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Intracellular Gradients of Ion Activities in the Epithelial Cells of the *Necturus* Gallbladder Recorded with Ion-Selective Microelectrodes

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Summary. In *Necturus* gallbladder epithelial cells the intracellular electrical potential, as recorded with microelectrodes, varied from −28 mV in the mucosal end to about −50 mV in the serosal end of the transporting cell. The Na^+ activity varied concurrently from about 39 mM to between 8 and 19 mM. Thus, within the cell both the recorded electrical and chemical gradients caused Na^+ to move towards the serosal end. Serosal addition of ouabain (5×10^{-4} M) caused the intracellular Na^+ activity to attain electrochemical equilibrium within 30 min. However, the intracellular electrical potential gradient was only slowly affected. In cells from animals stored at 5 °C, the Cl^- activity varied from about 55 mM in the mucosal end to 28 mM in the serosal end, and the K^+ activity from 50 mM to between 95 and 131 mM. Both ions were close to electrochemical equilibrium within the cytoplasm but were too concentrated to be in equilibrium with the mucosal solution. Bubbling CO_2 through the mucosal solution caused the intracellular gradients to vanish. When Na^+ in the bathing solutions was exchanged for K^+, the intracellular electrical potential became roughly constant at about −5 mV. The Cl^- activity became constant at 65 mM, and the K^+ activity became constant at 109 mM, both close to equilibrium with the mucosal solution. The Na^+ activity was reduced to about 1 mM. The ratio of the cytoplasmic resistivities between cells bathed in K^+-rich saline to cells bathed in Na^+-rich saline was measured by means of triple-barreled electrodes and compared to the same ratio as assessed from the activity measurements. The two values were equal only if one assumes the mobility of Na^+ inside the cell to be less than 1/10 of the mobility of K^+ or Cl^-. The same conclusion was reached by comparing the intracellular Na^+ flux calculated from the gradient of electrochemical potential to that flux assessed from the net solute absorption. Animals kept at 15 °C had lower intracellular Na^+ activities, higher Cl^- and K^+ activities, and higher rates of absorption than animals stored at 5 °C. Finally, the degree to which the intracellularly recorded electrical and chemical potentials could reflect an electrode artefact is discussed.

The technique of using double-barreled ion-selective microelectrodes is relatively new and has been used in only a few intracellular studies of absorbtive epithelia. Characteristic of these studies is the large variation not only in the values of the electrical potentials, but also in the values of the recorded activities.

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Table 1. Intracellular ion activities of epithelial cells, measured with ion-selective microelectrodes

Electrical potential (mV)	Na^+ (mM)	Cl^- (mM)	K^+ (mM)	Author(s), tissue
−45	14		85	Lee and Armstrong (1972) Bullfrog small intestine (vitro)
−5 to −30		30–80		Henriques de Jesus, Ellory and Smith (1975) Rabbit small intestine (vitro)
−5 to −36		8–60	30–120	Zeuthen and Monge (1975)[a] Rabbit small intestine (vivo)
−30 (−15 to −45)			40	White (1976) *Amphiuma* small intestine
−24		81		Armstrong, Wojtkowski and Bixenman (1977) Frog small intestine
−56			59	Khuri *et al.* (1972) Proximal renal tubule, *Necturus*
−68			54	Khuri (1976) Proximal renal tubule, Rat
−59		19		Khuri, Agulian, Bogharian and Aklanjian (1975) Proximal renal tubule, *Necturus*
−6 to −67	12	30	102	Palmer and Civan (1977)[a] Salivary gland, *Chironomus*
−28 to −56	19–39	28–58	49–131	This study Gallbladder, *Necturus* (Group *I*)

[a] These authors reported apparent concentration values. For the sake of comparison, I have multiplied these values with an activity coefficient of 0.76.

It was suggested earlier that these variations could arise because, by using microelectrodes, an intracellular gradient of electrical and chemical potential was found in the absorbing cells of the rabbit *ileum* (Zeuthen & Monge, 1975) and in the *Necturus* gallbladder (Zeuthen, 1976*a*, 1977*a*). It was argued that no obvious artefact could have caused this effect. It was the purpose of this study to examine the intracellular distributions of Na^+, and Cl^- and K^+ activities relative to the distribution of electrical potential in the *Necturus* gallbladder by means of double-barreled ion-selective microelectrodes and to see (i) how these distributions relate to the anatomical and functional asymmetry of the tissue, and (ii) to

reassess whether these distributions could arise from an electrode artefact. A preliminary account of this work has been presented (Zeuthen, 1976*b*, 1977*b*).

Materials and Methods

Tissue Preparation

The excised gallbladder from *Necturus maculosus* was mounted in a modified Ussing chamber and the electrode was advanced into the epithelial cells from the mucosal side, as described previously (Zeuthen, 1977*a*). The tissue was bathed with oxygenated Na^+-saline which contained (mM): 115.4 Na^+; 3.0 K^+, 2.7 Ca^{++}, 121.4 Cl^- and 2.4 HCO_3^-, or with K^+-saline which was similar, except that all Na^+ was replaced with K^+. pH was ~7.5.

Animals

Two batches of animals were used. 30 animals were obtained in the beginning of March, 1975, and another 30 in March, 1976. The first batch was kept at British room temperature (15 °C) and used within 8 weeks; these will be referred to as the animals from group *II*. The last batch were kept at 5 °C and used within 20 weeks; these will be called group *I*.

Ion-Selective Microelectrodes

Electrodes for simultaneous recording of electrical potential and Na^+ activity were constructed as described by Zeuthen (1975). Electrodes for recording electrical potential and K^+ or Cl^- activity were made as described by Zeuthen, Hiam and Silver (1974). Both types of electrodes had double-barrelled tips with a total tip diameter of about 0.5 μm and a taper of about 1:10. The impedance of each barrel was 3–70 MΩ when filled with and measured in 2M KCl. One barrel was used to record the electrical potential, and the other, fitted with the appropriate ion-selective membrane, to record the ion activity.

The ion-selective membrane in the Na^+ electrodes were made from glass (NaS_{11-18}, kindly supplied by N.C. Herbert) and was recessed 3–20 μm from the tip. This electrode, therefore, recorded the activity of those Na^+ ions which diffused into the deadspace formed between the Na^+ membrane and the tip. The average electrode had a deadspace of not more than 100 μm^3, sometimes as low as 20 μm^3 as judged from microscopic observation. Compared to this, the cell size of the *Necturus* gallbladder was about 16,000 μm^3 ($20 \times 20 \times 40$ μm). If all the cell volume was available to Na^+ and the electrodes were advanced from Na^+-saline into the cells, the Na^+ activity would increase at most 0.8 mM, if the cells were completely impermeable to Na^+. This would be undetectable. The electrodes had a response time of 30 sec to 3 min (typically 45 sec) where the response time was defined as the time needed for the electrode to record a new steady potential after an external change has been made[1]. There was a transient in the

1 The 1–5 sec response time mentioned by Thomas (1970) is the 50% response time. His tip diameters were about 1 μm, compared to the 0.5 μm used in this study. The intracellular response time of the electrode was similar to that recorded extracellularly. Therefore, there is no reason to assume that the tip of the electrode should be plugged during intracellular recording.

recording of the Na^+ activity due to the difference in the response time of the ion-selective barrel and the reference barrel. Thus, when there were simultaneous changes in the electrical and the electro-chemical potential at the tip, and when the two signals were subtracted in order to achieve the chemical potential and Na^+ activity, then there appeared a transient at the onset; the true Na^+ activity was obtained only after the Na^+-selective barrel has reached a steady state. The ion-selective membranes in the K^+ and Cl^- electrodes were made from liquid ion-exchangers (Corning 477317 and 477315, respectively). In these electrodes the sensitive interphase was at the very tip of the electrode. Thus, the ion activity was recorded at only the tip. The K^+ electrodes had response times of about 0.1 sec, the Cl^- electrodes of about 0.3 sec.

The reference barrel was filled with a concentrated salt solution in order to minimize changes in junction potentials when the electrode was advanced from the extracellular phase into the cell. The choice of salt solution presented a special problem, due to the close proximity of the ion-selective barrel to the reference barrel. If the salt solution contained the ion being measured the concentration in the media around the tip might rise, but if the salt solution was devoid of this ion the media might be depleted of the ion under study. The measurements of Cl^- and K^+ were therefore performed with either 2M KCl, 2M NH_4NO_3 or 1M Na_2SO_4 in the reference barrel and the results compared. K_{K,NH_4} and K_{Cl,NO_3} were high, about 0.3. Thus, if NH_4NO_3 or KCl accumulated in the vicinity of the tip it would be seen as an increase in K^+ and Cl^- activity, as compared to recordings performed with electrodes where the reference barrel was filled with Na_2SO_4. The reference barrel of the Na^+ electrode was always filled with 2M KCl.

The recording system was the same as that used by Zeuthen, Hiam and Silver (1974). The electrodes were connected by means of chlorided silver wires to electrometer inputs which had an input impedance of $10^{14}\,\Omega$. The preparation was earthed by means of a Ag/AgCl electrode and an agar bridge placed in the serosal solution.

Treatment of Data

The barrel that contained the ion-selective membrane recorded the sum of the electrical potential (ep) and a term cp_i^* related to the activity (a_i) of the ion

$$E_1 = ep + cp_i^*. \tag{1}$$

The other barrel (the reference barrel) was filled with a salt solution and recorded the electrical potential ep:

$$E_2 = ep. \tag{2}$$

By subtraction, cp_i^* was obtained. This relates to the activity of the ion (a_i) via an empirical expression (Walker, 1971)

$$E_1 - E_2 = cp_i^* = S_i \log(a_i + K_{ij} a_j) \tag{3}$$

where S_i is the sensitivity of the electrode expressed as mV, and K_{ij} is the selectivity to other ions present in activities a_j.

