J. Membrane Biol. 62, 7–17 (1981)

The Journal of
Membrane Biology

Chloride Distribution in the Proximal Convoluted Tubule of *Necturus* Kidney

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Summary. To assess the mechanism(s) by which intraluminal chloride concentration is raised above equilibrium values, intracellular Cl^- activity (α_i^{Cl}) was studied in the proximal tubule of *Necturus* kidney. Paired measurements of cell membrane PD (V_{BL}) and Cl-selective electrode PD (V_{BL}^{Cl}) were performed in single tubules, during reversible shifts of peritubular or luminal fluid composition. Steady-state α_i^{Cl} was estimated at 14.6 ± 0.6 mmol/liter, a figure substantially higher than that predicted for passive distribution. To determine the site of the uphill Cl^- transport into the cell, an inhibitor of anion transport (SITS) was added to the perfusion fluid. Introduction of SITS in peritubular perfusate decreased α_i^{Cl}, whereas addition of the drug in luminal fluid slightly increased α_i^{Cl}; both results are consistent with basolateral membrane uphill Cl^- transport from interstitium to the cell. TMA^+ for Na^+ substitutions in either luminal or peritubular perfusate had no effect on α_i^{Cl}. Removal of bicarbonate from peritubular fluid, at constant pH (a situation increasing HCO_3^- outflux), resulted in an increase of α_i^{Cl}, presumably related to enhanced Cl^- cell influx: we infer that Cl^- is exchanged against HCO_3^- at the basolateral membrane. The following mechanism is suggested to account for the rise in luminal Cl^- concentration above equilibrium values: intracellular CO_2 hydration gives rise to cell HCO_3^- concentrations above equilibrium. The passive exit of HCO_3^- at the basolateral membrane energizes an uphill entry of Cl^- into the cell. The resulting increase of α_i^{Cl}, above equilibrium, generates downhill Cl^- diffusion from cell to lumen. As a result, luminal Cl^- concentration also increases.

Key words: Renal transport, proximal tubule, selective microelectrodes, chloride, intracellular activity

Several recent studies have provided evidence that a significant fraction of PCT[1] absorption in mammalian kidney is mediated by passive processes. The main driving force producing passive (diffusive) NaCl and fluid absorption in rat PCT is the transmural chloride concentration gradient, $[Cl]_L/[Cl]_P$ (Frömter, Rumrich & Ullrich, 1973; Barrat, Rector, Kokko & Seldin, 1974; Frömter & Gessner, 1974*a*; Neumann & Rector, 1976; Anagnostopoulos, 1980). It is generally accepted that the observed $[Cl]_L/[Cl]_P$ ratio of about 1.20 generates a transmural Cl^- diffusion potential, lumen positive: the chloride equilibrium potential, $E_{Cl,TE}$, corresponding to this ratio is +4.8 mV. The transepithelial distribution of Na^+ ($[Na]_L/[Na]_P = 1$) yields a transepithelial sodium equilibrium potential, $E_{Na,TE}$, of 0 mV. Accordingly, the transmural PD, V_{TE}, is established at an intermediate value of some 2 mV, lumen positive (Frömter & Gessner, 1974*a*). The fundamental consequence of this particular distribution is that the driving forces for chloride ($V_{TE} - E_{Cl,TE} = -2.8$ mV) and for sodium ($V_{TE} - E_{Na,TE} = +2$ mV) are adequately directed to elicit Cl^- *and* Na^+ passive transport from lumen to interstitium. Thus, the diffusive moiety of NaCl absorption is directly related to the particular distribution of chloride across the tubular wall. This statement is not intended to downgrade the importance of the active Na^+ transport. However, since active Na^+ transport does not contribute to the es-

* C.N.R.S. Greco 24. Part of this work was presented at the 12th annual meeting of the American Society of Nephrology, Boston, Mass. (Edelman et al., 1979).

[1] *Abbreviations used:* PCT, proximal convoluted tubule; $[i]_P$, $[i]_L$, peritubular and intraluminal ionic concentration of the i_{th} ion species; α_P^i, α_i^i, α_L^i, peritubular, intracellular and intraluminal activities of the i_{th} species: $\Delta\alpha_i^{Cl}$, absolute change in intracellular Cl^- activity during perfusion (luminal or peritubular) with a test solution; V_{BL}, V_{TE}, intracellular and intraluminal potential with reference to interstitium; V_{BL}^{Cl}, Cl-selective electrode potential (intracellular); $E_{i,TE}$ $E_{i,BL}$, electrochemical potential difference of the i_{th} ion species across the whole epithelium and across the basolateral membrane (reference interstitium); SITS, 4-acetamido-4′-isothiocyanostilbene-2, 2′-disulfonic acid; TMA^+: tetramethylammonium.

0022-2631/81/0062-0007 $02.20

tablishment of the transmural Cl^- concentration gradient, it will not be given further consideration hereafter.

Two mechanisms may account, *a priori*, for the increase of luminal Cl^- concentration above plasma Cl^- concentration: (i) isosmotic $NaHCO_3$ absorption in excess of NaCl absorption and/or (ii) HCO_3^- for Cl^- exchange across the epithelial wall; the latter may be attained by a variety of different cellular processes (Rector, 1976). The two basic mechanisms cannot be and have never been experimentally distinguished by conventional micropuncture techniques, Yet, the $[Cl]_L > [Cl]_P$ inequality is ascribed exclusively to luminal NaCl "concentration", secondary to preferential $NaHCO_3$ and water absorption. However, several experimental observations suggest that the traditional view may not entirely account for the establishment of the transmural Cl^- concentration difference in mammalian PCT. (i) The difference of about 3 mV between V_{TE} and $E_{Cl,TE}$ is by definition a nonequilibrium state. In the absence of an inwards active (primary or secondary) Cl^- transport, transepithelial Cl^- distribution should decline towards equilibrium, especially in view of the high transepithelial Cl^- permeability (Frömter et al., 1973). Yet, V_{TE} (Frömter & Gessner, 1974*a*) and the $[Cl]_L/[Cl]_P$ ratio (Le Grimellec, 1975) remain remarkably stable over the last 9/10ths of the PCT, yielding an $E_{Cl,TE}$ value above equilibrium, despite declining HCO_3^- absorptive rates as one proceeds from glomerulus to late PCT (Sohtell, 1979). (ii) PCT microperfusion experiments have established that acetate for HCO_3^- substitution in the perfusion fluid does not prevent the rise of $[Cl]_L$ at the collection site; the collected $[Cl]_L$ figure is higher than the nominal $[Cl]_L$ of the perfusate, whether the perfusate contains HCO_3^- or acetate (Green & Giebisch, 1975*a*; Neuman & Rector, 1976; Green, Bishop & Giebisch, 1979). The rise of $[Cl]_L$ in control experiments is ascribed to the decrease of $[HCO_3]_L$ which is believed to result from reciprocal neutralization of luminal HCO_3^- with secreted protons (Pitts, 1968). Luminal Na-acetate could also neutralize H^+ secretion by titration to acetic acid. However, to obtain a rise of the $[Cl]_L/[Cl]_P$ ratio above 1.00 by this mechanism, acetic acid would have to be transported from the lumen to interstitium at a higher rate than NaCl. Although, this contingency cannot be ruled out, *a priori*, it cannot be accepted without reservations unless experimentally documented. Alternatively, Cl^- could be secreted into the lumen in exchange for acetate. (iii) PCT bicarbonate absorption is believed to be stoichiometrically coupled to H^+ ion secretion (Pitts, 1968). If, as generally claimed, the rate of bicarbonate absorption (i.e., that of H^+ secretion) determines the magnitude of the transepithelial Cl^- concentration difference (which controls the passive moiety of NaCl absorption), then changes in H^+ ion secretion should affect the rate of NaCl absorption. Yet, these two processes can be experimentally dissociated (Green & Giebisch 1975*b*; Ullrich et al., 1977). Such observations suggest again that an alternative mechanism, not coupled to the H^+-secretion/HCO_3^--absorption process, may contribute to the rise of $[Cl]_L$ and to the resulting passive NaCl absorption.

