2)	−21±2 (7)	29	14	2.1
4	−44±4 (7)	−20±2 (10)	35	18	1.9
5	−59±4 (8)	−22±6 (3)	21	10	2.1
6	−40±1 (4)	−15±6 (2)	34	21	1.6
7	−55±3 (10)	−19±1 (9)	22	12	1.8
8	−54±2 (12)	−29±2 (10)	34	12	2.8
9	−56±3 (12)	−20±1 (22)	22	11	2.0
10	−58[a]	−28±1 (10)	28[a]	11[a]	2.6[a]
11	−34±2 (13)	−25±2 (7)	65	26	2.5
Avg.	−49±2 (10)	−23±2 (11)	35±4 (10)	16±1 (10)	2.3±0.2 (10)

Numbers in parentheses indicate the number of successful impalements

[a] None of the measurements of ψ_{mc} fulfilled our criteria for a successful impalement. The value of 58 mV was calculated from the value of ψ_{mc} determined in the absence of Na (47 mV) (Table 2) corrected for the average percent depolarization (23%) observed in tissues 4, 7, 8 and 9. These values were not included in calculating the means.

impalements (−23 mV). The electrochemical potential difference for Cl across the mucosal membrane

$$\Delta\tilde{\mu}_{mc}^{Cl} = \frac{RT}{\mathcal{F}} \ln\left(\frac{(Cl)_m}{(Cl)_c}\right) + \psi_{mc}$$

averaged -23 ± 3 mV indicating that intracellular Cl is at a greater electrochemical potential than Cl in the surrounding solutions. The intracellular Cl activity, $(Cl)_c$ calculated from Eq. (5), with $(Cl)_m = 93.7$ mM, averaged 35 ± 4 mM. The intracellular Cl activity predicted for an equilibrium distribution, $(Cl)_c^e$, averaged 16 ± 1 mM. Thus, the average $(Cl)_c$ is 2.3 times greater ($P < 0.001$) than that predicted for a passive distribution; a similar ratio was calculated by Armstrong, Wojtkowski and Bixenman (1977) for bullfrog small intestine.

Intracellular Chloride Activities in the Absence of Sodium

In 5 tissues, ψ_{mc} and ΔE_t were first determined in the presence of the normal Ringer's solution and then determined after the Na-Ringer's was replaced with a Na-free, choline-Ringer's. The data obtained after 30 min in the absence of Na are given in Table 2. Two points should be noted. First, in the absence of Na there is a significant ($P < 0.01$) depolarization of ψ_{mc}; ψ_{mc} in the absence of Na averaged -43 ± 1 mV compared to the average value of -53 ± 3 mV observed in the same tissues in the presence of Na. Second, in the absence of Na, ΔE_t averaged -7 ± 1 mV compared to the average value of -24 ± 2 mV observed in the same tissues in the presence of Na ($P < 0.001$). In each tissue the calculated value of $(Cl)_c$ slightly exceeded the calculated value of $(Cl)_c^e$, but the average values 21 ± 2 and 18 ± 1, respectively, do not differ significantly ($0.2 > P > 0.1$). The small difference between $(Cl)_c$ and $(Cl)_c^e$ could arise from a small contribution of intracellular HCO_3 (and perhaps other anions) to ΔE_t. In the absence of Na, $\Delta \tilde{\mu}_{mc}^{Cl}$ averaged only -3 mV.

Changes in these directions were observed within 5 min after replacing Na with choline, and the depolarization of ΔE_t had a halftime of approximately 10 min.

Table 2. Intracellular chloride activities in the absence of extracellular sodium

Tissue	ψ_{mc} (mV)	ΔE_t (mV)	$(Cl)_c$ (mM)	$(Cl)_c^e$ (mM)	$(Cl)_c/(Cl)_c^e$
4	-41 ± 8 (3)	-10 ± 1 (10)	26	20	1.3
7	-42 ± 2 (7)	-5 ± 0.3 (9)	21	19	1.1
8	-43 ± 5 (6)	-8 ± 0.7 (6)	22	18	1.2
9	-43 ± 3 (9)	-4 ± 0.3 (4)	19	18	1.1
10	-47 ± 3 (13)	-7 ± 0.5 (11)	18	16	1.1
Avg.	-43 ± 1 (5)	-7 ± 1 (5)	21 ± 1 (5)	18 ± 1 (5)	1.2 ± 0.0 (5)

