

CO₂-stimulated NaCl Absorption in the Mouse Renal Cortical Thick Ascending Limb of Henle

Evidence for Synchronous Na⁺/H⁺ and Cl⁻/HCO₃⁻ Exchange in Apical Plasma Membranes

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ABSTRACT These experiments evaluated salt transport processes in isolated cortical thick limbs of Henle (cTALH) obtained from mouse kidney. When the external solutions consisted of Krebs-Ringer bicarbonate (KRB), pH 7.4, and a 95% O₂-5% CO₂ gas phase, the spontaneous transepithelial voltage (V_e , mV, lumen-to-bath) was ~ 8 mV; the net rate of Cl⁻ absorption (J_{Cl}^{net}) was $\sim 3,600$ pmol s⁻¹ cm⁻²; the net rate of osmotic solute absorption J_{osm}^{net} was twice J_{Cl}^{net} ; and the net rate of total CO₂ transport ($J_{CO_2}^{net}$) was indistinguishable from zero. Thus, net Cl⁻ absorption was accompanied by the net absorption of a monovalent cation, presumably Na⁺, and net HCO₃⁻ absorption was negligible. This salt transport process was stimulated by (CO₂ + HCO₃⁻): omission of CO₂ from the gas phase and HCO₃⁻ from external solutions reduced J_{Cl}^{net} , J_{osm}^{net} , and V_e by 50%. Furthermore, 10⁻⁴ M luminal furosemide abolished J_{Cl}^{net} and V_e entirely. The lipophilic carbonic anhydrase inhibitor ethoxzolamide (10⁻⁴ M, either luminal or peritubular) inhibited (CO₂ + HCO₃⁻)-stimulated J_{Cl}^{net} , J_{osm}^{net} , and V_e by $\sim 50\%$; however, when the combination (CO₂ + HCO₃⁻) was absent, ethoxzolamide had no detectable effect on salt transport. V_e was reduced or abolished entirely by omission of either Na⁺ or Cl⁻ from external solutions, by peritubular K⁺ removal, by 10⁻³ M peritubular ouabain, and by 10⁻⁴ M luminal SITS. However, V_e was unaffected by 10⁻³ M peritubular SITS, or by the hydrophilic carbonic anhydrase inhibitor acetazolamide (2.2 \times 10⁻⁴ M, lumen plus bath). We interpret these data to indicate that (CO₂ + HCO₃⁻)-stimulated NaCl absorption in the cTALH involved two synchronous apical membrane antiport processes: one exchanging luminal Na⁺ for cellular H⁺; and the other exchanging

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luminal Cl^- for cellular HCO_3^- or OH^- , operating in parallel with a $(\text{CO}_2 + \text{HCO}_3^-)$ -dependent apical membrane NaCl cotransport mechanism.

INTRODUCTION

This paper describes some of the transport characteristics of single cortical thick ascending limbs of Henle (cTALH) obtained from mouse kidney, with particular emphasis on the fact that CO_2 enhances the rate of conservative NaCl absorption in this nephron segment, although net CO_2 transport is negligible, and a description of the functional heterogeneity between the mouse cTALH, where CO_2 but not antidiuretic hormone (ADH) accelerates the rate of net NaCl absorption, and the mouse medullary thick ascending limb (mTALH), where ADH but not CO_2 accelerates the rate of net NaCl absorption (Hebert et al., 1981a).

The functional role of the renal thick ascending limb was deduced from micropuncture studies (Walker et al., 1941; Gottschalk and Mylle, 1959), in which it was shown that, under conditions of either extreme hydropenia or volume loading, tubular fluid appearing at the first accessible portion of the distal convoluted tubule was hypo-osmotic. Because this dilution of tubular fluid was due to absorption of salt in excess of water, the thick ascending limb has been commonly referred to as the diluting segment.

The *in vivo* clearance experiments of Wallin et al. (1973a, b) indicated that net bicarbonate absorption by the thick ascending limb was insignificant, and that this nephron segment might be negligibly permeable to HCO_3^- . More recent observations on the *in vitro* rabbit cTALH are also consistent with the possibility that net HCO_3^- transport by the cTALH is small, since there appears to be virtually no net total CO_2 transport by the isolated rabbit cTALH (Burg and Iino, 1979; Iino and Burg, 1981). For these reasons, it is generally accepted that the diluting power of the mammalian cTALH is referable almost entirely to net NaCl absorption rather than to net bicarbonate transport.

