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The Contribution of a Chloride Shunt to the Transmucosal Potential of the Rabbit Submaxillary Duct

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Summary. Chloride movement across the wall of the rabbit submaxillary duct has been studied. It was shown that the chloride diffusion from blood to luminal side was determined primarily by the existing transmucosal potential difference. From the fact that the ouabain-poisoned duct showed symmetrical behavior with respect to the chloride diffusion potentials in both directions and the fact that the measured chloride flux behaved as predicted according to the Goldman equation, it was suggested that a single barrier, rather than a series membrane system, determined the chloride movement. The permeability coefficients for chloride, in the order of 5.5×10^{-5} cm sec^{-1} are much larger than would be expected for cell membranes. These findings in combination with the observation that mannitol permeability is higher during chloride perfusion than during sulfate perfusion and the observed electron-microscopic changes favor the concept of the existence of an extracellular route in chloride diffusion. An equivalent electrical circuit is given in order to evaluate the contribution of the chloride shunt more quantitatively. Calculations showed that the ductal resistivity during sulfate perfusion has a value in the order of 434 Ω cm^2, while during chloride perfusion this value is lowered to 48 Ω cm^2, indicating that the ductal wall can change from a tight to a leaky epithelium. The implications of these findings are discussed.

The rabbit submaxillary duct extensively reabsorbs sodium and chloride. Since water does not follow these ions in isosmotic amounts the final outflowing perfusion fluid becomes hypotonic (Knauf, 1972*a*; Schneyer, Young & Schneyer, 1972; Young, 1973). If the duct is perfused with a sulfate-Ringer's solution a high transmucosal potential difference can be measured (Knauf & Frömter, 1970*a*). Because these features resemble those measured in frog skin it seemed logical to explain the experimental data in terms of the Ussing model (Knauf & Frömter, 1970). These authors showed that the luminal membrane behaves like a sodium-selective electrode, the serosal membrane being a potassium-electrode.

But a substantial difference from frog skin is that the luminal membrane, at least during in vivo perfusion, also shows ideal chloride-electrode characteristics (61 mV per decade) in the rat as well as the rabbit (Martin, Frömter, Gebler, Knauf & Young, 1973; Young, 1973). For this reason these authors postulated a chloride shunt, although this is doubted by Frömter, Gebler, Shopow and Pockrandt-Hemstedt (1974). A second difference from frog skin is that the duct has a low electrical resistance (Knauf, 1972*a*; Frömter *et al.*, 1974) and in this respect belongs to the category of leaky epithelia, whereas frog skin has a high electrical resistance. This low electrical resistance in combination with the high transmucosal p.d. (about 140 mV) cannot be easily understood in terms of the Ussing model (Schultz, 1972). Therefore, this study was undertaken to reinvestigate the problem and to elucidate the existence and contribution of a possible shunt pathway for chloride.

Materials and Methods

Salivary ducts of the rabbit submaxillary gland were dissected out carefully. Parts as long as 0.5–0.9 cm were mounted on two holding pipettes according to the method previously described by Knauf and Frömter (1970*a*). Both pipettes were mounted into a lucite chamber containing the circulating bathing fluid (37 °C). The left holding pipette was used as the perfusion pipette with six thin polythene tubings fixed inside. A microperfusion pump containing six syringes was connected with these tubings which allows rapid changes of the perfusion fluid. The right pipette collected the perfusion fluid after passing the duct but also allowed penetration with a glass micropipette into the lumen to measure the transmucosal electrical potential difference. This electrode was filled with a 3 M KCl solution. The reference electrode was a Ringer's-agar bridge mounted in the bathing chamber. During the substitution experiments the Ringer's-agar bridges had the same composition as the bathing fluid. Both the micropipette and the agar-bridge were connected to calomel half-cells, the signal of which was fed into a Keithley differential amplifier (Type 604). Normally the ductal lumen is negative with respect to the bathing fluid. All measured potentials are corrected for junction potentials. The highest values, up to 8.7 mV, were measured if chloride was perfused and sulfate was the main anion of the bathing fluid.

The bathing fluids used in this study were essentially the same as those of Knauf (1972*b*) containing sodium pyruvate and acetate (each 10 mM/liter) and glucose (6 mM/liter) as substrate for maintaining active sodium transport. Haemaccel (3 g %) was also used to get an oncotic pressure comparable to that of plasma. The sodium, potassium, chloride, (sulfate), and bicarbonate concentrations were, respectively, 126, 4, 105, (101), and 25 mEquiv/liter. Since Knauf (1972*b*) reported that calcium and magnesium were ineffectual on sodium transport and on the permeability properties of the duct, these ions were omitted.

In the Figures and the text the bathing fluid is always denoted by R, Cl-R being the chloride-Ringer's and SO_4-R the sulfate-Ringer's bathing solutions. In a few experiments the outside bathing fluid was rabbit serum. The perfusion fluids did not contain the substrates nor the Haemaccel unless explicitly stated. These fluids are denoted as Cl or SO_4. Isotonicity of all the fluids was reached by adding mannitol. The osmolarity was measured by determination of the freezing point depression on an Advanced Osmometer (Type 3A). The radio-

isotope ^{36}Cl (Radiochemical Centre Amersham) was used to measure the chloride flux from bath to lumen. Ten μCi was added to 10 ml bathing fluid. The collected perfusion fluid was sampled in Instagel (Packard Instrument Company Inc.) and counted in glass vials in a Philips counter (model PW-4003/4251). The perfusion rate in these experiments was 12.5 μl/min and the sample time 5 min. Background activity never exceeded 14% of that of the sample with the lowest activity. Mannitol permeation was measured by adding ^{14}C-mannitol (20 μCi) to a smaller sized bath, containing 3 ml of bathing fluid. Isotonicity of the solutions used in these experiments was reached by adding sucrose instead of mannitol.

Chloride permeability coefficients have been calculated according to the Goldman (1943) equation:

$$J_{Cl} = P_{Cl}\, C_o \frac{F \cdot \psi ms/RT}{1 - \exp(-F\psi ms/RT)} \tag{1}$$

where J_{Cl} is the influx of chloride ($mol \cdot cm^{-2} \cdot sec^{-1}$), C_o the chloride concentration of the bathing fluid, ψms the transmucosal potential difference and RT/F equals 26.5 mV at 37 °C. The mean length of 144 ducts was 0.66 cm ($\pm$SEM = 0.01 cm). The diameter was measured by perfusing with colored oil at the desired perfusion rate. Then photographs were taken and the diameter measured from strong enlargements. At a perfusion rate of 12.5 μl/min the mean diameter was found to be 297 μ which gives a surface area of 6.69×10^{-2} cm.

Because only unidirectional fluxes are measured the problem arises whether these may be used to calculate the permeability for reason of back diffusion. Adding ^{36}Cl to the perfusion fluid and perfusing fluid columns enclosed by oil showed that at perfusion rates higher than 2 μl/min back diffusion became neglectable. At 12.5 μl/min no decrease in counts of the perfusion fluid could be detected. At this speed the volume of the duct is about 0.5 μl which means that the content is refreshed 25 times every minute. For this reason we feel permitted to use the unidirectional flux to calculate permeability.

