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Chloride Reabsorption by Renal Proximal Tubules of Necturus

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Summary. Movement of Cl from the lumen of *Necturus* proximal tubule into the cells is mediated and dependent on the presence of luminal Na. Intracellular Cl activity was monitored with ion selective microelectrodes. In Cl Ringer's perfused kidneys, cell Cl activity was 24.5 ± 1.1 mM, 2 to 3 times higher than that predicted for passive distribution. When luminal NaCl was partially replaced by mannitol (capillaries perfused with Cl Ringer's) cell Cl decreased showing a sigmoidal dependence on luminal NaCl. Peritubular membrane potential was unaltered. Sulfate Ringer's perfusion of the kidneys washed out all cell Cl but did not alter peritubular membrane potential. Chloride did not enter the cell when the tubule lumen was perfused with 100 mM KCl, LiCl, or tetramethylammonium Cl. Luminal perfusion of NaCl caused cell Cl to rise rapidly to the same value as the controls in the Cl Ringer's experiments. Perfusion of the tubule lumen with mixtures of NaCl and Na_2SO_4, while the capillaries contained sulfate Ringer's yielded a sigmoidal dependence of cell Cl on luminal NaCl activity. Chloride movement from the lumen into the proximal tubule cells required approximately equal concentrations of Na and Cl. Current clamp experiments indicated that intracellular chloride activity was insensitive to alterations in luminal membrane potential, suggesting that chloride entry was electrically neutral. The transcellular chloride flux was calculated to constitute about one half of the normal chloride reabsorption rate. We conclude that the cell Cl activity is primarily determined by the NaCl concentration in the tubule lumen and that Cl entry across the luminal membrane is mediated.

Reabsorption of chloride by the *Necturus* proximal tubule has been explained on the basis of passive driving forces. The transepithelial potential difference (PD), oriented lumen negative, of 10 to 15 mV is thought to provide sufficient driving force to move chloride across the permeable extracellular shunt pathway [5, 6, 17, 18]. However, the ratio of the chloride concentration in the tubule lumen to that of the plasma is always near 1.0, which does not agree with the value predicted from the transepithelial PD for a passively distributed ion [2, 6, 8]. In addition, intracellular chloride activity in *Necturus* proximal tubule cells is about

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three times greater than the value predicted for passive distribution. This observation led Khuri [8] to suggest that chloride entry into the tubule cell from the lumen was active, possibly in combination with sodium.

Evidence has been accumulated in other epithelial tissues (intestine, gallbladder) which points to a neutral entry of NaCl into epithelial cells across the mucosal cell boundary [4, 7, 10, 12]. Entry of chloride ions into *Necturus* proximal tubule cells from the luminal solution is against a sizeable electrical gradient since the cell interior is about 50 mV negative to the lumen. However, a neutral complex of sodium and chloride would enter the cell from the lumen along its favorable chemical gradient and would be uninfluenced by the adverse electrical field.

We investigated the relationship between intracellular chloride activity, measured with ion selective microelectrodes, and the composition of the luminal perfusate. We found that intracellular chloride was rapidly responsive to changes in the luminal NaCl concentration but independent of variations in the PD across the luminal membrane. In addition, we observed that the entry of chloride into the tubule cell from the lumen required an approximately equal concentration of luminal sodium. Other cations such as K, Li, or tetramethylammonium could not substitute for Na and did not enable chloride to enter the cell from the luminal perfusate. The exit of chloride from the cell across the peritubular membrane appeared to be a wholly passive process.

Materials and Methods

Perfusion Methods

Adult *Necturus maculosus* were obtained from Mogul Ed (Oshkosh, Wis.) and stored in aquaria at 15 °C. They were fed goldfish and were held for at least one month after receipt from the supplier before being used in experiments. They were anesthetized by immersion in 0.1% tricaine methane sulfonate (Finquel, Ayerst, N.Y.) and the kidneys were isolated and perfused as previously described [5]. The composition of the perfusion solutions is given in Table 1. Luminal perfusion with test solutions was accomplished by insertion of a pipet into the glomerulus. The pipet was connected to a gravity flow microperfusion apparatus and the tubule perfused at about 50 nl/min.

Electrical Methods

Potential differences were recorded with conventional 3M KCl-filled glass microelectrodes connected to a Ag-AgCl half cell (W.P. Instruments, Hamden, Conn.). The ground electrode was a calomel cell connected to the peritoneal cavity by a 3 M KCl-agar bridge. The half cell was connected to the input of a dual electrometer (Model 750, W.P. Instru-

Table 1. Composition of perfusion solutions[a]

Chloride Ringer's		32.5 Chloride Ringer's		Sulfate Ringer's	
NaCl	90 mM	NaCl	22.5 mM	Na_2SO_4	50 mM
$NaHCO_3$	10	$NaHCO_3$	10	Na_2HPO_4	1.0
NaH_2PO_4	0.5	NaH_2PO_4	0.5	K_2HPO_4	1.0
KCl	2.5	KCl	2.5	KH_2PO_4	0.4
$CaCl_2$	1.8	$CaCl_2$	1.8	Ca-Lactate	3.6
$MgCl_2$	1.0	$MgCl_2$	1.0	$MgSO_4$	1.0
Glucose	2.2	Glucose	2.2	Glucose	2.2
Glutamine	0.5	Glutamine	0.5	Glutamine	0.5
Na-Butyrate	3.0	Na-Butyrate	3.0	Na-Butyrate	3.0
		Mannitol	118	Mannitol	50
99% O_2, 1% CO_2		99% O_2, 1% CO_2		Air	
2 g% PVP		2 g% PVP		2 g% PVP	
pH 7.6, Osm 214[b]		pH 7.6, Osm 207[b]		pH 7.6, Osm 210[b]	

[a] All solutions contain Heparin 1500 U/liter.
[b] Osmolality units mosmol/kg.

ments, Hamden, Conn.); electrode impedance was constantly monitored by the injection of small test pulses into the input of the amplifier. Outputs from the electrometer were displayed on a digital panel meter (Model 200/A, Newport Instruments, Newport, Calif.), amplified and recorded on magnetic tape (Model A, Vetter Instruments, Rebersburg, P.), and digitized, sampled and recorded by a computerized data acquisition system (Tek 31/153, Tektronix, Beaverton, Oreg.). When the tubule lumen contained a solution of ionic composition different from that in the capillaries, measurements of transepithelial PD were subject to changes in liquid junction potential. No corrections were made for these small liquid junction potentials since they were less than 1.7 mV when measured with large 3 M KCl electrodes. An additional source of error in the transepithelial PDs in tubules with altered luminal perfusate was a variation in electrode tip potential of about ± 5 mV. Since we did not systematically check the tip potential of all the voltage electrodes, we did not correct transepithelial PDs for changes in tip potential.