The mechanisms of NaCl absorption have been studied less extensively in the proximal tubule of *Necturus*. It is generally acknowledged that the $[Cl]_L/[Cl]_P$ ratio is slightly higher than unity, i.e., 1.05–1.07 (Walker, Hudson, Findler & Richards 1937; Bott, 1962), when $[Na]_L/[Na]_P$ is not significantly different from 1.00 (Bott, 1962; Garland et al., 1973). The lumen has been reported to be negative with respect to interstitium by 9 to 12 mV (Boulpaep, 1972; Spring & Paganelli, 1972; Spring & Kimura, 1978) or by about 1 mV (Anagnostopoulos, 1975; Edelman & Anagnostopoulos, 1976). Clearly, luminal chloride concentration appears also to be above equilibrium, in *Necturus*, despite a reported lack of luminal acidification (Giebisch, 1956). The failure of *Necturus* PCT to acidify the urine does not rule out that HCO_3^- could be still reabsorbed at the same rate as Cl^-, but in this case $[Cl]_L$ could not rise above $[Cl]_P$. Stated in another way, the lack of H^+ secretion in the lumen of *Necturus* PCT precludes an indirect decrease of $[HCO_3]_L$ by a mechanism similar to that described in mammalian PCT (Pitts, 1968). Therefore, the hypothesis of preferential HCO_3^- absorption and ensuing osmotic water removal cannot account for the observed transepithelial Cl^- distribution in *Necturus*, unless the luminal HCO_3^- ions are directly reabsorbed in the anionic form. However, the movement of HCO_3^- ions from lumen to cell is opposed by an important energy barrier. Indeed, intracellular HCO_3^- activity has been shown to be ten times higher than luminal HCO_3^- activity (Khuri et al., 1974). Hence the puzzling question: what is the driving force which establishes and subsequently maintains luminal chloride concentration above equilibrium values?

The similarities between mammalian and amphibian PCT are obvious. In both structures luminal Cl^- activities exceed those predicted for passive distribution (i.e., $V_{TE} - E_{Cl,TE} < 0$); yet no satisfactory explanation has been provided for this observation. More generally, the processes of transepithelial Cl^- transport have not been defined with sufficient accuracy in the PCT of mammalian and amphibian kidney. Conceivably, an answer to these questions

may not be achieved without (i) prior knowledge of the state of intracellular chloride activity α_i^{Cl} and (ii) some information on the factors which influence α_i^{Cl}. To adequately determine intracellular chloride activity, both V_{BL} and V_{BL}^{Cl} must be assessed, preferentially in the same cell and at the same time. This is achieved with double barreled microelectrodes. We have so far been unable to construct double barreled microelectrodes devoid of electrical coupling between the two channels. Thus, we determined α_i^{Cl} by means of two separate microelectrodes. We chose the proximal tubule of *Necturus*, rather than the rat PCT, because kidney impalement with two microelectrodes is technically feasible in amphibia. The amphibian tubule offers the additional advantage of allowing such impalements in association with perfusions of the lumen or peritubular capillaries with artificial solutions, without compromising the stability of microelectrode recordings. Thus, we were able to monitor the state of intracellular chloride activity continuously during reversible shifts of luminal or peritubular fluid composition.

Materials and Methods

Adult *Necturi*, purchased from Mogul-Ed Co. (Oshkosh, Wisc.), were maintained in aquaria at 15 °C and fed for periods of 1 to 3 months. They were anesthetized by immersion in 1.5% tricain solution (Prolabo, France). All experiments were performed in the proximal tubule of *Necturus* kidney, *in vivo*. The kidney preparation for micropuncture was described elsewhere (Anagnostopoulos, 1975; Edelman & Anagnostopoulos, 1976).

Perfusion Techniques

Artificial solutions of various compositions (Table 1) were used to perfuse peritubular capillaries or the lumen of single tubules.

a) Peritubular capillary perfusion experiments. A double barreled micropipette (15–30 μm outer tip diameter) was inserted into a portal vessel recognized by the inward direction of its blood flow. One channel of the pipette was filled with the control Ringer's solution, the second channel with one of the test solutions listed in Table 1. Before microelectrode impalement a few flushes were performed to verify correct localization of the micropipette tip and determine the area in which red blood cells reversibly disappeared upon application of pressure on either of the pipette channels; microelectrode impalements were performed within this area. The switching from blood perfusion to artificial solutions or from one solution to another was achieved by means of a high-speed perfusion technique (Frömter & Gessner, 1974*b*) based on the principle of two independent gravimetric pressure systems, each connected to one channel of the micropipette (Fig. 1, bottom).

b) Luminal perfusion. The tubule selected for micropuncture was impaled with a single, empty micropipette, and air was injected through it into the lumen. The tubule filled with air was sharply delineated, allowing the insertion of the double barreled micropipette, two to four loops distally from the initial puncture site. Then, the perfusion of the lumen was begun. The single barreled pipette was used to aspirate the column of air separating the two tips and, subsequently, to continuously collect perfused fluids. In this way tubular distension and intraluminal mixing of the two solutions, resulting from incomplete draining, were avoided. The collecting pipette was carefully watched, and in case of accidental interruption of fluid collection the study of the tubule was discontinued. In preliminary experiments we had observed that the forging of a large hole across the wall of the tubule was insufficient for adequate fluid drainage, presumably because spontaneous coalescence of the thick interstitial layer resulted in superficial obliteration of the tubular opening; under these circumstances the amplitude of the change in membrane PD, elicited by switching the perfusate from the control to a high-K solution, was progressively reduced with time as the procedure was repeated. No such dampening of the signal (change in PD) was observed when the perfusate was collected downstream. Switching from one solution to another was achieved as described above, in peritubular perfusion experiments (Fig. 1, top).

