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Relations Between Intracellular Ion Activities and Extracellular Osmolarity in *Necturus* Gallbladder Epithelium

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Summary. The interactions between ion and water fluxes have an important bearing on osmoregulation and transepithelial water transport in epithelial cells. Some of these interactions were investigated using ion-selective microelectrodes in the *Necturus* gallbladder. The intracellular activities of K^+ and Cl^- in epithelial cells change when the epithelium is adapted to transport in solutions of a low osmolarity. In order to achieve new steady states at low osmolarities, cells lost K^+, Cl^- and some unidentified anions. Surprisingly, the apparent K^+ concentration remained high: at an external osmolartity of 64 mOsm the intracellular K^+ concentration averaged 95 mM. This imbalance was sensitive to anoxia and ouabain. The effects of abrupt changes in the external osmolarities on the intracellular activities of Na^+, K^+ and Cl^- were also investigated. The gradients were effectuated by mannitol. The initial relative rates of change of the intracellular activities of Na^+ and Cl^- were equal. The data were consistent with Na^+ and Cl^- ions initially remaining inside the cell and a cell membrane L_p of 10^{-3} cm sec^{-1} osm^{-1}, which is close to the values determined by Spring and co-workers (K.R. Spring, A. Hope & B.-E. Persson, 1981. *In*: Water Transport Across Epithelia. Alfred Benzon Symposium 15. pp. 190–200. Munskgaard, Copenhagen). The initial rate of change of the intracellular activity of K^+ was only 0.1–0.2 times the change observed in Na^+ and Cl^- activities, and suggests that K^+ ions leave the cell during the osmotically induced H_2O efflux and enter with an induced H_2O influx. The coupling is between 98 and 102 mmoles $liter^{-1}$. Various explanations for the anomalous behavior of intracellular K^+ ions are considered. A discussion of the apparent coupling between K^+ and H_2O, observed in nonsteady states, and its effects on the distribution of K^+ and H_2O across the cell membrane in the steady states, is presented.

Key words intracellular ion activities · intracellular osmolarity · ion-selective microelectrodes · water permeability of cell membrane

Introduction

This paper deals with two related problems:

1) Leaky epithelia form a secretion which is isotonic to the bathing solutions. This applies not only to external solutions of normal physiological osmolarities but also to a range of hypo- and hyperosmolar solutions: rabbit gallbladder 60–550 mOsm (Diamond, 1964); pancreas 110–600 mOsm (Case, Harper & Scratcherd, 1968); *Rhodnius* malpighian tubule, 60–380 mOsm (Maddrell, 1969); *Necturus* gallbladder 1–400 mOsm (Hill & Hill, 1978) and 40–225 mOsm (Zeuthen, 1981*a*).

The finding raises several problems: what are the intracellular osmolarity and ion activities when cells are transporting at low external osmolarities; and how do cells maintain intracellular salts to sustain organelle function?

2) High osmotic water permeabilities (L_p) for mucosal and serosal cell membranes of *Necturus* gallbladder have been recently reported (10^{-3} cm sec^{-1} osm^{-1}; Spring et al., 1981). This was found by exposing cells to osmotic gradients, maintained by mannitol, while observing changes in their shape. This raises the question: Do the osmotically induced fluxes of water induce movements of ions across the cell membrane? Such effects might complicate the interpretation of the osmotic behavior of the cells.

These questions have been investigated by means of ion-selective microelectrodes. In one group of experiments, epithelia were adapted to transport at low external osmolarities and the rate of transport and intracellular ion activities were investigated. In a second group of experiments, the transiential changes in intracellular ion activities in response to abruptly imposed osmotic gradients, were examined.

Materials and Methods

Microelectrodes

Microelectrodes were made as described elsewhere (Zeuthen, Hiam & Silver 1974; Zeuthen, 1980) and had a double-barrelled tip with a total diameter of less than 0.3 μm. The reference barrel was pulled from a glass with an outer diameter of 1 mm, an inner diameter 0.5 mm and contained an internal fiber

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for easy filling. It had an impedance of 40–200 MΩ, when filled with 2 M KCl and measured in physiological saline. The other barrel, designed to contain the ion-selective membrane, was pulled from a glass with an outer diameter of between 2.0 and 1.5 mm and an inner diameter of 1.0 mm. When filled with 2 M KCl the impedance averaged 20–40 MΩ. By choosing glass tubes of different diameters for the two barrels the outflow of reference solution into the cell was minimized; in fact no vacuoles were observed in the cytoplasm at the tip of the electrode. This effect may be observed with electrodes of a larger tip diameter (Nelson, Ehrenfeld & Lindemann, 1978). K^+-sensitive electrodes were made from Corning® Code 477317 ion exchanger, Cl^--sensitive electrodes from Corning Code 477315 and Na^+-sensitive electrodes form an ion exchanger modified from one supplied by Professor Simon (Bindslev & Hansen, 1981; Garcia-Diaz & Armstrong, 1980). The reference barrel was filled with 2 M KCl and in some control experiments with saturated Na^+ citrate or Na_2SO_4. This caused no difference in the recorded activities or electrical potentials.

Electrodes were used within 8 hr from the time of filling with aqueous solutions. The siliconized wall of the electrodes maintained a resistance of at least $10^{12}\,\Omega$ (Engbaek & Guld, 1971). Thus, the shunt through the wall of an electrode with an impedance of $10^{10}\,\Omega$ (which is equal to or larger than the resistance of the ion-selective electrode) was insignificant.

The results are presented as apparent concentrations, i.e. the recording from the electrode is directly compared with an external reference solution of a known concentration. This means that the apparent intracellular ion concentrations: Na_c^+, K_c^+, Cl_c^-, are equal to the true concentrations if the intracellular activity coefficients are similar to those of the external solution.

Electrodes were calibrated before the experiment in a separate set-up. After cell impalement, in the experimental chamber, K^+ and Na^+ electrodes were calibrated with solutions of equal ionic strength as that of the perfusion solution, but containing various proportions of Na^+ and K^+. In order to minimize any systematic error from nonlinearity of electrodes, the concentration of the ion in the test solution was kept close to the value measured inside the cell. Due to the large sensitivity of Na^+ electrode to Ca^+ (Bindslev & Hansen, 1981) calibration of the electrode was performed in Ca^{++}-free solutions. As intracellular Ca^{++} is negligible, Na^+ can be assessed from such calibrations. Cl^- electrodes were calibrated in solutions of different proportions of Cl^- and HCO_3^-. The sensitivity of Na^+ and K^+ electrodes was 50–55 mV per 10-fold change in activity, and selectivity to K^+ and Na^+ was 0.05 and 0.01, respectively. The sensitivity of Cl^- electrodes was 44–50 mV, and selectivity to HCO_3^- was about 0.1. The HCO_3^- concentration inside the cells was assumed to be about 10 mM (Khuri, Bogharian & Agulian, 1974) and the measurements of Cl_c^- were corrected accordingly. Ion-selective barrels had a response time of less than 1 sec.