S_i and K_{ij} were obtained by calibration in standard solutions before and after experiments. The Na^+ electrodes were calibrated in solutions mixed from NaCl and KCl to an ionic strength of 150 or 200 mM; thus, only the influence of K^+ was considered. S_{Na} was an average 56 mV and $K_{Na,K}$ about 0.01. This means 100 mM K^+ looks to the electrode as 1 mM Na^+. The K^+ electrodes were tested in similar solutions. S_K was about 55 mV and $K_{K,Na}$ an average 0.02. The Cl^- electrodes were tested in solutions mixed from

NaCl and $NaHCO_3$ or Na_2SO_4 to an ionic strength of 150 mM. S_{Cl} was an average 52 mV and K_{Cl,HCO_3}, 0.03.

The chemical potential cp_i (mV)[2] can be obtained at 25 °C as

$$cp_i = 59.2\,\text{mV} \cdot \log_{10}(a_i). \tag{4}$$

All measurements were referred to the mucosal solution; thus, the electrical and chemical potential of any ion was zero there by definition. The translation of the activities into concentrations was done by using an activity coefficient of 0.76 (Robinson & Stokes, 1959). The effect of the presence of Ca^{++} on the activity coefficients of the measured ions was ignored. The chemical potentials (cp) were plotted as a function of the corresponding electrical potential (ep) (Figs. 2, 5, and 7). In the case of tissues bathed in Na^+ saline the linear regression line of cp *vs.* ep was calculated. The chemical potential ($\pm$SE) of the ions in the mucosal end of the cell was then obtained as the value of this regression line at the electrical potential recorded just inside the mucosal membrane (about -28 mV, Zeuthen, 1976*a* 1977*a*). The chemical potential in the serosal end was obtained in a like fashion, using the electrical potential recorded just inside the serosal membrane[3].

The condition for electrochemical equilibrium of an ion is the constancy of the electrochemical potential (ecp):

$$ecp = z \cdot ep + cp = \text{constant} \tag{5}$$

where z is the valency of the ion, ($+1$ for Na^+ and K^+, -1 for Cl^-). As the electrochemical potential was defined as zero in the mucosal solution, the condition for equilibrium between the cell interior and this solution was that the right hand side of Eq. (5) was zero inside the cell.

The condition for electrochemical equilibrium within the cytoplasm was that the electrochemical potential was constant, or

$$\frac{1}{z} \cdot \frac{\Delta cp}{\Delta ep} = -1. \tag{6}$$

Numbers are given with SE or SEM.

Mode of Advance

The electrodes were advanced by means of a hand-driven micromanipulator (Huxley, 1961). It was not possible to use the piezo-electrical micromanipulator described previously (Zeuthen, 1977*a*), as the nonsymmetrical distribution of weight of the ion-selective electrodes along the axes of movement caused a sideways as well as a forward movement of the tip when the piezo-electrical crystal was activated.

Net Transport Rate

The rate of solution transport away from mucosal solution was measured by means of a sac preparation by a gravimetric technique (Diamond 1962*a*).

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2 The chemical potential usually has the symbol μ, the units being cal mol^{-1}. However, in this paper the chemical potential is recorded as a voltage and it is, therefore, convenient to present the chemical potential by the symbol cp in mV. For conversion, $\mu = F \cdot cp$ where F is Faraday's constant.

3 It is incorrect to present data as activity $\pm$ SEM, according to Waddell and Bates (1969); the recorded parameter cp is, by and large, proportional to the chemical potential and only relates to the activity *via* a logarithmic transformation.

The Intracellular Resistivity

The cytoplasmic resistivity of cells bathed in K^+ saline ($\rho_{K^+ saline}$) and the resistivity of cells bathed in Na^+ saline were determined from the potentials evoked at the tip of a triple-barreled electrode (Zeuthen, 1977*a*) when the current was injected via one of the barrels and a large external electrode. The barrel used for current injection was filled with either 2M tri-K^+-citrate, 2M tri-Na^+-citrate or 2M NH_4NO_3. The barrels used for sensing the evoked potentials were filled with either 2M NH_4NO_3 or 2M KCl. Current pulses of $1\text{–}5 \cdot 10^{-9}$ A lasting 1 sec were applied every 10 sec in order to evoke the potentials[4].

The Voltage Divider Ratio

A current of $1\text{–}5 \cdot 10^{-5}$ A, lasting 1 sec, was passed across the tissue from the mucosal solution to the serosal solution. With an electrode placed intracellularly the induced changes in the potentials across the mucosal membrane ΔV_m and the serosal membrane ΔV_s could be assessed and their ratio $\Delta V_m/\Delta V_s$, the voltage divider ratio, calculated. The series resistance of the serosal and mucosal compartment plus the current passing electrode was assessed when the electrode was positioned just in front of or behind the cell-layer; it was less than 10% of the transepithelial resistance.

Results

The Intracellular Electrical Potential and the Positioning of the Electrode

Electrodes were advanced in steps of 1–10 μm through the mucosal solution until the tip just touched the mucosal membrane. When this happened the impedance of the reference barrel increased by about 10% and the recorded electrical potential changed about 1 mV (*see* Fig. 1*a*, 4 and 6). Additional stepwise advances of a total of 31 ± 4.7 μm (SE $n = 11$, 4 animals) were required before the electrode penetrated into the cell. A further advance of only 19 ± 1.9 μm ($n = 13$, 4 animals) caused the electrode to penetrate the serosal membrane. This compares well with the results obtained by means of a single-barreled electrode with impedances in the range 5–60 MΩ, (Zeuthen, 1977*a*); here the dimpling was 37 μm and the additional advance needed to penetrate the serosal membrane was 14 μm. Thus, the first measurement must usually have been recorded below the middle of the cell. This was also reflected in the fact that in about 65% of the penetrations, the initial electrical potential recorded was more negative than −40 mV, which was previously found to be the

4 The maximum current used, 5×10^{-9} A would case a maximum of 5.2×10^{-14} M of Na^+ K^+ or NH^+ to enter the cell during the 1-sec pulse. With a cell volume of 16,000 μm^3 ($20 \times 20 \times 40$ μm) this would mean an increase in concentration of about 3 mM. Most experiments were performed with current pulses of 2×10^{-9} A, although no difference in results was observed with increasing amplitudes.

potential in the middle of the cell (Zeuthen, 1977*a*). However, in the remaining 35% of the penetrations the initially recorded potential was less negative than −40 mV; in these cases the dimpling of the mucosal membrane prior to penetration was of the order of 10–20 μm. The electrodes recorded the largest intracellular potential of -49 ± 1.1 mV ($n = 45$, 10 animals) for the animals of group *I* and -56 ± 1.9 mV ($n = 44$, 9 animals) for the animals of group *II*, immediately before penetrating the serosal membrane. This was independent of the choice of the salt solution in the reference barrel. The potential in the mucosal end was taken as −28 mV (Zeuthen, 1977*a*).

Tissues Bathed in Na^+-Saline. Animals from Group I

A) The Na^+ activity. The Na^+ activity decreased with depth below the mucosal membrane. At each stepwise advance towards the serosal membrane a more negative electrical potential was recorded simultaneously with a more negative electrochemical potential of Na^+, and, therefore, the Na^+ activity decreased. An example of a recording is shown in Fig. 1*a*. As the decrease in chemical potential was proportional to the decrease in electrical potential and as the proportionality was constant throughout the cell, linear regression analysis of the data was considered appropriate.

For each decrease in intracellular electrical potential (ep) of 1 mV the chemical potential (cp) was recorded an average 0.9 mV lower. As this ratio was positive [*see* Eq. (6)], Na^+ was not distributed in equilibrium with the intracellular gradient of electrical potential. The chemical potentials in the mucosal end of the cell (cp_m) and in the serosal end (cp_s) were both negative; so were the electrical potentials. Thus, Na^+ was not

Table 2. The chemical potentials (cp) and activities (act) for Na^+ in the mucosal (m) and serosal (s) end of the cell in animals from group *I*[a] *(see text)*[b]

$\frac{1}{z}\frac{\Delta cp}{\Delta ep}$	cp_m (mV)	cp_s (mV)	act_m (mM)	act_s (mM)	Number of observations	Number of animals
0.9 ± 0.17	-21 ± 6.8	-40 ± 6.8	39	19	19	3

[a] ±SE.

[b] The term $\Delta cp/z \cdot \Delta ep$ is defined on p. 189.