Perfusing Solutions

Each of the solutions appearing in Table 1 was applied in single tubules, either from the luminal or from the peritubular side. Fresh solutions were prepared every day. They were equilibrated with a 97% O_2–3% CO_2 gas mixture for about 10 min, and their pH was measured and subsequently adjusted to 7.4 by addition of small amounts of NaOH or HCl (generally of the order of 1 mmol/liter). The solutions buffered with Hepes were not equilibrated with CO_2.

Table 1. Composition of the perfusing solutions, in mmol/liter

	Control	High K	Low K	Low Cl	Low Cl (sucrose)	Low HCO_3	Low Na	SITS
NaCl	82.0	50.5	82.0	10.0	45.0	82.0	–	82.0
KCl	3.5	35.0	2.0	3.5	3.5	3.5	3.5	3.5
$CaCl_2$	1.8	1.8	1.8	1.8	1.8	1.8	1.8	1.8
$MgCl_2$	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0
$NaHCO_3$	13.0	13.0	13.0	13.0	13.0	–	13.0	13.0
Hepes NaOH	–	–	–	–	–	4.0/1.0	–	–
NaF or Na gluconate	–	–	–	72.0	–	–	–	–
Sucrose	–	–	–	–	90.0	–	–	–
TMACl	–	–	–	–	–	–	82.0	–
SITS	–	–	–	–	–	–	–	1.0

In the low-HCO_3 solution the buffer was Hepes 4 mmol/liter and NaOH about 1 mmol/liter, adjusted to pH 7.4.

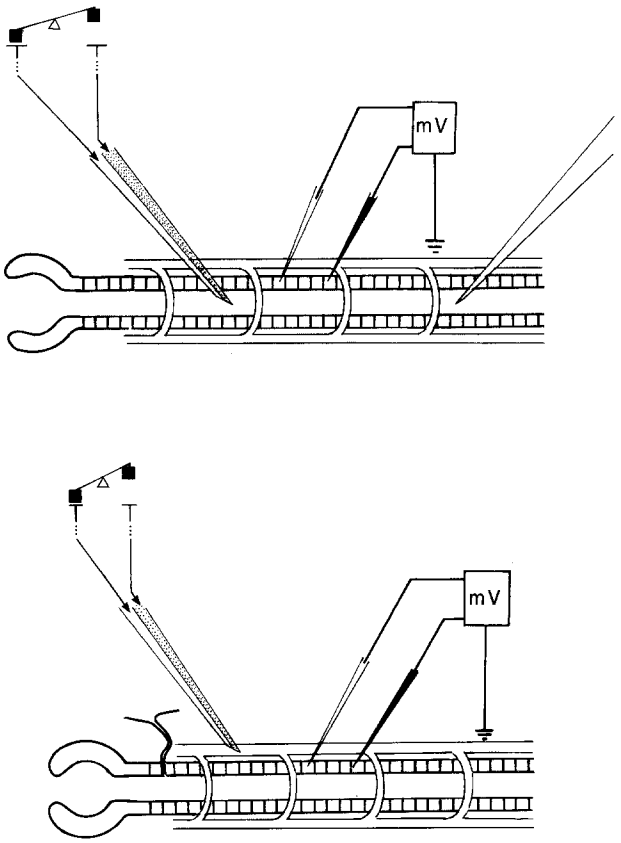

Fig. 1. Schematic representation of the technique used for perfusion and recording. Double barreled micropipettes were employed for reversible perfusion with a control and a test solution; in addition, a single barreled pipette collected all solutions introduced into the lumen (top); no collection pipette was necessary in peritubular perfusion experiments (bottom). Cell membrane PD (V_{BL}) and selective Cl^- electrode PD (V_{BL}^{Cl}) were simultaneously monitored by means of a conventional and a selective microelectrode, respectively

Electrical Measurements

Cell membrane potential was measured with conventional Ling-Gerard microelectrodes, pulled from pyrex capillary tubing, (OD, 2 mm; ID, 1 mm) bearing an internal fiber (Clark Electromedical Instruments, Pangbourne Reading, England) and filled with a 3 mol/liter KCl solution; their tip resistance was 15–50 MΩ. The electrodes were connected to the input of an electrometer (WP Instruments model 750) and all measurements were recorded on a Linseis chart recorder. Cl-selective electrodes were connected to the input of a WP Instruments F223 electrometer (input impedance 10^{15} Ω). The construction of selective electrodes is described elsewhere (Edelman, Curci, Samarzija & Frömter, 1978*a*; Thomas, 1978). Briefly, the aforementioned Clark capillaries were cleaned with detergent, washed carefully with bidistilled water, and boiled for at least 2 hr. The capillaries were dried in an oven at 200 °C for 15 min and pulled (Clark Electromedical Instruments) to a tip length of about 11–12 mm. The siliconization was achieved by exposing the inside of the electrodes to dichlorodimethyl silane vapors for 35–60 sec. The time of exposure to silane vapors was longer when air humidity was higher. After siliconization the microelectrodes were placed again in the oven at 130 °C for 40 min and the tip was filled with the chloride ion exchanger (Corning 477315) by means of a Hamilton syringe; they were stored overnight. Then the reference solution (0.095 mol/liter NaCl) was injected into the shank. In our *in vitro* determinations the reference electrode was a 0.095 mol/liter NaCl solution completed with agar 1%. However, measurements were also performed with a 3 mol/liter KCl-agar reference electrode, but the observed slopes were identical.

Table 2. Properties of Cl-selective electrodes (at 22 °C)

Resin	Corning N° 477315
Tip resistance	$2-8\times10^{11}$ Ω
Electrical slope	
in NaCl 10–100 mmol/liter	57.3 ± 1.3 mV/decade
in NaCl 5– 10 mmol/liter	47.4 ± 2.6 mV/decade
Selectivity coefficient ($1/K_{HCO_3}^{Cl}$) in NaCl 105 mmol/liter against NaCl 50 mmol/liter + $NaHCO_3$ 55 mol/liter	7–10
Rise time	< 0.5 sec