The *relative* rate of change of ion concentration or activities $da/a\,dt = dc/c\,dt$ was derived from equations of the type:

$$E_{Na} = E_o + S \ln(a_{Na} + k a_K) \tag{1}$$

where Na^+ is the ion measured, and K^+ the interfering ion, S the sensitivity and k the selectivity constant, E_{Na} the potential measured by the electrode, a the activity, and c the concentration. By differentiation:

$$\frac{1}{c_{Na}} \cdot \frac{dc_{Na}}{dt} = \frac{1}{a_{Na}} \cdot \frac{da_{Na}}{dt}$$

$$= \left(\frac{1}{S} \frac{dE_{Na}}{dt} - \frac{k}{a_{Na} + k a_K} \frac{da_K}{dt} \right) \frac{a_{Na} + k a_K}{a_{Na}} \tag{2}$$

$$= \frac{1}{S} \cdot \frac{dE_{Na}}{dt} \quad \text{(for small } k\text{)}. \tag{3}$$

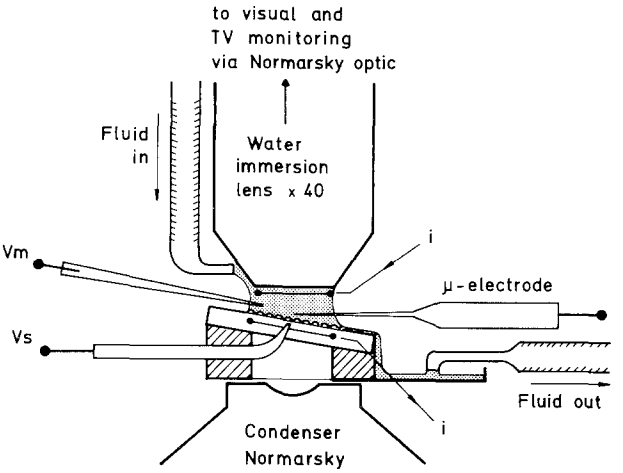

Fig. 1. Schematic drawing of the chamber in which the gallbladder epithelium (small semi-circles) could be probed with double barrelled microelectrodes under microscopic supervision. Current i could be passed across the epithelium via two silver wires shaped as rings. The electrical potential of the mucosal solution V_m and the potential of the connective tissue and the secreted solution (the serosal compartment) V_s were recorded via agar bridges

When Cl_c^- and K_c^+ are recorded the influence from other ions is small and the initial relative rate of change is approximated by the rate of change of the potential, measured by the ion-selective barrel, divided by the sensitivity of the electrode. Na^+ recordings were corrected for the influence from K^+.

Chamber for Microscopic Observation

To study the electrophysiology of the unilateral preparation, the excised gallbladder was extended over a hole (OD 6 mm) in a plate of Plexiglass and placed between a water immersion lens with Normarski attachment (Zeiss 40*x*, *n.a.* 0.63) and a Normarski-condenser (Zeiss, Phako IV 21) with a long working distance about 7 mm. The plate was at an angle of about 20° with the horizontal plane (Fig. 1).

A drop of solution (~15 μl) held under the objective served as a mucosal solution and was renewed in less than a second by continuous flow from a peristaltic pump. Having superfused the tissue, the fluid flowed via a wick into secondary chamber from where it was aspirated.

The tip and shank of the microelectrode moved in the focal plane of the water immersion lens. The lens was moved relative to the microscope table. The microscope table onto which the tissue and chamber were fixed moved with the fine adjustment of the microscope. Cells in focus were penetrated by the electrode. Current pulses (up to 10^{-5} A, 1 sec) were passed transepithelially via two ring-shaped silver wires; one attached to the water immersion lens, the other to the Plexiglass plate onto which the tissue was fixed. The wire contacted with the connective tissue. The electrical potentials of mucosal and serosal solutions were monitored by agar bridges connected to Ag/AgCl electrodes; the serosal electrode contacted the connective tissue. The microelectrode was positioned by means of a Huxley-micromanipulator (Huxley, 1961), which was equipped with stepping motors. The electrode could be moved in steps of 0.2 μm in three perpendicular directions by remote control. The position of the tip of the electrode was recorded digitally.

The microscope was equipped with a Philips TV camera (type LDH 26, vidicon tube), the picture monitored on a 12-inch screen (Philips) and recorded on a Sony video recorder (U-matic type VO 2630).

Chamber for Transport Studies

The rate of transport of unilateral (sweating) preparation was studied in bladders which were tied, connective tissue downwards, over the end of a (1 cm OD) plastic tube which was cut at an angle of 45°. The other end of the tube was closed by a stopper. The secretion was sampled from inside the tube. The osmolality and weight of the secretion was determined, to assess transport rates. The tube was placed vertically in a petri dish and the mucosal solution was applied from a 1-mm tube at the apex of the plastic tube and allowed to flow rapidly (>1 cm/sec) in a thin (~100 μm) layer over the mucosal surface, before it was sampled from the petri dish.

A small (0.2 mm) hole in the side of the plastic tube prevented hydrostatic pressure gradients to develop across the epithelial wall.

When the tissue was to be tested in a new solution, it was initially bathed on both sides for 45 min in the new solution. In order to expose the tissue to ouabain, the serosal side was washed in a saline containing 10^{-3} M ouabain for 30 min.

Perfusion

The perfusing solution could be changed within 1–3 sec by means of a valve. This valve was placed 2 cm from the chamber and connected to it via a narrow (0.5 mm ID) tube in order to minimize delay. Both the perfusing solution and the perfusion solution in waiting were pumped continuously by a peristaltic pump (AEG), but whereas the perfusing solution reached the tissue, the solution in waiting was spilled through a side branch consisting of a narrow glass tube, 1.0 mm ID, length 20 cm. The mouth of this tube was placed 2–3 cm above the level of the tissue. The rest of the system consisted of polystan tubing (i.e. 2 mm). Once the valve was switched between the two solutions, the previous perfusion solution was spilled through its side branch, while the new solution was perfused. By adjusting the outlet height of the side branches, the flow rates of solutions over the tissue could be matched exactly and peristaltic movements could be reduced. At an appropriate height the side branch of the solution being perfused remained filled with solution. The valve was a conventional syringe valve made from metal and was operated manually by two pieces of string.

Tissues

Gallbladders were removed from *Necturus maculosus* (Nasco®), which were maintained in running tap water at 10 to 15 °C for more than a month. Connective tissue and mesothelial membranes were removed by dissection. By applying a pressure of 8–10 cm^2 of H_2O to the serosal compartment of a tissue in which the mesothelium had been removed, blisters or domes of epithelium were formed (Bundgaard & Zeuthen, 1981). These domes were about 100 cell diameters in diameter. The underlying connective tissue could be removed by dissection. Usually 5 to 30 domes were formed. Sometimes, the dissection was unsuccessful and small holes of 3–10 cell diameters were formed in some of the blister. These holes were too small to form any shunt for fluid, but shunted electrically. For this reason, all tissues from which the connective tissues were removed, were short-circuited externally.

Osmolarities were measured in an Advance® osmometer or a Ramsay-Osmometer (Ramsay & Brown, 1955).

All numbers are ±SEM unless otherwise stated.

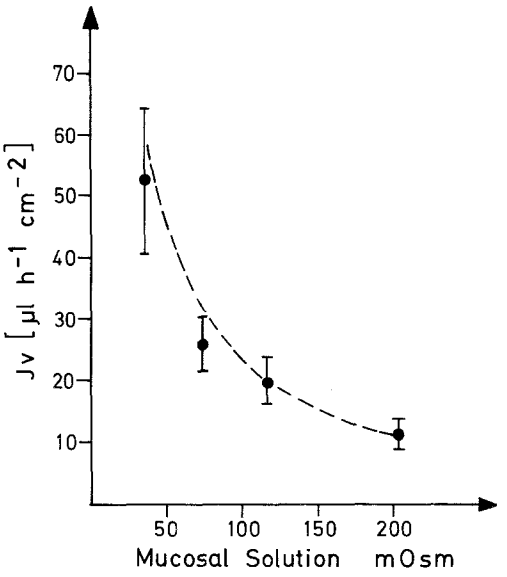

Fig. 2. The rate of transepithelial transport J_v as a function of the osmolarity of the mucosal solution. The bladder was mounted unilaterally (sweating) (*see* Materials and Methods). J_v increased with decreasing mucosal osmolarity following an almost inverse proportionality (broken line)

Table 1. Solutions[a]

	[mM] NaCl	[mOsm] mannitol	[mOsm]
1	28.8	0	71
2	57.5	0	119
3	28.8	80	146
4	28.8	120	187
5	28.8	160	225
5a	28.8	150	215
6	57.5	105	225
7	115.0	0	225
8	115.0	40	260
9	115.0	80	305
10	115.0	160	379
11	13.0	0	39
12	26.0	0	64
13	52.0	0	117
14	104.0	0	204
15	104.0	20	225
16	104.0	40	245
17	104.0	70	271
18	104.0	100	302
19	104.0	150	355
20	104.0	160	362

[a] In addition the solutions contained (in mM): 3 KCl, 2.7 $CaCl_2$, 2.4 $NaHCO_3$. Solutions were bubbled with O_2 and had a pH of about 7.6. Osmolarities were measured in an Advance® osmometer or a Ramsay-Osmometer (Ramsay & Brown, 1955).