For the potential dependence experiments an axial electrode [13] was inserted into the lumen of a free flow tubule. Constant current pulses were injected across the tubular epithelium using triangular waves generated by a function generator (Model FG101, Tektronix, Beaverton, Oreg.).

Ion Selective Electrode Methods

Single barrel chloride sensitive microelectrodes were constructed as follows. Capillary tubing (1 mm OD, 0.75 mm ID) was fabricated with an internal glass fiber by the National Institutes of Health glass shop. Similar capillary glass tubing is now commmercially available known as "omega dot" tubing. Pipets were pulled on a vertical puller (David Kopf, Tujunga, Calif.) and allowed to hydrate in air for 10 min. They were then exposed for 60 sec from the back end to the vapors of dimethyl-dichloro silane (Supelco, Inc., Bellefonte, Pa.). After heating for one hour at 100 °C to polymerize the silane, the electrodes were filled with chloride ion exchange resin (Orion, Cambridge, Mass.). Sufficient resin was injected into the electrode with a long 30-gauge needle to fill the electrode to the shoulder portion. The glass fiber in the electrode readily conducts the ion exchange resin down

to the tip. Electrodes were then left for 24 hr with their tips immersed in 1 M NaCl. No reference filling solution was placed above the resin; instead a chlorided silver wire was inserted directly into the resin when the electrode was ready to be used [11]. The silver wire was connected to the input of a very high impedance electrometer (Model F-23, input impedance $> 10^{15}\ \Omega$, W.P. Instruments, Hamden, Conn.). The output from the electrometer was displayed and recorded as described for the PD measurements.

Cl-electrodes constructed as described above exhibited the following characteristics: resistance $4 \times 10^{10}\ \Omega$, electrical time constant 0.1 sec, average slope 59.0 ± 0.6 mV/decade (at 23° C), correlation coefficient between PD and log Cl activity > 0.999, response time constant to a step change in chloride activity about 1 sec. Typical storage life of an electrode was 4 to 5 days. When solutions with known activites were presented to the electrodes *in vitro* the measured values agreed to within 1 mM (e.g., Cl Ringer's has a calculated chloride activity of 75.0 mM and a measured value of 76.0 ± 1.3, $n = 40$). Approximate selectivity of the electrode for chloride over other anions was as follows: Cl^-/HCO_3^- 10:1, $Cl^-/HPO_4^=$ 25:1, $Cl^-/SO_4^=$ 5:1. The observed sensitivity of the chloride ion exchanger to other anions introduced an artifact into measurements of intracellular chloride activity. A fraction of the apparent cell chloride activity was due to the electrode response to other intracellular anions. Perfusion of the kidney with sulfate Ringer's solution washed out all cell chloride (*see* Results) yet the chloride electrodes gave an apparent chloride of 5 to 8 mM (mean value 6.2). We attribute this reading to electrode sensitivity to intracellular anions other than chloride. When the tubule lumen was perfused with NaCl solutions, the apparent cell chloride activity increased rapidly above the background level. These increments in cell chloride then represent the true changes in the intracellular chloride activity.

Experimental Protocol

The early portion of a surface tubule was selected for micropuncture, and the PD measurements made first, peritubular membrane potential as well as transepithelial PD. These measurements were considered to be satisfactory if there was no change in electrode impedance or tip potential in surface fluid. The results were then stored in computer memory. A chloride electrode was placed on the surface of the kidney and a record of the steady-state reading was obtained. (Achievement of the steady state was ascertained by the computer which sampled the voltage readings repetitively until two successive readings taken 8 sec apart agreed to within 0.1 mV. The time interval and voltage tolerance were chosen after study of electrode behavior in standard solutions showed that a steady-state value had been reached. It took from 15 to 100 sec to achieve this steady-state voltage.) The chloride electrode was then inserted into a proximal tubule cell in the same region of the tubule where the PD measurements had been made. It was not possible to impale the same cell as had been used for the PD determination. The cell was considered damaged and the voltage record unacceptable if the chloride electrode reading did not suddenly jump at least 30 mV negative after the electrode penetrated the peritubular membrane. The initial negative voltage deflection is primarily due to the peritubular membrane PD and would be expected to be about -60 mV. A smaller initial deflection is actually observed because chloride electrodes develop positive voltages at physiologic chloride activities. This positive reading is typically about 70 to 80 mV. The electrode response to these two opposing voltages is an initial 30 mV or larger negative deflection. After a successful recording the electrode was removed from the kidney and calibrated in standard NaCl solutions (concentrations $-$ 10, 50, 100 mM). The electrode was also immersed in a reservoir of perfusion solution to test for spurious liquid junction potentials. The entire procedure was then repeated on the same tubule while the tubule was perfused with the test solution. The test solutions contained a low concentration (0.05%) of lissamine green to indicate the adequacy of tubule perfusion. PD and chloride activities were recorded after at least two

Table 2. Luminal NaCl substitution-chloride Ringer's perfusion

Luminal perfusate	Perfusate Cl^- activity (mM)	Peritubular PD (mV)	Transepithelial PD (mV)	Intracellular Cl^- activity (mM)
100 NaCl Ringer's (control)	75.0	-62.1 ± 1.2 (37)	-9.7 ± 0.5 (33)	24.5 ± 1.1 (41)
Control	75.0	-65.0 ± 2.1 (9)	-9.4 ± 1.0 (7)	23.9 ± 3.4 (9)
55 NaCl Ringer	42.9	-62.1 ± 2.9 (8)	-13.2 ± 0.9 (7)	24.8 ± 2.4 (9)
	p	n.s.	<0.02	n.s.
Control	75.0	-59.7 ± 3.2 (8)	-10.2 ± 1.0 (8)	25.4 ± 2.3 (10)
32.5 NaCl Ringer	25.2	-61.5 ± 1.9 (9)	-15.4 ± 1.2 (9)	11.8 ± 0.5 (9)
	p	n.s.	<0.01	<0.001
Control	75.0	-61.6 ± 3.0 (4)	-11.8 ± 2.8 (4)	21.5 ± 2.7 (6)
O NaCl	0	-61.3 ± 1.9 (6)	–	10.7 ± 0.6 (13)
	p	n.s.		<0.001

Mean $\pm$ SEM shown with number of tubules and p values for t-test. Control solution is 100 NaCl Ringer's. 55 and 32.5 solutions are Ringer's with mannitol replacement of the missing NaCl. O NaCl solution is pure mannitol (183 mosmol).

minutes of luminal perfusion with the test solutions since it was ascertained that a steady-state cell chloride was achieved in this short time. As will be seen in the results section of this paper, peritubular membrane potential was unchanged during all of the experiments involving luminal perfusion. Thus we chose not to construct double barrel electrodes with one Cl sensitive and one potential sensitive barrel as utilized by Khuri [8].

All data are presented as the mean value $\pm$SEM. The number of observations are in parentheses.