The slope of each microelectrode was tested in three different ways: (i) By immersion in pure NaCl solutions; their concentrations were 9.5, 50.0, and 95.0 mmol/liter. (ii) In solutions of same NaCl composition completed with mannitol to a total osmolality of 95.0 mmol/liter; no significant differences were observed in the slope of single electrodes tested with these two procedures. (iii) In 9.5, 50.0, and 95.0 mmol/liter NaCl solutions in which 10 mmol/liter $NaHCO_3$ was added: a small decrease of the slope was observed in this set. The selectivity coefficient of each electrode (Cl^-/HCO_3^-) was separately assessed by measuring in succession the potential developed in a pure 105 mmol/liter NaCl solution and in a 50 mmol/liter NaCl + 55 mmol/liter $NaHCO_3$ mixture. Since these two solutions display the same ionic strength, the Nicolsky equation (Nicolsky, 1937, *quoted by* Lev & Armstrong, 1975) was used to estimate the selectivity coefficient $K_{HCO_3}^{Cl}$. This and other properties of our Cl-selective electrodes are summarized in Table 2. Each microelectrode was individually calibrated and its slope was determined as indicated above. Simultaneous measurements of V_{BL} and V_{BL}^{Cl} (V_{BL}^{Cl} in the control state is the algebraic sum of V_{BL} plus $E_{Cl,BL}$) were performed in single tubules. Thus, intracellular chloride activity was directly calculated from the difference $V_{BL}-V_{BL}^{Cl}$.

Experimental Protocols

Perfused tubules (intraluminally or peritubularly) were impaled with two microelectrodes, one conventional and one Cl-selective. In each experiment the tips were placed into the layer of a single superficial tubule located within the perfused area. In this way both potentials, V_{BL} and V_{BL}^{Cl} were simultaneously monitored during shifts of peritubular or luminal perfusate from the control to an experimental solution and back to the control perfusion fluid. The measurements were considered acceptable only when both recordings were stable within ±3 mV for at least 2 min. The minimum stability requirement of 2 min for V_{BL} and V_{BL}^{Cl} was not always achieved prior to the exposure of the tissue to a test solution; it was usually shared between control and recovery steps bracketing exposure to test solutions, because in this way the reversibility of experimentally induced changes in PD was also confirmed. Spurious changes of V_{BL} and/or V_{BL}^{Cl}, not related to the effects of the test solution but rather to microelectrode displacements (both could occur during changes of fluid composition), were averted since in the latter case the shifts in PD did not comply with the above required reversibility criterion. We computed the control intracellular chloride activity in these experiments from the mean of plateau α_i^{Cl} values recorded before and after

exposure of each tubule to a test solution. This α_i^{Cl} figure was substracted from the peak change in α_i^{Cl} obtained in the same tubule during the experimental stage. The resulting $\Delta\alpha_i^{Cl}$ difference represents the change in intracellular Cl^- activity. Average values are given below as mean ±SEM. The significance of $\Delta\alpha_i^{Cl}$ changes was evaluated by the paired student's t test.

Results

Intracellular Cl^- Activity in the Control State

Paired impalements in which V_{BL} and V_{BL}^{Cl} were simultaneously measured in single tubules yielded average values of -68.1 ± 0.7 mV and -27.7 ± 0.6 mV, respectively, $n=90$. Cell membrane PD's of the same magnitude have already been observed in the *in vivo Necturus* kidney (Khuri, Agulian, Bogharian & Aklanjian, 1975; Spring & Kimura, 1978). Our Cl-selective electrode readings are slightly lower than those reported in another paper (Spring & Kimura, 1978), but in that particular study V_{BL}^{Cl} readings lower than $|-30|$ mV were discarded. We choose instead to include all our experimental determinations; their distribution is shown in Fig. 2. The average α_i^{Cl}, derived from these measurements may be estimated at 14.9 ±0.6 mmol/liter (range 10 to 29 mmol/liter; $n=90$); it is higher than the theoretical α_i^{Cl} of 3.6 mmol/liter required for electrochemical equilibrium. This latter figure is computed from a peritubular Cl^- activity of 63 mmol/liter, corresponding to a peritubular Cl^- concentration $[Cl]_P$ of 81 mmol/liter (Bott, 1962, and *unpublished observations*). In agreement with others, we conclude that the measured α_i^{Cl} is significantly greater than the equilibrium Cl^- activity (Khuri et al., 1975; Spring & Kimura, 1978); i.e., chloride is transported uphill into the cell. Intracellular Cl^- activities higher than equilibrium values have also been reported in other epithelia (Duffey, Turnheim, Frizzell & Schultz, 1978; Duffey, Thompson, Frizzell & Schultz, 1979; Reuss & Weinman, 1979).

The Effect of Peritubular Perfusions with Low-Cl Solutions

To test the ability of our microelectrodes to detect swift variations of α_i^{Cl}, low-Cl solutions were perfused in peritubular circulation. We have anticipated that the perfusion of the vascular bed with such low-Cl solutions would decrease intracellular Cl^- activity. F^- for Cl^- substitution reduced α_i^{Cl} by 3.43 ±0.30 mmol/liter, $n=6$, $P<0.001$; gluconate for Cl^- substitution and partial sucrose for NaCl substitution resulted in smaller but significant decreases of α_i^{Cl}, $\Delta\alpha_i^{Cl}$, 1.08 ± 0.23 mmol/liter, $n=6$, $P<0.01$, and 1.42 ± 0.31 mmol/liter, $n=6$, $P<0.01$, respectively.

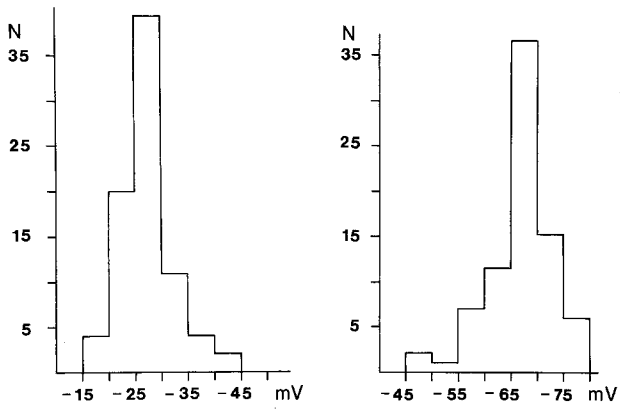

Fig. 2. Histogram distribution of 90 paired impalements, performed in 28 animals, with Cl-selective (left) and conventional (right) microelectrodes, in single tubules. N: number of observations (impalements)

The differing magnitudes of $\Delta\alpha_i^{Cl}$ with the three artificial solutions probably reflect different exchange rates between intracellular Cl^- and its extracellular substitute across the basolateral membrane. Fluoride permeability, P_F, has been found greater than P_{Cl} (Anagnostopoulos, 1977) when the opposite applies to gluconate (Anagnostopoulos & Planelles, 1979); it is also reasonable to assume that sucrose diffusion into the cell in exchange for NaCl is a slow process. A representative recording illustrating the effects of low-Cl solutions on α_i^{Cl} is shown in Fig. 3.