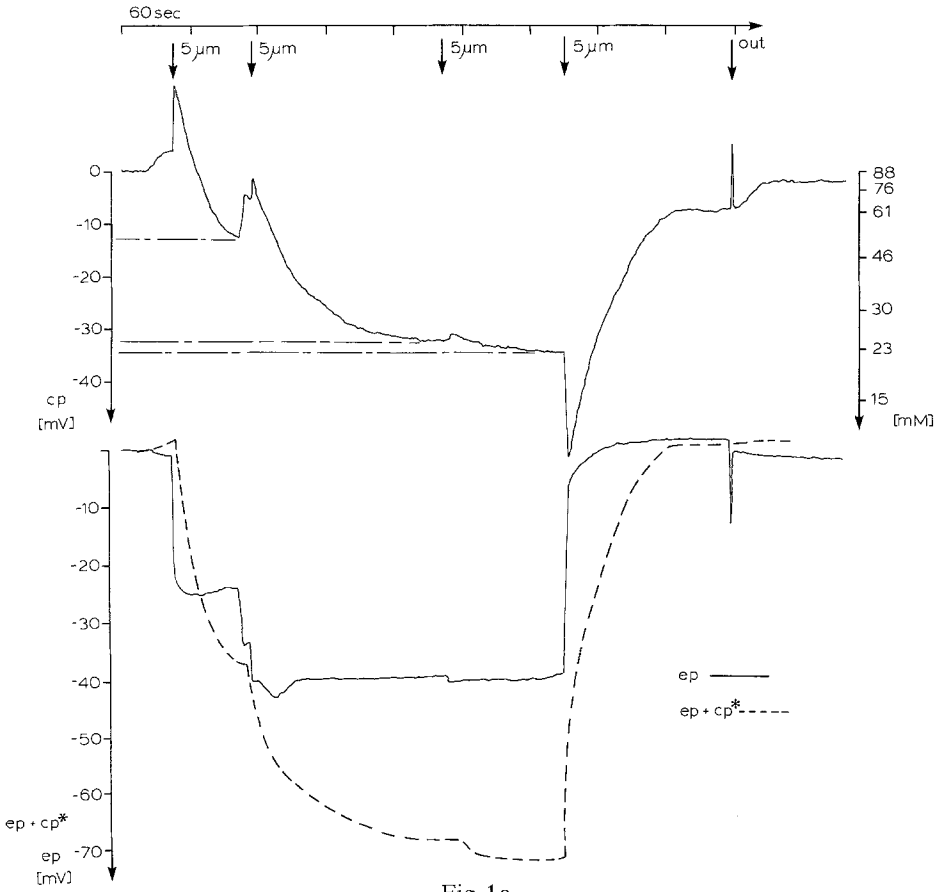

Fig. 1a

Fig. 1. (*a*): Simultaneously recorded intracellular profiles of electrical potential (*ep*), chemical potential (*cp*), and activity of Na^+ (mM) obtained when the tissue was bathed in Na^+-saline. The electrodes were advanced in steps of 5–10 µm (marked by arrows) from the mucosal solution through the cytoplasm towards the serosal side. At *out* the electrodes were retracted to the mucosal solution. (*a*): The Na^+-sensitive barrel of the double-barreled electrode recorded a potential ($ep+cp^*$, broken line, lower panel), which was closely related [Eq.(3)] to the electrochemical potential of the ion. The reference barrel recorded the electrical potential (*ep*, solid line, lower panel). By subtraction cp^* could be obtained, and by correcting for the slightly non-Nernstian behavior of the electrode the cp^* scale was transformed into a *cp* scale (*see* p. 189), upper panel. This could be translated into activities [Eq.(4)]. The transient in the recording of the Na^+ activity (*cp*) was due to the difference in the response time of the ion-selective barrel (about 45 sec in this case) and the reference barrel (<0.5 sec). Thus, the levels attained after 45 sec indicates the true Na^+ activity (stippled lines). The Na^+ activity was found to decrease towards the serosal side. (*b*): Obtained with ouabain added to the serosal solution for 15 min; the Na^+ activity was constant, but still too low to be in equilibrium with the outer solutions. (*c*): When ouabain had been present for more than 30 min (this penetration was performed after 65 min), the Na^+ activity was roughly in electrochemical equilibrium with the electrical potential gradient

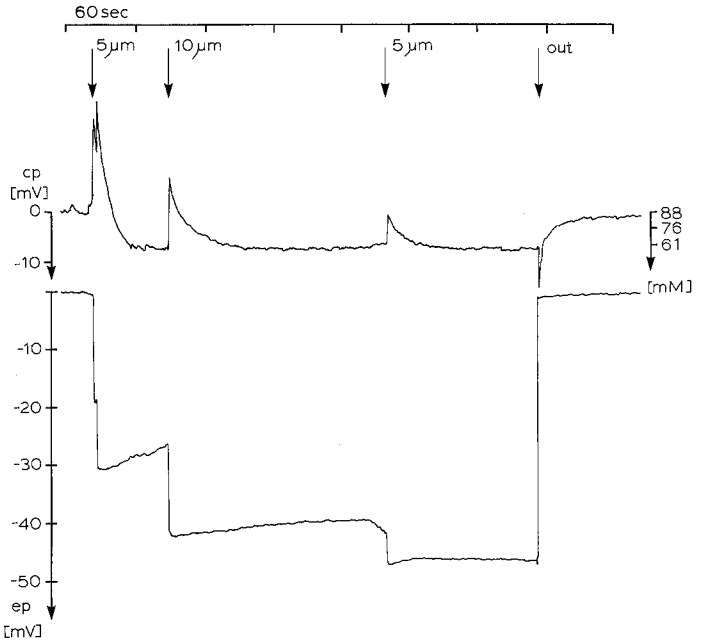
60 sec
5 µm
10 µm
5 µm
out
cp
[mV]
0
-10
88
76
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Fig. 1b

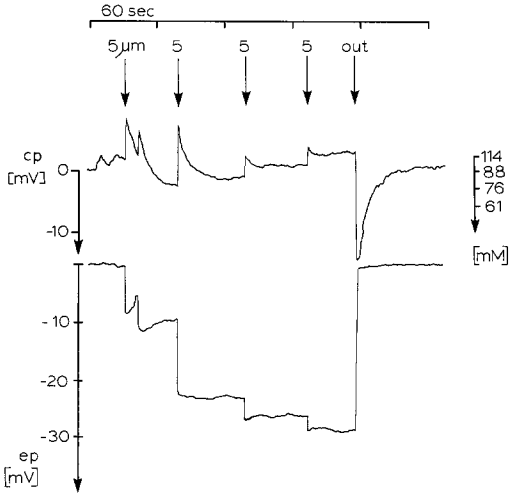
60 sec
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Fig. 1c

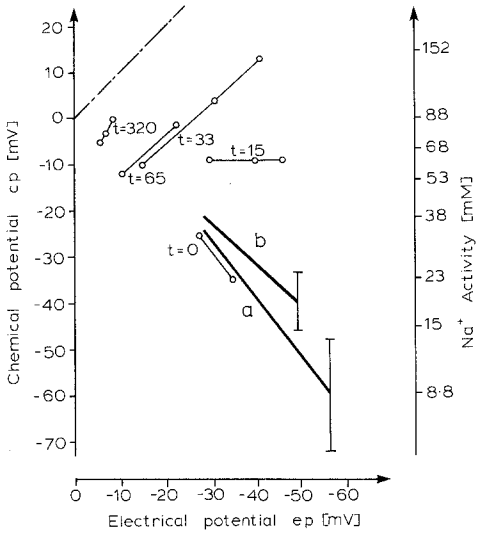

Fig. 2. The intracellular chemical potential (*cp*) and Na^+ activity as a function of the intracellular electrical potential (*ep*). The heavy line marked *a* collects the results found for animals in group *I*, and that marked *b* represents the values obtained for animals in group *II*. The tissues were bathed in Na^+-saline. The vertical bars represent the SE (*see* Tables 2 and 5 for statistics). The theoretical line (stippled) represents electrochemical equilibrium with the mucosal solution [*see* Eqs. (5) and (6)]. The results of individual penetrations, after addition of ouabain, are represented by open circles connected by thin lines, the values are shown for various times (*t*, in min) after the addition of ouabain to the serosal solution

in equilibrium between the cytoplasm and the mucosal solution [*see* Eq. (5)]. The activity in the serosal end (act_s) was about 20 mM lower than the activity in the mucosal end (act_m). A graphical presentation of the regression analysis is shown in Fig. 2.

B) The Cl^- activity. The Cl^- activity also decreased as a function of depth below the mucosal membrane. At each stepwise advance towards the serosal membrane a more negative electrical potential was recorded simultaneously with a decrease in the chemical potential and the Cl^- activity. An example of a recording is shown in Fig. 4, and the results of the linear regression analysis are presented numerically in Table 3 and graphically in Fig. 5.

The ratio of the increment in chemical potential to the simultaneous increment in electrical potential times the valency of Cl^- ($\Delta cp/z \cdot \Delta ep$) was close to -1 [*see* Eq. (6)]; thus, the intracellular Cl^- is distributed close to equilibrium within the intracellular gradient of electrical poten-

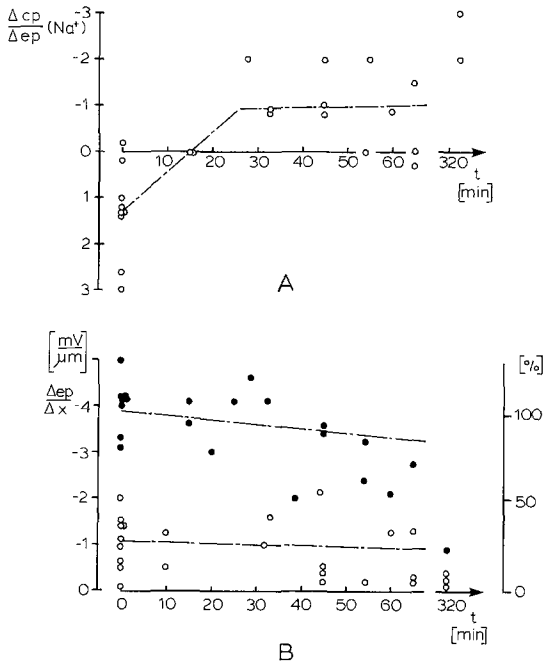

Fig. 3. (*A*): The ratio of increment in the chemical potential to increment in electrical potential ($\Delta cp/z \cdot \Delta ep$) as a function of time after the addition of ouabain to the serosal solution is shown. After approximately 30 min a value of about -1 was attained which shows that the Na^+ activity was now in electrochemical equilibrium with the intracellular gradient of electrical potential [*compare* Eq.(6)]. The stippled lines are the linear regression lines obtained by considering the values recorded before $t = 35$ min as one group, and values recorded after 25 min as another. (*B*): The decrease in the electrical potential in the serosal end of the cell (closed circles) as a percentage of the value measured before ouabain was added ($t = 0$). The open circles show the intracellular electrical gradient $\Delta ep/\Delta x$ (in mV/µm) as a function of time. This gradient was based upon the advance x of the electrode and was not corrected for effects of dimpling of the tissue. The stippled lines are linear regression lines

tial. There were no significant differences between the results obtained with various filling solutions. The chemical potentials in the mucosal end (cp_m) as well as the serosal end (cp_s) were negative; but with an electrical potential of -28 mV in the mucosal end and -49 mV in the serosal end the electrochemical potential [Eq. (5)] was positive and constant, about 16 mV throughout the cytoplasm. The intracellular Cl^- was therefore not in equilibrium with the Cl^- in the mucosal solution. The activity in the serosal end (act_s) was about 30 mM lower than the activity in the mucosal end (act_m).