Results

Part One: Steady States

Rate of Isoosmotic Transport. The rate of volume transport J_v (Fig. 2) and osmolarity of the transport-

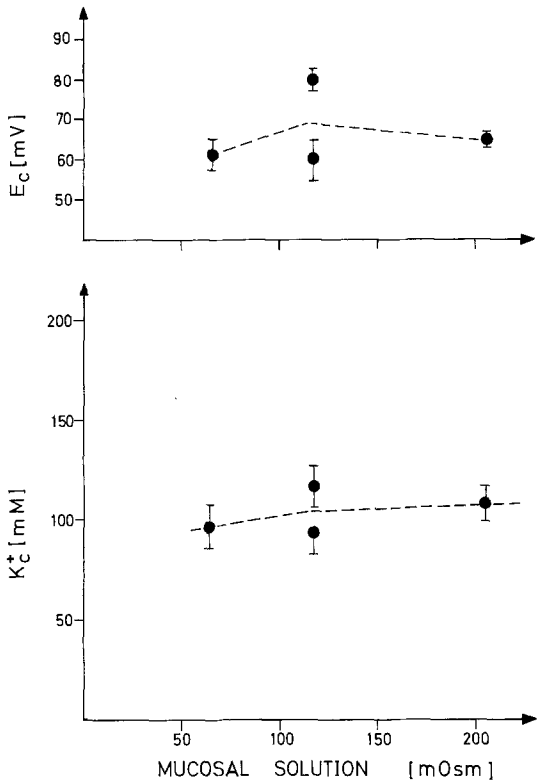

Fig. 3. Intracellular electrical potential (E_c) and apparent intracellular K^+ concentration (K_c^+) as a function of the osmolarity of the mucosal solution. Each point is the average from 3 to 12 animals, each based on 3 to 20 cells. The points obtained at 117 mOsm are from one animal each

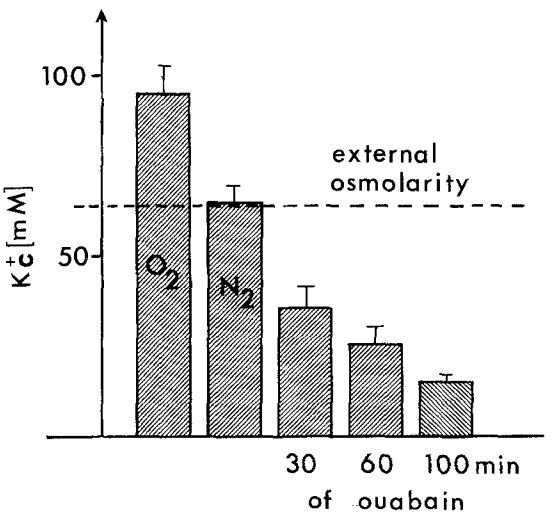

Fig. 4. The effects of ouabain (10^{-3} M) in the serosal solution were tested at an external osmolarity of 64 mOsm. The intracellular K^+ concentration (K_c^+) fell progressively with time as indicated by the bars marked 30, 60, and 100 min. Good oxygenation was essential for the maintenance of the high K_c^+, when the mucosal solution was bubbled with N_2 instead of O_2, K_c^+ was reduced (bar marked N_2)

ed solution were measured in unilateral preparation as described on p. 110. Measurements were performed in normal saline (solution 14, Table 1) and in three solutions where osmolarity had been reduced by removing NaCl (solutions 13, 12 and 11, Table 1). When the epithelium was bathed in saline with an osmolarity of 204 mOsm the secretion had an osmolarity of 203 ± 5 mOsm ($n=19$); in solution no. 13 (117 mOsm) an osmolarity of 108 ± 3 mOsm ($n=12$); in solution no. 12 (64 mOsm) an osmolarity of 64 ± 4 mOsm ($n=15$). If the mucosal side was bathed in solution no. 11 (39 mOsm) the epithelial cells were damaged: the rate of volume transport became insignificant from zero; the intracellular electrical potential decreased below -10 mV; and the intracellular K^+ concentration K_c^+ decreased below 20 mM. These tissues did not recover when returned to normal osmolarities. If, however, the K^+ concentration of solution 11 was reduced from 3 to 1.5 or 0.38 mM cells remained alive and produced a secretion of an osmolarity of 36 ± 4 mOsm ($n=3$).

The rate of volume-transport (J_v, Fig. 2) was, at normal osmolarities (204 mOsm, solution 14) 11.7 ± 1.8 µl hr^{-1} cm^{-2} ($n=11$). If the osmolarity of the mucosal solution was reduced to 117 mOsm, by removing NaCl (solution 13), J_v increased to 19.7 ± 3.0 µl hr^{-1} cm^{-2}. In solution 12 (64 mOsm) J_v was 26.3 ± 3 µl hr^{-1} cm^{-2} and in solution 11 (modified by reducing the K^+ concentration below 1.5 mM (36 mOsm) J_v was 52.5 ± 8.6 µl hr^{-1} cm^{-2}. Thus, J_v is almost exactly inversely proportional to the osmolarity of the mucosal solution.

Intracellular Electrical Potential E_c, Apparent Concentrations K_c^+ and Cl_c^- as a Function of External Osmolarity in Steady States. Gallbladders were mounted unilaterally and bathed on the mucosal side (p. 111) in saline with an osmolarity of 204 mOsm (solution 14, Table 1), or in salines where the osmolarity was reduced by removing NaCl: 117 mOsm (solution 13, Table 1); and 64 mOsm (solution 12, Table 1). Cells were probed by double-barrelled microelectrodes, and exhibited stable and uniform electrical and chemical potentials for periods up to 30 min. The recordings fulfilled the usual criteria of abrupt change at penetration (*see* Zeuthen, 1981*b*, for an example). The potentials were related to the mucosal solution. Electrical potential and apparent concentration were determined in 3 to 20 cells in 3 to 12 tissues. The average value of the averages from each tissue is summarized in Fig. 3.