The chloride conductance g_{Cl} has been calculated with the equation

$$g_{Cl} = \frac{I_{net}}{\psi ms - E_{Cl}} \tag{2}$$

where ψms is the transmucosal p.d. and E_{Cl} the Nernst potential of chloride is $\frac{RT}{F} \ln \frac{C_i}{C_o}$. I_{net} is the net chloride current defined as: $I_{net} = F \cdot J_{Cl(net)}$ or according to Ussing's ratio in the absence of exchange diffusion

$$I_{net} = F \cdot J_{Cl} \frac{C_i}{C_o} (1 - e^{-F\psi ms/RT}) \tag{3}$$

where F is 1 Faraday (96,500 Coulombs). C_i and C_o are the chloride concentrations in the perfusion and bathing fluid, respectively. J_{Cl} is again the unidirectional chloride flux from bath to lumen ($Mol \cdot cm^{-2} \cdot sec^{-1}$).

To facilitate comparison of flux measurements obtained on different ducts with probably different permeability coefficients, a flux ratio was calculated. If ψms in Eq. (1) is 0, then J_{Cl} equals $P_{Cl} \cdot C_o$. $J_{Cl(\psi ms = 0)}$ was found by extrapolating p.d. to zero mV. Thus the unidirectional Cl-flux relative to $J_{Cl(\psi ms = 0)}$ is:

$$\frac{J}{J(0)} = \frac{F\psi ms/RT}{1 - \exp(-F\psi ms/RT)}. \tag{4}$$

This function is plotted for positive values of ψms.

For electron-microscopy, the ducts were fixed by perfusion fixation, using the microperfusion system mentioned above. The fixation was carried out immediately following the

perfusion of the chloride or sulfate Ringer's solutions. As fixative an isotonic (300 mOsmol), cacodylate buffered (pH 7.2–7.4), 1.5% glutaraldehyde solution was used, prepared from a freshly distilled glutaraldehyde stock solution. The perfusion rate was 12.5 μl/min. After 15 min the salivary ducts were taken from the holding pipettes and immersed in the glutaraldehyde solution for an additional 2-hr fixation period. Postfixation was carried out with a 2% OsO_4 solution (2 hr). From the fixed ducts only the mechanically undisturbed mid-portions were used. These were cut longitudinally into four wedge-shaped strips.

For transmission electron-microscopy (TEM) the ducts were washed in 0.2 M cacodylate buffer (pH 7.4) + 0.15 M sucrose and subsequently dehydrated and embedded in accordance with standard procedures in Epon 812. Thin sections were studied in a Philips EM 300 electron-microscope. For scanning electron-microscopy (SEM) the tissue strips were treated according to the critical point-drying method (Anderson, 1951), coated with a carbon-gold layer and studied in a Philips PSEM 500 scanning electron-microscope.

Results

In order to evaluate the influence of the perfusion velocity on the transmucosal potential difference (p.d.) the duct was perfused at different rates with sulfate-Ringer's (bathing medium is rabbit serum, Fig. 1). It is obvious that the p.d. is largely dependent on the perfusion rate especially at the lower rates of perfusion. The pro rata decline in p.d. with time after stopping the perfusion is given in Fig. 2 for three different bathing solutions. The new steady state with chloride in the outside medium is reached only after about 30 min. The observation suggests that Cl diffusion from bath to lumen contributes to a larger extent to this phenomenon than the sodium reabsorption in the opposite direction. Nearly maximal values are obtained at a perfusion rate of 12.5 μl/min and since Knauf and Frömter (1970*a*) also used this rate of perfusion in most of their studies this speed was chosen for the following experiments.

What first attracted our attention can probably best be shown by the following set of experiments. In all four cases shown in Fig. 3 the duct is continuously perfused with sulfate-Ringer's. At a high transmucosal p.d. in sulfate-Ringer's as bathing medium, replacement of sulfate by chloride Ringer's did not alter the p.d. (upper left). Application of ouabain to the bathing sulfate medium resulted in a sharp decrease of the p.d.; subsequent substitution with chloride Ringer's partially restored the initial p.d. (upper right). The same phenomenon was seen when DNP was added to the bathing medium (lower right). If the perfusion fluid also contained amiloride a drop in p.d. was observed, but less than in the previous case and substitution of outside sulfate by chloride now resulted in a much smaller effect (lower left). Thus at a high transmucosal p.d. no chloride diffusion potential (from outside to inside) can be evoked, while at lower

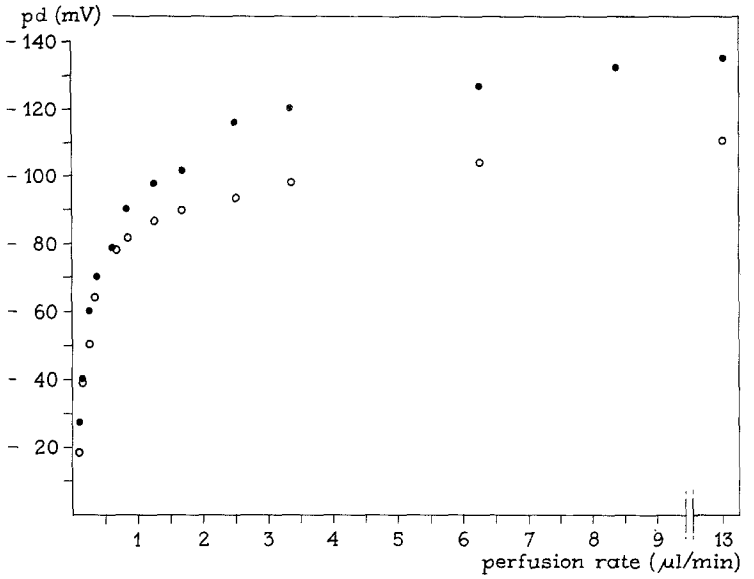

Fig. 1. Relationship between perfusion rate and transmucosal potential difference (p.d.) as measured in two main ducts

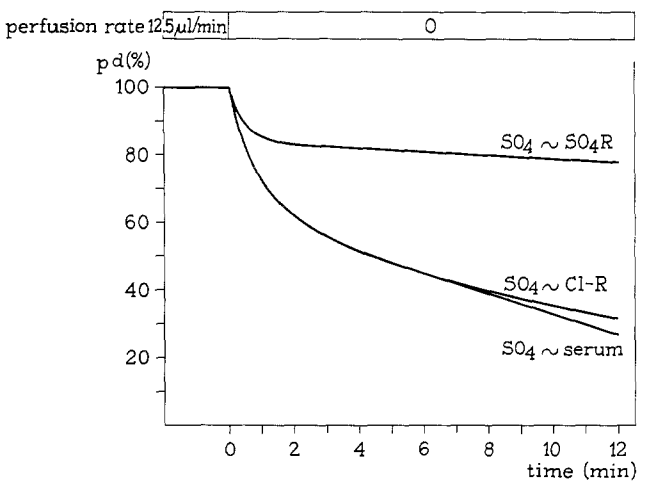

Fig. 2. Percentage drop in potential difference with time after stopping the ductal perfusion for three different bathing solutions

levels the magnitude of the chloride diffusion potential seems to be dependent on the existing p.d.