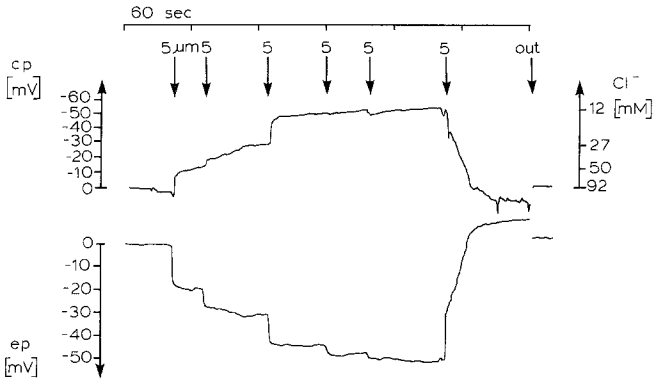

Fig. 4. Simultaneously recorded intracellular profiles of electrical potential (*ep*) and Cl^- activity. The electrode was advanced in steps of 5 µm (at arrows) from the mucosal end of the cell towards the serosal. At *out* the electrode was retracted to the mucosal solution. The cell was transversed after a total advance of 30 µm. The potentials in the subserosa were attained after an abrupt decline, followed by a slower one

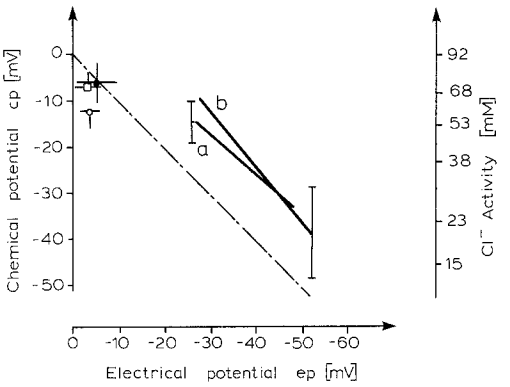

Fig. 5. The intracellular chemical potential (*cp*) and Cl^- activity as a function of the intracellular electrical potential (*ep*) when the tissue was bathed in Na^+-saline or K^+-saline. The heavy line marked *a* collects the results found for animals in group *I* when the tissues were bathed in Na^+-saline and when the reference barrel was filled with 2 M KCl; *b* represent the values obtained for animals in group *II*, in which case the electrodes were filled with 2 M NH_4NO_3. The vertical bars represent the SE (*see* Tables 3 and 5 for statistics). The value obtained with the Na_2SO_4-filled electrodes in tissues from group *I* bathed in K^+-saline is given by the open circle, with the KCl-filled electrodes by the square, and the NH_4NO_3-filled electrodes by the filled circle. These points are given with SE (*see* p. 202). The theoretical values representing electrochemical equilibrium with the mucosal solution are shown by the stippled line

Table 3. The chemical potentials (cp) and activities (act) for Cl^- in the mucosal (m) and serosal (s) ends of the cell in animals from group I [a] *(see text)* [b]

Filling solution	$\frac{1}{z}\frac{\Delta cp}{\Delta ep}$	cp_m (mV)	cp_s (mV)	act_m (mM)	act_s (mM)	Number of Observations	Number of Animals
Na_2SO_4	-0.72 ± 0.07	-16 ± 5.6	-31 ± 5.6	49	28	38	3
KCl	-0.85 ± 0.14	-15 ± 4.8	-33 ± 4.8	52	26	26	2
NH_4NO_3	-0.82 ± 0.18	-12 ± 2.5	-30 ± 2.5	58	29	14	3

[a] $\pm$SE.
[b] The term $\Delta cp/z\cdot\Delta ep$ is defined on p. 189.

When the electrode dimpled the membrane prior to penetration, the KCl and NH_4NO_3-filled electrodes recorded a larger Cl^- activity than that of the luminal solution (*see* Fig. 4), whereas the chemical potential recorded by the Na_2SO_4 filled electrodes was usually similar to that of the mucosal solution. A similar effect was sometimes seen prior to penetration of the serosal membrane: the NH_4NO_3 and KCl-filled electrodes would record a sudden increase in the chemical potential (the electrical potential was unchanged), whereas the chemical potential recorded by the Na_2SO_4 filled electrodes remained constant.

In about 5% of the penetrations the electrodes recorded on entry an electrical potential which was less negative than -25 mV. In these cases the recorded chemical potential was dependent on the choice of the filling solution and the potential was recorded as $+2.4\pm3.9$ mV ($n=8$, 3 animals, $\pm$SEM), which corresponds to an activity of 101 mM, when the reference barrel was filled with KCl or NH_4NO_3. When the electrodes were filled with Na_2SO_4, the chemical potential was recorded as -3.6 ± 1.6 mV ($n=9$, 3 animals) which corresponds to an activity of about 76 mM. The difference was significant ($P<0.01$, students t test).

C) The K^+ activity. The K^+ activity increased with depth beyond the mucosal membrane. At each stepwise advance towards the serosal membrane a more negative electrical potential was recorded simultaneously with an increase in the K^+ activity. An example of a recording is shown in Fig. 6. As the stepwise increase in chemical potential was proportional to the decrease in electrical potential and as the proportionality was constant through the cell, a linear regression analysis of the data was appropriate (Table 4). The ratio of the increments in

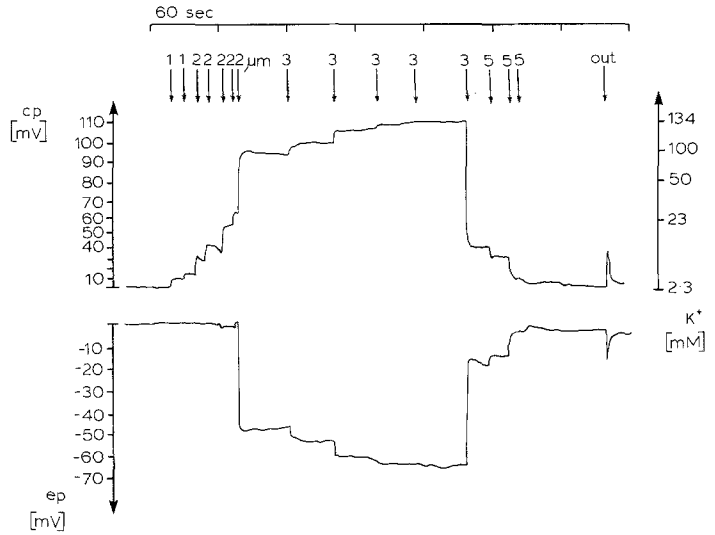

Fig. 6. Simultaneously recorded intracellular profiles of electrical potential (*ep*) and K^+ activity. The electrodes were advanced in steps from 1 to 5 µm (at arrows) through the cell towards the serosa. At *out* the electrode was retracted to the mucosal solution. Prior to penetrating the mucosal membrane (during the first 6 advances) the electrode (the reference barrel of which was filled with NH_4NO_3) recorded an increasing K^+ activity. This may be an artefact due to accumulation of the filling solution in the space between the tip and the dimpled mucosal membrane (*see* Discussion)

Table 4. The chemical potentials (*cp*) and activities (act) for K^+ in the mucosal (*m*) and serosal (*s*) ends of the cell in animals from group *I* [a] *(see text)* [b]

Filling solution	$\frac{1}{z}\frac{\Delta cp}{\Delta ep}$	cp_m (mV)	cp_s (mV)	act_m (mM)	act_s (mM)	Number of Observations	Number of Animals
Na_2SO_4	-0.86 ± 0.08	79 ± 6.5	96 ± 6.5	49	95	46	3
NH_4NO_3	-1.3 ± 0.16	80 ± 7.8	104 ± 7.8	51	131	18	4

[a] ±SE.
[b] The term $\Delta cp/z \cdot \Delta ep$ is defined on p. 189.

chemical potential to the simultaneous increments in electrical potential times the valency of K^+ ($\Delta cp/z \cdot \Delta ep$) was close to -1 [*see* Eq. (6)]. Thus, the intracellular K^+ was distributed close to equilibrium with the intracellular gradient of electrical potential, i.e., the electrochemical potential was constant throughout the cytoplasm. The chemical potentials in the mucosal (cp_m) and the serosal end (cp_s) were large and

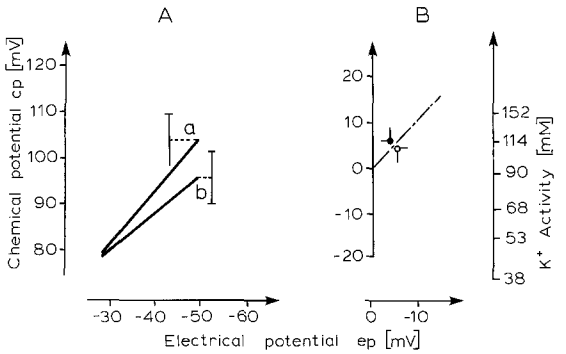

Fig. 7. The intracellular chemical potential (cp) and K^+ activity as a function of the intracellular electrical potential (cp). (A): Tissues bathed in Na^+-saline. The heavy line marked a collects the values found for animals in group I with the reference barrel filled with NH_4NO_3; b collects the values when the electrode was filled with Na_2SO_4. The vertical bars represents the SE (*see* Table 4 for statistics). (B): Tissues from group I bathed in K^+-saline. The values obtained with the Na_2SO_4-filled electrodes are given by open circles, and those with the NH_4NO_3-filled electrode by filled circles. These points are given with SE (*see* p. 202). These values were close to the theoretical values representing electrochemical equilibrium with the lumen (stippled line)

positive. Thus, within the range of intracellular electrical potentials, -28 to -49 mV, the electrochemical potential [Eq. (5)] was positive and constant about 50 mV throughout the cell. The intracellular K^+ was therefore not in equilibrium with the K^+ in the mucosal solution. There were differences between the results obtained with various filling solutions. With the Na_2SO_4-filled electrodes, the activity in the serosal end (act_s) was recorded 46 mM larger than the activity in the mucosal end (act_m) and 90 mM larger when the reference barrel was filled with NH_4NO_3. A graphical presentation of the regression analysis is shown in Fig. 7.

When the electrodes dimpled the membrane prior to penetration, those electrodes with reference barrels filled with NH_4NO_3 recorded a K^+ activity, which was higher (about 23 mM) than the mucosal activity, which was 2.3 mM (*see* Fig. 6). This effect was rarely seen with the electrodes with reference barrels filled with Na_2SO_4.

