Cotransport of K^+, Cl^- and H_2O by membrane proteins from choroid plexus epithelium of *Necturus maculosus*

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1 The interaction between K^+, Cl^- and H_2O fluxes was studied in the ventricular membrane of the choroid plexus epithelium from *Necturus maculosus* by means of ion-selective microelectrodes.

2. Three experimental strategies were adopted: the osmolarity of the ventricular solution was increased abruptly by addition of (i) mannitol or (ii) KCl; (iii) Na^+ in the ventricular solution was replaced isosmotically by K^+.

3. The mannitol experiments showed that H_2O had two pathways across the ventricular membrane. One was purely passive, with a water permeability, L'_p, of $0{\cdot}64 \times 10^{-4}$ cm s^{-1} (osmol $l^{-1})^{-1}$. This operated in parallel with an ion-dependent pathway of similar magnitude which was abolished in Cl^--free solutions.

4. When KCl was added there was a flow of H_2O *into* the cell. Surprisingly, this took place despite the osmotic gradient which favoured an efflux of H_2O. The effect was blocked by frusemide (furosemide), in which case KCl had the same effects as applications of NaCl or mannitol.

5. Replacement of Na^+ with K^+ caused an influx of H_2O. This flux could proceed against osmotic gradients implemented by mannitol.

6. The present data and those of earlier publications show that the interdependence of the fluxes of K^+, Cl^- and H_2O in the exit membrane can be described as cotransport. The fluxes have a fixed stoichiometry of 1:1:500, the flux of one species is able to energize the flux of the two others, and the transport exhibits saturation and is specific for K^+ and Cl^-.

7. A molecular model based upon a mobile barrier in a membrane spanning protein gives an accurate quantitative description of the data.

Leaky epithelia such as kidney proximal tubules, small intestine, gall-bladder, gland acini, etc. daily transport up to 200 l of water in humans. Yet the mechanism of transepithelial transport of water in these epithelia is not well understood. There are at least two problems. (i) How can leaky epithelia transport water despite large opposing osmotic gradients? Dog small intestine has been reported to be able to transport against 200 mosmol l^{-1}, while at the lower end kidney proximal tubule can transport against 30–60 mosmol l^{-1} (see recent reviews by Tripathi & Boulpaep, 1989; Zeuthen, 1992; Zeuthen & Stein, 1994). Isotonic transport, the special case with no external driving forces, is not understood either. (ii) There is no relation between the epithelial water permeability and transport rate. In small intestine, for example, the water permeability is low and it would require a transepithelial gradient of some 100 mosmol l^{-1} to pull water across the epithelium at physiological rates. Yet such gradients are not observed *in vivo*. On the other hand, the mammalian proximal tubulus possesses a high water permeability and transepithelial transport of water at physiological rates could in principle be obtained by gradients of a few milliosmoles per litre. However, the more permeable the epithelium, the less able it is to transport against differences in osmolarity because of the osmotic backflux. Models which attempt to circumvent these problems by assuming an internal diffusional barrier and small intraepithelial osmotic or hydrostatic gradients (i.e. Weinstein & Stephenson, 1981) are also untenable (see Zeuthen & Stein, 1994). This brings us back to (i).

Transport of water is secondary to the transport of ions so the central question is: which particular ion transport

mechanism or mechanisms energize the transepithelial transport of water? I have shown (Zeuthen, 1991*a*, *b*, 1992, 1993*a*, *b*) that one mechanism could be located in the basolateral membrane (ventricular membrane in choroid plexus epithelium) where electroneutral cotransport of K^+ and Cl^- couples to the flux of water in membrane proteins in which the fluxes are co-localized.

Experimental evidence for this scheme was obtained mainly from choroid plexus and to a lesser degree from gall-bladder (Zeuthen, 1981, 1982, 1983). The choroid plexus epithelium is similar to other leaky epithelia (Johanson, 1988) but has the advantage that the membrane across which K^+, Cl^- and H_2O leave the cell (and enter the cerebrospinal fluid) is freely available for rapid shifts in bathing solutions and for application of microelectrodes.

The description of the interaction between K^+, Cl^- and H_2O is completed in the present investigation where it is shown that the interaction can be described, at least formally, as cotransport: a downhill flux of each of the involved species can energize the uphill flux of each of the other and the stoichiometry is fixed. Thermodynamic considerations show that there is sufficient energy in the K^+ and Cl^- gradients to explain observed movements of H_2O. Finally, the quantitative nature and extent of the present data allow a realistic molecular model to be set up based on the mobile barrier model for primary and secondary active transport suggested by Mitchell (1957, 1990). Preliminary reports have been presented (Zeuthen, 1993*a*, *b*).

METHODS

The experimental approach described in this paper was to insert double-barrelled ion-selective microelectrodes into the epithelial cells of the choroid plexus. Once a stable recording of intracellular values of either K_i^+, Cl_i^-, Na_i^+ or cell volume, V, (via Ch_i^+, see below) was obtained, the composition of the ventricular solution was changed abruptly and the initial rate of change was recorded (Fig. 1).

The animals and experimental chamber are identical to those previously described (Zeuthen, 1982, 1991*a*, *b*). Briefly, the plexus from the fourth ventricle was taken out and sandwiched between two nylon nets in one of which a triangular hole was cut. The epithelial side of the plexus facing upwards protuded through this hole into the ventricular chamber, which consisted of the drop of solution held under the water immersion objective lens. The drop was renewed more than 10 times every minute. At the surface of the epithelial cells the initial rate of change of solution was 13–20% s^{-1}. For comparison the initial rate of change intracellularly was typically about 1·4% s^{-1} (Zeuthen, 1991*a*). Consequently the rate-limiting step is the ventricular membrane and rates of changes measured intracellularly reflect largely properties of this membrane (see below). The change in ventricular solution was 90% complete in, typically, 6 s. It can be estimated that unstirred layers are about 40 μm thick which gives a diffusional delay of about 0·5 s. Thus the rate of change of solution is largely determined by convection. For a detailed discussion, see Zeuthen (1991*a*) and LaCour & Zeuthen (1993). Using *Necturus* gall-bladder, I obtained the same values of the permeability, L_p, of the apical membrane as Cotton, Weinstein & Reuss (1989) and Spring, Hope & Persson (1981) (see Zeuthen, 1991*a*).

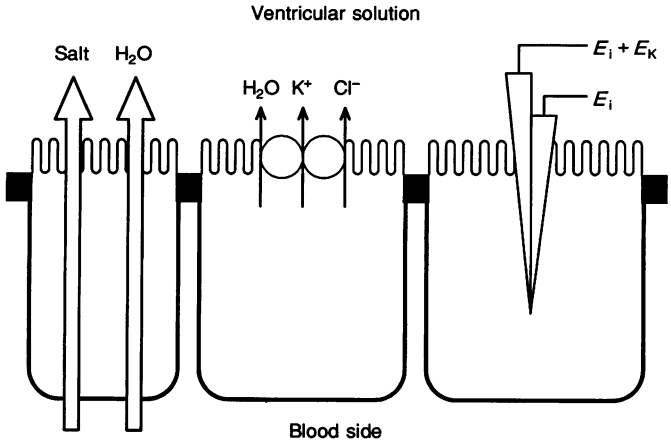

Figure 1. The thesis and the experimental approach
The epithelial cells from the choroid plexus transport salt and H_2O from the blood side into the ventricle (left). According to the localization of the brush border and the tight junctions this epithelium is sometimes designated backward facing as compared with, for example, gall-bladder. The thesis of this paper is that the exit of H_2O and KCl is mediated by cotransport by the same protein (middle). The coupling between salt and water transport is studied by means of double-barrelled microelectrodes which record the membrane potential (E_i) and the ion concentrations (expressed by the electrode potential, E_K); changes in cell volume can also be recorded. Fluxes are initiated by abrupt changes in the composition of the ventricular solution. Initial intracellular changes largely reflect the properties of the ventricular membrane; the imposed gradients across the blood-facing membrane are small and the membrane is relatively impermeable.

The double-barrelled ion-selective microelectrodes were identical to those previously described (Zeuthen, 1980). In the present studies I used the Corning ion exchanger 477317 for K^+ measurements, Corning 477913 for Cl^- measurements (New York, NY, USA) and Fluka No. 71176 (Fluka Chemie, Buchs, Switzerland) for Na^+ measurements. The Na^+ recordings were corrected for the influence of the extracellular Ca^{2+} concentrations (Zeuthen, 1982), which means that the intracellularly recorded values were multiplied by 0·94. When changes in cell volume were to be recorded, 1–3 mmol l^{-1} choline chloride (ChCl) was added to the ventricular solution. After about 30 min the intracellular concentration of Ch^+ (Ch_i^+) had attained values between 2 and 8 mmol l^{-1}, which were readily detectable with the K^+ electrode; the electrode 'sees' Ch^+ more than 66 times better than K^+. Intracellular Ch_i^+ therefore served as a marker of cell volume (Zeuthen, 1991*a*).

The tips of the electrodes were ground in a jet of water into which SiO_2 (silicic acid, 100 mesh; Mallinckrodt Chemical Works, St Louis, MO, USA) was suspended, 1:5 v/v. The solution was stirred magnetically in a conical flask and was allowed to flow out of the flask via a silicone tube (i.d. 1 mm), the end of which was held 50 cm below the level of the solution in the flask. The tips of the electrodes were held at a right angle for 2–4 s in the emerging jet of fluid. Electrodes initially had an impedance of the reference barrel of 200–400 MΩ when filled with 1 mol l^{-1} KCl or potassium acetate and measured in control solution. The grinding reduced the impedance by a factor of no more than 10 and optimized the electrode's sensitivity to Ch_i^+. The grinding also resulted in more stable and longer lasting cell penetrations.

The amount of a given ion will be taken as the cell volume, V, times the concentration of the ion. Using K^+ as an example, the amount of readily exchangeable intracellular K^+ is $V \times K_i^+$. The derivate of this in relation to time t equals the flux:

$$J_K = V K_i^+ \left(\frac{dK_i^+}{K_i^+ dt} + \frac{dV}{V dt} \right). \quad (1)$$

V can be expressed as cell volume per square centimetre of epithelium, h, which was taken as 10 μm. In that way J_K^+ is expressed per square centimetre of epithelium. $dK_i^+/K_i^+ dt$ equals $dE_K/S dt$ where dE_K is the change in potential of the K^+-sensitive barrel and S is the sensitivity of the electrode which was about 23 mV (25·3 mV for an ideal electrode). $dV/V dt$ was obtained from eqn (2) described below. The flux of water J_{H_2O} is expressed per square centimetre of epithelium:

$$J_{H_2O} = -\frac{h}{V}\frac{dV}{dt} = \frac{h}{S}\frac{dE_{Ch+K}}{dt}, \quad (2)$$

where E_{Ch+K} is the electrode potential due to Ch^+ plus the K^+ ions: $E_{Ch+K} = S \ln (a Ch_i^+ + K_i^+)$. Here a is the ratio of the sensitivities to Ch^+ and K^+. It is sometimes required to correct for changes in K_i^+ when the true changes in Ch_i^+ and thereby V are to be evaluated (see Zeuthen, 1991*a*).