The Site of Uphill Cl^- Transport

A convenient way to determine the site (membrane) at which Cl^- enters into the cell against its electrochemical potential difference is to study the effects of anion transport inhibitors on α_i^{Cl}. SITS is a well-known inhibitor of anion fluxes in a variety of tissues. There is also indirect evidence that SITS may depress anionic permeabilities at the basolateral membrane of the perfused *Necturus* kidney (Edelman, Teulon & Anagnostopoulos, 1978*b*). Finally, SITS has been reported to inhibit organic anion entry into the cell across the basolateral membrane of rabbit kidney tubules (Hong et al., 1978) and to decrease volume absorption in rat PCT when applied peritubulary (Ullrich et al., 1977). We anticipated this inhibitor to depress chloride fluxes in *Necturus* PCT, too. If so, the addition of SITS at the pole of the cell bearing the uphill Cl^- transport mechanism would lower the Cl^- transport rate and, thus, decrease α_i^{Cl}; conversely, the application of the inhibitor at the opposite membrane, i.e., at the site where only passive Cl^- exit occurs, would increase α_i^{Cl}. Addition of SITS in peritubular fluid brought about a decrease of α_i^{Cl} by 3.38 ± 1.20 mmol/liter, n

$=5$, $p<0.05$; this effect is consistent with an uphill transport of Cl^- directed from interstitium to cell, across the basolateral membrane. The introduction of SITS in the luminal perfusate elicited a small but insignificant increase of α_i^{Cl}, by 0.80 ± 0.51 mmol/-liter, $n=10$. Two representative recordings illustrating the effects of SITS on V_{BL} and V_{BL}^{Cl} are shown in Fig. 4.

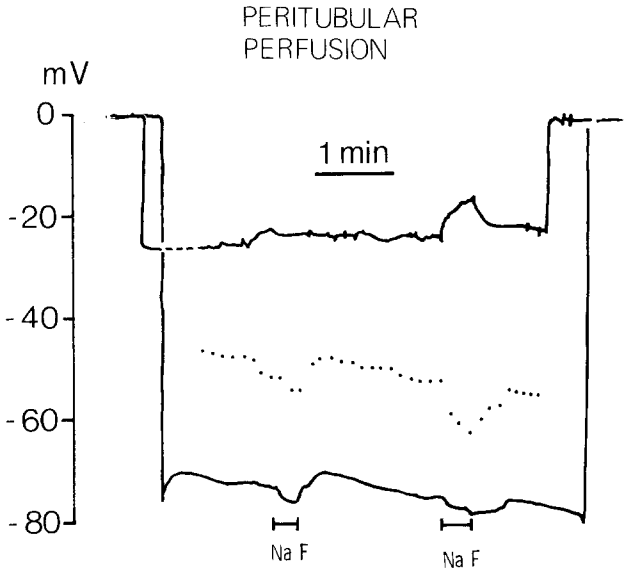

Fig. 3. Representative recording illustrating the effects of peritubular perfusion with low-Cl solutions on membrane potential, V_{BL} (bottom), and chloride electrode potential, V_{BL}^{Cl} (top). The difference $V_{BL}-V_{BL}^{Cl}$ (dotted line, middle) was obtained manually by substration at regular time intervals. This difference yields the value of α_i^{Cl} (*see Methods*). In this and subsequent figures, a shift of the $V_{BL}-V_{BL}^{Cl}$ curve in the depolarizing direction (upwards) amounts to an increase of α_i^{Cl}, when the opposite (hyperpolarizing or downwards shifts of the dotted line) indicates a decrease of α_i^{Cl}. Paired V_{BL} and V_{BL}^{Cl} recordings were always obtained in single tubules. In this as in other similar experiments in which NaF was substituted for NaCl in peritubular circulation ($n=6$), the absolute value of α_i^{Cl} was always reduced

The Effects of Na-free Solutions

Since it has been recently proposed that the uphill Cl^- entry into the cell takes place at the luminal border and is coupled to passive Na^+ influx across the luminal membrane (Spring & Kimura, 1978), the effects of Na-free solutions on α_i^{Cl} were also studied. Peritubular TMA^+ for sodium substitution resulted in an average 10 to 20 mV depolarization at both recording sites, V_{BL} and V_{BL}^{Cl}, an observation indicating that no change in α_i^{Cl} has occurred. The mean $\Delta\alpha_i^{Cl}$ difference in this series of experiments was 0.00 ± 0.26, $n=6$. TMA^+ for Na^+ substitution in the luminal fluid left both cell recordings unaffected (Fig. 5): as a result α_i^{Cl} remained stable, and the average $\Delta\alpha_i^{Cl}$ difference was not different from zero, 0.00 ± 0.00, $n=6$. The observation that complete removal of sodium as NaCl salt from the perfusing solutions did not alter α_i^{Cl} does not support the hypothesis that the entry and exit of Cl^- into and out of the cell, respectively, are sodium-dependent in the PCT of *Necturus*. Prolonged exposure of the tissue to essentially Na-free solutions may ultimately affect intracellular ionic activities; we argue, however, that if the diffusion of Cl^- across the luminal membrane were physiologically coupled to that of Na^+, α_i^{Cl} should have been sufficiently lowered after 2 min of complete NaCl removal to be detected in our experiments.

The Effects and the Role of Bicarbonate

Chloride for bicarbonate exchange has been observed in cell membranes of various tissues (Russell & Boron, 1976; Thomas, 1977; Russell, 1978), including epithelia (Leslie, Schwartz & Steinmetz, 1973; Weiner, 1980). To determine whether the uphill entry of Cl^- into the cell, across the basolateral membrane, is mediated via a Cl^-/HCO_3^- ex-

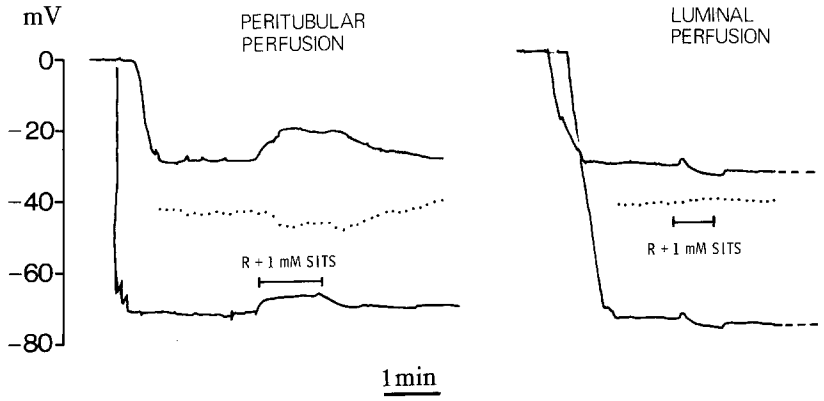

Fig. 4. Representative recordings, illustrating the effects of SITS on V_{BL}, V_{BL}^{Cl} and the difference $V_{BL}-V_{BL}^{Cl}$. Symbols are as in Fig. 3. Peritubular application of SITS (left) produces a shift of the $V_{BL}-V_{BL}^{Cl}$ dotted line downwards, indicating that this substitution results in a decrease of α_i^{Cl} ($n=5$). By contrast, the changes of the dotted line, elicited by the addition of SITS in the luminal perfusate (right) are minimal, and so is the corresponding shift of α_i^{Cl}($n=10$)