The results of a second set of experiments are given in Table 1. Here again there is no significant difference in p.d. between SO_4-R and Cl-R

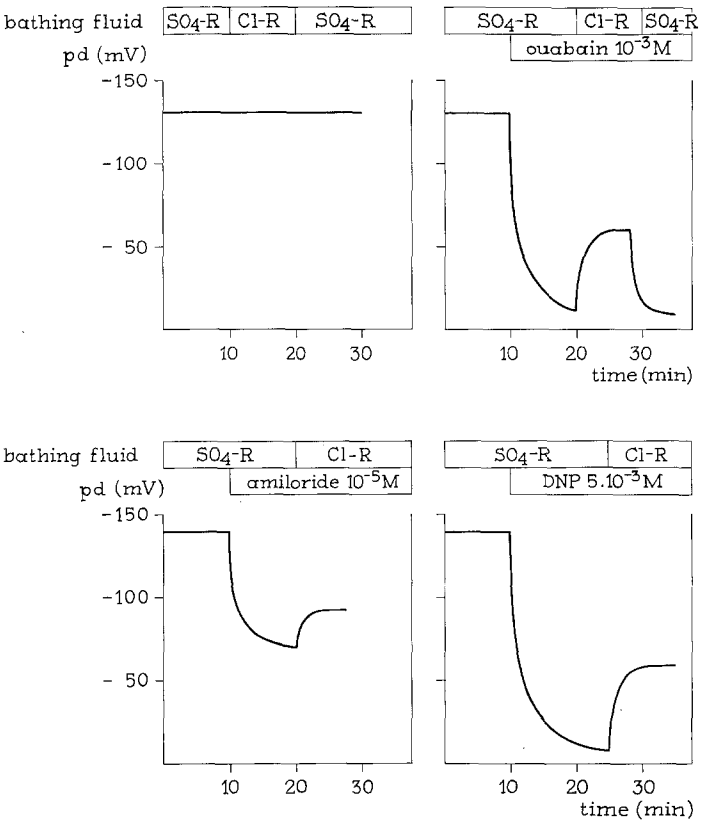

Fig. 3. Change in p.d. observed if the sulfate-Ringer's as bathing solution is replaced by chloride-Ringer's under different conditions. The perfusion fluid was sulfate-Ringer's in all four cases. The traces are from one experiment. The mean results are given in Table 1 and Fig. 5

Table 1. Mean values and the SEM's of the transmucosal potential difference measured with different perfusion as well as bathing solutions[a]

Perfusion fluid	Bathing fluid	Number of determinations	p.d. (mV)	SEM	
SO_4	Cl-R	54	−140.2	2.5	$0.3 > p > 0.2$
SO_4	SO_4-R	60	−144.2	2.6	
Cl	Cl-R	13	−12.8	0.8	$0.5 > p > 0.4$
Cl	SO_4-R	20	−14.8	1.3	
SO_4	Cl-R + *o*	20	−83.5	4.1	$p < 0.001$
SO_4	SO_4-R + *o*	14	−21.6	4.8	
SO_4 + *a*	Cl-R	8	−107.0	4.7	$p < 0.001$
SO_4 + *a*	SO_4-R	15	−63.9	5.1	

[a] Ouabain (5×10^{-5} M) added to the bathing fluid is indicated by + *o*. Amiloride (10^{-5} M) in the perfusate is denoted by + *a*.

as bathing medium when the duct is perfused with sulfate. The same holds true for chloride perfusion although the magnitude of the p.d. is drastically reduced in this case. On application of ouabain the transmucosal p.d. decreases but more so in the case of $SO_4 \sim SO_4$-R than $SO_4 \sim$ Cl-R. One would predict that the chloride diffusion potential would contribute about 62 mV under these circumstances at 10-fold concentration change if the membrane is ideally permeable for chloride.

If, on the other hand, the sodium entry into the cells is blocked by perfusion with amiloride one would expect to find in first instance the same effect as after ouabain. Since this is quantitatively not the case the above explanation cannot be valid unless the sodium entry from the lumen into the cells, and subsequently towards the sodium pumping sites, is not completely blocked by amiloride.

The situation is still more confusing when the duct is perfused with different chloride concentrations. Fig. 4 shows the results obtained when chloride in the perfusion fluid is substituted by sulfate. The slope is 61.9 ± 5.4 mV for a 10-fold change in chloride concentration. This finding in itself is not puzzling but becomes so if one remembers that Knauf and Frömter (1970*a*) also found a slope of 54 mV per decade change in sodium concentration for the same luminal membrane. These experiments were

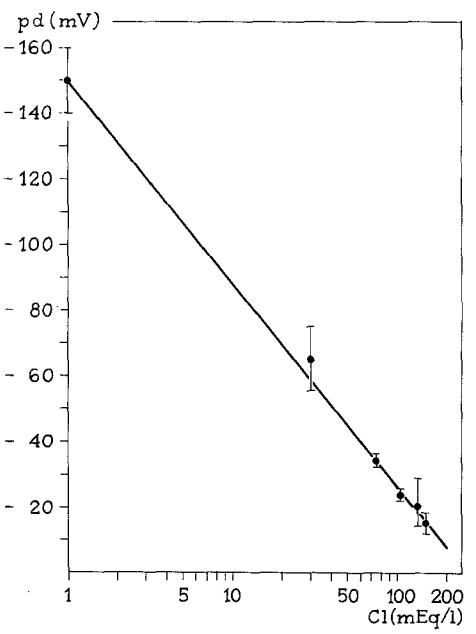

Fig. 4. Change in transmucosal potential difference in relation to the chloride concentration of the perfusion fluid. Sodium chloride was replaced by sodium sulfate. The SEM is given by bars

carried out under sulfate conditions, and assuming that the chloride permeability largely exceeds the sodium permeability, such a behavior could be explained. A second possibility is that chloride and sodium ions pass as completely independent fluxes. If for instance sodium were to pass the cell membrane and chloride the junctional route as a highly anion-selective barrier. Moreover, if the sodium pump is electrogenic in nature and contributes directly to the observed transmucosal potential difference, such a phenomenon could also be found.

Therefore, chloride substitution experiments were carried out after adding 5×10^{-5} M ouabain to the bathing fluid. The results are given in Fig. 5. The solution in which the chloride concentration is varied is indicated by a Δ sign. The straight line referring to $\Delta Cl \sim Cl\text{-}R$ is the same as that in Fig. 4. After ouabain the original slope of 61.9 mV per decade is reduced to a value of 40.2 ± 2.8 mV which is statistically significant ($p < 0.01$). Thus, inhibiting the sodium pumps at the apical cell membranes seems to influence the chloride permeability characteristics at the luminal side of

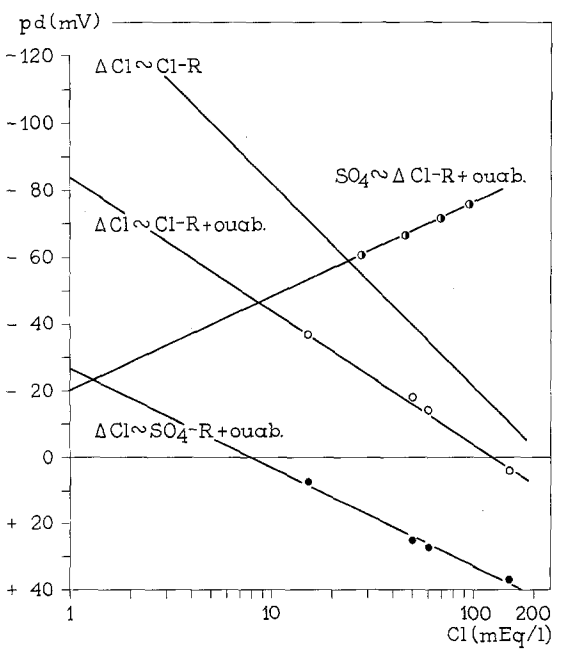

Fig. 5. Transmucosal potential difference in relation to chloride concentration of the bathing medium (Δ Cl-R) or of the perfusion fluid (Δ Cl) after adding ouabain (5×10^{-5} M). Only mean values are given. The calculated slopes $\pm$SEM are given in the text. In these experiments the composition of the perfusion fluid was equal to that of the bathing fluid except for the substituted chloride

the duct. A similar observation, but for sodium, has already been described by Knauf and Frömter (1970*a*).