The initial rate of change measured intracellularly is determined by the properties of the ventricular membrane

This follows from two facts. Firstly, the perturbation in the driving forces implemented by the abrupt changes in the ventricular solution is presented fully across the ventricular membrane. However, at the same time, the changes in driving forces across the basolateral or the abluminal membrane are small; they depend on the slow changes inside the cell. Secondly, the permeabilities to ions and H_2O across the abluminal membrane are not significantly larger, in most cases actually smaller, than across the ventricular membrane.

Consider the experiments where L_p is determined. Here the osmolarity of the ventricular solution is changed abruptly, say by the addition of 100 mmol l^{-1} mannitol (Fig. 2 in Zeuthen, 1991*a*). This induces an immediate efflux of water from the cell which is apparent a few seconds after the change of the ventricular solution, i.e. at a time when the intracellular osmolarity has changed less than about 4 mosmol l^{-1}. At that time, the change in the ventricular solution osmolarity is about 50% complete, equivalent to a gradient of 50 mosmol l^{-1}. Thus at any time the gradient across the ventricular membrane is more than 10 times larger than that across the abluminal membrane. Therefore, the L_p of the abluminal membrane should be 10 times higher than that of the ventricular membrane in order for our estimate to be in error by a factor of two. A realistic estimation of the L_p of the abluminal membrane shows that it is close to that of the ventricular membrane: a steady-state water flux across the epithelium is obtained when the osmotic gradient across the ventricular membrane is close to that across the abluminal membrane (see Fig. 2, Zeuthen, 1991*a*). Consequently the initial rate of change in cell volume obtained when the ventricular osmolarity is altered mainly reflects the L_p of the ventricular membrane. For a more elaborate discussion, see La Cour & Zeuthen (1993). Unstirred layers at the basolateral membrane are unlikely to affect this argument. Firstly, they are not likely to be quantitatively significant (Pedley & Fischbarg, 1980; Hill, 1980). Secondly, if unstirred layers should build up at the basolateral membrane during the application of mannitol and in effect decrease its water permeability, then the rate of volume change recorded on application of mannitol should be far smaller than the one measured on removal of the mannitol, which it is not.

Consider next the experiments where K^+ is increased in the ventricular solution. This gives rise to a cellular depolarization and the question is whether there is any influx across the basolateral membrane as a result. This was dealt with in some detail by Zeuthen, Christensen, Bærentsen & La Cour (1987*a*) who showed that the flux of K^+ across the basolateral membrane was about 30 times smaller than the flux across the ventricular membrane.

Consider finally the movement of Cl^-. The conductivity of the ventricular membrane is mainly due to K^+ ions (Zeuthen & Wright, 1981) and it can be estimated that the Cl^- conductance is about 25% of the K^+ conductance (Zeuthen *et al.* 1987*a*; Zeuthen, Christensen & Cherksey, 1987*b*). Furthermore, there is no reason to suspect that the basolateral membrane possesses any Cl^- conductance. It is therefore fair to neglect electrodiffusion of Cl^-. The evaluation of the Cl^- transport is, however, complicated by the existence of Cl^-–HCO_3^- cotransport in the ventricular membrane (Zeuthen, 1987).

The compositions of the solutions are given in Table 1. The salts are of analytical quality and the osmolarities were checked using a freezing point depression osmometer (Labex-Roebling, Berlin, Germany). Frusemide (furosemide) was used in concentrations of 1–2 × 10^{-4} mol l^{-1}.

Statistics

Values are given as means ± S.E.M. All comparisons were performed by means of Student's t test. The statistics of the fluxes were calculated from the statistics of the changes in the ion concentrations under the assumption that the changes in volume are a constant correction.

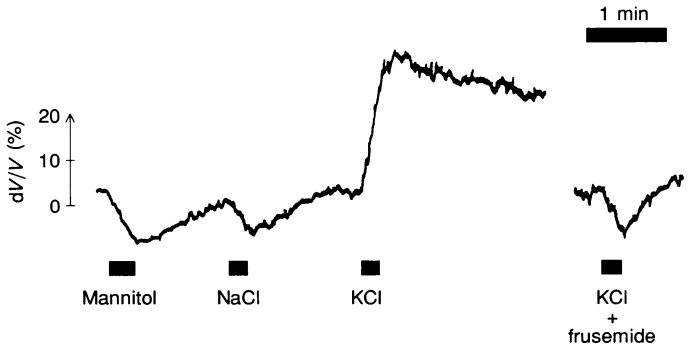

Figure 2. Osmotic behaviour of the epithelial cells
Cellular volume changes in response to the addition of 100 mmol l^{-1} mannitol, 50 mmol l^{-1} NaCl, 50 mmol l^{-1} KCl and 50 mmol l^{-1} KCl to which 0·1 mmol l^{-1} frusemide had been added. The recordings are from the same cell. The last event was recorded 7 min after the KCl event, at which time the cell had returned to its control volume. Please note that KCl causes a cell swelling, although the osmotic gradient favours a cell shrinkage.

RESULTS

In order to study the osmotic properties of the exit membrane of the choroid plexus epithelium, the osmolarity of the ventricular solution was increased by the addition of NaCl, KCl or mannitol. A surprising finding was that whereas NaCl or mannitol caused a normal osmometric shrinkage of the cell, increases in the osmolarity induced by KCl caused an immediate cell *swelling* (Fig. 2). This means that H_2O under those conditions moved from a higher to a lower osmolarity. The following experiments were designed to clarify the mechanism behind this effect and are in five parts. (1) There is a short description of the electrochemical parameters of the cell in steady state. The fluxes of H_2O, K^+ and Cl^- ions were studied when mannitol (2), NaCl or KCl (3) were added to the ventricular solution to increase its osmolarity or when Na^+ (4) in the ventricular solution was replaced isosmotically by K^+. (5) Finally I tested whether Na^+ has any role in the mechanisms studied.

(1) Electrochemical state of the cell

The electrochemical parameters of the cell in steady state bathed in control solution (Solution 1, Table 1) were the same as in previous studies (Zeuthen, 1991*a*, *b*, 1993*b*). The intracellular electrical potential, E_i, was $-82{\cdot}4 \pm 1{\cdot}9$ mV ($n = 26$). The transepithelial potential was less than 2 mV in open circuit (ventricular side negative) (Zeuthen, 1991 *a*, *b*); however, the epithelium was short-circuited in the present experiments. The apparent intracellular K^+

Table 1. Composition of solutions (mmol l^{-1})

	Solution No.	Na^+	Cl^-	K^+
Control	1	106	110	2
ΔKCl	2	106	111·5	3·5
	3	106	113·1	5·1
	4	106	116·3	8·3
	5	106	122·5	14·5
	6	106	135	27
	7	106	160	52
ΔK	8	93	110	15
	9	80	110	28
	10	53	110	55
Cl^- free	11	106	0	2
Na^+ free	12	0	110	2

The solutions also contained (mmol l^{-1}): 1 Mg^{2+}, 1 Ca^{2+}, 1 SO_4^{2-}, 10 glucose. In the Cl^--free solution Cl^- was replaced by isethionate and had 4 mmol l^{-1} calcium gluconate added. The Na^+-free solution was based on NMDG-Cl. The solutions were buffered with 5 mmol l^{-1} Hepes and bubbled with atmospheric air to give a pH of 7·48.

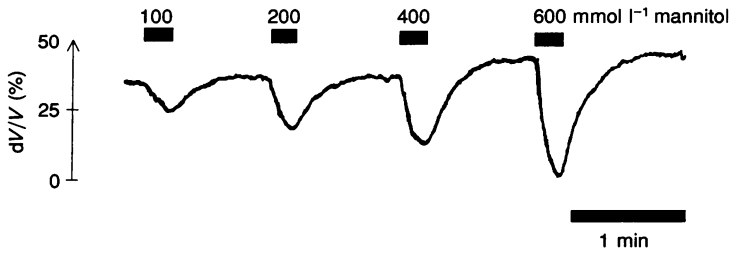

Figure 3. Cell shrinkage in Cl^--free solutions
Shrinkage was induced by 100, 200, 400 and 600 mmol l^{-1} mannitol added to the ventricular solution. The membrane potential was −96 mV throughout and changed only slightly during the application of the mannitol: a hyperpolarization of 0·5 mV at 400 mmol l^{-1} and one of 3 mV at 600 mmol l^{-1} were observed (data not shown).

concentration (K_i^+) was 83·6 ± 5·0 mmol l^{-1} ($n = 21$). With an equilibrium potential for K^+ of −94 mV, K^+ was accumulated intracellularly against the electrochemical gradient. The apparent intracellular Cl^- concentration (Cl_i^-) was 38·7 ± 2·6 mmol l^{-1} ($n = 15$). The equilibrium potential for Cl^- was −26·4 mV; thus Cl_i^- is also actively accumulated. The apparent intracellular Na^+ concentration (Na_i^+) was 15·0 ± 1·5 mmol l^{-1} ($n = 30$) while in cells adapted to Cl^--free solutions (Solution 2, Table 1) it was 12·9 ± 1·3 mmol l^{-1} ($n = 28$). In either case there is an active extrusion of Na^+ from the cell.

(2) Effects of addition of mannitol

The water transport across the ventricular membrane took place via two parallel pathways: a passive osmotic pathway which could be determined in Cl^--free ventricular solutions and one in which the water permeation depended on the presence of Cl^-.

Water fluxes (J_{H_2O}) with Cl^- absent

The cells were bathed on the ventricular side in Cl^--free solution (Solution 2, Table 1) for more than 5 min. Then the ventricular solution was changed abruptly to a Cl^--free solution to which mannitol had been added. An example of a measurement is given in Fig. 3. The effects on cell volume V (or J_{H_2O}, eqn (3)) are compiled in Table 2 and in Fig. 4 (●). J_{H_2O} was proportional to the osmotic gradient; additions of 100, 200, 400 or 600 mmol l^{-1} mannitol were tested. The experiments define a water permeability of $0{\cdot}64 \times 10^{-4} \pm 0{\cdot}03 \times 10^{-4}$ cm s^{-1} (osmol l^{-1})$^{-1}$ which shall be called L_p'.