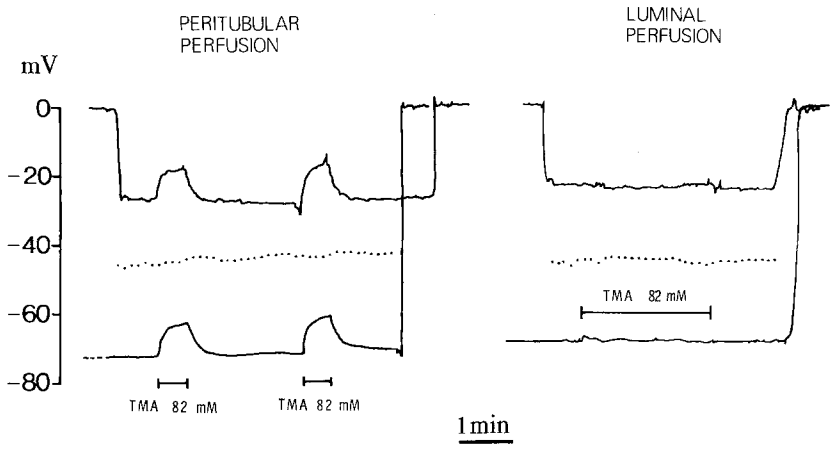

Fig. 5. Representative recordings illustrating the effects of low-Na^+ solutions on paired V_{BL} and V_{BL}^{Cl} determinations. Symbols are as in Figs. 3 and 4. *Left:* peritubular TMA^+ for Na^+ substitution depolarizes both V_{BL}^{Cl} (top) and V_{BL} (bottom) by the same magnitude, so that the difference $V_{BL}-V_{BL}^{Cl}$ (middle) and the corresponding α_i^{Cl} remain unaffected ($n=6$). *Right:* low-Na^+ solutions do not alter V_{BL}, V_{BL}^{Cl}, nor do they affect the difference $V_{BL}-V_{BL}^{Cl}$; α_i^{Cl} is not altered by the removal of luminal Na^+ ($n=6$)

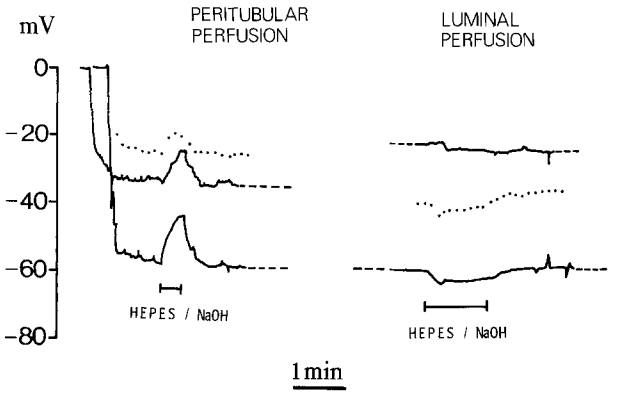

Fig. 6. Representative recordings from two experiments in which the effects of bicarbonate-free solutions were tested on V_{BL} and V_{BL}^{Cl}. Bicarbonate was replaced in peritubular fluid by an appropriate mixture of Hepes/NaOH so as to maintain the pH of the solutions unaltered (left). It is seen that V_{BL}^{Cl} is affected less than V_{BL} and that the difference $V_{BL}-V_{BL}^{Cl}$ shifts in the depolarizing direction, indicating that this substitution resulted in an increase of α_i^{Cl} ($n=9$). The introduction of the same HCO_3^--free solution into the lumen of the tubule (right) altered V_{BL} and V_{BL}^{Cl} by about the same magnitude; the $V_{BL}-V_{BL}^{Cl}$ difference in this series of experiments remained unaffected ($n=6$)

change mechanism, bicarbonate was removed from peritubular fluid and replaced by Hepes buffer, 4 mmol/liter. Preliminary experiments in which Hepes alone was added in the perfusate ($[HCO_3]_P$ remained unchanged) demonstrated that this buffer alone did not alter V_{BL} nor V_{BL}^{Cl}: α_i^{Cl} remained stable. By contrast, removal of peritubular HCO_3^- resulted in a reversible increase of α_i^{Cl} by 3.08 ± 0.97 mmol/liter, $n=9$, $P<0.02$. A representative recording is shown in Fig. 6. These results are interpreted as follows: removal of peritubular bicarbonate enhances the electrochemical gradient for HCO_3^- exit from the cell, thus stimulating HCO_3^- efflux towards the interstitium; the attendant increase of α_i^{Cl} is tentatively ascribed to an increased Cl^- influx into the cell, which is stoichiometrically coupled to the HCO_3^- efflux.[2] Consonant with this interpretation are recent findings in the perfused *Necturus* kidney, suggesting that the value of α_i^{Cl} is higher than the theoretical one predicted for equilibrium when the perfusate contains bicarbonate, but it is brought to an apparent equilibrium when the tissue is perfused with a HCO_3^--free, tris-maleate buffered solution (Edelman et al., 1978*b*).

Upon removal of bicarbonate from the luminal perfusate and its replacement by Hepes (Fig. 6), V_{BL} and V_{BL}^{Cl} were affected by less than 1 mV each: the mean absolute change of α_i^{Cl} was not statistically different from zero, -0.08 ± 0.40 mmol/liter, $n=6$.

The Effects of Potassium

Since sodium replacement by TMA^+ did not alter α_i^{Cl}, in the last series of experiments we tested the effects of potassium on intracellular Cl^- activity. An increase of $[K]_P$ from 3.5 to 35.0 mmol/liter elicited depolarization at both recording sites, but the depolarization was always larger with the conventional microelectrode than with its selective counterpart, indicating an increase of α_i^{Cl} (Fig. 7). On the average, α_i^{Cl} increased reversibly by 13.51 ± 2.28 mmol/liter, $n=10$, $P<0.001$, after 20–60 sec exposure to high-K solutions. Reduction of $[K]_P$ from 3.5 to 2.0 mmol/liter decreased the average α_i^{Cl} by 1.92 ± 0.26, $n=5$, $P<0.01$. The simplest interpretation for these experiments is that an alteration of the steady-state transmembrane K^+ distribution, secondary to an experimental modification of $[K]_P$, results in net K^+ in-

[2] Removal of HCO_3^- from peritubular fluid could also affect intracellular pH, which could secondarily alter α_i^{Cl}. However, since the solutions used to perfuse peritubular capillaries were buffered, extracellular pH was not affected. Under these circumstances, it is unlikely that intracellular pH would decrease sufficiently within 20 sec of exposure to Hepes-buffered, HCO_2-free solutions, to significantly depress P_{Cl} so as to indirectly increase α_i^{Cl}.