We also studied the chloride diffusion potentials following the addition of ouabain but in both directions as did Frömter *et al.* (1974), in order to establish whether or not symmetrical behavior could be found. In the case of $SO_4 \sim \Delta Cl$-R the duct was continuously perfused with sulfate and the chloride concentration in the bathing solution was varied. In $\Delta Cl \sim SO_4$-R the chloride concentration of the perfusion fluid was varied. The slopes of both lines are 28.4 ± 3.7 mV and 29.6 ± 2.4 mV, respectively, which are identical. This means that a symmetrical chloride diffusion potential exists if we accept the 23 mV line as a reference point. This value corresponds with the value of 21.6 mV in Table 1 for the case of $SO_4 \sim SO_4R$ + ouabain. Only after a period of about 3 hr this value does decrease to 0 mV which implies that the redistribution of intracellular ion concentrations is a slow process and that the nonequilibrium state between intra- and extracellular solutions in combination with the different permeability characteristics of the luminal and apical membranes is responsible for the initial transmucosal p.d. of about 23 mV. This symmetry strongly suggests that chloride diffusion exists through a single barrier unless both cell membranes happen to have identical relative permeabilities for chloride. To get more information about this possibility we studied the isotope chloride flux from bathing to luminal fluid in order to establish a permeability coefficient and to test whether this flux was indeed dependent on, or was determined by, the transmucosal potential difference. Table 2

Table 2. The chloride flux measured from bath towards lumen at several transmucosal p.d.'s in two ducts[a]

	p.d. (mV)	$J_{Cl} \times 10^{12}$ (Mol cm^{-2} sec^{-1})	$p \times 10^5$ (cm sec^{-1})
(1)	−125.5	186	4.26
	−103	254	3.09
	− 89.5	577	4.77
	− 85	741	5.36
	− 55	1,309	4.33
	− 55	1,298	4.30
	− 32.5	2,594	5.06
	− 22	2,487	6.80
(2)	−105	197	2.51
	− 75 (ouabain)	825	4.44

[a] The calculated chloride permeability coefficients are given in the third column. For explanation *see text*.

gives an example of two of these experiments. The outside bathing fluid was Cl-R containing the isotope. In the first experiment the chloride concentration of the perfusion fluid was varied between 0 and 100 mEquiv/liter. The expected transmucosal p.d. can be read from Fig. 4 and is given in the first column. The calculated permeability coefficients are given in the third column.

Three remarks have to be made: *i*) relatively high chloride permeabilities are found as compared with data obtained for other cell membranes. *ii*) The chloride permeability seems to be independent of the p.d. *iii*) The transmucosal p.d. is the primary determinant of unidirectional chloride flux. In the second experiment where the duct was perfused with sulfate-Ringer's and where ouabain was used to lower the p.d. to 75 mV, the flux reached a value comparable with the flux that would have been obtained if chloride had been perfused (first experiment).

The data regarding the flux measurements can also be used to calculate the chloride conductance (*see* Materials and Methods). Since Knauf (1972*a*) and Frömter *et al.* (1974) have measured the specific resistance of the duct for reasons of comparison we have plotted in Fig. 6 the reverse values of the chloride conductance (R_{Cl} expressed in $\Omega\,cm^2$) against the chloride concentrations of the perfusion fluid. With sulfate (or only 1 mEquiv Cl) as perfusion fluid a high chloride resistance was found which sharply decreased when the chloride concentration increased to about 10 mEquiv/liter. Then the decline slowed down. R_{Cl} in this experiment can

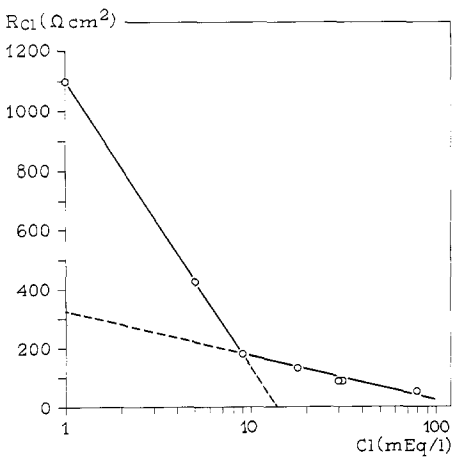

Fig. 6. Chloride resistance (R_{Cl}) plotted against the chloride concentration of the perfusion fluid

be described by the following equation

$$R_{Cl}(\Omega\,cm^2) = 1{,}100\,e^{-0.19\,(Cl)} + 330\,e^{-0.06\,(Cl)}. \quad (5)$$

The resistance for chloride measured in this way is much larger than the electrically determined resistance reported by Knauf (1972*a*) who found a value of 70 $\Omega\,cm^2$ while a value of 53 $\Omega\,cm^2$ was measured by Frömter *et al.* (1974).

In 26 experiments we found a value for the basic chloride flux ($SO_4 \sim$ Cl-R) of 160×10^{-12} Mol $cm^{-2}\,sec^{-1}$ with an SEM of 8.8. This equals a chloride resistance of 1,228 $\Omega\,cm^2$ at a mean p.d. of 140 mV. The sharp decrease to about 10 mEquiv/liter also seems to be statistically significant. Five additional measurements with 10 mEquiv Cl/liter as perfusion fluid resulted in a mean chloride flux of 437×10^{-12} Mol $cm^{-2}\,sec^{-1}$ yielding a R_{Cl} of 259 Ωcm^2. The luminal chloride concentration seems to have evoked this effect because the bathing fluid always contained the same chloride concentration. This points to the fact that somewhere at the luminal border chloride ions can largely influence the chloride resistance of the duct wall as measured from unidirectional fluxes. The flux data also obey the Goldman equation which is primarily derived for a single diffusion barrier. The ordinate in Fig. 7 is the ratio of the measured flux at a given transmural p.d. over the flux at p.d. = 0 mV (*see* Materials and Methods). The continuous line is calculated on the basis of the Goldman equation. The experimental data from three experiments agree fairly well with the predicted relationship. This finding, together with the symmetrical chloride diffusion potential, the high chloride conductance, and the influence of luminal chloride concentration on chloride resistance directed our attention more specifically to the study of the paracellular pathway. Additional information was derived by the following experiments.

Substitution of sodium by choline at the blood side has no influence on the p.d. (Knauf & Frömter, 1970*a*). Therefore, these authors state that the contraluminal membrane is impermeable to sodium. At the luminal membrane they found no permeability for potassium but a slope of 53.8 mV per decade of sodium concentration. If we substitute sodium for potassium in the duct fluid then after treatment with 5×10^{-5} M ouabain we find a slope of only 12 mV per decade concentration change in $SO_4 \sim SO_4$-R (Fig. 8), but no reaction in the case of Cl ~ Cl-R. If sodium is replaced by choline in Cl ~ Cl-R then again no reaction is observed. Therefore, the luminal membrane responds to a change in sodium concentration only if sulfate is used and thus only if there is a high chloride resistance. This again

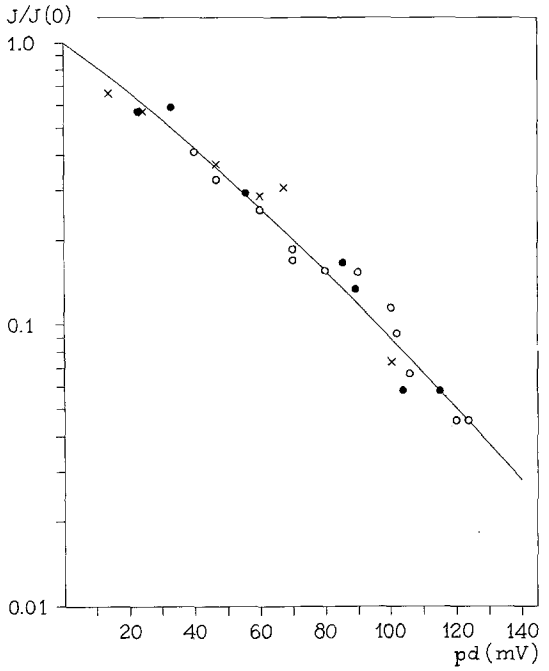

Fig. 7. Ratio of chloride flux at given p.d. over the chloride flux obtained at p.d. = 0 plotted against the transmucosal potential difference. The continuous line gives the predicted relationship if only one barrier is involved