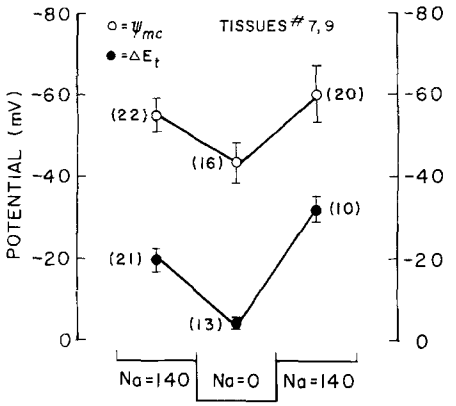

Fig. 5. Data obtained on two tissues (#7 and #9) 30 min before and 30 min after replacement of the normal Ringer's (Na = 140 mM) with Na-free, choline Ringer's. Numbers in parentheses are the number of successful impalements

Finally, in 2 experiments the Na-free, choline-Ringer's was replaced with the normal Ringer's and ψ_{mc} rose from an average value of -43 mV to one of -59 mV while ΔE_t rose from an average value of -4 mV to -32 mV. $(Cl)_c$ increased from 20 to 31 mM and $(Cl)_c/(Cl)_c^e$ increased from a value near unity to 2.7. These data are summarized in Fig. 5.

Discussion

Intracellular Electrical Potentials and Chloride Activities

At the outset it should be stressed that, ideally, double-barrel microelectrodes should be employed to determine intracellular ionic activities so that ψ_{mc} and ΔE_t can be obtained simultaneously on the same cell. This is particularly important and perhaps indispensable if (i) the epithelium is characterized by two or more distinct populations of cells and (ii) there is a wide variation in measurements made on a single tissue and among different tissues. Fortunately, rabbit gallbladder is comprised of a single layer of one cell type; histologically the only difference noted is that the columnar cells lining the crests of the mucosal folds are taller than those lining the valleys between folds (Kaye *et al.*, 1966; Tormey & Diamond, 1967; Blom & Helander, 1977). Further, we and other investigators have noted relatively little scatter in ψ_{mc} within a given tissue and among different tissues. Thus, one can place reasonable faith in a

comparison of separate determinations of ψ_{mc} and ΔE_t in rabbit gallbladder and need not resort to the use of double-barrel microelectrodes which can potentially introduce serious artifacts when used to impale small animal cells.

The results of these studies indicate conclusively that in the presence of 140 mM Na, the movement of Cl from the mucosal solution into the cell is directed against an adverse electrochemical potential difference and thus must be coupled to a nonconjugate source of energy. These findings confirm the earlier inference arrived at independently by Cremaschi *et al.* (1974) and Frizzell *et al.* (1975) based on measurements of ψ_{mc} and intracellular Cl concentrations. Intracellular Cl activities that exceed those predicted for an equilibrium distribution have also been reported for epithelial cells of bullfrog (Armstrong *et al.*, 1977) and *Amphiuma* small intestine (White, 1977); de Jesus, Ellory and Smith (1974) reported that $(Cl)_c$ sometimes exceeds $(Cl)_c^e$ in rabbit ileum, but their impalements were unstable and would not satisfy the criteria we have outlined above.

The value of 35 mM for $(Cl)_c$ contrasts with the value of 84 mM for the exchangeable intracellular Cl concentration determined by Frizzell *et al.* (1975) using ^{36}Cl and inulin for the extracellular space marker and with the value of 66 mM for the total cell [Cl] determined by Cremaschi *et al.* (1974) using sucrose for the extracellular space marker. Assuming that these values are not markedly distorted by errors in estimating the extracellular spaces, the apparent activity coefficient of intracellular Cl is only 0.42–0.53, a value much lower than the predicted value of 0.84 if all of the cell Cl were homogeneously distributed in free solution. Thus, if the measured cell Cl is truly intracellular, it follows that a substantial fraction is bound in the cytoplasm and/or sequestered within intracellular organelles.[2] White (1977) reported that the mean cytoplasmic $(Cl)_c$ in *Amphiuma* proximal small intestine (during the summer months) was 28 mM and that the intracellular Cl concentration determined using

2 If cell Cl were homogeneously distributed in free solution, the intracellular concentration should be approximately 41 mM (assuming an activity coefficient of approximately 0.85); this value is less than half the observed value (84 mM). From the data given by Frizzell *et al.* (1975) it can be shown that if this difference were due to the fact that inulin underestimates the extracellular space (and thus yields spuriously high values for cell Cl content and cell H_2O content), the "true" extracellular space would have to be almost *twice* the inulin space. Although this possibility cannot be excluded, it does not seem very likely and, at present, the conclusion that cell Cl is not homogeneously distributed in free solution seems to be the most reasonable explanation for the low apparent activity coefficient.

inulin as the extracellular space marker was 53 mM; thus the apparent activity coefficient of intracellular Cl in these studies was only 0.53.