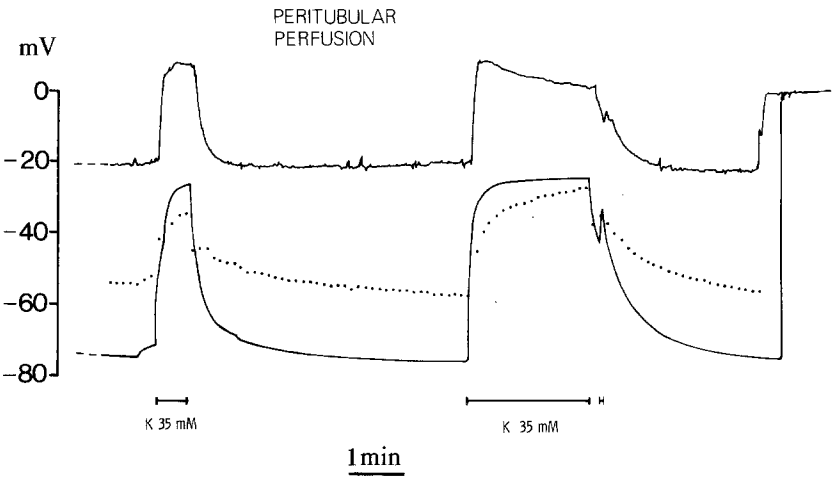

Fig. 7. Effects of an increase of peritubular K^+ concentration on V_{BL}, V_{BL}^{Cl} and $V_{BL}-V_{BL}^{Cl}$. In this and other recordings from similar experiments ($n=10$), the depolarization elicited by high-K solutions at the site of the conventional microelectrode (bottom) is clearly larger than the companion change recorded by the Cl-selective electrode (top); as a result, the $V_{BL}-V_{BL}^{Cl}$ curve shifts upwards, denoting an increase of α_i^{Cl}. Moreover, prolonged exposure of the tissue to high-K media produces further dissociation of V_{BL} and V_{BL}^{Cl}, and further depolarization of the $V_{BL}-V_{BL}^{Cl}$ line, indicating continuous (yet slower) increase of α_i^{Cl} during this substitution

flux into or efflux out of the cell, accordingly shifting α_i^K. Similar changes of transmembrane Cl^- net fluxes and α_i^{Cl} are elicited, to preserve electroneutrality. We acknowledge that our interpretation is entirely speculative; the data on the effects of potassium on α_i^{Cl} can be accounted for by other mechanisms, such as alterations of membrane permeabilities or, possibly, a reversal of the orientation of the electrochemical gradient for Cl^- in high-K^+ media. However, the potassium experiments do not deal directly with the main topic of this work; therefore, we did not undertake further studies to elucidate the mechanism(s) by which peritubular K^+ concentration may affect α_i^{Cl}. The potassium data are considered at this stage only as an additional validation of our technique and of the ability of the Cl-selective microelectrodes to detect reversible changes of α_i^{Cl}.

Discussion

The present study indicates that the intracellular Cl^- activity in the PCT of *Necturus* kidney is significantly higher than the theoretical value required for electrochemical equilibrium. Similar findings have been reported by other investigators (Khuri et al., 1975; Spring & Kimura, 1978). However, contrary to the conclusions of another study in which the uphill step of Cl^- transport was ascribed to a mechanism located at the apical membrane (Spring & Kimura, 1978), our experiments suggest that Cl^- enters the cell against an energy barrier across the basolateral membrane. We shall consider hereafter some differences in experimental design which may account for the differing conclusions between these two studies and then speculate on the possible implications of an inwards Cl^- transport across the basolateral membrane.

The Uphill Site of Cl^- Transport: Possible Explanations of Divergent Conclusions

Four differences in technique between this and a previous study on α_i^{Cl} deserve a brief comment. (i) Spring and Kimura (1978) computed α_i^{Cl} from separate steady-state measurements of V_{BL} and V_{BL}^{Cl}, first in the control state and then under various experimental conditions. Steady state was achieved after 20–30 min of kidney perfusion with a test solution. It is conceivable that prolonged exposure of the tissue to essentially NaCl-free solutions depletes the intracellular compartment from its NaCl content; in addition, membrane permeabilities to Na^+ and Cl^- could be altered after some time of tissue exposure to these solutions, possibly via an irreversible process. A sudden readmission of NaCl in luminal fluid may then produce a swift increase of α_i^{Cl}, as observed by Spring and Kimura (1978). Such observations, however, do not provide information on the mechanisms by which α_i^{Cl} is regulated under physiologic conditions, nor do they indicate how the cell responds to small alterations of extracellular steady state concentrations. Our own short-term perfusion experiments with essentially NaCl-free solutions constitute an important aggression to cell homeostasis, yet they failed to produce detectable α_i^{Cl} changes. It is doubtful that the effects of similar long-term aggressions are applicable to the conditions prevailing *in vivo*.

(ii) Intraluminal introduction of artificial solutions through a single glomerular pipette, without end proximal collection, may produce tubular distension and inadequate perfusion downstream, at the recording site.

(iii) Chloride selective electrode resistances in the range of $10^{10}\,\Omega$, such as those reported by Spring and Kimura (1978), correspond to tip diameters of

1 μm (Deisz & Lux, 1978), a size which may be damaging for the cells of the proximal tubule of *Necturus*.

(iv) Finally, the criterion for acceptable chloride electrode PD readings in the control state was arbitrarily set at −30 mV in the work of Spring and Kimura (1978); i.e., control V_{BL}^{Cl} values lower than |−30| mV were systematically discarded. This selection obliterates the lower portion of the control α_i^{Cl} population and tends to make it divergent from any similar group of data not subject to the same manipulation. Although discarding data may not necessarily introduce artificial differences between control and experimental states, any disparities obtained by such procedures must be regarded with extreme caution.