At the lowest osmolarity tested, 64 mOsm, K_c^+ averaged 95 mM. In solution 13 (117 mOsm) K_c^+ was

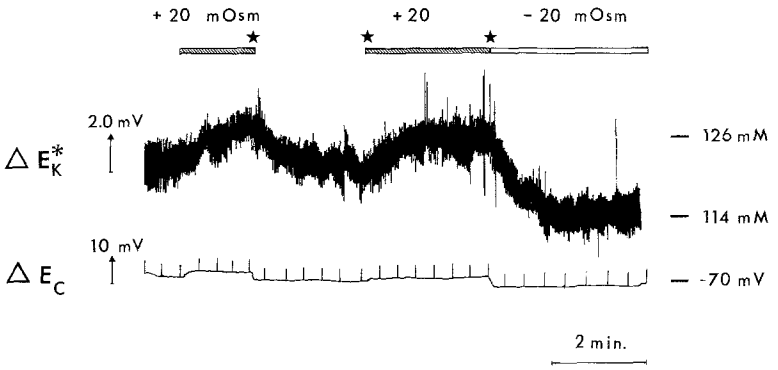

Fig. 5. The change in the apparent intracellular K^+ concentration K_c^+, given by the potential of the ion-selective barrel ΔE_k^*, and the electrical potential ΔE_c in response to abrupt changes in the osmolarity of the mucosal solution. The tissue was initially bathed in a solution of osmolarity 225 mOsm (solution 15, Table 1), then the osmolarity was increased +20 mOsm by adding mannitol (solution 16). This was done twice in this Figure. Finally, mannitol was completely removed as (solution 14) indicated by −20 mOsm. This situation represents the *smallest* changes that were attempted in this study. The changes in ΔE_k^* which were well defined (black stars) were used for evaluation of the initial relative rate of change in K_c^+. This was obtained simply as $\Delta E_k^*/S\Delta t$ [Eqs. (2) and (3)] where S is the sensitivity of the electrode. The intracellular electrical potential ΔE_c was measured relative to the serosal solution in this record; the transepithelial potential (*not shown*) changed less than 2 mV in this recording. The impedance of the reference barrel of the microelectrode was monitored every 25th sec; in this case it was 80 MΩ. The noise originates mainly from frictional electrostatics in the fluid pump

on average 108 mM. At normal external osmolarities, 204 mOsm, K_c^+ was 109 mM.[1]

K_c^+ was sensitive to anoxia and ouabain. If the mucosal solution was equilibrated with N_2 instead of O_2, K_c^+ decreased by about 40%. If ouabain was present on the serosal side, K_c^+ decreased gradually to about 10 mM in 60 min. Figure 4 shows these effects at an external osmolarity of 64 mOsm.

The apparent intracellular concentration of chloride (Cl_c^-) was 39 mM at normal external osmolarities (204 mOsm) and the chemical potential was -27.1 ± 3.1 mV relative to solution 14. At external osmolarities of 117 mOsm, Cl_c^- decreased to 11 mM (17.2 ± 1.9 mV relative to solution 11) and at 64 mOsm to 9 mM (-21.9 ± 4.1 mV relative to solution 11). These values are not corrected for artificial background recording (the amount of Cl^- apparently recorded by the electrode in a tissue bathed in a chloride-free solution). This can be as high as 6 mM (Machen & Zeuthen, 1980), which means that Cl_c^- may be as low as 2 mM at the lowest osmolarities tested.

Intracellular electrical potential (E_c) was −65 mV (Fig. 3) when the mucosal side of the tissue was superfused by normal saline (204 mOsm; solution 14). Cells hyperpolarized to as much as −80 mV when bathed in osmolarities of 117 mOsm (solution 13, Zeuthen, 1981*a*), but depolarized to −62 mV when bathed in low osmolarities of 64 mOsm (solution 12) (Fig. 3).

[1] If newly purchased animals were used the author obtained results which were more pronounced than those obtained with tap water-adapted animals (Zeuthen, 1981*a*). K_c^+ was 172 mM at normal external osmolarities and 144 mM at an external osmolarity of 112 mOsm and about 100 mM when the external osmolarity was 64 mOsm. However, the rate of volume transport was only 12 μl cm^{-2} hr^{-1} at an external osmolarity of 64 mOsm. The cells from these animals lost intracellular K^+ during adaptation to the low osmolarities, but K_c^+ remained higher than could be expected from osmotic equilibrium.

Part Two: Nonsteady States

The Initial Changes in Intracellular Electrical Potential E_c and Ion Activities Na_c^+, K_c^+ and Cl_c^- in Response to Step Changes in the External Osmolarity. Gallbladders were mounted unilaterally, as described in Materials and Methods, and the initial relative rate of change in concentrations, $dc/c\,dt$, Eqs. (2) and (3), and in intracellular potential were measured when the mucosal or serosal solution was abruptly changed. Bladders were initially bathed in normal saline (solution 7, Table 1), or in a saline that only contained 90% of the NaCl of normal saline, but to which 20 mOsm of mannitol was added (solution 15, Table 1). Both solutions had an osmolarity of 225 mOsm. A stepwise increase in the superfusion solution was performed by changing to a solution to which larger amounts of mannitol had been added (changing from solution no. 7 to 8, 9, 10 or changing from 15 to 16, 17, 18, 19 or 20). Stepwise decreases of −20 mOsm were made by removing mannitol from the perfusion solution (changing from solution 15 to 14). The rate of change of K_c^+ in response to gradients of ±20 mOsm (Fig. 5) was the smallest that could be detected with certainty (by eye), with the given signal-to-noise ratio. Figure 6

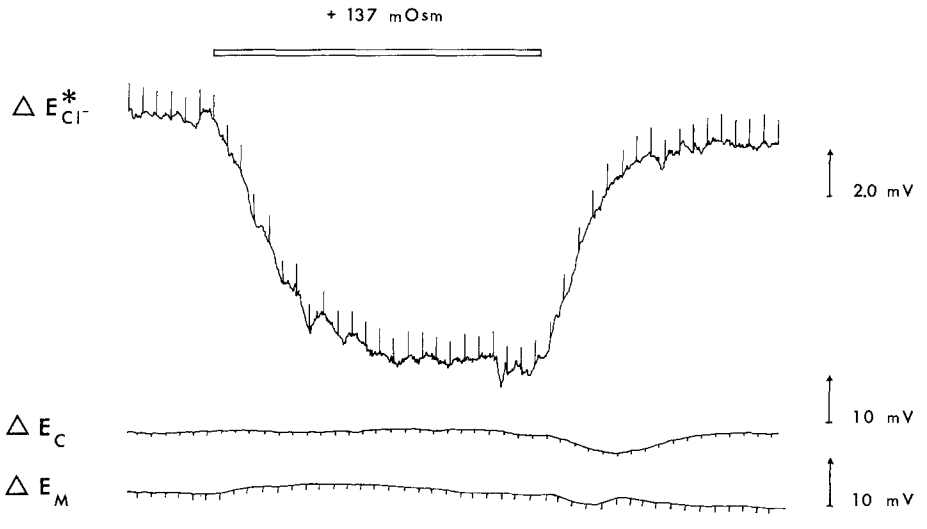

Fig. 6. The change in the apparent intracellular Cl^- concentration measured as the change in the electrical potential recorded by the Cl^--sensitive electrode $\Delta E^*_{Cl^-}$ [mV] in response to an increase of the osmolarity of the mucosal solution of 137 mOsm. The intracellular potential E_c and the mucosal potential ΔE_M were measured relative to the serosal solution. Every 20 sec a current pulse was passed across the epithelium in order to record the impedance of the tissue and the voltage divider ratio α (*see* p. 116). This situation represents the *largest* changes that were attempted in this study (*compare* Fig. 5)