Results

Chloride Ringer's Experiments

Perfusion of the lumen and capillaries with identical chloride Ringer's solutions gave the results shown in the top portion of Table 2. Peritubular and transepithelial PDs agree well with previously published values [3, 8, 13, 14, 16, 17]. Reduction of the luminal NaCl concentration to 55 mM had no effect on peritubular PD or cell Cl activity. As has been previously reported [1] the tubule P_{Na}/P_{Cl} is around 0.3 and dilution of luminal NaCl results in a lumen-negative dilution PD. The lower portion of Table 2 shows that the transepithelial PD became significantly more negative when the luminal NaCl concentration was reduced to 55 mM. Perfusion of the tubule lumen with 32.5 NaCl Ringer's greatly lowered cell Cl activity but did not influence the peritubular membrane potential.

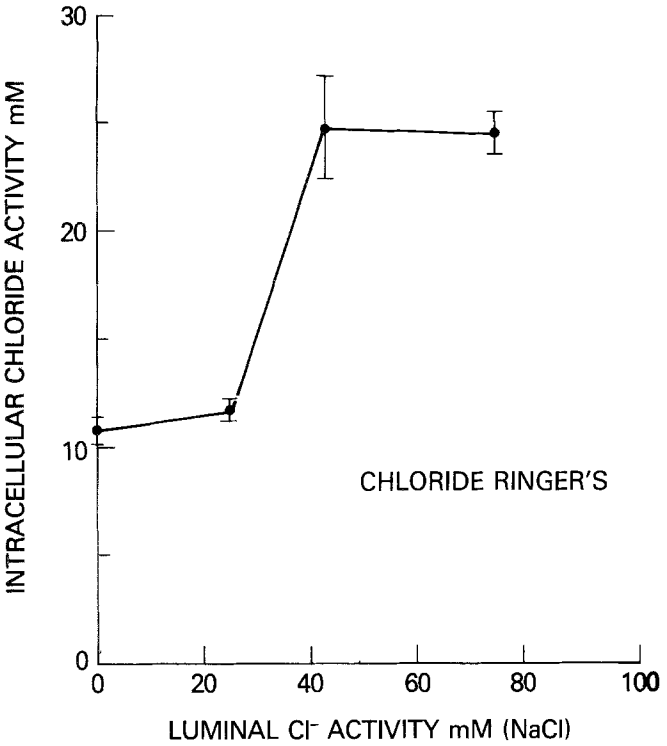

Fig. 1. Intracellular chloride activity on the ordinate is plotted as a function of luminal chloride activity on the abscissa. The capillaries were perfused with normal chloride Ringer's (chloride activity 75 mM). The luminal perfusates were NaCl Ringer's solutions in which some of the NaCl was replaced by mannitol. The 0 NaCl solution was pure mannitol. The line connecting the points was drawn by eye. Values shown are mean with SE. Paired control data are not included but are listed in Table 2

The decrease in cell chloride activity occurred rapidly. Repetitive measurements of different cells within the same tubule indicated that a new steady-state cell chloride was achieved in less than two minutes. The transepithelial PD became more negative as expected from the increased dilution potential. Complete removal of luminal NaCl and perfusion of the tubule with pure mannitol reduced cell Cl activity to 10.7 mM but did not affect the peritubular PD. Transepithelial PD values were unreliable because of the low conductivity of the mannitol solution. The cell Cl activity of 10.7 mM when the lumen is chloride-free is not very different from the value predicted for passive Cl distribution across the peritubular membrane (7.0 mM). Since, as described in Methods, the chloride electrode senses other anions as well as chloride, the apparent chloride activity of 10.7 mM probably represents a true intracellular chloride activity of 5 to 6 mM.

Table 3. Low NaCl lumen and capillary compared to low NaCl lumen only

NaCl luminal perfusate	NaCl capillary perfusate	Peritubular PD (mV)	Transepithelial PD (mV)	Intracellular Cl^- activity (mM)
32.5	32.5	-43.7 ± 1.4 (7)	-9.1 ± 0.8 (7)	12.6 ± 1.0 (18)
32.5	100	-61.5 ± 1.9 (9)	-15.4 ± 1.2 (9)	11.8 ± 0.5 (9)
	p	<0.001	<0.01	n.s.

Mean $\pm$ SEM with number of tubules and p value for t-test. Perfusion solutions are listed in Table 1.

Fig. 1 depicts intracellular chloride activity in mM as a function of the luminal Cl activity. Cell chloride activity shows a sigmoidal dependence on luminal Cl.

The dependence of cell Cl activity on the NaCl concentration of the capillary perfusate was also tested. The lumen and capillaries were perfused with 32.5 mM chloride Ringer's and cell chloride activity determined. Table 3 compares the results obtained to those of the 100 mM chloride Ringer's experiments (Table 2) in which the tubule lumen was perfused with 32.5 mM Cl Ringer's while the capillaries contained 100 Cl Ringer's. Perfusion of the capillaries with 32.5 mM Cl Ringer's decreased the peritubular PD significantly as has been previously reported [15]. Intracellular chloride was unchanged in spite of the large change in electrochemical gradient across the peritubular membrane.

Sulfate Ringer's Experiments

Perfusion of the kidneys with the sulfate Ringer's solution (Table 1) for one hour completely washed all chloride out of the kidney. Chemically measurable chloride was less than 1 mM/kg wet weight of kidney. Chloride concentration of the venous effluent fell below 0.1 mM within the first hour of sulfate Ringer's perfusion.

Table 4 gives the values of peritubular and transepithelial PD after one hour of sulfate perfusion. Control intracellular chloride activity is also listed, however, the results presented above indicate that no chloride is present in the tubule cells. As described in Methods this chloride electrode reading is the result of electrode sensitivity to intracellular anions such as phosphate, sulfate, bicarbonate, and organic anions.

In the lower portion of Table 4 the results of luminal perfusion experiments are listed. All the perfusates contained 100 mM Na and mixtures

Table 4. Luminal NaCl-substitution-sulfate Ringer's perfusion

Luminal perfusate	Perfusate Cl^- activity (mM)	Peritubular PD (mV)	Transepithelial PD (mV)	Intracellular Cl^- activity (mM)
Sulfate Ringer's	O	$-61.7 \pm 2(34)$	$-16.6 \pm 0.7(34)$	$6.2 \pm 0.5(44)$
Control	0	$-63.6 \pm 3.0(8)$	$-15.7 \pm 1.4(9)$	$5.8 \pm 0.8(14)$
100 NaCl	77.2	$-60.9 \pm 2.3(10)$	$+14.3 \pm 2.2(7)$	$21.3 \pm 2.9(16)$
	p	n.s.	<0.001	<0.001
Control	0	$-63.7 \pm 1.8(9)$	$-11.3 \pm 1.4(9)$	$7.9 \pm 1.1(10)$
50 NaCl + 25 Na_2SO_4	37.9	$-62.0 \pm 0.9(7)$	$+10.2 \pm 1.0(7)$	$17.8 \pm 1.2(12)$
	p	n.s.	<0.001	<0.001
Control	0	$-65.6 \pm 1.6(8)$	$-19.1 \pm 2.1(8)$	$4.6 \pm 0.6(10)$
25 NaCl + 37.5 Na_2SO_4	18.8	$-59.8 \pm 1.9(10)$	$-1.6 \pm 2.9(9)$	$10.0 \pm 0.9(12)$
	p	n.s.	<0.001	<0.001