Active Chloride Transport and the Sodium-Gradient Hypothesis

As shown in Table 2 and Fig. 5, replacement of Na in the bathing media with choline leads to a reversible decrease in $(Cl)_c$ and, after approximately 30 min, $(Cl)_c$ declines to the value consistent with an equilibrium (passive) distribution across the mucosal (and baso-lateral) membrane. *Thus, the accumulation of Cl by the cell against an electrochemical potential difference is dependent upon the presence of Na* as suggested by the data of Frizzell *et al.* (1975). This finding and the finding that the unidirectional influx of Cl is coupled to the influx of Na (Frizzell *et al.*, 1975; Cremaschi & Henin, 1975; Rose & Nahrwold, 1977) constitute compelling evidence that the Na-gradient across the mucosal membrane *contributes* energy for the uphill accumulation of Cl by the cell and for transepithelial Cl transport in the absence of a transepithelial electrochemical potential difference.

The only remaining question is: Is the electrochemical potential difference for Na across the mucosal membrane *sufficient* to energize the uphill movement of Cl into the cell? Since $(Na)_c$ is not known, $\Delta\tilde{\mu}_{mc}^{Na}$ cannot be calculated. Measured values of the intracellular Na concentration range between 66 mM (Frizzell *et al.*, 1975) and 88 mM (Cremaschi *et al.*, 1974) so that it is a virtual certainty that $(Na)_c$ is considerably lower than these estimates (Lev & Armstrong, 1975). However, *even* if the intracellular Na activity were equal to that in the mucosal solution, $\Delta\tilde{\mu}_{mc}^{Na}$ derived entirely from ψ_{mc} (49 mV) is far more than sufficient to energize the movement of Cl against an electrochemical potential difference of 23 mV.

We conclude that Cl absorption by rabbit gallbladder can be entirely energized by coupling to the electrochemical potential difference of Na across the mucosal membrane which, in turn, is established and maintained by a mechanism that is directly coupled to a source of metabolic energy and actively extrudes Na from the cell across the baso-lateral membranes; no direct coupling between Cl transport and metabolic energy need be invoked. A pictorial summary of this model is illustrated in Fig. 6.

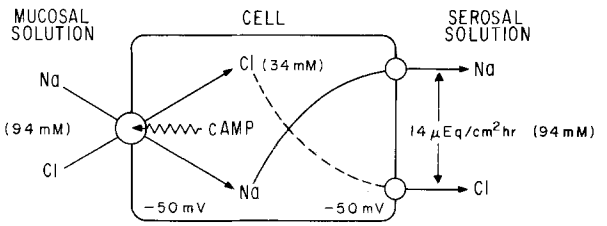

Fig. 6. Model of coupled NaCl transport by rabbit gallbladder, based on data from present study and those published previously (Frizzell *et al.*, 1975). Values in parentheses are thermodynamic activities

The Mechanism of Chloride Exit Across the Baso-lateral Membrane

According to the present data, if the movement of Cl out of the cell across the baso-lateral membrane is not coupled to an additional, nonconjugate source of energy, the driving force for this flow is 23 mV; this is true regardless of the mechanism of exit. The rate of transcellular Cl transport under these conditions (Fizzell *et al.*, 1975) is approximately 14 μEq/cm²hr or 374 μA/cm². Thus, the partial conductance of the exit process $G^s_{Cl} \cong 16$ mmhos/cm² (or the partial resistance of the exit process is $R^s_{Cl} \cong 60\,\Omega \cdot cm^2$). The electrical resistance of the baso-lateral membrane of rabbit gallbladder has not been determined directly; however, using an indirect approach Henin *et al.* (1977) have arrived at a value of 143 $\pm 24\,\Omega$ cm². If this value is correct, the *total conductance* of the baso-lateral membrane would be too low to permit the exit of Cl in a conductive form even if the *entire conductance* of this barrier is assigned to the movement of Cl. Further, Henin and Cremaschi (1975), Cremaschi and Henin (1975) and van Os and Slegers (1975) have presented evidence suggesting that the baso-lateral membrane is relatively impermeable to Cl. These observations await confirmation; however, if they are correct they suggest that Cl exit from the cell, albeit down an electrochemical potential gradient, cannot be due to simple diffusion or a carrier-mediated facilitated diffusion process that results in the transfer of charge.