Cell swellings were also tested. The water permeability obtained when the Cl^--free ventricular solution was diluted abruptly by a factor of two was $0{\cdot}44 \times 10^{-4} \pm 0{\cdot}08 \times 10^{-4}$ cm s^{-1} (osmol l^{-1})$^{-1}$ ($n = 5$).

Water fluxes (J_{H_2O}) with Cl^- present

The cells were initially bathed in control solution (Solution 1) whereafter the osmolarity of the ventricular solution was increased abruptly by the addition of 50, 100, 200, 400 or 600 mmol l^{-1} mannitol. The results are given in Table 3 and in Fig. 4 (○).

J_{H_2O} was proportional to the mannitol gradient for additions up to 200 mmol l^{-1}, equivalent to a water permeability of around $1{\cdot}3 \times 10^{-4}$ cm s^{-1} (osmol l^{-1})$^{-1}$. Surprisingly, additions of 400 mmol l^{-1} mannitol produced an efflux of H_2O which was not significantly larger than that obtained with 200 mmol l^{-1}. However, additions of 600 mmol l^{-1} mannitol produced an efflux which was significantly larger (with each cell as its own control) than

Table 2. Fluxes of ions and H_2O across the ventricular membrane in response to mannitol challenges (Cl^- absent)

Solution change	J_{H_2O} (10^{-9} l cm^{-2} s^{-1}) / dV/Vdt (10^{-3} s^{-1})	dK_i^+/dK_i^+dt (10^{-3} s^{-1})	J_K [a] (10^{-9} mol cm^{-2} s^{-1})
11 → 11 + 100 Man	7·2 ± 0·78 (8)	6·6 ± 0·84 (7)	0·06 ± 0·08 (8)
11 → 11 + 200 Man	12·8 ± 1·0 (7)	11·3 ± 1·0 (9)	0·15 ± 0·10 (7)
11 → 11 + 400 Man	21·6 ± 1·6 (12)	16·1 ± 1·6 (9)	0·57 ± 0·16 (12)
11 → 11 + 600 Man	34·9 ± 3·1 (6)	23·4 ± 2·5 (7)	1·18 ± 0·32 (6)

[a] J_K is calculated from eqn (1) using K_i^+ of 103 mmol l^{-1} obtained for cells adapted to Cl^--free solutions (Zeuthen, 1991*b*). Quantities of mannitol (Man) added to the indicated solutions are given in millimoles per litre. Here and in subsequent tables, numbers of experiments (n) are given in parentheses.

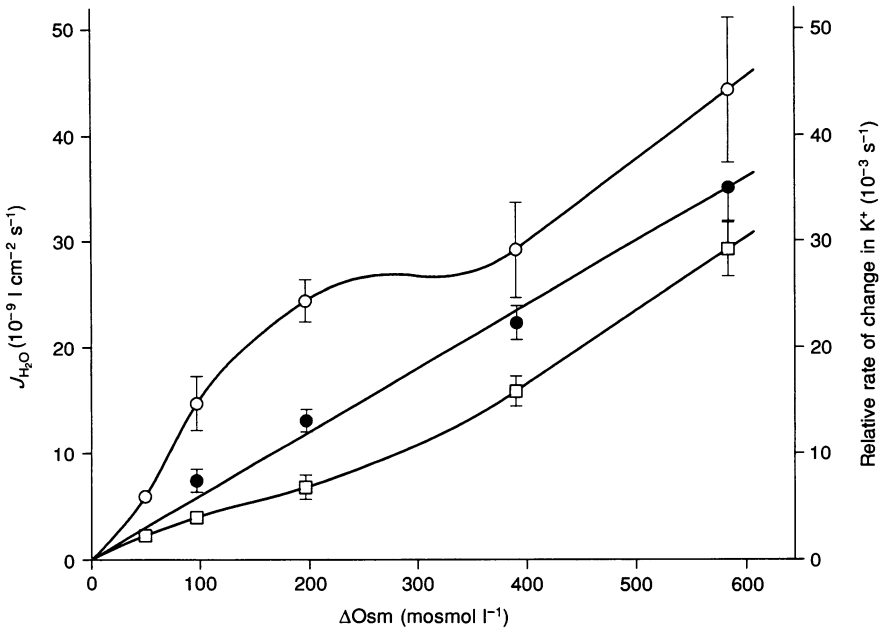

Figure 4. Cell water and K^+ changes during shrinkage
○, water flux J_{H_2O} in response to osmotic gradient (ΔOsm) across the ventricular cell membrane in cells bathed in control solutions (Table 1); ●, data from cells bathed in Cl^--free solutions (Solution 11, Table 1). The lower curve (□) shows the initial relative rates of change in K_i^+ (dK_i^+/K_i^+dt) obtained as a function of ΔOsm in control solutions.

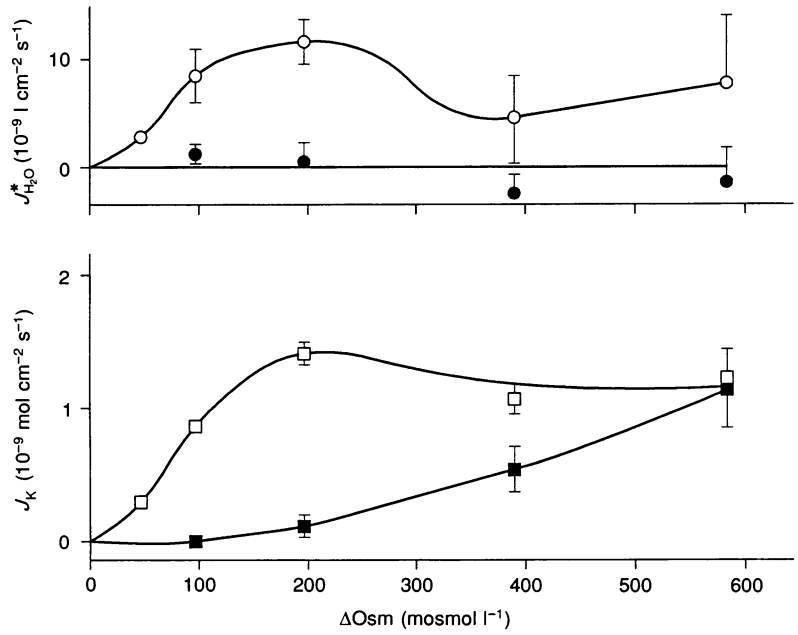

Figure 5. H_2O and K^+ fluxes in the ion-dependent pathway
Upper panel, the water flux, $J^*_{H_2O}$ (eqn (4)), in the presence (○) and absence (●) of Cl^-. Lower panel, the K^+ flux associated with the osmotic efflux of water, calculated from eqn (1), in the presence (□) and absence (■) of Cl^-.

the one obtained with both 200 and 400 mmol l^{-1}. Interestingly the line defined by the J_{H_2O} obtained at 400 and 600 mosmol l^{-1} goes through the origins of the axes and defines an L_p of 0.77×10^{-4} cm s^{-1} (osmol l^{-1})$^{-1}$ with an S.E.M. of about 15%.

The *additional* transport of water made possible by the presence of Cl^- shall be called $J^*_{H_2O}$ (○ in Fig. 5). Quantitatively, it is obtained as the difference between the total water transport J_{H_2O} and L'_p (determined in Cl^--free solutions) times the osmotic gradient applied (ΔOsm):

$$J^*_{H_2O} = J_{H_2O} - L'_p \Delta Osm. \quad (3)$$

K^+ fluxes (J_K) with Cl^- absent

When 100 or 200 mmol l^{-1} mannitol was added in the Cl^--free case, the initial relative change of K_i^+ reflected precisely the volume changes (Table 2), which means that $dK_i^+/K_i^+dt = -dV/Vdt = dCh_i^+/Ch_i^+dt$. From eqn (1) it is seen that J_K in this case is zero which means that K^+ remains inside the cell during the cell shrinkage. For the larger gradients tested, 400 and 600 mosmol l^{-1}, the difference between dK_i^+/K_i^+dt and $-dV/Vdt$ was significant ($P < 0.05$ and < 0.02 respectively). Consequently K^+ can leave the cell during shrinkage for large osmotic gradients. J_K in the Cl^--free case is compared in Fig. 5 (■) with J_K obtained in the presence of Cl^- (□). Apparently Cl^- removal from the ventricular solution abolishes or reduces J_K.

K^+ fluxes (J_K) with Cl^- present

The initial relative rate of change in K_i^+ (□ in Fig. 4) was much smaller than could be expected from the cell shrinkage. Most probably the K^+ ions are lost during the cell shrinkage via the ventricular membrane. This is demonstrated by the finding that frusemide applied simultaneously with the mannitol causes the relative rate of change in K_i^+ to approach the change in volume (Table 3). Application of frusemide this way ensures that the drug only has time to block transport processes at the ventricular membrane. The efflux of K^+ initiated by the cell shrinkage can be calculated from eqn (1). The result is shown in Fig. 5 (□). J_K exhibits saturation for gradients higher than 200 mosmol l^{-1}. When J_K is saturated, the amount of K^+ which remains inside the cell is independent of the osmotic gradient. Consequently the changes in K_i^+ during saturation reflect the volume changes and can be used to compute a water permeability of $0.68 \times 10^{-4} \pm 0.07 \times 10^{-4}$ cm s^{-1} (osmol l^{-1})$^{-1}$.

The coupling ratio $J_K/J^*_{H_2O}$

The coupling ratio, that is, the ratio between the osmotically induced K^+ flux, J_K (□, Fig. 5), and the water flux via the cotransport system, $J^*_{H_2O}$ (eqn (3)), was relatively constant for osmotic gradients implemented by additions of 50, 100, and 200 mmol l^{-1} mannitol. It ranged from 106 to 127 mmol l^{-1} (Table 3). For larger gradients (400 and 600 mmol l^{-1}) the coupling ratio was much larger. In these cases its precise value is impossible to determine from the present data since it depends critically on the precise value of L'_p. If L'_p was taken as 0.64×10^{-4} cm s^{-1} (osmol l^{-1})$^{-1}$ then the ratio was 300–400 mmol l^{-1}. If the L'_p was 0.77×10^{-4} cm s^{-1} (osmol l^{-1})$^{-1}$ the ratio calculated as infinite.