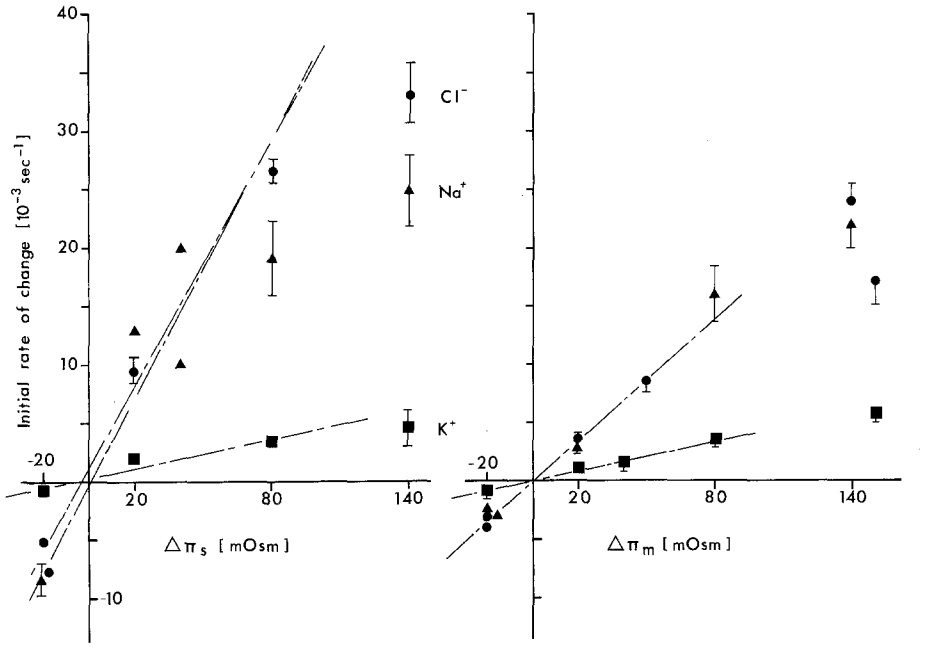

Fig. 7. The initial rate of change (in units of $10^{-3}\,sec^{-1}$) of Cl^-_c (circles), Na^+_c (triangles) and K^+_c (squares) as a function of the osmotic gradient imposed abruptly by changing the serosal solution $\Delta\pi_s$ or the mucosal solution $\Delta\pi_m$. The tissue was initially bathed in solution 7 or 15, 225 mOsm (Table 1). The increased osmolarities were achieved by perfusing salines to which mannitol was added; the decrease was achieved by removing mannitol. (For details of solutions, *see text* and Table 1.) For K^+ SEM is not shown if smaller than symbols; each point represents the average of 3 to 12 measurements from at least 2 animals. For Na^+ and Cl^- single points represent single measurements. The stipled lines are the lines of regression for measurements in the range -20 to 80 mOsm except for Na^+ in the serosal experiment where the range -20 to 40 mOsm was used. The statistics of the regression lines are given in Table 2. The intercepts with the ordinate were never significantly different from zero

shows a recording of Cl^-_c during a change in mucosal solution of ± 137 mOsm. The effects of stepwise increases and decreases in osmolarity on the initial changes in ion concentrations are compiled in Fig. 7. The larger the change in osmolarity $\Delta\pi$, the larger the initial relative rate of change in the concentration although large gradients seemed to be less efficient at effecting the concentrations. In order to compare the effects of the external osmolarities on the intracellular concentrations the author used the values obtained with smaller gradients, i.e. where the responses were linearly related to the change in osmolarity. The following analysis is therefore based on gradients of -20 to 80 mOsm. An exception is

Table 2.

		Na_c^+	K_c^+	Cl_c^-	X^-
Steady states	measured from mucosa	7.9 mM[a] (−6.0 mV ± 2.6, $n=6$)	123 mM[b] (−5.0 mV ± 1.0, $n=47$)	31 mM[c] (32 mV ± 1.6, $n=15$)	99.9 meq[d]
	measured from serosa	11.6 mM (3.9 mV ± 2.3, $n=6$)	113 mM (−7.3 mV ± 2.9, $n=8$)	32 mM (31 mV ± 1.0, $n=7$)	92.6 meq[d]
Transients, 10^{-3} sec^{-1} mOsm^{-1}	measured from mucosa	0.185	0.039	0.163	0.012[d]
	measured from serosa	0.413	0.042	0.318	0.007[d]
Transients,[e] mM sec^{-1} × mOsm^{-1} × 10^3	measured from mucosa	1.46	4.80	5.05	1.21[d]
	measured from serosa	4.79	4.75	10.18	− 0.64[d]

The chemical potential in mV is:

[a] relative to 140 mM KCl + 10 mM NaCl [b] relative to 150 mM KCl
[c] relative to solution 6, 110 mM Cl^- [d] calculated from electroneutrality
[e] calculated from the steady-state values and the transients listed above.

Table 3. Statistics for Fig. 7

		Intercept ordinate (10^{-3} sec^{-1})	Slope (10^{-3} sec^{-1} mOsm^{-1})	r	Number of observations
Serosal	Na^+	0.06 ± 1.4	0.413 ± 0.056	0.94	9
	Cl^-	1.39 ± 1.2	0.318 ± 0.022	0.98	9
	K^+	0.22 ± 0.73	0.042 ± 0.011	0.83	8
Mucosal	Na^+	0.08 ± 1.7	0.185 ± 0.028	0.89	13
	Cl^-	0.16 ± 0.33	0.163 ± 0.012	0.96	17
	K^+	0.15 ± 0.20	0.039 ± 0.004	0.89	28

the changes in Na^+ induced from the serosal side which are analyzed in the range −20 to 40 mOsm.

The initial relative change in K_c^+ was 0.039×10^{-3} sec^{-1} mOsm^{-1} when gradients were imposed from the mucosal side and 0.042×10^{-3} sec^{-1} mOsm^{-1} when they were imposed from the serosal side. There was no significant difference between the initial relative rate of change induced in Na_c^+ and Cl_c^-. When the gradients were imposed from the mucosal side, Na_c^+ and Cl_c^- changed on average 0.12×10^{-3} sec^{-1} mOsm^{-1} and when gradients were imposed from the serosal side, Na_c^+ and Cl_c^- changed on average 0.36×10^{-3} sec^{-1} mOsm^{-1}. (*See* Tables 2 and 3.) In each experiment the initial rate of change was the same whether the external osmolarity was changed to larger or smaller osmolarities.

The induced changes in intracellular electrical potential were small in all these experiments. Changes were less than 2 mV across either cell membrane in experiments where the mucosal solution was changed (Fig. 8). Up to one mV could be induced, transepithelially (mucosa positive) when the mucosal solution was hyperosmolar. In the experiments where the serosal solution was changed, the tissue was short-circuited; in this case the induced change in E_c, similar to that observed at mucosal substitutions (Fig. 8), was typically less than 2 mV.

The effects on K_c^+ of large negative and zero gradients of osmolarity were also studied (Fig. 9). The gradients were imposed from the mucosal side only. Gradients were produced either by removing NaCl (solutions 1 and 2, Table 1) or by removing NaCl and adding mannitol (solutions 3, 4 and 5*a*). In some cases 50 or 75% of the NaCl was removed, but isoosmotic amounts of mannitol were added (solutions 5 and 6). As can be seen from Fig. 9, the initial relative rate of change is a function of the osmolarity of the solution only and is independent of its composition. Furthermore, the rate of change is numerically equal to the change obtained by positive osmotic gradients.

The removal of 50 to 75% of NaCl from the mucosal perfusion solution (with or without mannitol added) induced 10 to 15 mV hyperpolarization of the cells and negative potentials of 10–15 mV of