Mean ± SEM shown with number of tubules. 50 Na_2SO_4 Ringer's is control perfusate in all experiments. All other perfusates are pure salt solutions whose Na^+ concentration is 100 mM. *p* values are for *t*-test.

of the anions $SO_4^=$ and Cl^-. When the luminal NaCl concentration was 100 mM (no sulfate), cell chloride activity rose rapidly (<2 min) to 21.3 mM without any change in peritubular PD. Transepithelial PD became strongly positive due to a chloride/sulfate biionic potential across the chloride selective shunt pathway. Perfusion of the lumen with 50 mM NaCl and 25 mM Na_2SO_4 did not alter peritubular PD. Transepithelial PD was slightly lower than the 100 mM NaCl perfusion in agreement with a reduction in the magnitude of the biionic potential. Intracellular Cl activity rose to a value slightly lower than that obtained in 100 mM NaCl perfusion. Luminal perfusion with 25 mM NaCl and 37.5 mM Na_2SO_4 did not alter peritubular PD. Transepithelial PD was near zero because of a balance between the biionic potential and the spontaneous PD. Cell chloride activity increased from the chloride-free situation. Fig. 2 shows the relationship between intracellular chloride activity and luminal Cl activity for the sulfate Ringer's experiments. The Na concentration of both luminal and capillary perfusates remained at 100 mM in all of the sulfate Ringer's experiments, yet the cell chloride was determined by the luminal NaCl concentration. In addition, it appeared that

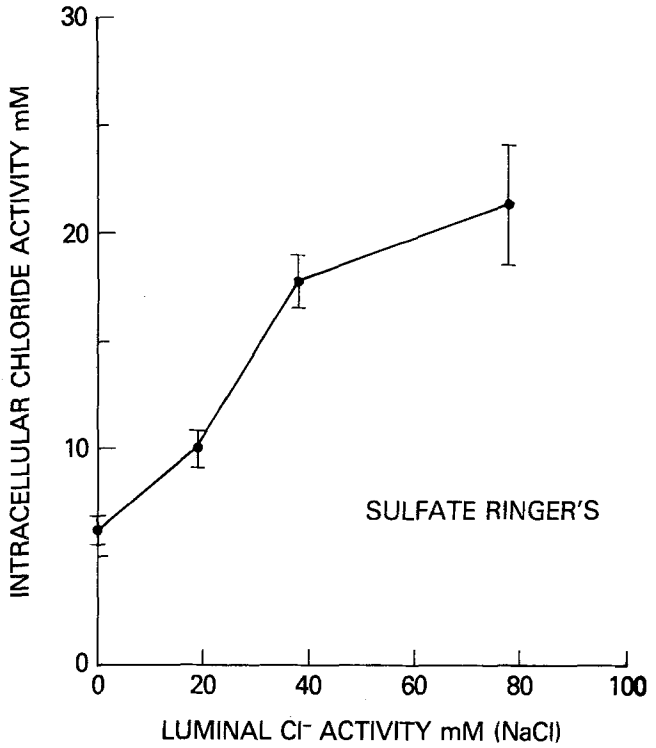

Fig. 2. Intracellular chloride activity on the ordinate, as a function of luminal chloride activity on the abscissa, is shown with mean values and their standard errors. The capillaries were perfused with sulfate Ringer's and the lumen with mixtures of NaCl and Na_2SO_4. The luminal Na concentration was constant at 100 mM. The 0 chloride solution contained 50 mM Na_2SO_4 alone, while the 77 mM perfusate contained only 100 mM NaCl. The line connecting the points was drawn by eye. Paired control data are not indicated but are listed in Table 3

chloride must be present in the lumen at a concentration greater than 25 mM to maintain elevated cellular Cl activity.

We tested the cation sensitivity of the luminal chloride entry step by perfusing the tubule lumen with 100 mM KCl, LiCl or tetramethylammonium chloride (TMACl). The results are listed in Table 5 and depicted in Fig. 3. The 100 mM NaCl result is reproduced for comparison. Luminal perfusion with 100 mM KCl, LiCl or TMACl did not alter peritubular PD or intracellular chloride activity. In spite of the large chemical concentration gradient for chloride, no chloride entered the proximal tubule cells unless Na was present in the lumen. Large biionic potentials developed due to the concentration differences in both anions and cations across the shunt pathway.

Table 5. Luminal cation substitution-sulfate Ringer perfusion

Luminal perfusate	Peritubular PD (mV)	Transepithelial PD (mV)	Intracellular Cl^- activity (mM)
Control	$-63.6 \pm 3.0(8)$	$-15.7 \pm 1.4(8)$	$5.8 \pm 0.8(14)$
100 NaCl	$-60.9 \pm 2.3(10)$	$+14.3 \pm 2.2(7)$	$21.3 \pm 2.9(16)$
p	n.s.	<0.001	<0.001
Control	$-58.8 \pm 1.6(8)$	$-12.4 \pm 1.7(8)$	$9.6 \pm 2.4(9)$
100 TMACl	$-56.7 \pm 1.8(8)$	$+26.8 \pm 3.0(8)$	$7.2 \pm 1.0(12)$
p	n.s.	<0.001	n.s.
Control	$-57.8 \pm 2.0(10)$	$-15.6 \pm 1.1(10)$	$7.2 \pm 1.0(11)$
100 LiCl	$-62.0 \pm 1.7(8)$	$+17.5 \pm 3.5(7)$	$6.5 \pm 0.9(12)$
p	n.s.	<0.001	n.s.
Control	$-61.6 \pm 1.9(6)$	$-15.5 \pm 0.8(6)$	$9.2 \pm 1.7(6)$
100 KCl	$-62.2 \pm 5.5(5)$	$+12.9 \pm 2.6(5)$	$6.1 \pm 0.9(10)$
p	n.s.	<0.001	n.s.

Mean $\pm$ SEM shown with number of tubules and p values for t-test. Control solution is 50 Na_2SO_4 Ringer's, test perfusates are pure salt solutions whose chloride activities are 77.2 mM.