However, carbonic anhydrase activity is particularly striking in the mouse cTALH, both within the cytoplasm and along the inner surface of the luminal membrane (Dobyan et al., 1982). Moreover, the presence of bicarbonate and CO_2 in external solutions has been noted to enhance rates of net NaCl transport in a number of different epithelia, both urinary (Ullrich et al., 1971; Chen and Walser, 1977; Green and Giebisch, 1975) and nonurinary (Field et al., 1971; Podesta and Mettrick, 1977; Wright, 1977; Heintze et al., 1981). In certain instances, this effect has obtained in the absence of net total CO_2 transport (Heintze et al., 1981; Petersen et al., 1981), and in some circumstances, the $(\text{CO}_2 + \text{HCO}_3^-)$ -mediated enhancement of NaCl transport has depended, at least in part, on carbonic anhydrase activity (Field et al., 1971; Ullrich et al., 1971; Green and Giebisch, 1975).

For these reasons, we evaluated the effects of $(\text{CO}_2 + \text{HCO}_3^-)$ on the rate of net NaCl absorption by the isolated mouse cTALH. Our observations indicate that net NaCl absorption in this nephron segment was enhanced dramatically by $(\text{CO}_2 + \text{HCO}_3^-)$ in the absence of net total CO_2 transport. This $(\text{CO}_2 +$

HCO₃⁻)-stimulated NaCl absorption was abolished by inhibition with ethoxzolamide, but not by acetazolamide.

These data are in accord with the possibility that (CO₂ + HCO₃⁻)-stimulated NaCl absorption in the cTALH may involve two synchronous apical cell membrane antiport processes, one exchanging extracellular Na⁺ for cellular H⁺, and the other exchanging extracellular Cl⁻ for cellular HCO₃⁻ or OH⁻. These events result in apical membrane NaCl uptake from luminal fluid, and simultaneously, H₂CO₃ formation in luminal fluid. Our calculations indicate that luminal carbonic acid dehydration is sufficiently rapid, without luminal carbonic anhydrase activity, to allow zero net CO₂ transport, and that intracellular carbonic anhydrase activity was necessary for (CO₂ + HCO₃⁻)-stimulated NaCl absorption, since the rate of uncatalyzed CO₂ hydration to H₂CO₃ was significantly smaller than the (CO₂ + HCO₃⁻)-stimulated rate of net NaCl absorption.

It is also possible that (CO₂ + HCO₃⁻)-independent salt transport involved a coupled NaCl entry step of indeterminate stoichiometry. And for both (CO₂ + HCO₃⁻)-stimulated and (CO₂ + HCO₃⁻)-independent salt transport, it is probable that basolateral (Na⁺ + K⁺)-ATPase was required to maintain the electrochemical Na⁺ gradient between cytoplasm and extracellular fluid. A preliminary account of these findings has been published elsewhere (Friedman and Andreoli, 1981).

METHODS

The techniques used for studying transport processes in single in vitro microperfused mouse cTALH segments were similar to those described originally by Burg et al. (1966; Burg and Green, 1973) for isolated rabbit nephron segments and to those used in this laboratory (Hebert et al., 1981a) for the study of transport processes in segments of mouse mTALH. Stated briefly, 20–30-d-old (~25 g) male Swiss white mice were killed by cervical dislocation and rapid exsanguination. The kidneys were removed and cortical thick limbs were dissected freehand, without use of collagenase or other enzymatic treatment, from coronal sections of renal cortex immersed in Krebs-Ringer bicarbonate buffer (KRB) (see Table I for composition). After transfer to a thermoregulated lucite chamber, the tubule segments, 0.5–1.0 mm in length, were connected to concentric glass pipettes and perfusion was initiated either by hydrostatic pressure or with an infusion pump (Sage Instrument Co., Cambridge, MA). The tubules were perfused at rates of 2–15 nl/min at 37°C; the specific perfusion rate is indicated in each group of experiments.