(3) Effects of addition of KCl

The osmolarity of the ventricular solution was increased by the addition of KCl. This caused the cell to swell abruptly (Fig. 6) and not to shrink as would be expected from the direction of the osmotic gradient. The rates of swelling are given in Table 4 (as $-dV/Vdt$ or J_{H_2O}) and in Fig. 7*A*. In the presence of frusemide (10^{-4} mol l^{-1}) (or bumetanide, 10^{-5} mol l^{-1}; Zeuthen, 1993*b*) the cell behaved as a normal osmometer and shrank in response to the addition of KCl (Fig. 6). The cells depolarized in response to the addition of KCl. When 1·5 mmol l^{-1} KCl was added, in the presence of frusemide, ΔE was 1.6 ± 0.5 mV ($n = 8$); with 3·1 mmol l^{-1} KCl, ΔE was 4.3 ± 0.6 mV ($n = 10$); with 6·3 mmol l^{-1} KCl, ΔE was 7.4 ± 0.8 mV ($n = 12$); with 12·5 mmol l^{-1} KCl, ΔE was 15.8 ± 1.2 mV ($n = 13$); with

Table 3. Fluxes of H_2O and ions across the ventricular membrane in response to mannitol challenges (Cl^- present)

Solution change	$\frac{J_{H_2O}}{dV/Vdt}$ (10^{-9} l cm^{-2} s^{-1}) / (10^{-3} s^{-1})	dK_i^+/K_i^+dt (10^{-3} s^{-1}) Control	dK_i^+/K_i^+dt (10^{-3} s^{-1}) +Frusemide	J_K (10^{-9} mol cm^{-2} s^{-1})	$J^*_{H_2O}$[a] (10^{-9} l cm^{-2} s^{-1})	Coupling J_K/J_{H_2O} (mmol l^{-1})
1 → 1 + 50 Man	6·2 ± 0·1 (3)	2·3 ± 0·8 (4)	4·4 ± 0·7 (6)	0·32 ± 0·06	3·0 ± 0·1 (3)	106
1 → 1 + 100 Man	14·7 ± 2·6 (7)	4·1 ± 0·6 (16)	11·5 ± 1·7 (8)	0·86 ± 0·05	8·6 ± 2·6 (7)	100
1 → 1 + 200 Man	24·3 ± 2·2 (12)	6·8 ± 0·9 (16)	15·6 ± 2·0 (6)	1·42 ± 0·07	12·1 ± 2·2 (12)	117
1 → 1 + 400 Man	29·1 ± 4·5 (10)	15·9 ± 1·4 (17)	22·3 ± 2·0 (8)	1·07 ± 0·11	4·8 ± 4·5 (10)	223[b]
1 → 1 + 600 Man	44·3 ± 6·7 (10)	29·1 ± 2·7 (10)	—	1·23 ± 0·22	7·8 ± 6·7 (10)	157[b]

Quantities of mannitol (Man) added to the indicated solutions are given in millimoles per litre.
[a] $J^*_{H_2O} = J_{H_2O} - L'_p \Delta Osm$. [b] These values were difficult to determine, see text.

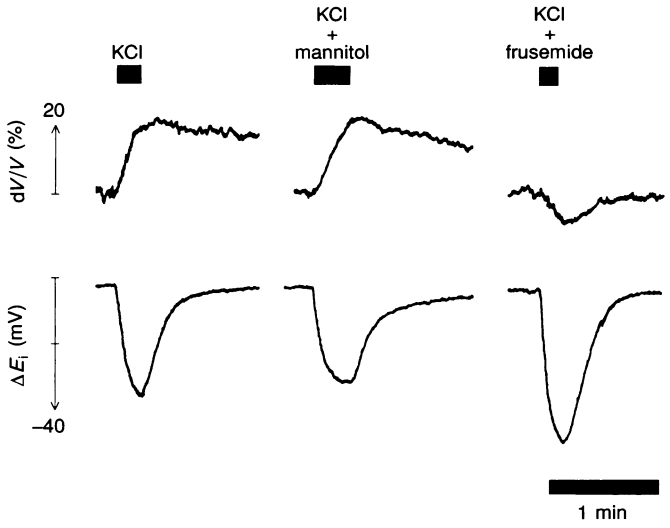

Figure 6. Effects of additions of KCl
Recordings of the relative changes in cellular volume (V) and intracellular potential (E_i) as a function of time. The first set of curves shows the effect of the increase of the ventricular osmolarity induced by 50 mmol l^{-1} KCl, the second set the effect of the addition of 50 mmol l^{-1} KCl + 50 mmol l^{-1} mannitol, and the third set the addition of 50 mmol l^{-1} KCl + 10^{-4} mol l^{-1} frusemide. Please note that the KCl causes a cell swelling in the absence of frusemide, while in the presence of this inhibitor a cell shrinkage develops.

Table 4. Initial intracellular changes in activities and fluxes across the ventricular membrane in response to osmotic changes implemented by KCl

Solution change	J_{H_2O} (10^{-9} l cm^{-2} s^{-1}) / dV/Vdt (10^{-3} s^{-1})	dK_i^+/K_i^+dt (10^{-3} s^{-1})	dCl_i^-/Cl_i^-dt (10^{-3} s^{-1})	J_K (10^{-9} mol cm^{-2} s^{-1})	J_{Cl} (10^{-9} mol cm^{-2} s^{-1})	$J^*_{H_2O}$ [a] (10^{-9} l cm^{-2} s^{-1})	Coupling J_K/J_{H_2O} (mmol l^{-1})
1 → 2 + 1·5 KCl	−1·8 ± 1·6 (3)	0·23 ± 0·15 (7)	13·1 ± 4·0 (10)	−0·17 ± 0·13	−0·60 ± 0·16	−1·8	94
1 → 3 + 3·1 KCl	−3·3 ± 1·1 (7)	2·4 ± 1·8 (6)	20·8 ± 2·6 (14)	−0·48 ± 0·15	−0·96 ± 0·10	−3·4	141
1 → 4 + 6·3 KCl	−8·0 ± 1·2 (8)	4·1 ± 2·3 (7)	31·7 ± 3·3 (11)	−1·0 ± 0·19	−1·6 ± 0·13	−8·1	123
1 → 5 + 12·5 KCl	−15·6 ± 2·1 (9)	6·6 ± 0·5 (3)	45·2 ± 5·6 (12)	−1·8 ± 0·0	−2·4 ± 0·22	−17·1	105
1 → 6 + 25 KCl	−14·4 ± 1·6 (17)	10·0 ± 1·0 (4)	65·8 ± 7·2 (11)	−2·0 ± 0·1	−3·2 ± 0·30	−17·4	114
1 → 7 + 50 KCl	−17·2 ± 2·0 (28)	7·5 ± 0·9 (27)	101·4 ± 11·4 (7)	−2·1 ± 0·1	−4·7 ± 0·46	−23·3	90
1 → 7 + 50 KCl + f	7·7 ± 1·6 (13)	7·5 [b]	24·3 ± 2·3 (5)	~0	−0·66 ± 0·09	0·3 ± 1·6	~0
1 → 1 + 50 NaCl	14·9 ± 2·8 (11)	—	30·7 ± 4·4 (3)	—	−0·63 ± 0·18	—	—
1 → 1 + 100 Man	10·6 ± 1·2 (12)	6·4 ± 1·6 (5)	—	—	—	—	—

[a] $J^*_{H_2O} = J_{H_2O} - L'_p \Delta Osm$. [b] This value is inferred from Zeuthen *et al.* 1987*a*, where it was shown that the relative rate of increase in K_i^+ was similar in control and in frusemide (f)-treated tissues. Quantities of KCl, NaCl and mannitol (Man) added to the indicated solutions are given in millimoles per litre.

25 mmol l^{-1} KCl, ΔE was 24·9 ± 2·2 mV ($n = 19$); with 50 mmol l^{-1} KCl, ΔE was 32·7 ± 1·5 mV ($n = 38$) and finally with 50 mmol l^{-1} KCl, ΔE was 36·0 ± 1·1 mV ($n = 22$). There is no reason to believe that the depolarization had any direct influence on the KCl-induced cell swelling; for one thing the depolarization was always larger in a given cell in the presence of frusemide, yet the cell shrank when the drug was present.

The direction and magnitude of the osmotic gradient across the ventricular membrane

When the osmolarity was increased by the addition of KCl there was an increase in K_i^+ and Cl_i^- but a small decrease in Na_i^+ (data not shown). Since the cell swells there must also be a dilution of other intracellular entities. Thus the rate of increase in intracellular osmolarity estimated from K_i^+ and Cl_i^- alone is probably an upper limit of the rate of increase in the total intracellular osmolarity. Using the addition of 50 mmol l^{-1} KCl as an example it can be calculated that the changes in K_i^+ and Cl_i^- (Table 4) increase the intracellular osmolarity at a rate of 4·5 mosmol l^{-1} s^{-1}. If we consider the dilution of the other intracellular constituents (about 70 mosmol l^{-1}), the intracellular osmolarity increases by 3·3 mosmol l^{-1} s^{-1}. In view of the fact that the extracellular osmolarity increases by 13% s^{-1} or 13 mosmol l^{-1} s^{-1} (Zeuthen, 1991*a*) the increase in extracellular osmolarity is always larger than the increase in the intracellular one, in the given example by a factor of about 4.

How fast should the intracellular osmolarity increase if the cell swelling was to be explained by simple osmosis? The cell *shrinks* at an initial relative rate of $10{\cdot}6 \times 10^{-3}$ s^{-1} when 100 mosmol l^{-1} mannitol is added to the ventricular solution. The cell *swells* at a rate of $17{\cdot}6 \times 10^{-3}$ s^{-1} when 50 mmol l^{-1} KCl is added, which is 1·7 times faster. If the flux of KCl into the cell should build up sufficient intracellular osmolarity to pull H_2O in at the observed rate, the intracellular osmolarity should increase by $(1 + 1{\cdot}7) \times 13\%$ s^{-1}, or 35% s^{-1}. This rate is one order of magnitude larger than the observed rate of KCl influx. The impossibility of such rapid increases in K_i^+ and Cl_i^- is also apparent from the simple fact that the driving force for KCl entry in this case would be abolished or even reversed.