D) The effects of ouabain. When ouabain (5×10^{-4} M) was added to the serosal solution, the distribution of the Na^+ activity was the first to be affected. After 15 min the activity of Na^+ was constant throughout the cell at about 60 mM; an example of a penetration is shown in Fig. 1*b*. However, the intracellular electrochemical potential of Na^+ was still

lower than the external potentials. After 30 min the Na^+ activity came into equilibrium with the gradient of intracellular electrical potential; an example of a penetration is shown in Fig. 1*c*. The chemical potential as a function of the electrical potential is shown for different penetrations at different times of the exposure to the action of ouabain in Fig. 2. Only in the few cases where the electrode recorded a small intracellular electrical potential in the mucosal end of the cell, of less than a third of the serosal potential, was the intracellular Na^+ activity too high to be in equilibrium with the cell electrical potential (e.g., the first step recorded in Fig. 1*c*). The ratio between the change in chemical potential and change in electrical potential ($\Delta cp/z \cdot \Delta ep$) at different times of exposure to ouabain is shown in Fig. 3.

The gradient of the electrical intracellular potential and the value of the intracellular electrical potential at the serosal end of the cell was only slowly affected by the ouabain (Figs. 2 and 3). In a 5-hr period the average gradient in the cell, obtained by dividing the change in potential by the corresponding advance of the electrode, decreased by $0.24 \pm 0.12\%$ /min ($\pm$SE, $n=25$, 3 animals). In the same period the electrical potential in the serosal end decreased by $0.28 \pm 0.07\%$/min ($\pm$SE, $n=14$, 3 animals).

E) The effects of CO_2. With the electrodes placed intracellularly, the mucosal solution was gassed with 100% CO_2 for up to 1 min. The intracellular electrical potential in the serosal end of the cell decreased from -50 to -20 mV within about 20 sec (10, 10, and 45 sec were recorded in three animals) after the onset of the gassing. Penetrations performed after the bubbling had ceased showed that the intracellular gradients of electrical and chemical potentials of K^+ and Cl^- had been largely abolished. It was rarely possible to obtain stable intracellular recordings in the CO_2-poisoned cells for longer than 10 sec. Recording of Na^+ was therefore not successful.

After the initial steep changes in the electrical and chemical potentials (*see* Fig. 8), the electrical potential continued to decrease at a slower rate, $6.4 \times 10^{-3} \pm 2.3 \times 10^{-3}$ mV/sec (SE; $n=56$; 5 animals) the chemical potential of K^+ by $27 \times 10^{-3} \pm 5 \times 10^{-3}$ mV/sec ($\pm$SE; $n=37$; 3 animals) and the chemical potential of Cl^- by $7 \times 10^{-3} \pm 3 \times 10^{-3}$ mV/sec ($\pm$SE, $n=18$, 2 animals).

F) The voltage divider ratio. The voltage divider ratio $\Delta V_m/\Delta V_s$ was measured in three tissues with four different electrodes advanced from the mucosal side. It had an average value of 2.15 ± 0.25 ($\pm$SEM; $n=60$),

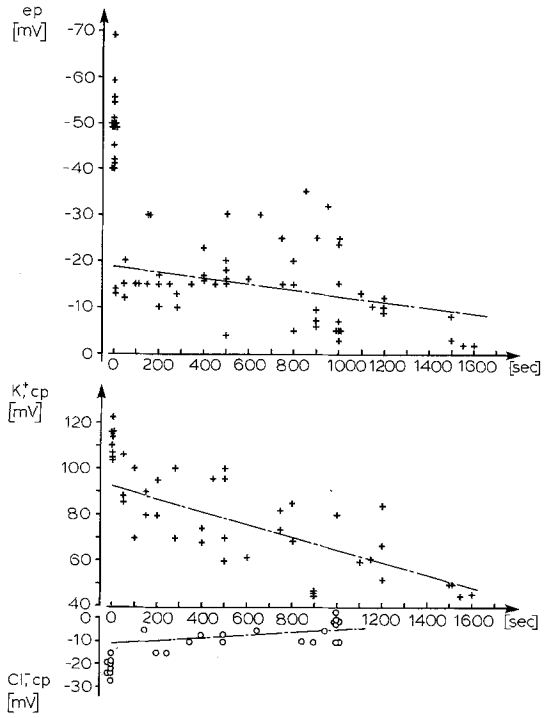

Fig. 8. The effect of CO_2 on the electrical chemical potential of Cl^- and K^+. At $t=0$ sec the mucosal solution was gassed with 100% CO_2 for 60 sec. This abolished the intracellular gradients and reduced the intracellular electrical potentials (*ep*) within 20 sec to less than -20 mV. The intracellular chemical potentials (*cp*) were also reduced, although not as steeply as the electrical potentials. The values at $t=0$ sec are the values from the serosal end of the cell. The stippled lines are the regression lines based on the values measured after $t=0$ sec, pooled from 5 animals

but was smaller, 1.7 ± 0.4, when the tip of the electrode was in the mucosal end of the cell, and larger, 3.2 ± 0.5, when the tip was in the serosal end. The same pattern was found in five penetrations performed from the serosal side in two tissues. The voltage divider ratio was small in the mucosal end, 1.6 ± 0.5, and larger in the serosal end 3.2 ± 1.2. During these penetrations the gradient in electrical potential was the same as that recorded with penetrations from the mucosal side (Zeuthen, 1977*a*).

G) Recordings from behind the cells. On penetrating the serosal membrane, i.e., on exit from the cell, the electrical potential returned to near zero values; at first abruptly decreasing about 30 mV (*compare* Figs. 1 *a*, 4 and 6) and then falling in an exponential manner. During this

last phase the impedance of the reference barrel was temporarily increased between 2 and 10 times. On the average the final stable electrical potential was recorded as -6.0 ± 1.4 mV ($\pm$SEM, $n=12$, 6 animals), with the activity of Na^+ recorded as about 84 mM, the K^+ activity as 11 mM, and the Cl^- activity as 96 mM. When the electrode was advanced into the serosal space no significant evoked potential was recorded as a result of transepithelial-induced current pulses. This applies also to the phase where the electrode potential returned exponentially towards near zero values. This strengthens the assumption that, even if the impedance of the electrode is temporarily increased in the connective tissue, the tip of the electrode has left the cell.

Tissues Bathed in K^+-Saline. Animals from Group I

The intracellular electrical potential was constant at -4.4 ± 2.8 mV ($\pm$SE, $n=69$, 7 animals) in the mucosal three quarters of the cell length, and only towards the serosal membrane did the potential decrease to about -8 mV (Zeuthen, 1976*b*). The Na^+ activity was constant and low; in eight recordings from 3 animals only values between 1.5 and 0.5 mM were recorded (average 1 mM). The Cl^- activity (*see* Fig. 5) was also constant; only towards the serosal membrane did the Cl^- activity decrease about 15%, which is equivalent to a decrease in the chemical potential of about 3 mV. Three different solutions were used in the reference barrels (*see* p. 188). The highest value of Cl^- activity recorded was 74 mM [$cp = -5.5 \pm 4.6$ mV ($\pm$SE, $n=4$, 2 animals)] when the reference barrel was filled with NH_4NO_3, in which case the intracellular Cl^- activity was in equilibrium with the external Cl^- activity. When the reference barrel was filled with KCl the Cl^- activity recorded was slightly lower 70 mM [$cp = -6.9 \pm 1.51$ mV ($\pm$SE, $n=10$, 3 animals)]. When the reference barrel was filled with Na_2SO_4 the Cl^- activity recorded was 53 mM [$cp = -12.3 \pm 3.51$ mV ($\pm$SE, $n=53$, 3 animals)].

The K^+ activity (*see* Fig. 7) was also constant, and only near to the serosal membrane did the activity increase about by 20%. The recorded activity depended on the choice of the solution in the reference barrel. The highest K^+ activity recorded was 113 mM [$cp = 6.1 \pm 1.9$ mV ($\pm$SE, $n=90$, 4 animals)] when the reference barrel was filled with NH_4NO_3. When the reference barrel was filled with Na_2SO_4, the average K^+ activity recorded was 105 mM ($cp = 4.8 \pm 2.8$ mV [$\pm$SE, $n=38$, 3 animals]). In both cases the intracellular K^+ activities were in equilibrium with the mucosal K^+.

The Intracellular Resistivity. Animals from Group I

It was reported earlier (Zeuthen, 1976*a*, 1977*a*), that the specific resistivity of cells bathed in Na^+-saline as recorded by triple-barreled electrodes varied with depth below the mucosal membrane. In the present measurements, only the average values of the resistivities in the cells of tissues bathed in K^+-saline ($\rho_{K^+\text{-saline}}$) were compared to the resistivities of cells bathed in Na^+-saline ($\rho_{Na^+\text{-saline}}$).

If the resistivities were measured with electrodes in which the current-injecting barrel was filled with 2 M tri-K^+-citrate or 2M NH_4NO_3 and the reference barrels with either NH_4NO_3 or KCl, the values had an average ratio $\rho_{K^+\text{-saline}}/\rho_{Na^+\text{-saline}}$ of 0.61 ± 0.035 (SEM, $n=467$, 6 animals). If the current-injecting barrel was filled with tri-Na^+-citrate, the ratio increased to 1.00 ± 0.038 (SEM, $n=354$, 4 animals), predominantly due to an increase in the measured value of $\rho_{K^+\text{-saline}}$.

Tissues Bathed in Na^+-Saline. Animals from Group II

The patterns of electrical and chemical potentials were the same as in the animals from group *I*.

The Na^+ and Cl^- activities in the mucosal end (act_m) decreased towards the serosal end (act_s), whereas the K^+ activity increased. The value of the parameter [$\Delta cp/z \cdot \Delta ep$, Eq. (6)] showed also that for this group of animals K^+ and Cl^- were roughly in equilibrium with the gradient of electrical potential, whereas Na^+ was not. With a gradient of electrical potential from -28 mV in the mucosal end to -56 mV in the serosal end and a gradient of chemical potential between the mucosal end (cp_m) and the serosal end (cp_s) of the cell, it was seen that none of the

Table 5. The chemical potentials (cp) and activities (act) for Na^+, Cl^-, and K^+ in the mucosal (m) and serosal (s) ends of the cell in animals from group *II*[a] (*see text*)[b]

	$\frac{1}{z}\frac{\Delta cp}{\Delta ep}$	cp_m (mV)	cp_s (mV)	act_m (mM)	act_s (mM)	Number of Observations	Number of Animals
Na^+	1.3 ± 0.3	-25 ± 11.8	-60 ± 11.8	33	8	16	4
Cl^-	-1.2 ± 0.08	-9.2 ± 10.2	-39 ± 10.2	65	20	84	5
K^+	-1.0 ± 0.08	81 ± 5.7	109 ± 5.7	53	160	28	3

[a] $\pm$SE.