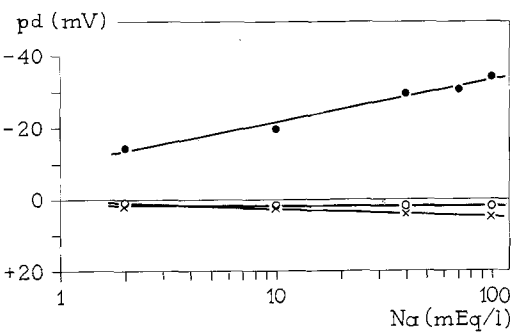

Fig. 8. Relationship between transmucosal p.d. and luminal sodium concentration after adding ouabain (5×10^{-5} M) to the bathing solution. If sodium is replaced by potassium in $SO_4 \sim SO_4$-R the symbol is ●. The same substitution in Cl ~ Cl-R is given by ○ and sodium replacement by choline in Cl ~ Cl-R by ×

could very well be explained by a shunt pathway which is more or less closed in sulfate and electrically open in a chloride medium.

Mannitol, frequently used as an extracellular marker, was added to the bathing fluid as ^{14}C-mannitol. The permeabilities, calculated from the

Table 3. Permeability of mannitol, mean values ±SEM, measured during perfusion with sulfate or chloride solution (10 ducts)

$P_{mannitol}(SO_4\text{-perfusion})$	$=0.015 \pm 0.001 \times 10^{-4}$ cm sec^{-1}
$P_{mannitol}$(Cl-perfusion)	$=0.067 \pm 0.004 \times 10^{-4}$ cm sec^{-1}

unidirectional isotope flux obtained at 12.5 µl/min, are depicted in Table 3. It is obvious that during chloride perfusion the permeability of mannitol is nearly four times greater as compared with the value obtained during sulfate perfusion. The value measured during chloride perfusion is nearly the same as the value established by van Os, de Jong and Slegers (1974) for the rabbit gallbladder (0.097×10^{-4} cm sec^{-1}). For this epithelium, as another leaky epithelium, we could demonstrate that mannitol permeation indeed followed the paracellular route. Although we recognize the differences between gallbladder and duct wall there is at the moment no realistic argument why, with respect to mannitol permeation, both epithelia could not be treated equally. More extensive studies on non-electrolyte permeability will be discussed in a subsequent paper.

In the context of this article only a brief presentation of our electron-microscopic findings will be given. A more complete description of our findings will be presented elsewhere. As can be seen from the survey SEM micrograph (Fig. 9), the mucosal surface of the salivary ducts shows broad semicircular folds, separated by shallow pits.

In thin TEM section of untreated ducts it appears that the epithelial cells show complex lateral cell membrane interdigitations, especially in the lower half of the cells. This interdigitation pattern is the more complex, the more the cells approach the top of the mucosal folds. After sulfate Ringer's perfusion as well as after chloride Ringer's perfusion the epithelium shows an extensive dilatation of the lateral intercellular spaces (*see* Fig. 10).

Fig. 9. Low magnification SEM-micrograph showing a quarter of a longitudinally sectioned salivary duct. Note the semicircular folds of the epithelial lining. 127 ×

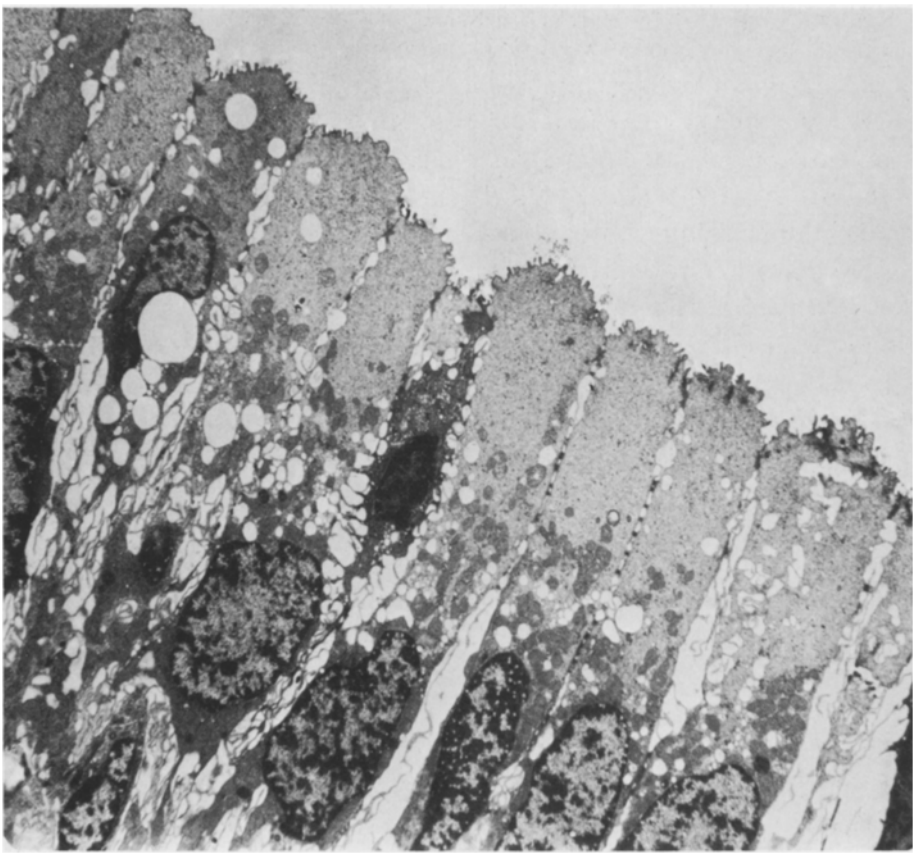

Fig. 10. In this micrograph taken from a chloride-perfused duct, the extensive dilatation of the intercellular channels is clearly seen. Note the irregularly bulged apical surface of the cells. 4,125 ×

By morphometric-stereological methods (Weibel, 1969) no definite difference in volume density (=fractional volume; the volume of the intercellular space related to the total volume of the epithelium) of this intercellular space between the sulfate and the chloride-treated ducts could be established.

On the other hand, two distinct and reproducible morphological differences occurred between the sulfate- and the chloride-perfused ducts:

1. In the sulfate ducts the surfaces of the cells retain their normal morphology as it is seen in untreated ducts, i.e. on the slightly convex apical surface of the cells a dense crowding of small regular microvilli occurs (Fig. 11*a*), which in TEM appear to carry a distinct extraneous coat (Fig. 12*a*). In the chloride perfused ducts, on the contrary, the apical

cell surface is distinctly bulged outwards (Fig. 11*b*), whereas the microvilli are far less regular in structure and often appear swollen (Figs. 11*b*, 12*b*).