It should also be noticed that when frusemide was present there was still an appreciable increase in K_i^+ and Cl_i^-. When, for example, 50 mmol l^{-1} KCl was added in the presence of the drug (Table 4), the changes were close to those obtained when 6·3 mmol l^{-1} KCl was added in the absence of the drug. However, in the case with frusemide the cells shrank while the cells swelled when 6·3 mmol l^{-1} KCl was added. This strongly suggests that the changes in

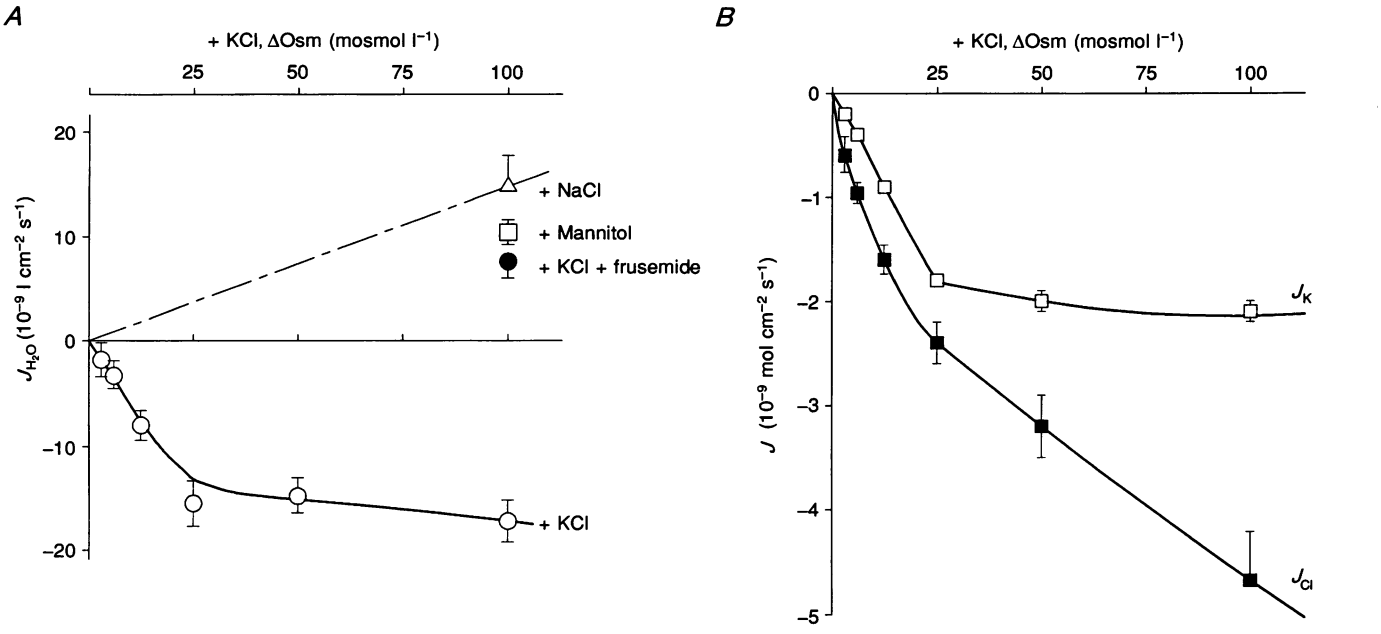

Figure 7. Effects of additions of KCl on fluxes of H_2O, K^+ and Cl^-
A, water flux, J_{H_2O}, is shown as a function of the osmotic gradient implemented by KCl, KCl + frusemide, mannitol or NaCl. Please note that the KCl causes a water influx and therefore cell swelling (negative J_{H_2O}) while the other osmotic experiments causes cell shrinkage, i.e. water efflux (positive J_{H_2O}). *B*, fluxes of K^+ and Cl^- in response to additions of KCl to the ventricular solution. The fluxes are negative, indicative of influx.

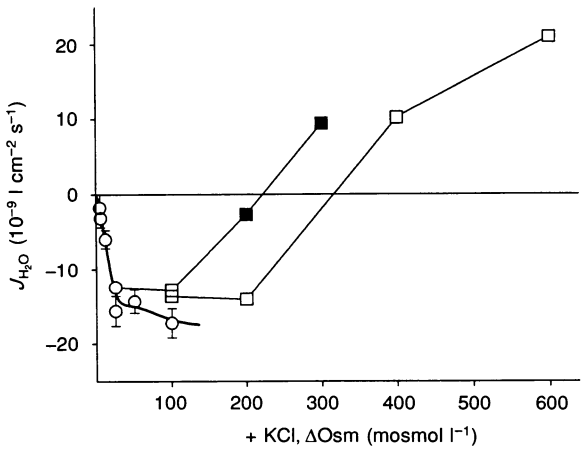

Figure 8. The effects of additions of KCl and mannitol on water fluxes
Water fluxes (J_{H_2O}) in response to osmotic gradients implemented by KCl. As in Fig. 7*A*, but here the effects of additional increases in osmolarity are shown. □, two cells where the KCl gradient was increased to 300 mmol l^{-1}. ■, two cells where 100 and 200 mmol l^{-1} mannitol were added to the 50 mmol l^{-1} KCl.

intracellular ion activities have nothing to do with the rate and direction of water transport across the ventricular membrane.

The adverse osmotic gradient is smaller the smaller the addition of KCl. In fact when 1·5 mmol l^{-1} KCl was added the intracellular osmolarity increased slightly faster than the extracellular one, by about 0·2 mosmol l^{-1} s^{-1}. However, H_2O moved into the cell at a rate which would require a transmembraneous gradient of about 18 mosmol l^{-1}, given the L_p determined in the mannitol experiments. Thus this finding could only be explained by simple osmosis if the L_p of the membrane was increased by a factor of about 100 due to the exposure of KCl. As will be outlined below, such an increase in L_p was not observed.

The underlying assumption that the L_p remains constant during the application of KCl is confirmed by experiments. Cells were exposed to an increase of 50 mmol l^{-1} KCl plus 50 or 100 mmol l^{-1} mannitol (Fig. 6). The reduction in cell swelling induced by mannitol was equivalent to an L_p of $2{\cdot}1 \times 10^{-4} \pm 0{\cdot}5 \times 10^{-4}$ cm s^{-1} (osmol l^{-1})$^{-1}$ ($n = 6$) which is close to the one determined when mannitol alone was applied.

In four cells even larger amounts of mannitol (or extra KCl) were added (Fig. 8). It was found that it took an additional amount of 100–200 mosmol l^{-1} KCl or mannitol to balance the influx of water. These data suggest that the coupling mechanism between KCl and H_2O is able to transport H_2O into the cell against transmembraneous gradients of up to about 300 mosmol l^{-1}.

In conclusion, when KCl is added to the ventricular solution the external osmolarity may increase above that of the cell. Despite this, the cell swells. Consequently there

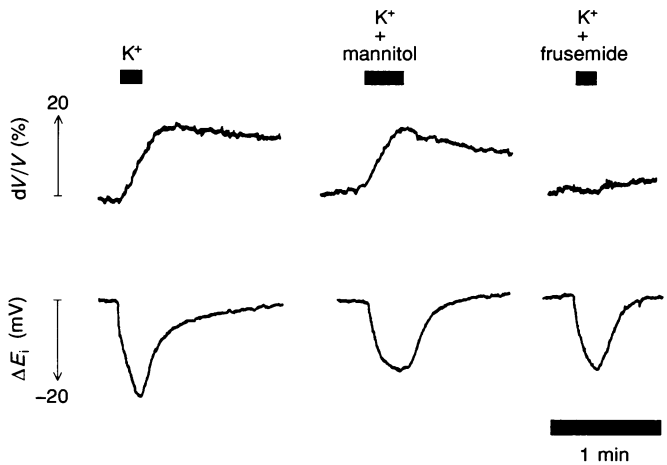

Figure 9. Effects of increases in K^+
Recordings of the relative changes in cellular volume (V) and intracellular volume potential (E_i) as a function of time. The first set of curves shows the effect of an isotonic replacement of 25 mmol l^{-1} Na^+ in the ventricular solution by K^+. The second set the replacement of 25 mmol l^{-1} Na^+ by K^+ plus the addition of 50 mmol l^{-1} mannitol. The third set shows the effect of the replacement of 25 mmol l^{-1} Na^+ by K^+ when 10^{-4} mol l^{-1} frusemide is added simultaneously. Please note that the replacement itself causes a cell swelling which is abolished in the presence of frusemide.

Table 5. Initial intracellular changes in activities and ion fluxes across the ventricular membrane in response to replacement of Na^+ by K^+

Solution change	J_{H_2O} (10^{-9} l cm^{-2} s^{-1}) dV/Vdt (10^{-3} s^{-1})	dK_i^+/K_i^+dt (10^{-3} s^{-1})	dCl_i^-/Cl_i^-dt (10^{-3} s^{-1})	J_K (10^{-9} mol cm^{-2} l^{-1})	J_{Cl} (10^{-9} mol cm^{-2} l^{-1})	Coupling J_K/J_{H_2O} (mmol l^{-1})
1 → 8 + 12·5 K^+	−14·6 ± 1·8 (9)	2·3 ± 1·0 (8)	25·3 ± 2·2 (4)	−1·4 ± 0·08	−1·3 ± 0·09	95
1 → 9 + 25 K^+	−19·7 ± 3·1 (9)	2·8 ± 1·8 (5)	34·1 ± 8·7 (3)	−1·9 ± 0·15	−2·1 ± 0·35	106
1 → 10 + 50 K^+	−26·1 ± 3·1 (7)	3·2 ± 1·6 (5)	42·1 ± 9·7 (3)	−2·5 ± 0·13	−2·7 ± 0·39	96
1 → 10 + 50 K^+ + f	−0·16 ± 0·4 (4)	—	—	—	—	—

Quantities of K^+ added to the indicated solutions are given in millimoles per litre.

is an *influx* of H_2O across the ventricular membrane despite the fact that the osmotic gradient is tantamount to an efflux of H_2O. The influx can even proceed in the face of an additional osmotic gradient created by mannitol.

Ion fluxes and the coupling ratio

If frusemide inhibition is taken as a measure of K^+–Cl^- cotransport then a major fraction of the ion fluxes must be mediated by cotransport: frusemide blocked J_K and reduced J_{Cl} to 14% of control (Table 4).

The water flux, $J^*_{H_2O}$, associated with the influx of K^+ and Cl^- was calculated from eqn (3) and is given in Table 4. The coupling ratio $J_K/J^*_{H_2O}$ is also given in Table 4. In view of the large variation in the ion and water fluxes, the ratio $J_K/J^*_{H_2O}$ remains remarkably constant within 85–137 mmol l^{-1}. This analysis of the ratio of ion to water transport is difficult to extend to the ratio $J_{Cl}/J^*_{H_2O}$ because Cl^- has other pathways than the K^+–Cl^- cotransporter (see Fig. 7*B*).