[b] The term $\Delta cp/z \cdot \Delta cp$ is defined on p. 189.

ions were in equilibrium with the mucosal solution. There were, however, significant numerical differences between the two groups of animals: The electrical potentials recorded from the serosal cell end of animals from group *II* were more negative, -56 ± 1.9 mV (SEM, $n=44$), when compared to the -49 ± 1.1 mV (SEM, $n=45$) obtained in the animals from group *I*. Also, the Na^+ activity was about 8 mM lower throughout the cell, and the difference between the Cl^- and K^+ activity between the two poles of the cells were about twice as large in the animals of group *II*. The reference barrels of the Na^+ electrodes were filled with KCl, the barrels of the Cl^- and K^+ electrodes with NH_4NO_3.

The rate of fluid-transport was recorded in three animals from group *II* as 15, 15 and 18 $\mu l\, h^{-1}\, cm^{-2}$.

Discussion

This paper describes the measurements of the activities of Na^+, K^+ and Cl^- in the epithelial cells of *Necturus* gallbladder by means of double-barreled ion-selective microelectrodes. It shows that K^+ ions and Cl^- ions were accumulated inside the cells against their electrochemical gradients when the tissue was transporting in Na^+-saline, and that these ions were passively distributed across the plasma membranes, when the tissue was bathed in K^+-saline. Na^+ ions were actively removed from the cell during transport but tended to attain electrochemical equilibrium when ouabain was added to the serosal solution. The results also show that these electrodes recorded intracellular gradients of electrical potential, as well as in the chemical potentials of Na^+, Cl^- and K^+.

I shall first discuss the distribution of ions in the penetrated cell and proceed in the paragraph headed *Electrode Artefacts* to discuss whether the measured distributions of ions originate from the presence of the electrodes.

Accuracy of the Activity Measurements

Whereas the recorded intracellular electrical potentials were independent of the choice of salt solution in the reference barrel, the recorded ion activities depended to some degree on whether the salt solution contained ions to which the ion-selective membrane was sensitive (*see* p. 188). Thus, the Cl^- activity recorded with electrodes filled with KCl or NH_4NO_3 must be regarded as an upper limit, as the Cl^- ion exchanger was sensitive to NO_3^-, whereas the activity recorded with electrodes filled

with Na_2SO_4 must be a lower limit. Inside the cell the differences obtained were insignificant (Table 3). However, the effect of the filling solution accumulating around the tip was pronounced when the cell dimpled prior to penetration. In the few cases where a low electrical potential (<25 mV *see* p. 197) was recorded after penetration of the mucosal membrane, the KCl- and NH_4NO_3-filled electrodes recorded a Cl^- activity of 101 mM, compared to the 92 mM in the lumen; whereas the Na_2SO_4 filled electrodes recorded only 76 mM. It is possible that recordings were from inside the mucus-filled vesicles situated mainly below the mucosal membrane (Zeuthen, 1977*a*, Fig. 11). Similar considerations in terms of the influence of the solution in the reference barrel apply to the measurements of K^+ activity; recordings obtained with the NH_4NO_3-filled electrodes must be an upper limit. The effect was most pronounced when the tip dimpled the mucosal membrane (*see* Fig. 6).

The impedance of the electrode was temporarily increased between 2 to 10 times when it was advanced into the subserosa. Concurrently there was a change in the tip potential. The ion activities and electrical potentials recorded in the subserosa were, therefore, unreliable.

The averaged value of the calculated intracellular Cl^- concentration, using an intracellular activity coefficient of 0.76, of cells bathed in Na^+ saline was about 60 mM, which was slightly lower than the average value reported from studies in whole tissue of other gallbladders. In fish 85 mM was reported by Diamond (1962*b*) and 65 mM by Cremaschi, Smith and Wooding (1973). In rabbit 65 mM was reported by Cremaschi, Henin and Ferroni (1974) and 90 mM by Frizzel, Dugas and Schultz (1975). The average value of the calculated K^+ concentration, 95–140 mM, is equal to or higher than the average values of the concentrations reported in the above-mentioned studies, whereas the average value of the calculated Na^+ concentration, 30 mM, was about half of the reported concentrations.

The averaged values of the recorded ion activities were in general agreement with other studies of epithelia using ion-selective microelectrodes (*see* Table 1).

The Intracellular Ion Activities

A) The Na^+-activity. It seems likely that the Na^+ activity recorded intracellularly was in some way linked to the transepithelial transport of Na^+. Firstly, with Na^+-saline in the lumen, both the intracellular electrical and the chemical gradient favored movement of Na^+ from the mucosal end of the cell towards the serosal end. Secondly, ouabain added

to the serosal solution abolished this intracellular electrochemical gradient within about half an hour, a time close to that in which ouabain stopped the transepithelial solute and solvent transport in this tissue (Dr. A.E. Hill, *personal communication*) and fish gallbladder (Diamond, 1962*a*). Thirdly, the electrochemical potential gradient across the serosal membrane of -106 mV assessed from Table 2 suggested that Na^+ was actively removed from the cell, in agreement with the prevailing view of an ATP-linked Na^+ pump situated at the serosal membrane (Kaye *et al.*, 1966).

If it is assumed that the Na^+ ions sensed by the electrode were free to move in response to the existing electrical, chemical and frictional forces, then the intracellular flux J_{Na^+} could be described by the Nernst-Planck flux equation as the product of the diffusional constant, the concentration and the gradient of the electrochemical potential (*see* Appendix B). As the two last factors are known, the diffusion constant (or mobility) of Na^+ can be assessed when the calculated intracellular flux is compared to the transepithelial flux which was determined from the fluid transport away from the lumen. In Appendix B it is shown that if one requires, in the limiting case, that the intracellular flux equals the transepithelial flux then one has to require that the average intracellular diffusion constant of Na^+ is 60 times smaller than that of K^+ and Cl^-.

On the other hand, there is no reason why the intracellular Na^+ flux should exactly equal the transepithelial flux. Although the principle of electroneutral transport applies to the whole heterogeneous membrane, it does not apply to its constituent elements alone; there is, therefore, the possibility of ionic recirculation between the elements. Thus, if part of the Na^+ ion flux pumped into the lateral intercellular spaces returned to the mucosal solution via the leaky junctions, the intracellular flux would exceed the transepithelial flux. In the *Necturus* gallbladder the impedance of the junctions has been calculated to be of the same magnitude as the impedance of the lateral spaces, about $150\,\Omega\,cm^2$ (Frömter, 1972). Then, for geometrical reasons alone, Na^+ ions in the mucosal end of the lateral spaces would have an equal probability of returning to the mucosal solution or of passing into the serosal solution. The number of Na^+ ions transported across the tissue in the rabbit gallbladder *per* oxygen molecule consumed (Martin & Diamond, 1967) or ATP hydrolyzed (Van Os & Slegers, 1971) was about half the theoretical value. These measurements do not therefore exclude the possibility of a recirculation of a factor of two, i.e., that the Na^+ flux into the cell exceeds the transmural flux by a factor of two.

The assumption of a low mobility (or diffusion constant) for Na^+ relative to Cl^- and K^+ is not contradicted by the values achieved for the ratio between the specific resistivities of the intracellular space of cells bathed in K^+-saline to cells bathed in Na^+-saline. The value predicted from the activity measurements (*see* Appendix A) under the assumption that Na^+ and other ions such as X^{n-} (*see below*) had a mobility which was more than 10 times smaller than K^+ and Cl^-, was between 0.59 and 0.82, which is comparable to the measured value. Also the finding that electrodes filled with Na^+ salts (as compared to those filled with K^+ salts) recorded significantly higher values for the resistivities of cells bathed in K^+-saline supports the notion of a low diffusion constant of Na^+. Probably these electrodes leaked Na^+ (or K^+) into the cell; an increase in the Na^+ activity, however, would then increase the resistivity, whereas an increase in the K^+ activity would not. A low mobility for Na^+ in the intracellular space of other secretory epithelia has been suggested (Rossier & Rothman, 1975). Their argument was based on the uptake kinetics of ^{22}Na.

It should be stressed that these comparisons of the intracellular resistivities apply only if the cell volume is the same, whether the tissue is bathed in Na^+-saline or K^+-saline. The volume of the lateral intercellular spaces of tissues bathed in Na^+-saline constituted about 1% of the volume of the epithelia as estimated from electronmicrographs (*see* Zeuthen, 1977*a*, Fig. 11)[5]. In some electronmicrographic studies (kindly made accessible to me by Mr. M. Steward) of tissues bathed in K^+-saline, the only observed change, if any, was the collapse of the lateral intercellular spaces. Consequently, the volume of the cells were virtually unchanged, and the density of organelles the same whether the cells were bathed in K^+-saline or Na^+-saline.

In summary: If one assumes that the intracellularly recorded Na^+ activities directly describe the transmural Na^+ flux, then one has to assume either a much larger recirculation than a factor of two, which would seem energetically impossible (*see above*), or a low mobility of Na^+ relative to K^+ and Cl^- in the intracellular space. Yet this last assumption will be at variance with the findings obtained in axoplasm from squid axons (Hodgkin & Keynes, 1965) and *Myxicola* (Baker & Schapiro, 1976). In these cells Na^+ behaved much as in free solution.

5 The width of the lateral intercellular spaces of the *Necturus* gallbladder, as judged from our electronmicrographs and from electrical measurements (Frömter, 1972) is of the order of 1,000 A. This is smaller than the average obtained in the gallbladder of the rabbit: 0.88 μm (Tormey & Diamond, 1967).

There are, however, differences between the two types of cells. Most of the intracellular Na^+ is in a chemically unbound state in the axoplasm, whereas about half of the total intracellular Na^+ could be chemically bound in epithelial cells, as found by Lee and Armstrong (1972) in the small intestine.