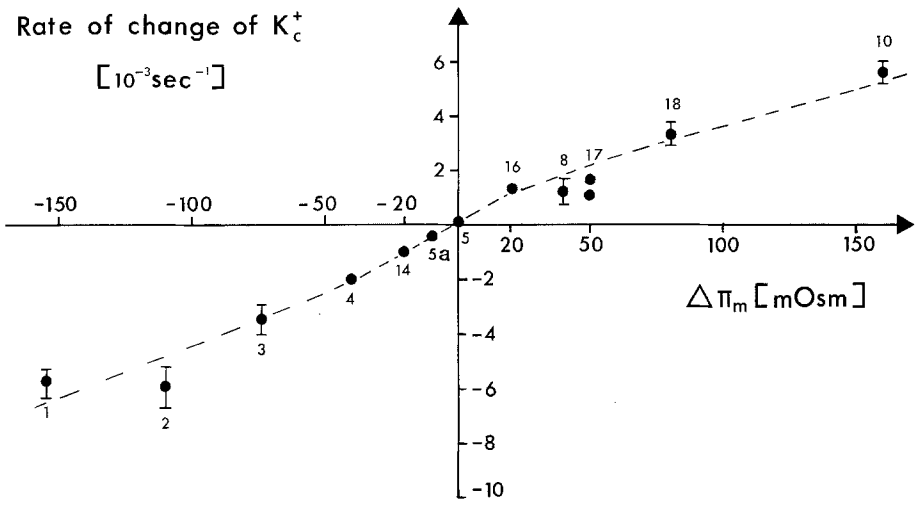

Fig. 8. The rate of change in the apparent intracellular concentration of potassium, K_c^+ [10^{-3}sec^{-1}] as a function of the osmotic gradient across the mucosal membrane caused by an abrupt change in the osmolarity of the mucosal solution. The tissue was initially bathed in solution 7 or 15, 225 mOsm (Table 1). The increased osmolarities were achieved by perfusing salines to which mannitol was added (solutions 16, 8, 17, 18 and 10). The decreases in osmolarities were achieved by perfusion with solutions from which NaCl had been removed (solutions 1, 2 and 14) or NaCl removed and mannitol added, (solutions 3, 4, 5a). Isoosmotic solutions (solution 5) were produced by removing 75% of the NaCl from solution 15 and by adding mannitol. The number at each point indicates the solution used (Table 1). The bars are SEM which is not shown if smaller than the symbols. The points are the average from 3 to 12 measurements

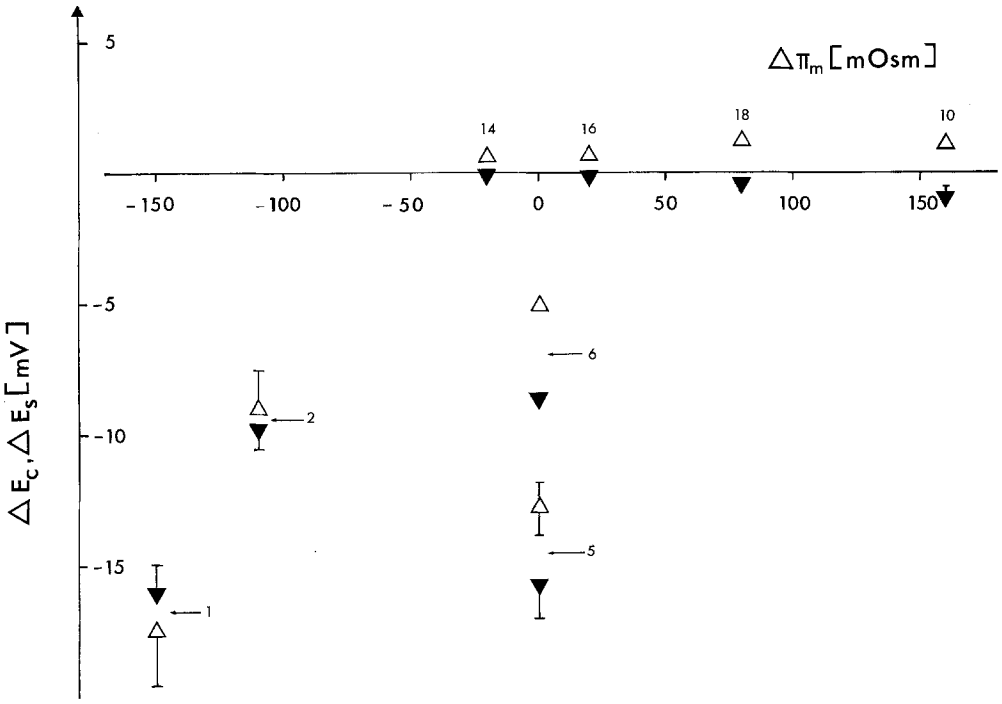

Fig. 9. The change in the intracellular electrical potential, (ΔE_c, open triangles) relative to the mucosal solution and the change in the electrical potential of the serosal solution (ΔE_c, closed triangles) relative to the mucosal solution both as a function of the change in the osmolarity of the mucosal solution. The osmotic gradients were produced by the solutions indicated by the numbers at each point (Table 1). SEM not shown if smaller than symbol; the average value was obtained from 3 to 12 measurements

the serosal compartment, both relative to the mucosal compartment (Fig. 8).

Resting values of the concentrations and potentials were the same whether cells were penetrated from the mucosal or the serosal side (*see* Table 3).

Epithelial Resistance R_T and Voltage Divider Ratio α. Under control conditions the resistance of the epithelium (R_T) was 337 ± 4.8 (13 determinations in 4 tissues), and the voltage divider ratio α (the voltage induced across the mucosal membrane divided by

the voltage induced across the serosal membrane) was 2.58 ± 0.24. In experiments where the osmolarity of the mucosal solution was increased abruptly both R_T and α increased. If the osmolarity was increased by 160 mOsm, R_T increased to 842 ± 80 ($n=7$) and α to 2.80 ± 0.21 ($n=4$); this increase was not significant.

Electroneutrality and Ion Fluxes. In order to preserve electroneutrality a negative charge-density X^- must exist in the cell. X^- [mEq liter^{-1}] can be calculated on the basis of measurements of Na^+, K^+ and Cl^- in the steady state. X^- was between 95 and 100 mEq liter^{-1} (Table 3) when based on data obtained with electrode penetrations from the serosal or the mucosal side. For simplicity the valency of X^- is assumed to be one.

Electroneutrality must also be fulfilled during the shrinkage or swelling of the cell:

$$\frac{dNa_c^+}{dt} + \frac{dK_c^+}{dt} = \frac{dCl_c^-}{dt} + \frac{dX^-}{dt}. \quad (3)$$

The initial relative rate of change of X^- can therefore be calculated as 0.0027×10^{-3} sec^{-1} mOsm^{-1} when gradients are applied from the mucosal side, and -0.039×10^{-3} sec^{-1} mOsm^{-1} when applied from the serosal side. The negative sign indicates that the concentration of X^- decreases when the cell shrinks (Table 2).

Let us assume that Na^+ and Cl^- ions remain to a first approximation inside the cell when the cell shrinks or swells in response to the imposed osmotic gradients (*see also* Discussion). The fact that K_c^+ and X^- do not concentrate at the same rate as that observed for Na_c^+ and Cl_c^- indicates that K_c^- and X^- ions disappear from the compartment from which the electrodes measure. From the figures in Table 2 it can be calculated that 98 mmol [calculated as $(0.12-0.034) \times 123/0.17$] of K_c^+ and X^- leaves the cellular compartment for each liter of H_2O when the osmotic gradient is applied from the mucosal side, and 102 mmol of K_c^+ and X^- leaves the compartment for each liter of H_2O when the gradient is applied from the serosal side.

Discussion

The *apparent* intracellular concentrations and electrical potentials were similar to those obtained in some recent studies (Reuss & Weinman, 1979; Garcia-Diaz & Armstrong, 1980) using liquid ion-exchanger electrodes. Na_c^+: 8–12 mM; Cl_c^-: 31–32 mM; K_c^+: 123–113 mM; E_c: −50 to −80 mV. The Na_c^+ was lower than that obtained by glass microelectrodes (Zeuthen, 1978; Graf & Giebisch, 1979) which recorded 20–40 mM. One explanation is that liquid ion exchanger electrodes cause less damage to the cell wall and consequently less Na^+ influx. Another explanation is that glass electrodes, via their dead space, introduce Na^+ into the cell.

Effects of Low External Osmolarities in Steady State

When cells were adapted to transport in dilute media, Cl_c^- decreased but K_c^+ remained fairly constant (Fig. 3; *see also* footnote 1). Cell volume, measured stereologically, (Bundgaard & Zeuthen, 1981) appeared to be unaffected by osmolality. This is indicative of volume-regulation and agrees with the findings of Spring et al. (1981) who found that this regulation occurs within 15 min.