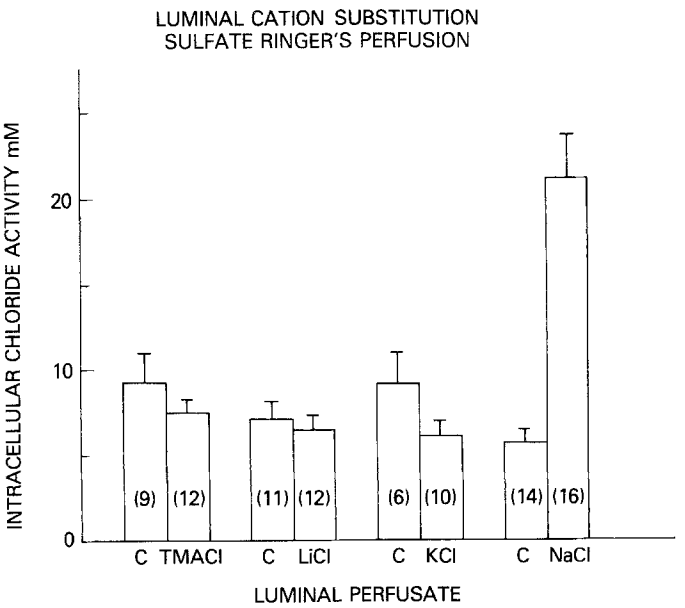

Fig. 3. Intracellular chloride activity on the ordinate when the tubule lumen is perfused with either Na_2SO_4 (control) or the chloride salt of tetramethylammonium (TMA), Li, K or Na. The capillaries were perfused with sulfate Ringer's. The bars depict the mean value and SE for these paired experiments. Chloride activity is not significantly changed during perfusion of TMACl, LiCl or KCl. The change during NaCl perfusion is highly significant (*see* Table 5)

Table 6. Luminal perfusion with low Na^+, high Cl^-

Luminal perfusate	Peritubular PD (mV)	Transepithelial PD (mV)	Intracellular Cl^- activity (mM)
10 NaCl + 90 TMACl	$-63.9 \pm 3.1(6)$	$-0.4 \pm 1.0(6)$	$10.7 \pm 0.9(8)$
0 NaCl	$-61.3 \pm 1.9(6)$	–	$10.7 \pm 0.6(13)$
p	n.s.		n.s.

p value for *t*-test. Capillary perfusion with normal chloride Ringer's.

Ionic Requirements for Normal Cell Chloride Activity

Entry of chloride into the proximal tubule cell required NaCl in the lumen; the tubule lumen was perfused with a solution containing 100 mM chloride but only 10 mM Na to test the Na concentration requirement. When a mixture of 10 mM NaCl and 90 mM TMACl was perfused through the lumen of proximal tubules, cell chloride activity fell to a value indistinguishable from the NaCl-free perfusion. The capillaries were perfused with 100 mM Cl Ringer's. Table 6 lists the results and compares them to the zero NaCl case from Table 2. It is of interest to note that the transepithelial PD fell to near zero when the tubule lumen was perfused with this low Na, high Cl solution.

Intracellular chloride activity remains at the minimum value of 10 to 11 mM when the lumen is perfused with a solution deficient in either Na or Cl. Fig. 4 summarizes the results of four different experiments in which tubules were perfused with solutions deficient in either Na or chloride. The results are compared to the NaCl-free perfusion experiment from Table 2. Cell chloride activities in Fig. 4 do not significantly differ from each other. Maintenance of normal cell chloride activity thus requires similar concentrations of Na and Cl in the tubule lumen at a minimum value of 50 mM.

Potential Dependence Experiments

Since cell chloride activity greater than 10 mM is solely determined by the entry of chloride from the tubule lumen, variation in steady-state chloride activity provides a measure of alterations in the rate of luminal chloride entry. A review of the results in Tables 2, 3, and 4 suggests

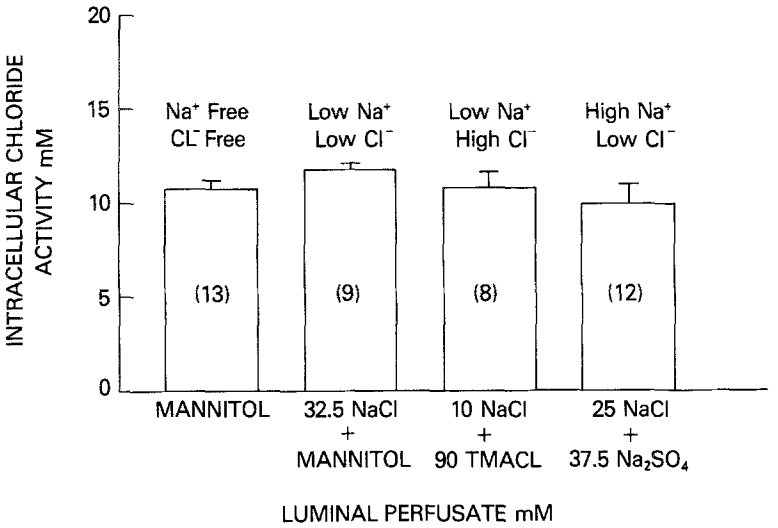

Fig. 4. Intracellular chloride activity on the ordinate is shown as the mean with SE for various test conditions. The first three bars, mannitol, 32.5 NaCl+mannitol, 10 NaCl+90 TMACl, show the results of experiments in which the capillaries were perfused with NaCl Ringer's. The luminal perfusates vary widely in ionic strength and composition yet cell chloride is not significantly altered. The last bar depicts the results when the capillaries are perfused with sulfate Ringer's while the lumen is perfused with a mixture of NaCl and Na_2SO_4. The cell choride activity obtained is not significantly different from any of the other conditions shown

Table 7. Cell chloride and luminal potential difference

Luminal perfusate	Capillary perfusate	Luminal PD (mV)	Intracellular Cl^- activity (mM)
100 NaCl	100 NaCl	-52.4 ± 1.0	$24.5 \pm 1.1(41)$
100 NaCl	50 Na_2SO_4	-45.1 ± 2.5	$21.3 \pm 2.9(16)$
	p	<0.01	n.s.
32.5 NaCl	100 NaCl	-46.1 ± 1.7	$11.8 \pm 0.5(9)$
32.5 NaCl	32.5 NaCl	-34.6 ± 1.2	$12.6 \pm 1.0(18)$
	p	<0.001	n.s.

Mean $\pm$ SEM together with number of tubules. p value for t-test. Luminal PD values calculated from data in Tables 2, 3 and 4.

that variations of the PD across the luminal membrane of the cell do not affect steady-state cell chloride activity. These data are summarized in Table 7. If chloride enters the proximal tubule cell from the lumen in a charged form, steady-state chloride activity should be affected by changes in the PD across the luminal membrane. Since the luminal

PD changes were secondary to alterations in the ionic composition of the luminal or capillary perfusates, the experiments in Table 7 do not directly resolve the question of the possible potential dependence of luminal Cl entry. We undertook, therefore, a series of experiments utilizing an axial electrode to pass current across the tubule epithelium and thus directly alter the luminal PD.