(4) Isosmolar replacements of Na^+ by K^+

The K^+ concentration in the ventricular solution was increased and the Na^+ concentration reduced without changing the osmolarity of the solution. This caused an immediate and constant rate of cell swelling, i.e. an immediate constant influx of H_2O; the swelling could be blocked by frusemide. K^+ changes from 2 to 15, 2 to 28 and 2 to 55 mmol l^{-1} were tested, i.e. changes from Solution 1 to Solutions 8, 9 or 10 (Table 1). A typical experiment is shown in Fig. 9. The results are compiled in Table 5, the water fluxes in Fig. 10*A* and the ion fluxes in Fig. 10*B*.

The associated increase in K_i^+ and Cl_i^- ranged from 1·2 mmol l^{-1} s^{-1} when 13 mmol l^{-1} Na^+ was replaced to 1·9 mmol l^{-1} s^{-1} when 53 mmol l^{-1} Na^+ was replaced. This increase in intracellular osmolarity was matched, however, by the dilution of other intracellular substances. In the steady state these amounts come to some 70 mosmol l^{-1} (see above). Given the swelling the rate of decrease in these substances ranged from −1·1 to

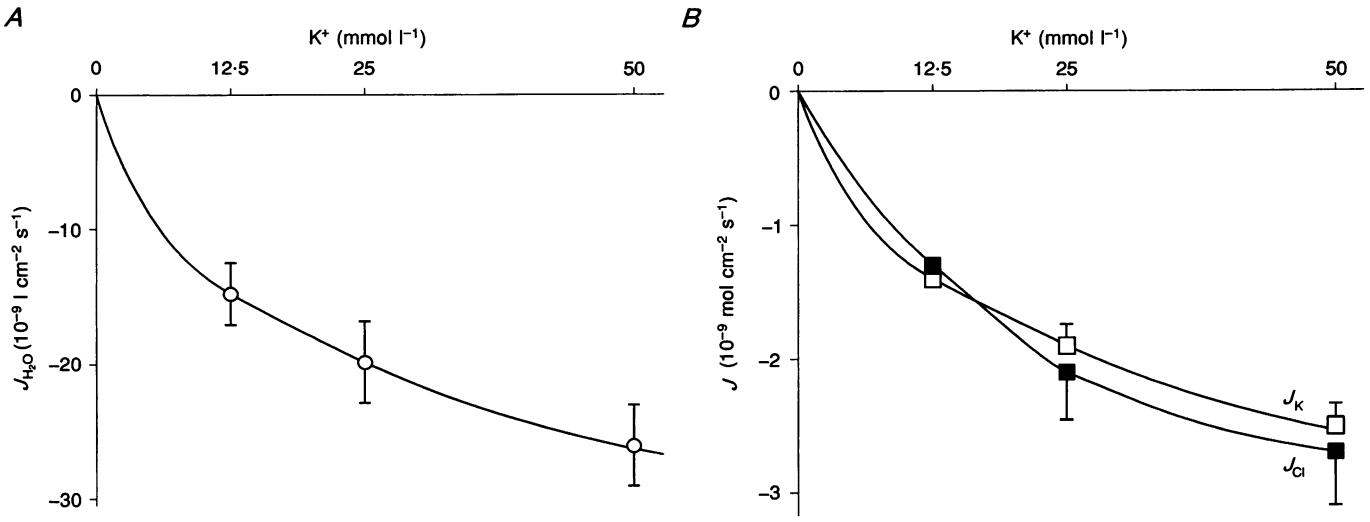

Figure 10. The effects of K^+ increases on fluxes of H_2O, K^+ and Cl^-
A, the water fluxes (J_{H_2O}) across the ventricular cell membrane when Na^+ is replaced isosmotically by K^+ in the same type of experiments as shown in Fig. 9. *B*, fluxes of Cl^- and K^+ into the cell (negative) in response to K^+ replacements of Na^+.

$-1{\cdot}8$ mosmol l^{-1} s^{-1}, the dilution of these substances may, therefore, roughly match the increases in K_i^+ and Cl_i^-.

Thus, there may be only minute differences between the intra- and extracellular osmolarities. What then is the driving force for the abrupt initiation of the inward directed flux of water? To answer this question experiments were performed in which 50 mmol l^{-1} mannitol was added simultaneously with the replacement of 26 mmol l^{-1} Na^+ by K^+. In other words the osmolarity of the ventricular solution was increased by about 50 mosmol l^{-1} (Fig. 9). In this case there was still an influx of H_2O of $11{\cdot}8 \times 10^{-9} \pm 1{\cdot}8 \times 10^{-9}$ l cm^{-2} s^{-1} ($n = 4$) which obviously proceeds against an osmotic gradient of some 50 mosmol l^{-1}. As a corollary we can obtain the L_p of the membrane: the water flux into the cell was $19{\cdot}7 \times 10^{-9}$ l cm^{-2} s^{-1} when the K^+ was replaced and no mannitol was added. The reduction of the water flux which results from the addition of 50 mmol l^{-1} mannitol defines an L_p for the ventricular membrane of $1{\cdot}1 \pm 0{\cdot}23$ cm s^{-1} (osmol l^{-1})$^{-1}$ ($n = 4$), close to the one determined in the other experiments.

The water flux correlates to the rate of influx of K^+ and Cl^-. J_K equals J_{Cl} (Fig. 10*B*), both are initiated abruptly together with J_{H_2O} and all three fluxes are inhibited by more than 80% with frusemide. Furthermore, the coupling ratio, $J_K/J^*_{H_2O} = J_{Cl}/J^*_{H_2O}$, is remarkably constant and falls in the range 95–106 mmol l^{-1} (Table 5). This is similar to the range found when mannitol or KCl were added. Please note that $J^*_{H_2O} = J_{H_2O}$ in these experiments since ΔOsm is zero (eqn (3)).

(5) The role of Na^+

Tests were conducted to see whether Na^+ ions participated in the apparent cotransport of K^+, Cl^- and H_2O. Although some movements of Na^+ ions could be elicited in some types of experiments, the general conclusion is negative: Na^+ does not participate in the cotransport. Four different types of experiments were performed.

(1) Mannitol (100 mmol l^{-1}) was added to the ventricular solution. This caused a cell shrinkage of $14{\cdot}7 \times 10^{-3}$ s^{-1} (Table 3) and an initial relative rate of increase in Na_i^+ (dNa_i^+/Na_i^+dt) of $10{\cdot}9 \times 10^{-3} \pm 2{\cdot}2 \times 10^{-3}$ s^{-1} ($n = 7$), which is slightly smaller than the rate of volume change. This means (eqn (1)) that there is a minute loss of Na^+ from the cell of $0{\cdot}057 \times 10^{-9}$ mol cm^{-2} s^{-1} during the osmotic challenge. This loss is 15 times smaller than the loss of K^+ (compare Table 2). Application of strophantidin, a Na^+–K^+-ATPase inhibitor, did not affect this result (data not shown).

(2) KCl (50 mmol l^{-1}) was added to the ventricular solution. This caused a minute decrease in Na_i^+. In view of the induced cell swelling of $17{\cdot}2 \pm 2{\cdot}0$ s^{-1} (Table 4) this is equivalent to an upper limit for the influx of Na^+ of no more than $0{\cdot}27 \times 10^{-9} \pm 0{\cdot}03 \times 10^{-9}$ mol cm^{-2} s^{-1} ($n = 28$) (eqn (1)). The result was not affected by blockers of Na^+–K^+-ATPase. This influx of Na^+ is eight times smaller than the influx of K^+ obtained in the same experiment.

(3) The effects of Na^+-free ventricular solutions (Solution 12 in Table 1) were tested. Changes in the ventricular Na^+ concentration had insignificant effects on cell volume; dV/Vdt was $3{\cdot}4 \times 10^{-3} \pm 2{\cdot}8 \times 10^{-3}$ s^{-1} ($n = 22$). On the other hand removal and subsequent return of Na^+ to the bathing solution caused significant influxes and effluxes of Na^+. When, for example, Na^+ was returned to the ventricular solution there was an influx of Na^+ of $0{\cdot}43 \times 10^{-9} \pm 0{\cdot}06 \times 10^{-9}$ mol cm^{-2} s^{-1} ($n = 16$). This was only slightly affected by frusemide which reduced the flux to $0{\cdot}35 \times 10^{-9} \pm 0{\cdot}07 \times 10^{-9}$ mol cm^{-2} s^{-1} ($n = 14$). The influx of Na^+ was associated with an influx of Cl^- of $0{\cdot}85 \times 10^{-9} \pm 0{\cdot}1 \times 10^{-9}$ mol cm^{-2} s^{-1} ($n = 6$).

(4) In an earlier paper (Zeuthen, 1991*b*) I tested the effects of a return of Cl^- to the ventricular solution of tissues adapted to solutions in which most Cl^- had been replaced by an inert anion (i.e. isethionate). That is a shift from solution 11 to solution 1 (Table 1). In the present experiments this caused an influx of Na^+ of $0{\cdot}36 \times 10^{-9} \pm 0{\cdot}02 \times 10^{-9}$ mol cm^{-2} s^{-1} ($n = 23$). Frusemide reduced the influx to $0{\cdot}15 \times 10^{-9} \pm 0{\cdot}04 \times 10^{-9}$ mol cm^{-2} s^{-1} ($n = 7$), but it was unaffected by strophantidin, in which case the influx was $0{\cdot}39 \times 10^{-9} \pm 0{\cdot}03 \times 10^{-9}$ mol cm^{-2} s^{-1} ($n = 4$). The influx of Na^+ was about one-third of the Cl^- and K^+ influxes obtained in the same experiment (Zeuthen, 1991*b*), which were $1{\cdot}03 \times 10^{-9}$ and $1{\cdot}05 \times 10^{-9}$ mol cm^{-2} s^{-1} respectively.

In conclusion, there is some evidence for cotransport between Na^+ and Cl^- (see part (3) above). This cotransport is not very sensitive to frusemide and is not associated with volume changes. Therefore it cannot be participating in the K^+–Cl^-–H_2O cotransport (for example as Na^+–K^+–$2Cl^-$ cotransport), as hypothesized in Zeuthen (1993*b*). On the other hand small amounts of Na^+ (see part (4) above) may be cotransported with the K^+ and Cl^-.

DISCUSSION

Previous investigations suggest that the fluxes of K^+, Cl^- and H_2O are co-localized in membrane proteins in the ventricular membrane of the choroid plexus and that energy can be exchanged between the fluxes in such a way that the downhill flux of ions can energize the uphill flux of H_2O and and vice versa. Specifically it was shown that (i) water fluxes induced by mannitol led to frusemide-dependent fluxes of K^+ and Cl^- (Zeuthen, 1991*a*), (ii) a Cl^- flux could energize an uphill flux of H_2O and K^+ (Zeuthen, 1991*b*) and (iii) osmotic gradients implemented by KCl caused uphill fluxes of H_2O (Zeuthen, 1993*b*). These earlier investigations suffer from two drawbacks. Firstly, the passive water permeability is not taken into account, which weakens the quantitative evaluation of the data. Secondly, the papers do not offer a realistic molecular model to explain the findings.