Perfusion and Bathing Solutions

The compositions of the various tubular perfusion and bathing solutions are indicated in Table I. All solutions were adjusted to pH 7.4 and 290 mosmol/kg H₂O. Bicarbonate-containing solutions were equilibrated with a 95% O₂-5% CO₂ gas phase; bicarbonate-free solutions (e.g., Krebs-Ringer phosphate [KRP]) were equilibrated with a 100% O₂ gas phase. The bathing solutions were exchanged at 10-min intervals to avoid significant evaporative losses. In experiments in which ²²Na⁺ or ³⁶Cl⁻ was added to the bath, 50 μl of water was added to the bath at 12-min intervals to maintain a constant bath osmolality.

Changes in the composition of perfusion and bathing solutions are indicated in the text. In experiments performed with bicarbonate-containing solutions, the perfusion

chamber was bubbled with 95% O₂-5% CO₂ at a rate sufficient to maintain the bath at pH 7.4. In experiments in which bicarbonate was omitted from tubule perfusion and bathing solutions, the system was gassed entirely with 100% O₂. In all experiments, the bath pH was monitored continuously with a miniature pH probe (MI-502; Microelectrodes, Inc., Londonderry, NH) fitted to the rear of the perfusion chamber and connected to an Orion 601A pH meter (Orion Research, Cambridge, MA).

Microchemical Measurements

The chloride concentrations in perfused and collected samples of tubular fluid were determined in triplicate using the electrotitrimetric procedure of Ramsay (Ramsay et al., 1955). Since the rate of net fluid absorption in our experiments was indistin-

TABLE I
COMPOSITION OF SOLUTIONS

Solutes	KRB	KRP	Cl ⁻ -free*	N ⁺ -free	K ⁺ -free	Low NaCl*	Low HCO ₃ ⁻
NaCl	120	145	—	—	120	30	142
Na isethionate	—	—	109	—	—	—	—
TEA Cl	—	—	—	109	—	—	—
TEA HCO ₃	—	—	—	25	—	—	—
NaHCO ₃	25	—	25	—	25	25	2.5
Na acetate	—	—	10	—	—	10	—
KCl	—	—	—	—	—	5	—
NaH ₂ PO ₄ /Na ₂ HPO ₄	—	—	—	—	2.8	1.2	—
KH ₂ PO ₄ /K ₂ HPO ₄	2.8	2.8	2.8	2.8	—	—	2.8
CaCl ₂	—	—	—	1.0	—	1.0	—
CaSO ₄	1.0	1.0	1.0	—	1.0	—	1.0
MgSO ₄	1.2	1.2	1.2	1.2	1.2	1.2	1.2
L-Alanine	5	5	5	5	5	5	5
Glucose	5.5	5.5	5.5	5.5	5.5	—	5.5
Mannitol	—	—	—	16	—	170	—

Solutions containing HCO₃⁻ were gassed with 95% O₂-5% CO₂ and HCO₃⁻-free solutions were gassed with 100% O₂. pH was adjusted to 7.4 at 37°C and osmolality to 295-300 mosmol. Perfusion and bathing solutions were identical with the exception that 0.4 mg/dl of exhaustively dialyzed bovine serum albumin was added to all bathing solutions and glucose and L-alanine were replaced isosmotically by urea or mannitol in the perfusate solutions.

* In studies using Cl⁻-free or low-NaCl solutions, the control solution (KRB) against which it was tested contained 10 mM Na acetate and 109 mM NaCl.

guishable from zero (*vide infra*), net chloride absorption (J_{Cl}^{net} , pmol s⁻¹ cm⁻²) was calculated according to the relation:

$$J_{Cl}^{net} = \frac{\dot{V}_L([Cl^-]_o - [Cl^-]_L)}{A} \quad (1)$$

where [Cl⁻]_o and [Cl⁻]_L are the concentrations of chloride in the perfused and collected fluid samples, respectively, \dot{V}_L is the fluid collection rate, and A is the apparent luminal surface area. The apparent surface area was calculated from the measured tubule length, determined with an eyepiece micrometer, and the luminal diameter, estimated from photomicrographs.