The apparent K^+ concentration measured at an external osmolarity of 64 mOsm was higher than the value expected from osmotic equilibrium across the plasma membrane (Fig. 3). This leads to several unpleasant alternatives. (*i*) The electrode is sensitive to some unknown substance released inside the cell at low osmolarities. (*ii*) The cell wall is impermeable to H_2O. (*iii*) The activity coefficient for K_c^+ is larger than one. (*iv*) The reflection coefficient of the intracellular K^+ salt is less than one. The two last points will be discussed in detail:

The apparent intracellular K^+ concentration determined by microelectrodes can be taken as the lower limit of the number of osmotic active ions, if it is assumed that the activity coefficient in the cell is equal to that in the external solutions, 0.76 to 0.83 (Robinson & Stokes, 1959). This seems justified, as the electrode only measured electrochemically free ions, and as the activity coefficient for K^+, in epithelial cells, has been found to be equal to, or smaller than 0.76 (White, 1976; Armstrong, Bixenman, Frey, Garcia-Diaz, O'regan & Owens, 1979; Zeuthen & Wright, 1981). This point can be elaborated further. In order to assess the osmotic effects of electrochemically free K^+ ions, the measured activity should be divided by the activity coefficient f and multiplied by the osmotic coefficient ϕ. The ratio ϕ/f is larger than one and is an increasing function of the valency of the co-ion (Robinson & Stokes, 1959). There are now two extreme cases: if an activity coefficient of 0.76 is assumed, the osmotic effect of K^+ ions would (in the case of Cl^- being the co-ion) be obtained by multiplying the apparent concentrations by an osmotic coefficient of about 0.93 (Robinson & Stokes, 1959). In that case, however, Cl^- ions would exert an equally large osmotic effect as K^+ ions and the osmolarity of K^+ ions

alone would indeed *be* a lower estimate of the total intracellular osmolarity. At the other extreme, where the co-ion is multivalent and exerts no osmotic effect, the ratio ϕ/f would be much larger than one, because f decreases faster with increasing valency than does ϕ. In this case the concentration would again be a lower estimate of the osmolarity.

The imbalance in K_c^+ across the cell membrane was dependent on the supply of O_2. If the mucosal solution was bubbled with N_2 instead of O_2, the K^+ concentration fell to what could be expected from osmotic equilibrium (Fig. 4). Ouabain applied to the serosal side also changed K_c^+ towards equilibrium values. The existence of an intracellular hyperosmolarity was corroborated by some preliminary measurement of the osmolarity of the tissue in a Ramsay-Osmometer (Zeuthen, 1981*a*).

If an osmotic imbalance exists across the cell membrane, the question is whether it will affect the fluxes of H_2O. This will depend on the properties of the membrane, especially the reflection coefficient of the intracellular K^+ salt. The apparent intracellular concentration of Cl^- was about 35 mM at normal osmolarities, but decreased well below 10 mM when cells were adapted to dilute solutions. Thus, the low intracellular concentration of Cl^- indicates that the majority of intracellular anions are large and organic. It is possible that these anions are impermeable to the cell membrane. Consequently the intracellular K^+ salt would be impermeant and have a reflection coefficient of one and an effective osmotic gradient would exist across the cell membrane and have the direction for transport of H_2O into the cell. It is known from a number of studies [*see reviews* by MacKnight & Leaf (1977) and Hoffmann (1977)] that osmotic equilibrium exists across cell membranes at plasma osmolarities. No studies, however, deal with low external osmolarities, nor do they use epithelial cells exclusively.

L_p Determined from the Rate of Change in Na_c^+ and Cl_c^-

When the osmolarity of the mucosal or serosal solution was changed abruptly, the initial relative rates of change in Na_c^+ and Cl_c^- were equal and linearly related to the magnitude of the osmotic gradient in the range -20 mOsm to $+80$ mOsm (Fig. 7). If it is assumed that Na^+ and Cl^- ions remain inside the cell during the first phases of swelling or shrinkage, then their rates of change will reflect the rate of H_2O efflux or influx. The L_ps which were obtained were 1.6×10^{-3} cm sec^{-1} osm^{-1} for the serosal membrane and 0.68×10^{-3} cm sec^{-1} osm^{-1} for the mucosal membrane, when a cell weight of 40 μm was assumed. These values are close to those obtained by Spring et al. (1981): 2.4×10^{-3} sec^{-1} osm^{-1} and 1.1×10^{-3} cm sec^{-1} osm^{-1}, which were derived directly by optically observing the rate of shrinkage or swelling of the cell. Thus the relative rates of change of Na_c^+ and Cl_c^- were equal. This supports the notion that Na^+ and Cl^- remain inside the cells during the initial phases of the cell shrinkage or cell swelling. The apparent intracellular concentration of the two ions are different (Table 2). Only if they remain intracellularly could their relative rates of change be expected to be equal.[2] If the small difference between the optical and the electrochemical estimates of the L_p is real, it means that Cl^- and Na^+ fluxes are induced across the cell membranes.

Rate of Change of K_c^+

The initial rate of change of intracellular concentration of K^+ was only one-tenth of the rate of change observed for Na_c^+ and Cl_c^- when the osmotic gradient was imposed from the serosal side, and one-fifth when the osmotic gradient was imposed from the mucosal side (*see* Fig. 7). This means that K^+ ions disappear from the compartment in which the electrode measures. Consequently K^+ ions are either compartmentalized or sequestered inside the cell or, as shall be discussed on p. 119, the H_2O fluxes cause movements of K^+ across the cell membrane. It can be calculated from Fig. 7, Table 3 and p. 117 that approximately 102 mmoles of K^+ leave or enter the cellular compartment per liter of H_2O in the experiment where H_2O fluxes are induced across the serosal membrane, and 98 mmoles liter^{-1} in the experiment where H_2O fluxes are induced across the serosal membrane.

It is unlikely that the induced K^+ fluxes are mediated directly by any change in the gradients of electrical potential across the membranes: (*i*) These changes were maximally ± 2 mV (Fig. 8 and p. 115) and membrane resistance would need to be as low as 1–5 Ω cm^2 to explain the fluxes. Current estimates are of the order 1000–4000 Ω cm^2 (Frömter 1972; Reuss & Finn 1975; 1977). This is probably an overestimate (Zeuthen, 1976; Boulpaep & Sackin, 1980; Zeuthen, 1981*c*) but hardly by three orders of magnitude, although Frömter, Suzuki, Kottra & Kampmann (1981) have now found that the resistance of the serosal membrane is as low as 130 Ω cm^2. (*ii*) The changes observed in K_c^+ were

[2] The serosal effects are probably underestimated due to the extensive foldings of the serosal membrane; the mucosal effects are probably overestimated, because in some preliminary experiments changes in the osmolarity of the mucosal solution are seen to be reflected instantaneously in the lateral spaces; thus it might be difficult to expose exclusively the mucosal membrane.

positive when the osmotic gradient was positive and equally large but negative when the osmotic gradient was negative (Fig. 9). The change in electrical potential inside the cell, however, was approximately +2 mV when positive osmotic gradients were used, but about −15 mV when negative gradients were used (Fig. 8). Thus the symmetry in the response of K_c^+ indicates that the change in electrical potential has no effect on the initial rate of change in K_c^+. (*iii*) When the mucosal solution was changed from normal saline to one with 75% of the NaCl replaced by mannitol and with an unchanged osmolarity, K_c^+ did not change even if E_c changed by −15 mV (Figs. 8 and 9). Thus, changes in E_c do not initially induce changes in K_c^+. It can be concluded that the driving force for removal of K^+ from the cellular compartment can not be explained by electrodiffusion alone.