Accordingly, the purposes of the present investigation were (i) to determine the purely passive component of the transmembraneous water transport in order to get a

precise quantitative picture of the ion-dependent part of the water transport, (ii) to complete the experimental basis for the proof of formal cotransport between K^+, Cl^- and H_2O, namely that each species can energize the flux of the two others and that a fixed stoichiometrical relation exists between the fluxes and (iii) to suggest a realistic molecular model.

Two pathways for water permeation

The present data show that the water permeability of the ventricular membrane can be divided into two components: an ion-dependent pathway in parallel with a passive pathway (Fig. 11). Firstly, the osmotic water flux in Cl^--free solutions was independent of the osmotic gradient and could therefore be described by one value of water permeability, $L'_p = 0{\cdot}64 \times 10^{-3}$ cm s^{-1} (osmol l^{-1})$^{-1}$ (Fig. 4). The flux through this pathway did not induce any concomitant K^+ flux (Fig. 5), at least not for smaller osmotic gradients. This pathway constitutes the ion-independent pathway for water and probably reflects water movement through the lipid bilayer and/or CHIP 28 water channel protein which has been demonstrated in the ventricular membrane of the choroid plexus epithelium from rat (Nielsen, Smith, Christensen & Agre, 1993). Secondly, the osmotic water flux was larger in the presence of Cl^- than in Cl^--free solutions. For an osmotic gradient of up to 200 mosmol l^{-1} it was larger by a factor of two ($1{\cdot}4 \times 10^{-3}$ cm s^{-1} (osmol l^{-1})$^{-1}$). This increment in water flow therefore depends on the presence of Cl^-. Furthermore it was associated with an appreciable flux of K^+ (Fig. 5) which was inhibited by frusemide (Table 3). This pathway therefore constitutes the ion-dependent one.

The saturation of the ion-dependent pathway

There are two lines of evidence which show that the ion-dependent pathway saturates at osmotic gradients larger than 200 mosmol l^{-1}: both the water flux ($J^*_{H_2O}$, Fig. 5) and the K^+ fluxes induced concomitantly (J_K, Fig. 5) exhibit saturation. (In fact the $J^*_{H_2O}$ tends to decrease for the larger osmotic gradients). The occurrence of saturation lends support to the notion that the ion-dependent pathway for water permeation takes place by cotransport and not by simple diffusion (see discussion on the molecular model). The slopes of the curves of J_{H_2O} and dK^+/K^+dt *versus* the osmotic gradient (Fig. 4) must after saturation reflect the passive water permeability (L'_p). This expectation is supported numerically (see Results).

Comparison with the earlier studies.

In the earlier investigations (Zeuthen, 1991*a*, *b*) I only applied osmotic gradients of up to 100 mosmol l^{-1} and obtained an L_p of $1{\cdot}4 \times 10^{-4}$ cm s^{-1} (osmol l^{-1})$^{-1}$. This was too narrow a range to reveal the two components of the water pathway. Furthermore, in the earlier papers I used too short a time in Cl^--free solutions (2 min) to obtain the full reduction in the water permeability probably because sufficient Cl^- remained in the preparation. What I needed at that time was to ensure that the L_p did not *increase* by two orders of magnitude in Cl^--free solutions, an assumption confirmed by the present study. Furthermore, the suggestion (Zeuthen, 1991*b*) that cell adapted to Cl^--free solutions for 30 min or more cannot shrink further is unrealistic. The present results show that cells adapted to Cl^--free solutions swell and shrink at the same rate when exposed to the same osmotic gradient.

Uphill transport of H_2O induced by K^+ gradients

When the K^+ concentration of the ventricular solution was increased either by the additions of KCl or by isosmotic replacements of Na^+ there was an *abrupt* initiation of an *influx* of H_2O despite the fact that the osmotic gradient was in the opposite direction or absent. The ability of the K^+ gradient to generate anomalous osmosis was proved further in those experiments where the adverse osmotic gradient was increased by simultaneous addition of mannitol (Figs 6 and 9). This decreased the influx of water by an amount given by the water permeability of the membrane, but still the water flux proceeded from ventricular solution of high osmolarity into the cell which had low osmolarity. It took additions of something like 200 mmol l^{-1} mannitol to match the influx of water induced by the increase of ventricular K^+

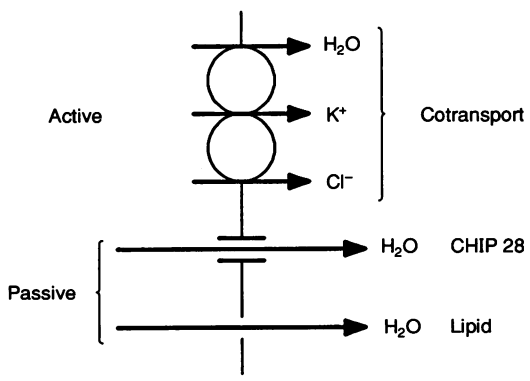

Figure 11. Two pathways for transport of H_2O
There are two pathways for H_2O across the ventricular membrane, one determined by cotransport of K^+ and Cl^- and another determined by the lipid bilayer and/or the CHIP 28 proteinaceous water channel.

concentration (i.e. Fig. 8); in other words the cotransport-induced influx of water was then equal to the osmotic efflux via the passive pathway and the total water flux was zero. All these effects of increased K^+-concentration in the ventricular solutions were abolished by frusemide in which case KCl acted as a normal osmoticum and isosmotic K^+ replacings had no effect on cell volume (Figs 6 and 9). This in itself strongly indicates that the effects are mediated by K^+–Cl^- cotransport.

Unstirred layers around the ventricular membrane cannot explain the findings: if the flux was evenly distributed across the membrane, the total concentration gradient inside the cell would be only 0·1 mmol l^{-1} (Zeuthen, 1991*b*). As an alternative I have previously tried to explain the uphill transport of water as an effect of unstirred layers around the mouths of the K^+–Cl^- cotransport protein (Zeuthen, 1991*b*, 1993*b*). Such an effect is not likely either. As pointed out in the discussion following Zeuthen (1993*b*), unstirred layers of KCl of the required magnitude, about 100 mosmol l^{-1}, would cause the driving force for the K^+–Cl^- cotransport itself to vanish or even to reverse. In any case the turnover number of the cotransporter is likely to be of a magnitude (10^3 s^{-1}) which is too low to result in fluxes large enough to create gradients in the vicinity of the proteins.

When the ventricular solution was returned to control solutions the cell volume recovered only slowly (Figs 2, 6 and 9). One reason could be that the increase of extracellular K^+ causes an influx of a solution with a composition close to 110 mmol l^{-1} KCl (see below). This is fairly close to the intracellular composition of 80 mmol l^{-1}. Consequently the driving forces for a return to control volume in this type of experiment will be smaller than the driving forces for the initial swelling.

The water fluxes into the cells obtained from isosmolar K^+ replacements of Na^+ were larger than those obtained from additions of KCl (compare the upper traces of Fig. 7*A* and Fig. 10*A*). The difference arises because the KCl increases the osmolarity and causes a superimposed efflux of water, while the K^+ replacement does not change the osmolarity. This interpretation agrees well with the finding that the differences between the two sets of measurements can be obtained from the product of the passive water permeability, L'_p, and the osmotic gradient arising from the KCl. It would therefore be a mistake to interpret the shape of the J_{H_2O}–ΔOsm curve (Fig. 7*A*) as if true saturation took place on addition of 25 mmol l^{-1} KCl. Rather the flattening of the curve reflects a balance between cotransport-driven entry and passive osmotic exit of water.

Evidence for cotransport of K^+, Cl^- and H_2O

Cotransport in a membrane protein can be defined by three things, exchange of energy between fluxes, specificity and stoichiometry. (i) A downhill flux of each of the participating species should be able to energize the simultaneous uphill (secondary active) transport of the other participating species. (ii) The transport should be specific; only certain ions can participate in the fluxes. (iii) The ratio between any two of the fluxes should be constant and given by the properties of the cotransport protein. This means that the ratio is independent of the type and magnitude of the external driving forces and therefore of the type of experiment.

(i) Each of the participating species, H_2O, Cl^- and K^+, were able to energize the uphill transport of the two others.

H_2O fluxes implemented by mannitol caused an efflux of H_2O, of K^+ and of Cl^- (Zeuthen, 1991*a*). These ion fluxes were downhill. But when the mannitol was removed again, there was downhill influx of H_2O and uphill fluxes of K^+ and Cl^-. There is no reason to expect a large electrodiffusive flux of K^+ in these experiments: The membrane potential changes only by a few millivolts when the mannitol is applied (Zeuthen, 1991*a*) and with an electrodiffusive permeability of the ventricular membrane of 20×10^{-6} cm s^{-1} (Zeuthen *et al.* 1987*a*), the electrodiffusive flux will be less than 1% of J_K in all cases.

Passive Cl^- fluxes generated by isotonic changes of Cl^- in the ventricular solutions caused uphill transport of K^+ and H_2O (Zeuthen, 1991*b*).

Passive K^+ fluxes generated by changes in K^+ in the ventricular solution caused uphill influxes of H_2O and of Cl^- (Figs 7 and 10). The nature of the Cl^- influx is not immediately clear, but given the fact that most of the Cl^- fluxes are inhibited by frusemide in low doses, 10^{-4} mol l^{-1}, it is a reasonable conclusion that the majority of the Cl^- influx is mediated by K^+–Cl^- cotransport, which means that it proceeds in an electroneutral fashion. This fraction is therefore uphill since it is unaffected by the intracellular electrical potential.

(ii) Specificity for K^+ and Cl^- was demonstrated in Zeuthen (1991*b*). When K^+ was removed from the ventricular solution cotransport into the cell was inhibited. Other anions, SCN^-, acetate and NO_3^- were unable to substitute for Cl^-. Substituting H_2O is experimentally difficult and was not attempted. Na^+ probably did not participate (see Results).