B) The Cl^- activity. With Na^+-saline as the bathing solution, the intracellular Cl^- activity was too high everywhere to be in equilibrium with the mucosal solution. This was also the case in the other studies compiled in Table 1. As the transepithelial transport of Cl^- was from the mucosal to the serosal solution, Cl^- possibly left the cell passively across the serosal membrane, and entered the cell actively across the mucosal membrane. Accepting the proposed neutral coupling mechanism of Na^+ and Cl^- at the mucosal membrane (Frizzel *et al.*, 1975; Henin & Cremaschi, 1975) it is seen from Tables 2 and 3 that the electrochemical potential difference of -50 mV for Na^+ across this membrane easily exceeds the electrochemical gradient of -10 to -20 mV, opposing the entry of Cl^-. The coupled NaCl entry is therefore energetically possible. When Na^+ was replaced by K^+ in the bathing solutions, the intracellular Cl^- activity increased, but in view of the simultaneous change in electrical potential, the Cl^- activity attained electrochemical equilibrium with the bathing solutions. This supports the notion that Na^+ is required for the accumulation of Cl^- in the transporting cell.

C) The K^+ activity. With Na^+-saline as the bathing solution, the K^+ activity was in electrochemical equilibrium within the cytoplasm, but the intracellular activity was too high to be in equilibrium with the mucosal and serosal solution. This also applies to the other studies listed in Table 1, except the kidney where the intracellular K^+ activity was reported to be in equilibrium with the bathing solutions. However, when Na^+ was replaced by K^+ in the bathing solutions, the intracellular activity attained equilibrium with the mucosal solution. This supports the idea of a Na^+-linked K^+ influx situated at the serosal membrane. As no significant K^+ influx into the mucosal solution has yet been reported, K^+ must predominantly return to the serosal solution via the serosal membrane.

The Requirement of Electroneutrality and Mechanisms of the Intracellular Electrical Gradient

The positive and negative charges in any microscopic volume must add up to zero. The sum of the calculated concentrations (taken equal to

Table 6. The equivalents of intracellular negative charge (nX^{n-}), other than Cl^-, that are required to balance the equivalents of intracellular Na^+ and K^+

Bathing solution	Animals	nX^{n-} (mEq)	
		Mucosal end	Serosal end
Na^+-saline	*Group I*	39–54	112–163
	Group II	28	195
K^+-saline	*Group I*	62	62

the measured activities divided by an activity coefficient of 0.76) of the recorded Na^+ and K^+, minus the Cl^-, at each point in the cell must therefore represent a density of charge (nX^{n-}) associated with an anion (X^{n-}) which was not measured. Calculated values of nX^{n-} (expressed as mEq) in the mucosal end and the serosal end of the cell are presented in Table 6 as derived from the activities of Na^+, Cl^- and K^+ (from Tables 2, 3, 4 and 5).

The changes in the calculated concentrations of Na^+, Cl^- and K^+ seemed to be logarithmic functions of the depth below the mucosal membrane. However, within the ranges observed, the apparent concentrations could be expressed as a linear function of depth with an error of about 5 mM. As a result, the charge density associated with X^{n-} varied almost linearly by about 100 mEq between the mucosal and the serosal end of the cells when bathed in Na^+-saline, and had a value between 50 and 70 mEq in cells bathed in K^+-saline.

Several factors will contribute to nX^{n-}. In the transporting cell the increased levels of ATP, ADP, PO_4^{2-} and HCO_3^- together with other metabolic intermediates, e.g., lactate, would contribute to nX^{n-}. On the other hand the H^+ production associated with the mitochondria which are predominantly situated in the mucosal end of the cell would cause nX^{n-} to diminish due to the protonation of X^{n-}.

In view of the possible nature of X^{n-}, it seems plausible to assume that X^{n-} has a lower mobility than that of K^+ and Cl^-. This is also suggested by the value of the ratio between the resistivities of cells bathed in K^+-saline and Na^+-saline, (*see* Appendix A). The asymmetric distribution of X^{n-} found in cells bathed in Na^+-saline could contribute to the intracellular gradient of electrical potential, if X^{n-} were associated with ions, the distribution of which were constant during the transport of Na^+. In this case a Donnan potential difference would exist between the two ends of the cell. This possibility has been discussed quantitatively

(Zeuthen, 1977*b*). This interpretation agrees well with the finding that the intracellular gradients of potentials were abolished when the tissue was acidified by means of CO_2; probably all the X^{n-} became protonated ($nH^+ + X^{n-}$) and the charge associated with X^{n-} was neutralized[6].

One can exclude diffusion-potentials and current-generated potentials within the cytoplasm generated by the Na^+ flux as the cause for the intracellular gradient of electrical potential: Ouabain reversed the intracellular gradient of the Na^+ activity and stopped transmural Na^+ and H_2O transport within 30 min, by which time the intracellular electrical gradient was only slightly affected. The maintenance of the electrical gradient was, therefore, not directly caused by the mechanism that caused the Na^+ gradient. An electrical gradient based on a nonuniform exit of H_2O from the cell (Lindemann & Pring, 1969) can also be excluded, as the electrical gradient remained when H_2O-transport had been arrested by the addition of ouabain.

Differences in Animals

The animals received in March, 1975, and kept at 15 °C (group *II*) possessed higher intracellular Cl^- and K^+ activities, higher rates of fluid transport, and more negative intracellular potentials but lower intracellular Na^+ activities than animals received in March, 1976, and kept at 5 °C (group *I*). The rate of fluid transport in group *II* was about $16\,\mu l\,h^{-1}\,cm^{-2}$ as compared to the values of about $5\text{–}10\,\mu l\,h^{-1}\,cm^{-2}$ obtained in animals from group *I* (Dr. A.E. Hill, *personal communication*). This difference in performance could be due to either an effect of the storage temperature or an inherent difference between the batches. However, the differences do suggest a positive correlation between rate of fluid-transport and the electrochemical differences of the ions across the mucosal and serosal membranes.

Electrode Artefacts

Up to now I have discussed the distribution of ions in the cell penetrated by an electrode. It will now be discussed to what degree these

6 When the X^{n-} are protonated by the CO_2 (let us assume this happens instantaneously) the hitherto nonuniform intracellular distributions of K^+ and Cl^- will approach a uniform intracellular distribution due to diffusion. We can estimate the time constant for this change from Fick's second equation as $\tau \simeq x^2/2D$ where x is the length of the cell (40 μm), and D is the diffusion constant. Even if D was 10 times less than the value in saline, $1.5 \times 10^{-5}\,cm^2\,sec^{-1}$, the time constant will be less than one sec.

distributions could arise from the presence of the electrode. Some obvious artefacts can be ruled out: Measurements showed that the intracellular electrical gradient could not be explained as a diffusion potential or ohmic potential caused by fluxes of Na^+, Cl^- or K^+ along the shaft of the electrode, fluxes which should originate from an incomplete seal between the penetrated membrane and the electrode. (i) Any contribution from active Na^+ fluxes could be ruled out since the electrical gradient largely remained when the active Na^+ flux had been stopped by the addition of ouabain to the serosal solution. (ii) Any contribution from passive K^+ and Cl^- fluxes (and passive Na^+ fluxes in the ouabain-poisoned tissue) could be ruled out since the measured electrochemical potential of these ions were constant throughout the cytoplasm. If a leak of these ions existed, one would expect the recorded electrochemical potential for K^+ to be higher in the serosal end than in the mucosal end of the cell, indicating a flux of K^+ out of the cell; and one would expect the recorded electrochemical potential for Cl^- to be lower in the serosal end of the cell than in the mucosal end, indicating a flux of Cl^- into the cell. These considerations complement both the previous observations that the intracellular electrical gradient existed in the impaled cell, as seen with two electrodes in the same cell (Zeuthen, 1977*a*), and that the current needed to maintain the electrical gradient as an ohmic potential was too large to be supplied by the charges in the tissue (Zeuthen, 1977*b*).

Arguments against a change in tip-potential as a cause for the recording of the intracellular gradients were presented earlier (Zeuthen, 1977*a*). Further evidence was supplied by the present measurements. If there were an intracellular substance which was able to change the tip potential of the reference barrel more in the mucosal end of the cell than in the serosal, then a gradient of electrical potential would artificially be recorded. This would not, however, explain the observed gradient of Na^+ activity, as this cannot be the result of an erroneously recorded electrical potential being subtracted from an otherwise constant electrochemical potential of Na^+ (*compare* Eqs. (1) and (2) and Fig. 1*A*); it would result in an increasing Na^+ activity being recorded towards the serosal membrane. Similarly, if one argues that both the tip of the reference barrel and the Na^+-sensitive barrel were subject to changes in tip potential, then these changes would cancel out by subtraction [Eqs. (1) and (2)] and the true Na^+ distribution would be recorded.

One type of artefact which cannot be ruled out by the present measurements is one where the presence of the electrode tip causes the

activity of X^{n-} to diminish relatively more in the mucosal than in the serosal end of the cell. This might be the case if the electrode penetration caused a leak which was maintained predominantly by H^+ ions. Such a leakage could take place either through the electrode glass, via the electrode-membrane interphase or via the filling solution of the electrode. This could produce the observed gradients in the charge density associated with X^{n-} if these ions became more protonated in one end of the cell than the other. The distributions of K^+ and Cl^- would then arise as a passive equilibrium distribution relative to the resulting charge distribution of X^{n-}. Such a gradient would also be abolished in the CO_2-poisoned tissue, as all X^{n-} would then be in the form: nHX^n, which would not be altered by the presence of any H^+-selective leak caused by the electrode.