2. A second difference exists in the "height" in which the lateral dilatation always approaches the tight junctions so that immediately below these junctions, large vacuole-like spaces are present (compare Fig. 12*a* with Fig. 12*b*). When the morphometric-stereologic analysis of the volume density is restricted only to the apical, supranuclear zone of the epithelium a distinct difference between chloride and sulfate ducts can be settled. It therefore appears that the "intercellular pathway" more closely and more "voluminously" approaches the ductular lumen.

Discussion

The very low transepithelial electrical resistance measured by Knauf (1972*a*) and Frömter *et al.* (1974) across the wall of the submaxillary duct classifies this epithelium as a "leaky" epithelium, to which also belong the proximal renal tubule, the gallbladder, the small intestine, and the choroid plexus. The latter structures are, however, very permeable to water while the duct epithelium is not. So also within the group of leaky epithelia differences remain. Although the physiological function of these leakages is still interpreted differently, most investigators agree that ions as well as nonelectrolytes can pass these epithelia via the extracellular route (Barry, Diamond & Wright, 1971; Wright, Barry & Diamond, 1971; Smulders, Tormey & Wright, 1972; Wright, Smulders & Tormey, 1972; Ullrich, 1973; Desjeux, Tai & Curran, 1974; van Os *et al.*, 1974; Schafer, Troutman & Andreoli, 1974). The direct consequence of the leakiness is that very low electrical potential differences are measured across these epithelia because the paracellular route can operate as an electrical shunt (Boulpaep, 1967; Shiba, 1970; Barry & Diamond, 1971; Schultz, 1972; Diamond, 1974). Frömter (1972) using a micropipette as a scanning electrode was able to demonstrate current sinks at the region of the cellular junctions in *Necturus* gallbladder, demonstrating that the leakage was located in the cell junctions.

The leaky tight junctions studied so far all have cation-selective pathways except those of the submaxillary duct (Knauf & Frömter, 1970*a*; Frömter *et al.*, 1974). Thus, in several respects the submaxillary duct forms its own category in the group of leaky epithelia.

The experimental results presented in this paper show: (1) that the chloride permeability of duct epithelium is high as compared with values

Fig. 11*a*

Fig. 11. (*a*) SEM micrograph showing the epithelial cells from a sulfate-perfused duct in surface view. The microvilli on the slightly convex surfaces of the epithelial cells are about equal in height and diameter. 5,640×. (*b*) The same from a chloride-perfused duct. The cell surfaces are bulged outwards and the microvilli are irregularly shaped and partly swollen. 5,640×

for cell membranes; (2) that the unidirectional flux of chloride has a voltage dependency according to the Goldman equation; (3) that after treatment with ouabain a symmetrical chloride diffusion potential can be measured which agrees with the observation of Frömter *et al.* (1974); (4) that the mannitol permeation across the epithelium can be increased significantly by perfusion with chloride as compared with sulfate perfusion; (5) that during perfusion with chloride the proximal parts of the extracellular spaces between the cells are widened.

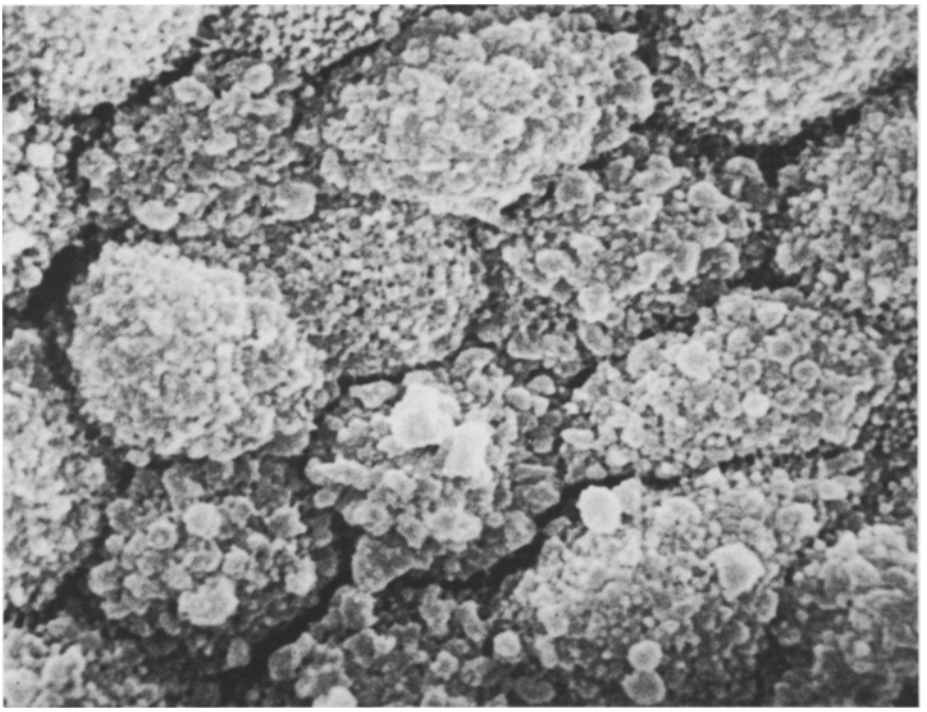

Fig. 11*b*

Although these results separately cannot be regarded as conclusive enough to postulate a single step barrier in chloride diffusion across the ductal wall, the combination of them strongly favors such a concept. Especially of interest is that, as already was shown for the electric resistances, also the permeability for mannitol can be changed depending on the anion which is perfused. The paracellular route therefore cannot be regarded as a static structure but is a more dynamic one, which can change from "leaky" to less leaky. The question remains whether this route is permeable for nonelectrolytes alone or also for chloride. In this respect we differ from the conclusion reached by Frömter *et al.* (1974) who explicitly state that the electrical conductance across the duct wall at least is due for 82% of chloride but they do not accept the anion-selective extracellular pathway. The main reason was that the halide ion selectivity sequence, as established for the duct, is comparable with that of frog muscle cell membranes. In the first place a junctional route can also have selectivity sequences as is demonstrated for the cation-selective junctional routes in gallbladder. Moreover these shunts could be made anion-selective