(iii) With the division of the water flux into a passive component via the lipid phase and/or CHIP28 and an active component via the K^+–Cl^-–H_2O system it is possible to give a precise quantitative statement about the stoichiometry. The present data show that the ratio between J_K and the water flux associated with the cotransport, $J^*_{H_2O}$ (eqn (3)), is remarkably constant. It is statistically the same in all three types of experiments (compare Tables 3, 4 and 5); collectively it is 107 ± 4 ($n = 12$) with a range from 90 to 141 mmol l^{-1}. Previously, (Zeuthen, 1991*a*, *b*) I presented the stoichiometries as the ratio J_K/J_{H_2O}. These values are too small because the total

water transport was included in the denominator. It is more difficult to evaluate the ratio $J_{Cl}/J^*_{H_2O}$ since Cl^- has pathways other than K^+–Cl^- cotransport, i.e. exchange with HCO_3^- (Zeuthen, 1987). In the experiments where Na^+ is replaced by K^+, however, $J_K/J^*_{H_2O}$ equals $J_{Cl}/J^*_{H_2O}$, which lends support to the argument for a fixed stoichiometry.

In conclusion, there is good formal evidence for K^+–Cl^-–H_2O cotransport. In addition, the specific inhibition in all types of experiments by frusemide and bumetanide (10^{-5} mol l^{-1}; Zeuthen, 1993) as well as the saturation of the osmotically induced ion and H_2O fluxes for gradients above 200 mosmol l^{-1} (Figs 4 and 5) points towards a coupling mechanism between the K^+, Cl^- and H_2O fluxes based upon cotransport in a membrane protein.

A molecular model for K^+–Cl^-–H_2O cotransport

The molecular model I shall present is based upon the volume changes that take place in a membrane-spanning protein during conformational changes. The model is derived from the mobile barrier model suggested for secondary and primary active transport across membranes by Mitchell (1957, 1990).

According to this model the K^+ and Cl^- ions bind to sites located in the hydrated access part of the protein (Fig. 12) which opens to one side of the membrane, the *cis* side, while a barrier is present between the substrates and the other side of the membrane, the *trans* side. When the protein changes its conformation, the *trans* barrier opens while the *cis* barrier closes. In the present context I make two additions to the Mitchell model: (i) a substantial number of water molecules are occluded in the access volume around the binding sites for the ions during the conformational change; (ii) the protein has a conformational state in which little or no water is bound (see lower part of Fig. 12). On this model the transition between the allosteric conformations of the protein and therefore the rate of transport will depend on the chemical potentials of K^+, Cl^- and H_2O in the outer solutions. The coupling ratio of the transported species will depend on the number of binding sites for K^+ and Cl^- and of the volume of the occluded access space. The ratio of coupling between KCl and H_2O in the present experiments was about 110 mmol l^{-1}, equivalent to a shift of 500 molecules of H_2O or a volume of 1530 nm^3 for each pair of ions. With a molecular weight of 260 kDa of the cotransporters isolated from bovine choroid plexus (Cherksey & Zeuthen, 1987, 1988) the volume is about 3% of the protein volume. The model accords with changes of the volume of membrane bound as well as soluble enzymes during conformational changes and hence in the amount of water attached to, or enclosed within, such proteins (see Zeuthen & Stein, 1994).

Frusemide blocked the ability of the protein to transport K^+ and Cl^-, yet the total water permeability of the membrane was not reduced by the drug (Table 3 and Zeuthen, 1991*a*). Apparently frusemide prevents binding of K^+ and Cl^- without interfering with the ability of the protein to transport water. Conversely, high doses of Ba^{2+}, (10 mmol l^{-1}; Zeuthen, 1991*b*), prevented the translocation of H_2O without interfering with the ability to cotransport K^+ and Cl^-. These two examples of uncoupling of the apparent cotransport must be addressed by a more refined molecular model.

Energetics of the K^+–Cl^-–H_2O cotransporter

Some transport properties can be estimated from the Gibbs relation, irrespective of the underlying molecular mechanisms. This relation says that equilibrium exists across the membrane when

$$K^+_c \times Cl^-_c [C_{w,c}]^n = K^+_t \times Cl^-_t [C_{w,t}]^n . \quad (4)$$

Here c indicates the *cis* compartment and t the *trans* compartment, C_w is the concentration of water and n the number of water molecules participating in the transport. Now the concentration of water is very much the same on the two sides of the membrane. Thus it can be calculated that even if the exponent, n, is equal to 500, the ratio of respective terms in C_w^n, across a membrane separating solutions which differ by, say, 100 mosmol l^{-1}, is only a factor of 2·3. With *cis* values for K^+ and Cl^- of 80 and 40 mmol l^{-1} (typical intracellular values) and *trans* values for K^+ and Cl^- of 2 and 110 mmol l^{-1} (typical extracellular values), clearly the concentrations of the ions are the main determinants of the direction of transport. Substituting these concentration gradients into eqn (4) shows that a water gradient equivalent to 320 mosmol l^{-1} can be held in balance.

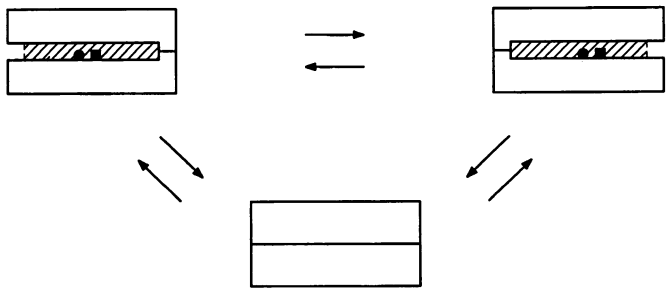

Figure 12. Molecular model
The mobile barrier hypothesis of Mitchell applied to the cotransport of K^+ (■), Cl^- (●) and H_2O (hatched). Hydration of the access volume allows the binding of K^+ and Cl^- (left-hand side). This induces a conformational change in the cotransport protein which causes the permeability barrier to shift from one side of the membrane to the other. The protein can also exist in a closed state where the access volumes are relatively small.

In all experiments discussed so far there seems to be enough energy in the dissipative flux of KCl to drive the uphill flux of H_2O. The available energy can be estimated from the product of the flux of KCl and the difference in chemical potential of the two ions across the membrane. As an example, leaky epithelia from amphibia typically transport isotonic volumes at a rate of 10 μl h^{-1} cm^{-2} or $2{\cdot}7 \times 10^{-9}$ l s^{-1} cm^{-2}. An isotonic flux will represent 110 mmol of cation per litre of water. Thus, it is not unreasonable to set the dissipative efflux of KCl via the cotransporter (J_{KCl}) to $0{\cdot}3 \times 10^{-9}$ mol cm^{-2} s^{-1}. With intracellular and extracellular values of free K^+ concentrations of 80 and 2 mmol l^{-1} and of Cl^- of 40 and 110 mmol l^{-1}, the chemical energy dissipated, given by:

$$J_{KCl} \times RT \ln (K_i^+ \times Cl_i^- / K_o^+ \times Cl_o),$$

amounts to 2×10^{-6} J s^{-1} cm^{-2}. To move a flux of H_2O of $2{\cdot}7 \times 10^{-9}$ l^{-1} s^{-1} cm^{-2} against a gradient of 100 mosmol l^{-1} (or 2·24 atm) an input of $2{\cdot}7 \times 10^{-9}$ l s^{-1} cm^{-2} $\times$ 2·24 atm $= 0{\cdot}6 \times 10^{-6}$ J s^{-1} cm^{-2} (1 l atm = 100 J) is required. Transepithelial transport of H_2O against gradients of 200 mosmol l^{-1} is clearly within the possible range.

Relevance for epithelial transport

The observed cotransport of K^+, Cl^- and H_2O cannot in itself explain transepithelial isotonic transport. Given the measured coupling ratio, KCl and H_2O leave the cell in a ratio of 110 mmol l^{-1}. Since the Na^+–K^+-ATPase which is located at the ventricular membrane (Zeuthen & Wright, 1981) takes up 2 K^+ ions for every 3 Na^+ ions released, the final secretion could have a concentration of about 165 mmol l^{-1}. This solution is hyperosmotic to the bathing solutions by about 90 mosmol l^{-1}. Additional mechanisms are therefore required to give a complete description of isotonic transport. Given the concept of the present paper, cotransport of H_2O with ions, it would be logical to test whether the transporters responsible for entry (i.e. Na^+–H^+ or Na^+–glucose) also translocate H_2O.

Nonetheless, the K^+–Cl^-–H_2O cotransport does explain a number of so far unexplained observations.

(1) Leaky epithelia can adsorb water from a compartment which has a higher osmolarity than the compartment into which the secretion takes place (the exit bath). It takes transepithelial osmotic gradients of between 30 and 200 mosmol l^{-1} to stop the water transport (for references see Zeuthen, 1991*b*, 1992, 1993*b*). If water moves into the cell by simple osmosis the efflux of water from the cell must take place in an uphill fashion. Now there is evidence for K^+–Cl^- cotransport in the exit membrane of most leaky epithelia (see Zeuthen & Stein, 1994), and the ability for uphill transport of water could be explained if cotransport of K^+, Cl^- and H_2O was operating. Thus, the energy dissipated by the efflux of KCl across the basolateral membrane could be utilized to energize a secondary active efflux of water. This agrees with the calculations above, which indicate that the cotransport can sustain transport against an osmotic difference of up to 300 mosmol l^{-1}.

(2) *Necturus* gall-bladder epithelium adapted to transport in 116 mosmol l^{-1}, about half of normal osmolarity, maintained an intracellular osmolarity of 180 mosmol l^{-1} as estimated using microelectrodes and a Ramsay osmometer (Zeuthen, 1981, 1982). Similar findings were obtained in the bullfrog gall-bladder (Zeuthen, 1983). If the cells were treated with ouabain or deprived of O_2, the osmolarity fell to 141 mosmol l^{-1}. It seems that the cells under the hyposmolar conditions are hyperosmolar relative to the surrounding solutions by at least 40 mosmol l^{-1}. This could be explained if the osmotic influx of water was balanced by a secondary active efflux mediated by the K^+–Cl^-–H_2O cotransport.

(3) The reflection coefficient (σ) for NaCl of the kidney proximal tubular epithelium and probably also for other leaky epithelia is significantly less than one: $\sigma_{ep,NaCl}$ has been found to be in the range 0·5–0·8 (for references, see Zeuthen, 1992). This could be explained if cotransport of K^+, Cl^- and water took place at the basolateral membrane. When the transepithelial osmotic gradient is made up from NaCl, a transepithelial gradient of Cl^- is imposed at the same time. This would increase the flux of Cl^- through the cell in the direction opposite to the direction of the osmotic flow. This in turn would increase the flux of KCl across the basolateral membrane and, via the coupling of KCl and water, a flux of water would be induced in the direction opposite to the osmotic flux. In effect, the net flux of water would be less than that expected from osmosis and consequently $\sigma_{ep,NaCl}$ would be calculated to be less than one.

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