These current clamp experiments were performed as follows: (i) a straight segment of early proximal tubule was chosen for micropuncture. The glomerulus was punctured and the tubule continuously perfused with 100 or 32.5 mM Cl Ringer's. Capillary perfusion was always 100 mM Cl Ringer's. A 22 μm diameter pipet was inserted parallel to the long axis of the tubule. (ii) The axial electrode was extended from this pipet – no oil blocks were used – and current was passed from the electrode to ground. (iii) A KCl electrode was inserted into a tubule cell in the region of the epithelium directly over the axial electrode. A record of the PD response to the current signal was then made. The current clamp command was a triangular wave of extremely low frequency (0.025 or 0.05 Hz) and amplitude 1.5 to 1.75 μA. The frequency and amplitude were selected after preliminary experiments based on the need to change the luminal PD a large amount symmetrically without inducing polarization artifacts. It was possible, by using a slow symmetrical triangular wave, to pass relatively large currents across the epithelium and thereby change the transepithelial PD by 50 to 70 mV without creating polarization potentials. Only a small fraction of the transepithelial current flows through the cells [13], and the change in peritubular PD is much smaller than the change in luminal PD (i.e., ca. 10 mV). (iv) The KCl electrode was next advanced into the tubule lumen and a record made of the transepithelial PD changes induced by the current clamp. (v) A chloride selective electrode was placed in a tubule cell over the axial electrode and a record made of the response to the same current clamp command.

A typical experiment in which lumen and capillaries contained 100 mM Cl Ringer's is shown in Fig. 5, where there are three voltage records each with its current-voltage diagram. The first record (left) is the response of the transepithelial PD to the current signal. It may be seen that the record is a faithful reproduction of the triangle wave command (0.05 Hz) and that the current-voltage diagram is linear. The spontaneous transepithelial PD was −7.0 mV and the magnitude of the PD excursion was 70 mV. The change in peritubular PD is shown in the center of Fig. 5. Again the voltage record is a duplicate of the current signal and the current voltage plot is linear. The peritubular PD prior to the current clamp was −61.5 mV, and the PD change during current flow

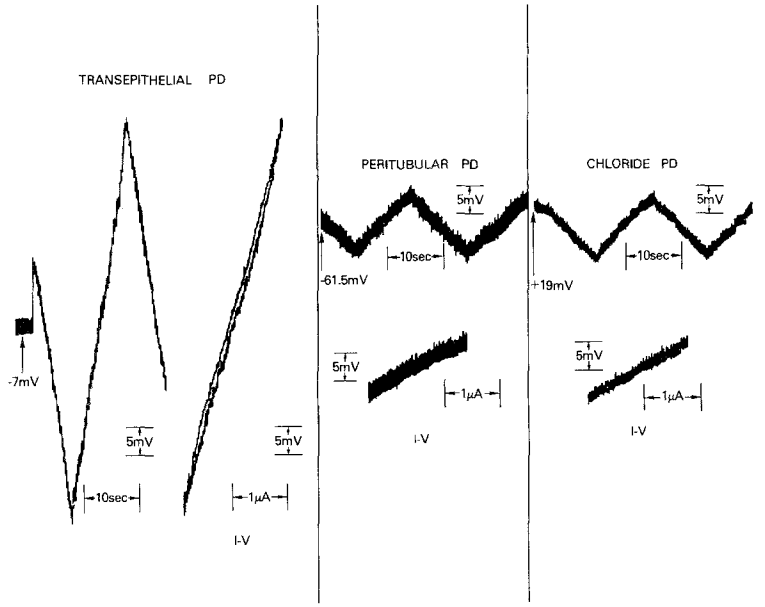

Fig. 5. Records of PD and current-voltage plots (I–V) of a typical experiment designed to test the potential dependence of the intracellular chloride activity. The left panel depicts the voltage change across the epithelium during the passage of a 1.75 μA current from an axial electrode placed within the tubule lumen. The triangular current varied symmetrically around the transepithelial PD of −7 mV. The transepithelial PD was changed 35 mV in each direction. To the right of the voltage record is the current-voltage diagram for the transepithelial PD. The center panel shows the change in peritubular PD in the same tubule and location as the transepithelial PD record. The voltage record is in phase with the transepithelial PD and the current-voltage plot is linear. The voltage divider ratio (the ratio of luminal PD change to peritubular PD change) is about 6 to 1. The right hand panel shows the record of peritubular PD obtained, for the same current in the same tubule, by the use of a chloride-sensitive electrode. The voltage record is identical to that in the middle panel and the current-voltage plot is similar in slope and linearity. The chloride electrode used had a slope of 59.9 mV and a correlation coefficient of 0.9993 in standard NaCl solutions. Cell chloride activity was 16.56 mM and did not change during current passage

was 12.5 mV. The luminal membrane potential was changed by the difference between the peritubular and transepithelial PD alterations. The spontaneous luminal PD of −54 mV was varied from −25 mV to −83 mV during each 20-sec cycle. The record on the right of Fig. 5 shows the response of the chloride electrode placed in a tubule cell. The chloride electrode responds to both voltage and chloride activity, yet the record is identical to that recorded with the KCl electrode (center panel). The current voltage plot is linear and similar in slope and amplitude to the PD record. The chloride electrode did not sense any change in cell chloride activity from the initial value of 16.5 mM.

Since it was possible that cell chloride was normally too high to be influenced by luminal PD changes, we did a number of experiments in which the tubule lumen was perfused with 32.5 Cl Ringer's to reduce cell chloride. Cell chloride activities ranging from 9.3 to 23.0 mM were achieved by the perfusion. In none of the 20 experiments conducted in this manner did the chloride electrode record differ significantly from the KCl electrode recording either in linearity or amplitude. In 6 experiments we were able to pass very low frequency current signals of 0.025 Hz (one cycle/40 sec) without polarization artifacts. No evidence of potential-induced change in cell chloride activity was obtained.[1] Chloride entry from the tubule lumen must be neutral because of the observed lack of potential dependence.