Thus, the available data suggest that Cl exit from the cell across the basolateral membrane is coupled to the movement of a cation in the same direction and/or an anion in the opposite direction and does not contribute to the electrical conductance of the barrier. In this respect it is

of interest that NaCl transport by rabbit gallbladder is dependent on the presence of bicarbonate in the bathing media (Diamond, 1964; Martin, 1974; M.C. Dugas *et al.*, *unpublished observations*). Further, the stimulatory effect of HCO_3 is evident if the anion is present only in the serosal bathing solution (M.C. Dugas, R.A. Frizzell & S.G. Schultz, *unpublished observations*). Martin and Murphy (1974) have argued that the effect of HCO_3 is due to its role as a precursor of carbamyl phosphate which serves as a source of metabolic energy for the Na pump. A possibility raised by our observations is that Cl exit from the cell involves an obligatory, electrically neutral Cl-HCO_3 exchange. If this, admittedly speculative, notion is correct, the downhill movement of Cl out of the cell would provide the energy for the uphill movement of HCO_3 into the cell; the HCO_3 which enters the cell in exchange for Cl may then enter the metabolic cycle described by Martin and Murphy and/or recycle across the baso-lateral membrane.[3]

Clearly, additional studies are needed to determine conclusively whether or not the conductance of the baso-lateral membrane is sufficient to permit Cl exit down an electrochemical difference of 23 mV in an uncoupled (conductive) form; if not, some nonconductive process must be invoked. Further speculation on this point seems unwarranted until these data are obtained.

Evidence for a Rheogenic Sodium Pump at the Baso-lateral Membrane

As discussed above and illustrated in Fig. 5, replacement of Na with choline leads to a significant reversible depolarization of ψ_{mc} and the

3 Two points should be stressed. First, clearly, some fraction of the total Cl efflux could leave the cell by means of a conductive pathway so that the nonconductive process need not account for the entire outflow. Second, the major restriction on any nonconductive process is that charge balance is preserved. The simplest nonconductive exit process that would comply with this restriction is a neural NaCl transport mechanism; this possibility has been entertained by Henin *et al.* (1975, 1976) and van Os and Slegers (1975). However, this would require coupling to a source of metabolic energy and nothing would be gained by the coupled entry step energy-wise; thus, although such a process cannot be excluded at this time, it is an unattractive possibility. On the other hand, there are many examples of coupled Cl-HCO_3 exchange processes in epithelial and nonepithelial cells, and the notion that the downhill movement of Cl drives the uphill movement of HCO_3 into the cell where it can enter a metabolic reaction that yields a high energy substrate for the Na pump is an attractive hypothesis, but it raises a problem with respect to charge balance. A Cl-HCO_3 exchange with recycling of HCO_3 across the basolateral membrane raises no problem with respect to charge balance and could explain the requirement for HCO_3 in the serosal solution.

electrical potential difference across the baso-lateral membrane, ψ_{cs}. This cannot be due to the fall in cell Cl since, if a Cl-diffusion potential contributed to either ψ_{mc} or ψ_{cs} a decline in $(Cl)_c$ would lead to a hyperpolarization of the electrical potential differences across these barriers (i.e., the cell interior would become more negative). Similarly, if the loss of cell Na and Cl is accompanied by a decrease in cell volume leading to an increase in $(K)_c$ and, if K-diffusion potentials contribute to ψ_{mc} or ψ_{cs} (Cremaschi *et al.*, 1974; van Os & Slegers, 1975), this would also lead to a hyperpolarization of these barriers.

The simplest and most direct interpretation of this finding is that the mechanism responsible for the extrusion of Na from the cell against a steep electrochemical potential difference is rheogenic and not simply a neutral Na-K exchange. Such a process would contribute directly to ψ_{cs} and, because of the presence of paracellular shunts, indirectly to ψ_{ms} (Schultz, 1972). These findings provide direct evidence for the inference drawn by Rose and Nahrwold (1976, 1977) from less direct studies.

Finally, Henin *et al.* (1977) have reported that in rabbit gallbladder the resistance of the mucosal membrane is approximately equal to that of the basolateral membrane ($R^m/R^s = 1.09$). Since the resistance of the paracellular shunt pathway is much less than that of either limiting membrane (Frizzell *et al.*, 1975), the 10-mV depolarization of ψ_{mc} and ψ_{cs} following removal of Na corresponds to a decrease of 20 mV in the electromotive force across the basolateral membrane. If our reasoning is correct, this is a minimum estimate of the electromotive force of the rheogenic component of the Na extrusion process since, as discussed above, concomitant changes in Cl and/or K diffusion potentials would tend to offset the depolarization.

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Note Added in Press

Spring and Kimura (*J. Membrane Biol.* **38**:233, 1978) have recently reported that $(Cl)_c$ in proximal renal tubular cells of *Necturus* are 2–3 times higher than that predicted

for a passive distribution and that it declined toward the "equilibrium" value when Na in the perfusate was replaced with K, Li or tetraethylammonium. These findings are in excellent agreement with those reported in the present paper and strongly point to the presence of a neutral, Na-coupled Cl entry mechanism at the luminal membrane of *Necturus* proximal renal tubule.

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