The average value of the voltage divider ratio measured when the electrodes were advanced from the mucosal side was similar to that measurement by others using single-barreled electrodes in this tissue (Frömter, 1972; Reuss & Finn, 1975). However, the voltage divider ratio was smaller (about 1.7) in the mucosal end of the cell, where the small intracellular potential was recorded, and larger (3.2) in the serosal end of the cell, where the higher negative intracellular potential was recorded. This pattern was also reported by Suzuki and Frömter (1977), who further reported that the gradation in potential as well as in the voltage divider ratio was an effect found only in connection with relatively large electrodes. Based on the available data, however, it is not clear at present to what degree a relatively low value of the voltage divider ratio reflects a leak caused by the electrode penetration: (i) Even if the change in voltage divider ratio suggests a leak, the values obtained also show that this leak is too small to explain the intracellular electrical gradient in terms of a leakage current. This can be deduced from the calculations of Lindemann (1975)[7]. (ii) The voltage divider ratio in the *Necturus* gallbladder is a function not only of the resistance of the mucosal and serosal membranes, but also of the dimensions of the lateral intracellular spaces. Thus, Frömter (1972) reported a 10-fold change (from 0.3 to 3) in

7 Assume that the value of the voltage-divider ratio recorded in the serosal end of the cell (3.8) reflects the ratio of the membrane resistances: $R\hat{o}/R\hat{\imath}$, where $R\hat{o}$ is the impedance (in Ω) of the mucosal membrane and $R\hat{\imath}$ the impedance of the serosal membrane. Further assume that the value recorded in the mucosal end (1.6) is lower because of a leak ($R\hat{s}$ in Ω) caused by the penetration of the mucosal membrane. $R\hat{s}$ will be in parallel with $R\hat{o}$, and the erroneously low voltage-divider ratio will calculate as $\Delta Vm/\Delta Vs = R\hat{o}/R\hat{\imath}(R\hat{s}/[R\hat{o}+R\hat{s}])$, Lindemann (1975). Thus, for the twofold change to occur, $R\hat{s} \simeq R\hat{o}$ when the tip of the electrode is in the mucosal end of the cell, and

the voltage divider ratio when the lateral intercellular spaces were opened or closed by means of transmurally imposed osmotic gradients. Any changes in the geometry of the penetrated cell caused by the penetration of the electrode could therefore cause a change in the voltage divider ratio. (iii) Also, the finding in this paper that the changes in the voltage divider ratio were independent of the orientation of the tract of the electrode suggests that the changes in this ratio cannot entirely be ascribed to a leak along the shaft of the electrode.

Concluding Remarks

The model of the epithelial cell, as seen by means of the ion-selective microelectrodes, was more elaborate than but not principally different from the classical one in which the cell is viewed as a membranous sac containing various organelles suspended in an aqueous solution of ions and proteins. The differences can be summarized as follows: (i) The average resistivity of the intracellular medium varied throughout the cell, probably due to the spatial distribution of organelles, i.e., the mucus-filled vesicles situated in the mucosal end of the cell (Zeuthen, 1977*a*); (ii) if one assumes that the intracellularly recorded Na^+ ions are those which are transported transmurally and that all the intracellular space is available to Na^+, then the ratio of the mobilities of the ions in the cytoplasm seem to be different from those found in free solution, the mobility of Na^+ being more than 10 times lower than that of K^+ and Cl^-; (iii) an appreciable intracellular gradient of electrical potential was recorded by the microelectrode in the transporting cell. The K^+ and Cl^- ions were passively distributed within this gradient. The measurement did not exclude the possibility that this electrical gradient arose as a result of an asymmetric distribution of ions (X^{n-}) which were immobilized during transport. But the question remains whether these distributions exist in the unpenetrated cell, or whether they arise as a result of the presence of the electrode tip; some, but not all, electrode artefacts could be ruled out. In this connection it is interesting to note that similar

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$R\hat{s} \gg R\hat{o}$ when the tip is in the serosal end of the cell. The resistance of the mucosal membrane is 4,470 Ωcm^2 (Frömter, 1972), and the area about $20 \times 20\ \mu m^2$; thus, $R\hat{o} \simeq R\hat{s} \simeq 10^9\ \Omega$. With an intracellular electrical potential of e.g. 50 mV, this leak would cause a leakage current of about 5×10^{-11} A to pass through the penetrated cell. However, in order to produce the intracellular electrical gradient of 25 mV which was observed, e.g., by means of two electrodes in the same cell (Zeuthen, 1977*a*), the leakage current should be at least 10^{-7} A, even if the resistivity of the cytoplasm was 10 times that of saline.

intracellular gradients of ion concentrations have been recorded from another epithelium (Malpighian tubule) using a different technique, *X*-ray microanalysis (Gupta *et al.*, 1976).

The present picture is quite different from models where the cells are viewed as an ordered phase in which the cations are strongly associated with fixed negative binding sites (Ling, 1962). As the ion-selective electrodes record activities, the model was based on the measurements of chemically free ions. Also the ions which were not accessible to direct measurement (X^{n-}) were also not fixed, as their concentration changed when the cell was exposed to different environments (Table 4).

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Appendix A

The specific resistivity of a solution is inversely proportional to the sum of the products of the concentrations (c), the mobility (u) and the numerical value of the valency of the ion (n).

Thus, for a solution of Na^+, K^+, Cl^- and an anion X^{n-}:

$$\rho \propto \frac{1}{u_{Na}\, c_{Na} + u_K\, c_K + u_{Cl}\, c_{Cl} + n u_X\, c_X}. \tag{A-1}$$

This expression deals only with that fraction of the intracellular ions that is free to move in the cytoplasmic solution according to the existing electrical and diffusional forces, i.e., that fraction which is sensed by the ion-selective electrodes. The ratio between the resistivities obtained in cells bathed in Na^+-saline and K^+-saline may therefore be calculated as:

$$\frac{\rho_{K^+\text{-saline}}}{\rho_{Na^+\text{-saline}}} = \frac{u_{Na}\, a_{Na}^{Ns} + u_{Cl}\, a_{Cl}^{Ns} + u_K\, a_K^{Ns} + n u_X\, a_X^{Ns}}{u_{Cl}\, a_{Cl}^{Ks} + u_K\, a_K^{Ks} + n u_X\, a_X^{Ks}}. \tag{A-2}$$

The activity coefficients $f = a/c$ of the ions concerned have been taken to be equal for all ion species, and f therefore cancels out in the expression (A-2) when the concentrations are replaced by activities. The activity coefficient used here should not be confused with that calculated on the basis of the total (bound and unbound) ion contents of the cell. The lower suffix denotes the ion species and the upper suffix denotes the bathing solution; Ns for Na^+-saline and Ks for K^+-saline. It has further

been assumed that $a_{Na}^{Ks}=0$ (*see* p. 202). The ratio [Eq.(A-2)] could be calculated for three cases for animals from group *I*. (i) If it was assumed that $u_{Na}=u_{Cl}=u_K=u_X$ then Eq.(A-2) reduced to the ratio of the sum of the ion activities. Using the average values of the activities calculated from Tables 2, 3, 4 and 5, the ratio was calculated to lie between 0.88 and 1.21, depending on whether the maximal or minimal values were used. (ii) Assuming that $u_X \ll u_{Na}=u_{Cl}=u_K$, the ratio was calculated to be about 0.87. (iii) If it is assumed that u_{Na}, $u_X \ll u_{Cl}=u_K$, then Eq.(A-2) can be reduced to the ratio of the Cl^- and K^+ activities alone, and calculated as 0.59 or 0.82 when the minimal or maximal values were used. The measured value was 0.61 (*see* p. 203).

Appendix B

The intracellular Na^+ flux, J_{Na}^+, can be determined from the Nernst-Planck flux equation.

$$J_{Na}^+ = -D_{Na} \cdot c_{Na} \cdot \frac{F}{RT} \cdot \frac{d}{dx}(z \cdot ep + cp). \tag{B-1}$$

The diffusion constant D_{Na} describes the frictional forces, c_{Na} the concentration and RT/F equals 26 mV at 20 °C. The last term is the derivative of the electrochemical potential [defined in Eq.(5)] with respect to the depth (x) below the mucosal membrane. z is the valency of Na^+ which equals +1. The equation can be rewritten as:

$$J_{Na}^+ = -D_{Na} \cdot c_{Na} \cdot \frac{F}{RT} \cdot \frac{d(ep)}{dx}\left(1+\frac{d(cp)}{d(ep)}\right). \tag{B-2}$$

If c_{Na} is taken equal to the measured activity divided by an activity coefficient of 0.76, all terms are known except D_{Na}. $d(ep)/dx$ was determined as -0.6 mV/μm (Zeuthen, 1977*a*) $d(cp)/d(ep)$ was 0.9, and the concentration varied from 47 to 21 mM between the mucosal and the serosal ends of the cell (Table 1).

Let us first tentatively assume that $D_{Na}=D_{Cl}=D_K$ (where $D=u \cdot kT$, k being Boltzmann's constant and T the absolute temperature). The expression (A-1), Appendix A is then reduced to:

$$\rho_{Na^+\text{-saline}} \propto \frac{kT}{D_{Na}} \cdot \frac{1}{a_{Na}^{Nas}+a_{Cl}^{Nas}+a_K^{Nas}}. \tag{B-3}$$

As the last factor was roughly constant through the cell (Tables 2, 3 and 4), $1/D_{Na}$ must vary through the cell as the resistivity which varied from 10 times that of Na^+-saline in the mucosal end to 6 times in the serosal end (Zeuthen, 1977*a*), D_{Na} must therefore vary from 1/10 to 1/6 of the value in free solution 1.3×10^{-5} M cm^2 sec^{-1} (Brockris & Reddy, 1970, p. 296). Inserting in Eq.(B-2) we obtain in the mucosal end of the cell $J_{Na}^+ = 1.2 \times 10^{-8}$ M cm^{-2} sec^{-1} and in the serosal end 0.86×10^{-8} M cm^{-2} · sec^{-1}.

The net flux across the epithelial cell layer was assessed from the rate of isotonic absorbtion about 10 µl/cm^{-2} h^{-1} (Dr. A.E. Hill, *personal communication*), which was equivalent to a Na^+ flux of 3.3×10^{-10} M cm^{-2} · sec^{-1}.

Thus, with the assumption that $D_{Na} = D_{Cl} = D_K$, the intracellular Na^+ flux was calculated to be about 60 times larger than the net flux across the epithelia. Conversely, it can be calculated that only if one assumes $D_{Na} = 1/60\ D_{Cl} = 1/60\ D_K$ will the transepithelial flux equal the calculated intracellular flux. It should be noted that an inherent assumption in these calculations is that all of the intracellular space is available to free Na^+ diffusion.

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