Induced Fluxes of Anions

The requirement of electroneutrality dictates that the loss (or gain) of K^+ from the cellular compartment is accompanied by an equivalent loss (or gain) in the intracellular density of negative charge from ions other than Cl^-. The rate of change of this unknown anion X^- (meq liter^{-1}) is calculated in Table 3; for simplicity it is assumed that this anion is univalent. It can be seen that X^- remains relatively constant when the cell shrinks or swells.

Due to electroneutrality the flux of K^+ from (or into) the cellular compartment must be accompanied by an equivalent flux of ions. An efflux may not simply be in the form of the salt KX; X^- may also combine with H^+, which would free HCO_3^- to cross the membrane together with K^+. Another possibility is a K^+/H^+ exchange. The finding that the neutral flux of K^+ and the unknown ion occurs without any appreciable change in the intracellular electrical potential E_c shows that the mechanism of permeation probably is not purely electrodiffusive. If so, a diffusion potential would be expected to arise in order to cause the flux of negative charge to equal the flux of positive charge across the membrane. In this context it is interesting to note that cell shrinkage, induced by removal of extracellular Na^+ (Spring & Hope, 1979), causes no appreciable change in E_c (Reuss, 1979; Garcia-Diaz & Armstrong, 1980).

Nature of the H_2O Fluxes Across the Cell Membrane

From the steady-state experiments it can be concluded that the influx of H_2O, at least the low external osmolarities, can be accounted for by osmosis. With an L_p of the order of 10^{-3} cm sec^{-1} osm^{-1} and an intracellular hyperosmolarity of 35 mOsm originating from the excess of K_c^+, the influx will be calculated as 126 µl cm^2 hr^{-1}. This flux is of the same order as the volume transport observed at the low osmolarities, about 50 µl cm^{-2} hr^{-1} (Fig. 2).

The nonsteady state experiments suggested that the osmotically induced fluxes of H_2O induced a neutral, co-flux of K^+ and anions. If the fluxes of ions and water were through the *same* pathway, irreversible thermodynamics (Kedem & Katchalsky, 1963*a*) show that a flux of K^+ ions and anions across the membrane will induce a flux of H_2O. Given the distribution of K^+ across the membrane, the magnitude of induced H_2O flux will be assessed below:

Consider a cell membrane consisting of a semipermeable cell membrane which separates two solutions of permeant K^+ ions, permeant anions B^-, impermeant Na^+ ions and impermeant ions of a charge density X^-. Kedem and Katchalsky (1963*a*) show that the permeation of ions and water across the membrane can be expressed by a rate of volume transport J_v, a rate of salt transport J_s, the salt being KB, and a current I. As the flux of KB did not generate any potential difference across the membrane, the current can be neglected:

$$J_v = L_{11}(-\Delta\pi) + L_{12}(\Delta\pi_s/C_s) \tag{1A}$$

$$J_s = L_{21}(-\Delta\pi) + L_{22}(\Delta\pi_s/C_s). \tag{2A}$$

The hydrostatic pressure difference across the membrane is assumed to be zero, $\Delta\pi$ is the osmotic pressure difference, and C_s is per definition

$$C_s = \Delta\pi_s/\ln(a_c/a_o)\cdot RT \tag{3A}$$

where $\Delta\pi_s$ is the osmotic pressure difference due to the permeable salt, a is the activities of the salt on the outside o, or on the inside c of the membrane.

L_{11} and L_{21} were determined from the nonsteady state experiments where an osmotic gradient $-\Delta\pi$ was imposed by means of mannitol; from Eq. (1A):

$$RT\cdot L_{11} = RT\cdot\Delta J_v/-\Delta\pi = 1.1\cdot 10^{-3}\ \text{cm sec}^{-1}\ \text{osm}^{-1} \tag{4A}$$

and from Eq. (1A) and (2A) and the fact that $L_{12} = L_{21}$:

$$L_{21}/L_{11} = L_{12}/L_{11} = \Delta J_s/\Delta J_v = 102\ \text{mmoles liter}^{-1}. \tag{5A}$$

Having determined the coefficients we can return to Eq. (1A) applied for the steady state, inserting Eq. (3A) and using $\Delta\pi = RT\Delta C$:

$$J_v = -RTL_{11}\,\Delta C + RTL_{12}\cdot\ln(a_o/a_c). \tag{6A}$$

The first term equals the osmotic influx +126 µl cm^{-2} hr^{-1} calculated above. The second term describes the H_2O efflux caused by the permeation of the salt. With $a_{KB} = \sqrt{a_K\cdot a_B}$ and HCO_3^- as the permeable anion B the following example can be calculated: $K_c^+ = 105$ mM, $HCO_{3,c}^- = 10$ mM (Khuri et al., 1974), $K_o^+ = 3$ mM and $HCO_{3,o}^- = 2.4$ mM. With these figures the last term of Eq. (6A) can be calculated as −975 µl cm^2 hr^{-1}. If Cl^- is the anion the last term calculates as −435 µl cm^{-2} hr^{-1}. As long as B^- is less than 150 mM either intra- or extracellularly, the efflux of H_2O will exceed the osmotic influx.

Thus the coupled efflux of H_2O could be of the same order as the osmotic influx; in the steady state K^+ and B^- should enter the cell via one pathway

(or be generated in the cell, $B^- = HCO_3^-$) and leave the cell together with H_2O.[3]

It should be emphasized that irreversible thermodynamics make no assumption about the physical structure of the membrane. It is also difficult to visualize a pore which could cause this large coupling between salt and water. The only context in which such couplings are described is in connection with the Teorell-Meyer-Sievers model of membranes discussed by Kedem and Katchalsky (1963*b*). According to this theory K^+ should permeate through an aqueous pore with fixed negative charges and B^- through another aqueous pore with fixed positive charges. In the present context the charge densities of the two pores should be matched and have a charge density of the order of 100 mEq/liter in order to account for the observed neutral coupling and the flux of water.

Conclusion

Na^+ and Cl^- remain inside the cell when H_2O fluxes are induced across the membrane. Thus their initial rate of change is a measure of the water permeabilities of the membranes.

Some irregularities in the behavior of the intracellular K^+ activity are, however, demonstrated. Various interpretations are possible. I have focused on the interpretation which accepts that the K^+ concentration inside the cell can be higher than the value expected from osmotic equilibrium across the cell membrane, and H_2O influx effected by this imbalance could be compensated by a H_2O efflux coupled to the passive efflux of a K salt. The driving forces for the loss of K^+ salt from the cell during shrinkage, or gain during swelling, are unknown. The forces are probably not electrical.

It is possible that the osmotic influxes and coupled effluxes of water described here are part of the cells' volume-regulatory mechanisms. Whether transepithelial volume transport is affected will depend on the exact distribution of L_p, coupling and reflexion coefficients at the two membranes. Obviously any coupled efflux of H_2O will predominate in the membrane across which the efflux of K^+ (and accompanying anions) is largest. In the model for K^+ transport described by Koefoed-Johnsen and Ussing (1958) K^+ ions are envisaged to enter the cell actively via a Na^+/K^+ pump, only to leak out passively across the serosal membrane; this mode of operation has been confirmed to operate in a leaky epithelia, e.g. choroid plexus (Zeuthen & Wright, 1981). The calculations above imply that the passive K^+ flux would be large enough to induce an efflux of H_2O which was of the same order as the transepithelial H_2O flux.

[3] One could argue that the coupling was indirect and mediated by an intracellular pressure. In that case the hydraulic permeability of the cell membrane would need to be at least two orders of magnitude larger than the osmotic permeability in order for the pressure to be in a physiologically acceptable range, say 5 cm H_2O. Such effects are suggested in the gastric mucosa (Moody & Durbin, 1969).

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