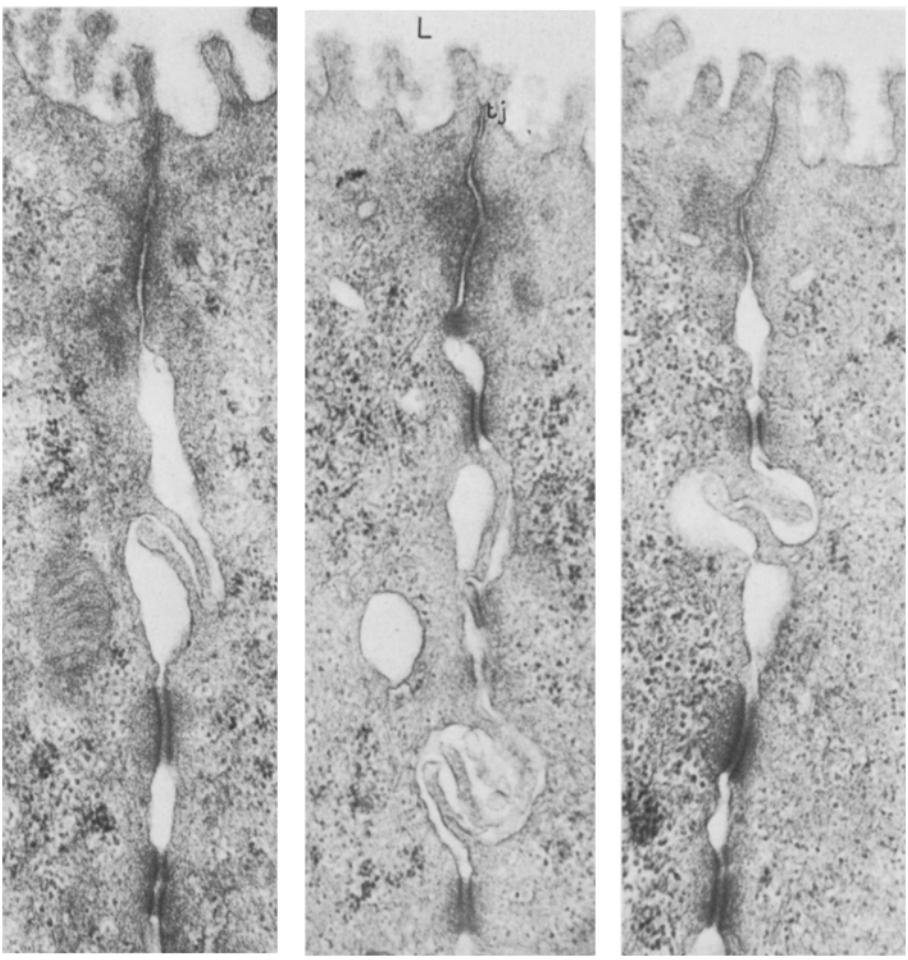

Fig. 12*a*

Fig. 12*a* and *b*. In these two sets of micrographs the difference in the degree of dilatation of the intercellular channels between the sulfate (*a*) and the chloride (*b*) perfused ducts is illustrated. For further explanation *see text*. *L*=lumen; *tj*=tight junction. 36,960×

by treating them with thorium which also holds for barnacle muscle (Machen & Diamond, 1972). In the framework of these observations and knowing that mannitol can pass the epithelium we feel permitted to interprete our other results as being caused mainly by a single step barrier which is anion-selective.

Assuming that the luminal cell membrane is permeable to sodium and that the serosal membrane is not and that the reverse holds true for potassium, then a simple equivalent electrical circuit can be used to describe the transmucosal potential (Fig. 13).

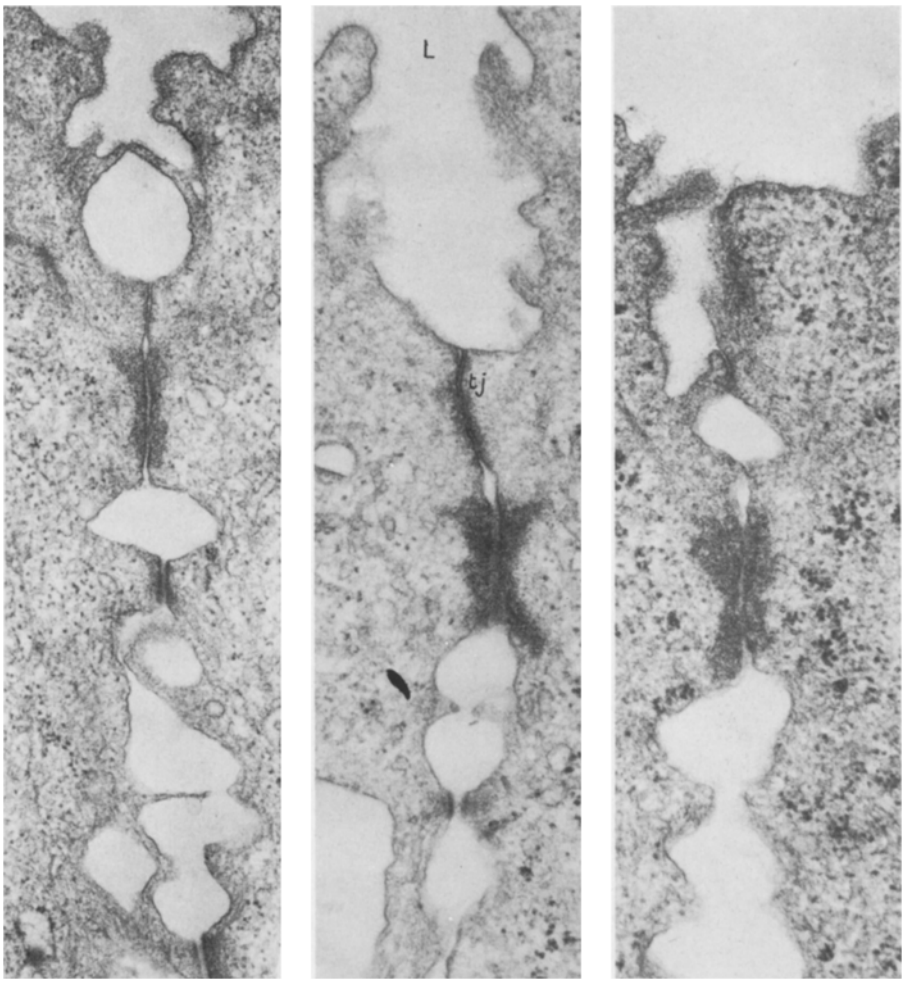

Fig. 12*b*

E_m and E_s are the sums of the mucosal and serosal EMK's respectively. E_m in our case is thought to be mainly a sodium diffusion potential and E_s a potassium diffusion potential according to the Ussing model. R_m and R_s are the resistances of the membranes to ion movements. The extracellular EMK is termed E_j and the corresponding resistance R_j. This model is, of course, only valid if the possibility of a contribution from an electrogenic mechanism can be excluded. Since this is not ruled out in our case, the results obtained following ouabain can only be used for an interpretation along these lines. The transmucosal potential difference can now

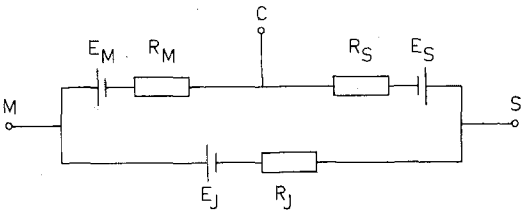

Fig. 13. Equivalent electrical circuit of the epithelium

be described by:

$$\psi_{ms}=\frac{R_j}{R_t}(E_m+E_s)+\frac{R_m+R_s}{R_t}E_j \tag{6}$$

where $R_t=R_m+R_s+R_j$. Then E_j is the chloride diffusion potential which becomes zero if the chloride concentration is equal on both sides or in the case of SO_4-SO_4R when no chloride is available. Under these circumstances, therefore, only the first term of Eq. (6) remains. From Fig. 5 we know that this first term is -3 mV if the chloride concentration on either side is 105 mEquiv/liter and -26 mV for SO_4-SO_4R. If we assume that during the substitution experiments $E_m+E_s=E_c$ and $R_m+R_s=R_c$ have not changed, then only two values for R_j have to be found to solve the equation. Reasons for believing that E_c has not changed substantially are: a) it takes only 3 min to reach a new steady-state value during Cl-substitution, and b) a symmetric chloride diffusion potential could hardly have been measured if E_c had changed to an appreciable extent. As a first approximation E_c and R_c are considered to be constants. But the difficulty is how to establish values for R_j in the case of $Cl-ClR=R_{j(Cl)}$ and $SO_4-SO_4R=R_{j(SO_4)}$. The calculated value for $R_{j(Cl)}$ is about 50 Ω cm^2 (Fig. 6) assuming that other anions do not contribute to R_j. With respect to pyruvate and acetate this seems permissible but for bicarbonate it is not so certain. Relative permeabilities given in the literature for P_{HCO_3}/P_{Cl} vary from 0.3 to 0.01. Preliminary experiments have shown that if metabolism of the duct is inhibited, bicarbonate permeates with as much difficulty as sulfate. Therefore, we feel permitted to neglect P_{HCO_3} with respect to P_{Cl} in our system, in which case R_j is almost $R_{j(Cl)}=50\,\Omega\,cm^2$. To establish the relationship between $R_{j(Cl)}$ and $R_{j(SO_4)}$ for a certain value of R_c the following calculations were carried out.