Chloride Flux Estimations

The results of the chloride Ringer's experiments indicated that chloride efflux across the peritubular membrane was probably passive, determined by the electrical potential difference between cell and extracellular fluid (ECF). The ionic current of chloride from cell to ECF across the peritubular membrane would be given by:

$$I_{Cl} = G_{Cl}(V_m - V_{eq}), \tag{1}$$

where I_{Cl} is the chloride current in amps/cm^2, G_{Cl} the partial chloride conductance of the peritubular membrane (mho/cm^2), V_m the peritubular membrane potential, V_{eq} the chloride equilibrium potential across the peritubular membrane. G_{Cl} may be calculated from the total membrane conductance, G_m, and the chloride transference number, T_{Cl}, as follows:

$$G_{Cl} = G_m T_{Cl}. \tag{2}$$

We have not measured the partial chloride conductance of the peritubular membrane, but G_m and T_{Cl} have been measured by Boulpaep [3] as 3.9×10^{-4} mho/cm^2 and 0.2 to 0.39, respectively. G^{Cl} is then 0.8 to 1.5×10^{-4} mho/cm^2. The maximum values of I_{Cl}, V_m, V_{eq} and the net

1 If chloride entered the cell as a monovalent, positively charged species, the change in luminal PD can be calculated to alter the chloride entry rate by about 50% at each extreme value. This would lead to a small, but readily observable, change in the chloride electrode voltage of about 2 to 5 mV. It is unavoidable that changes in peritubular PD also occur during current passage. In all cases these changes were small (ca. ± 5 mV) and the peritubular PD was always far above the chloride equilibrium potential. Thus the chloride flux through the peritubular membrane should not have been measurably affected by these small PD excursions.

Table 8. Transcellular chloride flux estimation

Luminal perfusate	V_m (mV)	V_{eq} (mV)	I_{Cl} μA/cm^2	ϕ_{Cl} pmoles cm^{-2} sec^{-1}
100 NaCl	−62.1	−28.5	5.0	52.6
55 NaCl	−62.1	−28.2	5.1	53.0
32.5 NaCl	−61.5	−47.2	2.1	22.4

Capillaries perfused with normal Ringer's. V_m is the peritubular membrane PD, V_{eq} the chloride equilibrium PD across the peritubular membrane, I_{Cl} the calculated chloride current across the peritubular membrane, ϕ_{Cl} the flux equivalent to I_{Cl} in picomoles cm^{-2} sec^{-1}.

chloride flux, Φ_{Cl}, are given in Table 8 for the chloride Ringer's experiments in Tables 2 and 3. Since the cell chloride is in a steady-state, the chloride efflux across the peritubular membrane must be matched by influx across the luminal membrane. The transcellular chloride flux thus calculated may be compared with the observed reabsorption rate of NaCl for only one condition – the experiments in which both lumen and capillaries contain 100 mM Cl Ringer's. Net NaCl reabsorption by the proximal tubules of a perfused *Necturus* kidney under comparable conditions to the present experiments is about 95 picomoles/cm^2-sec [15]. Accepting this combination of results from different laboratories, the calculated transcellular flux constitutes approximately 55% of the observed net NaCl reabsorption.

A second approach was also used to estimate the chloride flux into the cell across the luminal membrane. A chloride electrode was placed in a proximal tubule cell in a tubule whose lumen was perfused with sulfate Ringer's or 100 TMACl; the capillary perfusate was either sulfate Ringer's or chloride Ringer's. The luminal perfusate was switched from sulfate Ringer's (or 100 TMACl) to 100 NaCl and the time course of increase in cell chloride was recorded. Most of these experiments were technical failures because of displacement of the chloride electrode when the perfusate was changed or because of instability of the voltage record. Three successful experiments were performed and the tracings are shown in Figure 6. From the initial slope of the change in cell chloride and the surface to volume ratio of the cell it is possible to calculate the initial chloride flux into the cell. Since the cell chloride activity is low before the introduction of NaCl into the tubule lumen, the rate of luminal Cl entry may have been enhanced in these experiments. The average initial slope obtained is 0.38 mV/sec which corresponds to a Cl concentration change of 0.12 mM/sec. The corresponding flux per cm^2 of tubule may be calculated from the estimated cell volume per cm^2 of tubule

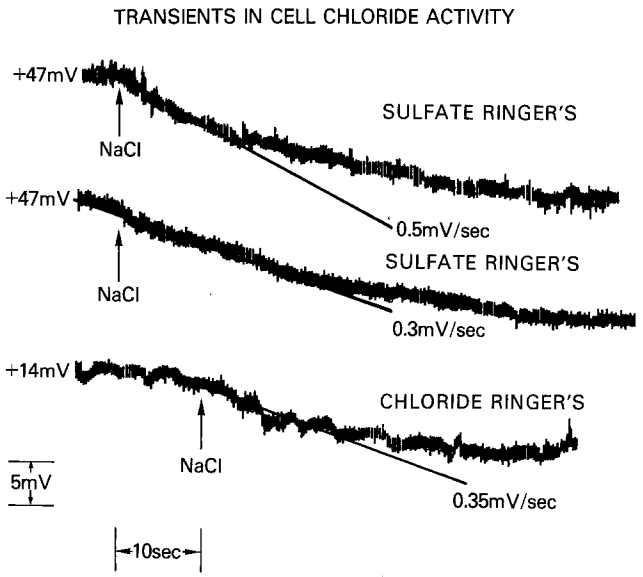

Fig. 6. Records from three successful experiments showing the increase in intracellular chloride activity when the tubule lumen is perfused with 100 mM NaCl. The top two tracings are from experiments in which the capillaries and lumen contained sulfate Ringer's until the point indicated by the vertical arrow. The initial slope of the change in chloride electrode PD is shown at the end of each of the fitted lines. The bottom tracing shows a result from a normal chloride Ringer's perfusion. The capillaries contained chloride Ringer's while the lumen was perfused with 100 mM TMACl until the point indicated by the vertical arrow when 100 mM NaCl was perfused through the lumen. Peritubular membrane PD did not change during these and other perfusions

(2.0×10^{-6} liter/cm^2). The flux value obtained is 240 pmoles/cm^{-2} sec^{-1} which is about five times the steady-state transcellular flux calculated from the conductance of the peritubular membrane.

Discussion

Our results show that intracellular chloride activity is largely a function of the NaCl concentration in tubule lumen. The nonlinear relationship between cell chloride activity and luminal chloride activity (or concentration) suggests an interaction with the luminal membrane. The fact that cell chloride activities are far in excess of the values predicted for passive distribution further supports the hypothesis of mediation of chloride entry by the luminal membrane. Additional evidence comes from the selectivity of the entry step for NaCl and the inability of the chloride salts of other cations (K^+, Li^+, TMA^+) to alter cell chloride.

Finally, two observations point to a neutral NaCl complex as the species which crosses the luminal membrane. First, similar concentrations of sodium and chloride are required to elevate cell chloride. (The presence of either ion in small amounts, 10 to 25 mM, does not influence the entry of chloride from the lumen.) Second, the lack of potential dependence of chloride entry is consistent with the movement of a neutral carrier complex of sodium and chloride from lumen to cell.

Neutral entry of NaCl into epithelial cells from the mucosal solution has been observed in other epithelia. Frizzell *et al.* [4] concluded that a substantial portion of the transepithelial NaCl flux across rabbit gallbladder was the result of the neutral movement of NaCl from the lumen into the cell. This conclusion was based on the results of both Na and Cl tracer experiments as well as electrophysiological data. Hénin and Cremaschi [7] also postulated neutral NaCl transport on the basis of electrical measurements in rabbit gallbladder. A similar conclusion was reached by Nellans *et al.* [10] for NaCl movement into the epithelial cells of the rabbit ileum. Although we have not measured Na fluxes or Na activities in the present investigation, our results suggest a close relationship between Na and Cl movement. It is highly likely that NaCl entry into *Necturus* proximal tubule cells from the tubule lumen is such a neutral process.