The Mechanisms and Significance of the Inward Cl^- Transport Across the Basolateral Membrane

The experiments in which SITS was used as a means to decrease transmembrane Cl^- transport suggest that the uphill entry of Cl^- into the cell proceeds across the basolateral membrane, i.e., against the direction of the net absorptive transport. The inhibitory effect of SITS on transmembrane anion exchange in PCT appears to be small, as estimated from the magnitude of the observed α_i^{Cl} changes. This is not surprising in view of the disparity in responsiveness to disulfonic stilbenes among various tissues: these compounds completely inhibit sulfate fluxes in red blood cells (Cabantchik & Rothstein, 1974), but fail to affect HCO_3^- secretion and Cl^- absorption in turtle bladder (Husted, Cohen & Steinmetz, 1979)

The occurrence of an uphill entry of Cl^- into the cell across the basolateral membrane, i.e., against the direction of net NaCl absorption, may appear at first sight as a useless and energetically wasteful process. To understand its possible role, one must consider in addition the unique pattern of transepithelial Cl^- distribution in PCT: luminal Cl^- activity is higher than the theoretical α_L^{Cl} predicted for transepithelial equilibrium in both amphibian and mammalian PCT. Our tentative interpretation for these observations is as follows: The primary event appears to be the uphill transport of Cl^- from interstitium to cell: it raises α_i^{Cl} above equilibrium. The resulting Cl^- electrochemical potential gradient across the luminal membrane favors a downhill Cl^- transport from cell to lumen, which would accordingly increase α_L^{Cl}. In this way, α_L^{Cl} values above equilibrium with regard to interstitium may be achieved; they can be maintained at steady state throughout the PCT, despite a continuous paracellular net Cl^- passive outflux, owing to the concomitant refueling from cell to lumen. This model is presented only as a plausible working hypothesis since one of its steps, the entry of Cl^- from cell to lumen, has not been yet experimentally documented.

Next, we shall consider the mechanism(s) by which chloride may enter the cell across the basolateral membrane, against an energy barrier. The experiments dealing specifically with this point were those in which removal of basolateral HCO_3^- resulted in an increase of α_i^{Cl}. Three explanations may account for this observation: (i) The first hypothesis is that the removal of bicarbonate brings about a decrease of P_{Cl} at the basolateral membrane, lowering the passive exit of Cl^- from cell to interstitium and accordingly increasing α_i^{Cl}. However, chloride equilibrium potential across the basolateral membrane may be tentatively estimated at −36 mV (from $\alpha_i^{Cl}=14.9$ and $\alpha_P^{Cl}=63$ mmol/liter), when $V_{BL}\simeq -68$ mV. Under these circumstances, a decrease of P_{Cl} should hyperpolarize the cell. We observed, instead, the removal of bicarbonate to produce cell depolarization (*see*, e.g., Fig. 6). (ii) Another alternative could be that the removal of peritubular HCO_3^- stimulates an inwards active Cl^- transport. This is an unlikely hypothesis, since it requires the additional postulate that the pump is inhibited by an ion (HCO_3^-) other than the substrate (Cl^-) carried by the pumping process, despite the fact that the substrate concentration remains unchanged. (iii) The most simple interpretation is that the exit of HCO_3^- from cell to intestitium is coupled to the entry of Cl^- from peritubular spaces to cell, accordingly increasing α_i^{Cl}. This exchange could be mediated via a specific carrier or proceed through diffusional independent pathways, in compliance with electroneutrality requirements. Cl^- may be exchanged with HCO_3^- as such or with its decarboxylated form, the base OH^-.

The concept of an operational exchange between Cl^- and HCO_3^- may be regarded as an acceptable hypothesis on the binding condition that the downhill gradient of HCO_3^- is sufficient to energize the uphill transport of chloride. α_i^{Cl} in *Necturus* is only two to four times greater than the theoretical figure predicted for electrochemical equilibrium (*see also* Khuri et al., 1975, and Spring & Kimura, 1978) when $\alpha_i^{HCO_3}$ is ten times larger than its own equilibrium figure (Khuri et al., 1974)[3]; thus, the premises

[3] It was recently reported by Boron and Boulpaep (1980) that the cytosolic pH of *Ambystoma* PCT is more acid than extracellular pH by 0.13 pH units. Such observations do not necessarily contradict the findings of Khuri et al. (1974). If both studies are correct and if the pH data obtained in *Ambystoma* are applicable to the *Necturus* kidney, then intracellular P_{CO_2} values must be substantially higher than those prevailing in extracellular fluid.

for a Cl^-/HCO_3^- counter-transport system, Cl^- entering the cell in exchange for HCO_3^-, are met. Cl^-/HCO_3^- exchange, not dependent on carbonic anhydrase activity and on oxidative metabolism, has been previously reported in toad bladder (Weiner, 1980). In *Necturus* kidney, the sequence of events leading to the constitution of the transepithelial chloride gradient may be entirely accounted for by passive processes, i.e., not coupled to metabolic work, since (i) intracellular HCO_3^- values above equilibrium result from the hydration of CO_2, catalyzed and/or uncatalyzed, (ii) the uphill entry of Cl^- into the cell is believed to be mediated by and coupled to the downhill exit of HCO_3^- across the basolateral membrane, and (iii) the postulated downhill transport of Cl^- from cell to lumen is consistent with the orientation of its electrochemical potential difference.

At last, we shall speculate on the purpose possibly accomplished by the particular orientation of the transepithelial Cl^- gradient. Nonequilibrium states in biological structures do not occur as random aberrations among otherwise orderly processes; they are subservient to the accomplishment of a physiologic function. The only useful purpose we can think of, regarding the transepithelial Cl^- distribution, is the promotion of passive (energy-free) absorption across the paracellular pathway, in *Necturus*, as in rat PCT (Frömter, 1977). Although teleology alone does not prove the merits of a model, $E_{Cl,TE}$ and $E_{Na,TE}$ were computed from available estimates of the $[Cl]_L/[Cl]_P$ and $[Na]_L/[Na]_P$ ratios (Bott, 1962; Garland et al., 1973), to test whether the above hypothesis is consistent with the appropriate experimental background. The theoretical V_{TE} values required to produce passive NaCl absorption lay in the range of 0 to +1 mV, lumen positive. Small positive transmural PD's are known to prevail in rat PCT (Frömter & Gessner, 1974*a*). By contrast, the figure of V_{TE} in *Necturus* is a matter of controversy (Boulpaep, 1972; Spring & Paganelli, 1972; Edelman & Anagnostopoulos, 1976; Spring & Kimura, 1978). Small positive V_{TE} readings have been occasionally reported in *Necturus* PCT, too (Willbrandt, 1938; Edelman & Anagnostopoulos, 1976), but the low frequency of their occurrence cannot be taken at present as sufficient supportive evidence in favor of the presently discussed teleological hypothesis. Further work is needed to elucidate the physiologic role of the transepithelial chloride gradient in *Necturus* PCT.

It is a pleasure to acknowledge the invaluable contribution of Dr. S. Curci during the course of this study. The study was supported by INSERM grants 78-116, 79-39 and DGRST grant 77-7-1288.

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Received 22 July 1980; revised 5 December 1980;
revised again 17 February 1981