As stated earlier, the first term of Eq. (6) can be written as

$$-3=\frac{R_{j(Cl)}E_c}{R_{j(Cl)}+R_c} \quad \text{for } Cl-ClR$$

and as

$$-26 = \frac{R_{j(SO_4)} E_c}{R_{j(SO_4)} + R_c} \quad \text{for } SO_4 - SO_4R.$$

Rearranging gives:

$$R_{j(SO_4)} = \frac{26 \cdot R_c R_{j(Cl)}}{3R_c - 23R_{j(Cl)}}.$$

This equation shows that R_c must at least be greater than $7.6 \cdot R_{j(Cl)}$. Rearranging yields $R_{j(SO_4)} > 8.6 \cdot R_{j(Cl)}$.

The equation was fed to the computer with the intention of calculating values for $R_{j(SO_4)}$ given a certain value for R_c over the range of 10 to 100 $\Omega\,cm^2$ for $R_{j(Cl)}$. Thus for a given value of R_c a graph can be constructed for the corresponding values of $R_{j(Cl)}$ and $R_{j(SO_4)}$. Fig. 14 shows the combined results.

These graphs show an almost linear relationship between $R_{j(Cl)}$ and $R_{j(SO_4)}$, if we neglect the extremely low R_c values, in the most critical range around $R_{j(Cl)} = 50\,\Omega\,cm^2$. If we accept 1,250 $\Omega\,cm^2$ to be the lowest reasonable value for R_c then we still have to find a highest value. For this reason the E_c values have been calculated. These are given in Table 4 together with the corresponding $R_{j(SO_4)}$ values for a smaller range of $R_{j(Cl)}$. The

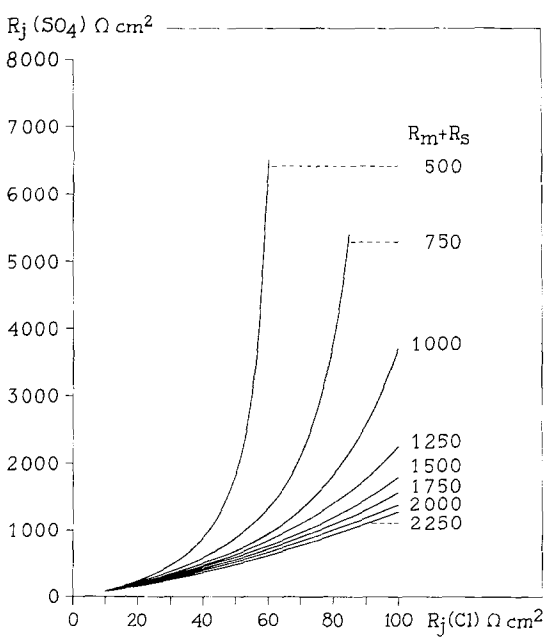

Fig. 14. Values of $R_{j(SO_4)}$ belonging to hypothetical values of $R_m + S_s$, over a range of $R_{j(Cl)}$ of 0 to 100 $\Omega\,cm^2$

Table 4. Calculated values of E_c for the assumed limits of R_c

	R_c (Ω cm^2) 1,250		R_c (Ω cm^2) 2,500	
$R_{j(Cl)}$ (Ω cm^2)	$R_{j(SO_4)}$ (Ω cm^2)	E_c (mV)	$R_{j(SO_4)}$ (Ω cm^2)	E_c (mV)
40	459	−96	395	−190
50	625	−78	511	−153
60	822	−65	637	−128

Table demonstrates that for all three values of $R_{j(Cl)}$ an unexpectedly high E_c value is obtained for the high R_c range. For this reason R_c must have a value lower than 2,500 Ω cm^2. If we do not permit E_c to exceed 100 mV then R_c can only vary between 1,250 and 1,600 Ω cm^2 (for $R_{j(Cl)}$ is 50 Ω cm^2). The corresponding range for $R_{j(SO_4)}$ then becomes: 564–625 Ω cm^2. The real values must be sought somewhere in between. The experiments reported here do not permit the establishment of more accurate values. Therefore, we have only used mean values to demonstrate some of the implications of the results. If $R_{j(Cl)}$, $R_{j(SO_4)}$ and R_c are 50, 594 and 1,425 Ω cm^2, respectively, then we find a value of −88 mV for E_c.

The voltage divider ratio for an EMF across the cell R_j/R_t, for the case of Cl−ClR, then becomes 0.033, and for SO_4-SO_4R, 0.29. This means that the slope of 12 mV measured in the experiment shown in Fig. 8 for a 10-fold change in sodium concentration without a shunt, should have been about 12/0.29 = 41 mV. In the case of Cl−ClR the same substitution experiment then yields a slope of $0.033 \times 41 = 1.3$ mV which is a value that cannot be detected by the experiment as is indeed shown by Fig. 8.

Using the approximate values to calculate the replacement resistances (RR) according to the equation $1/RR = 1/R_j + 1/R_c$ we then find for Cl−ClR a value of 48 Ω cm^2 and for the case of SO_4-SO_4R a value of 434 Ω cm^2. These values differ substantially from those found by Knauf (1972*a*): 11 and 70 Ω cm^2, respectively. For the rat and human submaxillary duct, however, more comparable values have been reported by Gruber, Knauf and Frömter (1973) and Knauf and Frömter (1970*b*): 400 and 365 Ω cm^2, respectively. We cannot explain the discrepancy between our values and those of Knauf satisfactorily at the moment, but since two different techniques have been used a possible explanation should be sought in terms of the methodology. Experiments are now in progress to evaluate this question more specifically. Our data suggests that the duct epithelium can change from a leaky to a tight epithelium. Using the mean value of the

26 chloride flux measurements in $SO_4 - ClR$, a mean value for the chloride permeability can then be calculated. This value is 5.51×10^{-5} cm/sec ($\pm$SEM = 0.34). By rearranging Eqs. (1), (2) and (3) we can find an expression for $R_{j(Cl)}$ which is dependent on ψ_{ms}, the chloride concentrations and the permeability coefficient for chloride. Using the previously determined value for $R_{j(Cl)}$ of 50 Ω cm^2 (both sides are bathed with 105 mEquiv/liter chloride) then gives a P_{Cl} of 5.0×10^{-5} cm/sec which is close to the mean value of 5.51×10^{-5} cm/sec. This again suggests that the calculated values are realistic. But this also implies that under the conditions described chloride ions move across the ductal wall, mainly extracellularly. Moreover, this extracellular pathway must be highly anion selective because potassium substitution at the luminal side and sodium substitution on the serosal side do not show any effect on the transmucosal potential. Preliminary electron-microscopic studies indeed show a dilatation of the proximal extracellular spaces after perfusion with chloride in contrast to sulfate perfusion. These studies will be extended in order to relate the morphologic changes with the physiological function in more detail.

The decrease in p.d. on lowering the rate of perfusion must largely be due to chloride diffusion from the bath towards the lumen. The high permeability coefficients for Cl enables the chloride concentration to increase rapidly if the perfusion rate is very low or if the perfusion is stopped. If, in the ductal lumen, the chloride concentration increases then the transepithelial potential decreases, which in turn favors the chloride flux (Table 2). It can be calculated that at zero perfusion rate the intraluminal chloride concentration has already increased from 0 to 80 mEquiv/liter within 10 min, which fully explains the drop in potential.

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