The elevated value of cell chloride activity during 100 mM Cl Ringer's perfusion has been used in the past as evidence for carrier mediation of chloride entry [8]. As previously described, it is unclear from measurements of apparent intracellular chloride activity alone how much of that activity is truly due to chloride ions. The results of the sulfate Ringer's perfusions (Tables 4 and 5) indicate that about 5 to 6 mM of the apparent cell chloride activity is due to the electrode sensitivity to other anions. The control cell chloride activity in Table 2 is 24.5 mM; from the preceding discussion the true chloride activity is probably closer to 20 mM. This would be equivalent to an intracellular chloride concentration of 26.7 mM if the cell chloride activity coefficient equals that in free solution (0.75). Khuri [8] measured the chloride activity of *Necturus* proximal tubule cells, *in vivo*, and calculated an intracellular chemical concentration of 18.7 ± 1 mM. Chemically measured chloride in *Necturus* kidneys gives a proximal tubule chloride of about 32 mM/kg cell H_2O [8,15, 18]. Uncertainty in the extracellular space determination and contributions by cells other than those of the proximal tubule could be sufficient to explain the difference between chemically measured concentration and that calculated from the chloride activity [8].

A remarkable observation in our experiments is the insensitivity of the *Necturus* peritubular membrane potential to changes in the luminal ionic composition. Peritubular PD (Tables 2, 4, 5, 6) is in excellent agreement with previous observations, and it remains unaltered during perfusion of the lumen with a wide variety of solutions. Thus luminal perfusion of pure mannitol, 100 mM KCl or 100 mM NaCl has no effect on peritubular PD. The constancy of peritubular PD enabled us to utilize single barrel chloride electrodes instead of the double barrel type, which are difficult to construct and may damage the cell membrane more than single barrel electrodes. Double barrel electrodes do have the advantage of recording voltage and chloride activity from the same cell. It is possible that we could have failed to detect small steady-state differences in peritubular PD or cell chloride activity because of our sampling of cell populations with single barrel electrodes. It is also possible that we failed to detect transient alterations in membrane potential or chloride activity since we recorded steady-state readings. The peritubular membrane of *Necturus* proximal tubule cells behaves as a K electrode [3, 5]. Measurements of cell K activity led Khuri [8] to conclude that K was in electrochemical equilibrium across the peritubular membrane. He suggested that the peritubular PD was created by active Na transport and K distributed itself passively in accordance with the electrical driving forces. The constancy of the peritubular PD in experiments in which the lumen is NaCl-free may seem to contradict Khuri's proposal, since no luminal Na would be available to maintain the peritubular membrane potential. It is possible, however, that the peritubular PD is kept constant by the existing potassium diffusion potential for a relatively long time period even when the lumen is devoid of NaCl and there is presumably no active Na transport.

The absence of large changes in peritubular PD when the lumen is perfused with 100 mM KCl of LiCl indicates that the luminal membrane of the tubule cell has virtually no passive permeability to these ions. The luminal PD has been reported to show minor dependence on the concentration of K or Cl in the tubule lumen [3]. Our observations suggest that the cell admits luminal NaCl only in a neutral form. The selectivity of this entry process is high and the rate of Cl entry into the tubule cell varies with the luminal Cl activity. Lindemann *et al.* [9] have suggested a negative feedback relationship between outside Na concentration and the Na permeability of the outer facing membrane of the cells of the frog skin. Spring and Giebisch [15] have observed a similar relationship in *Necturus* proximal tubules. Luminal membrane

Na permeability was calculated from the chemical Na gradient, luminal PD and net Na flux. This permeability was inversely proportional to luminal Na concentration; e.g., as luminal Na fell, luminal Na permeability increased. A similar conclusion may be reached on the basis of the present results. Assuming that Cl enters the cell from the lumen in a neutral form and exits passively across the peritubular membrane as a charged species, we may write the following equation:

$$\frac{dQ}{dt} = k\,\Delta C_L - P\left(\Delta C_P + \bar{C}_P \frac{F\Delta\Psi}{RT}\right) \tag{3}$$

where dQ/dt is the rate of change of the quantity of cell chloride, k the luminal membrane chloride entry rate constant, P the peritubular membrane chloride permeability, ΔC_L and ΔC_P are the chloride activity differences across the luminal and peritubular membranes, respectively, $\bar{C}_P$ is the average Cl activity across the peritubular membrane (arithmetic mean), $\Delta\psi$ the peritubular PD, F the Faraday, R the gas constant, T the temperature. In the steady-state $dQ/dt = 0$ and Eq. (3) may be rearranged to yield

$$\frac{k}{P} = \frac{C_{cell}\left(1 + \frac{F\Delta\Psi}{2RT}\right) + C_{cap}\left(\frac{F\Delta\Psi}{2RT} - 1\right)}{\Delta C_L} \tag{4}$$

where C_{cell} is the intracellular chloride activity, C_{cap} the chloride activity of the capillary perfusate and the other symbols are as previously defined. When the control values in Table 2 are substituted in Eq. 4, the ratio of luminal rate constant to peritubular permeability, k/P is 0.96. When the luminal NaCl concentration is 32.5 mM and the capillaries contain 100 mM NaCl, this ratio increases to 2.3. If we make the reasonable assumption that the peritubular Cl permeability does not change when the luminal chloride is reduced, then most of the change in the ratio is due to an increase of the luminal rate constant, k. The consequence of this increase in the luminal rate constant is that cell chloride decreases nonlinearly as the luminal chloride is reduced.

Since luminal Na and Cl are reabsorbed at the same rate by *Necturus* proximal tubule, it has been assumed that the chloride flux may be estimated from the rate of isosmotic fluid reabsorption. Our calculations (Table 8) suggest that about 50% of the net transepithelial chloride flux takes a transcellular path, the remainder traverses the extracellular shunt pathway. The driving force for chloride movement across the shunt path

is the negativity of the tubule lumen. Since the shunt path of *Necturus* proximal tubule is chloride selective (*see* Table 2, *also* [1]), chloride may diffuse out of the lumen driven by the transepithelial PD. The fraction of luminal chloride which leaves the tubule lumen by traversing the cellular pathway cannot be described by the equations for passive transepithelial ionic movements. Thus the use of the ratio of tubular chloride concentration to that in the plasma is not a measure of the transepithelial PD of the *Necturus* proximal tubule. The transepithelial movement of chloride ions does not appear to be a wholly passive process but involves considerable participation by the tubule cells.

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