The osmolalities of fluid samples were determined in parallel with chloride measurements in aliquots of perfusate and collected tubular fluid. The osmolalities in duplicate samples were measured using a Clifton nanoliter osmometer (Clifton

Technical Physics, Hartford, NY) according to a previously described procedure (Friedman and Roch-Ramel, 1977). The osmotic solute flux ($J_{\text{osm}}^{\text{net}}$, $\text{posm s}^{-1} \text{cm}^{-2}$) was then computed as:

$$J_{\text{osm}}^{\text{net}} = \frac{\dot{V}_L([\text{osm}]_O - [\text{osm}]_L)}{A} \quad (2)$$

where $[\text{osm}]_O$ and $[\text{osm}]_L$ refer to the osmolalities of perfused and collected fluid samples, respectively.

The total CO₂ in perfused and collected fluid samples was measured according to a previously described microcalorimetric technique (Lucci et al., 1979; Cogan et al., 1979) using a picapnotherm (Microanalytic Instruments, Bethesda, MD). Determinations of total CO₂ were made on duplicate aliquots of 20-nl samples. Standard curves, prepared using known concentrations of Na₂CO₃, were linear over the range of 0–25 mM total CO₂ ($r > 0.98$). The net total CO₂ flux ($J_{\text{CO}_2}^{\text{net}}$, $\text{pmol s}^{-1} \text{cm}^{-2}$) was computed as the difference in CO₂ concentrations between perfused ($[\text{CO}_2]_O$) and collected ($[\text{CO}_2]_L$) fluid samples from the expression:

$$J_{\text{CO}_2}^{\text{net}} = \frac{\dot{V}_L([\text{CO}_2]_O - [\text{CO}_2]_L)}{A} \quad (3)$$

The term total CO₂ refers to all CO₂ species present, at equilibrium, in bicarbonate-buffered solutions: dissolved CO₂, HCO₃⁻, H₂CO₃, and CO₃⁼. Evidently, at an ambient pH of 7.4, 95% of the total CO₂ will be in the form of HCO₃⁻.

Tracer Fluxes

The rate of net volume absorption (J_v , $\text{nl min}^{-1} \text{mm}^{-1}$) was measured using exhaustively dialyzed [³H-methoxy]-inulin (New England Nuclear, Boston, MA), as described previously (Schafer et al., 1974). J_v was computed from the difference between perfusion and collection rates and normalized for tubule length. J_v was measured concomitantly with ²²Na⁺ and ³⁶Cl⁻ fluxes and was uniformly indistinguishable from zero. Thus, this segment, like the mouse mTALH (Hebert et al., 1981a), the rabbit cTALH (Burg and Green, 1973), and the rabbit mTALH (Rocha and Kokko, 1973), was water impermeable.

The unidirectional bath-to-lumen tracer flux of sodium was measured by the addition of ²²NaCl (carrier free; New England Nuclear) to the bathing solution at an average activity of 2.4×10^{11} cpm/mol. Aliquots of bathing fluid were collected at the beginning and end of each tubular fluid sampling period to determine the activity of ²²Na⁺ in the bath. No significant change in the bath tracer activity was discernible during this interval. To avoid appreciable tracer accumulation in luminal fluid, we used relatively high perfusion rates, 20 nl/min, in these experiments. Under these conditions, the ²²Na⁺ activity of the collected fluid samples was uniformly <5% of that in the bathing solution.

³⁶Cl⁻ was obtained as an aqueous solution of [³⁶Cl]HCl (ICN Chemical and Radioisotope Division, Irvine, CA; specific activity ~13 mCi/g). This solution was titrated to pH 7.4 with NaOH and then evaporated to dryness. Because of the low specific activity of the original material, the powdered Na³⁶Cl was dissolved in a NaCl-free KRB bath solution. The final Na⁺, Cl⁻, and K⁺ concentrations, osmolality, and pH were then adjusted to correspond to those of the normal isotonic KRB bath. Using this procedure, the activity of ³⁶Cl⁻ in the bathing solution averaged 5.6×10^{11} cpm/mol. As with ²²Na⁺ fluxes, the bath-to-lumen ³⁶Cl⁻ fluxes were carried out when

the perfusion rates were ~ 20 nl/min, so that the $^{36}\text{Cl}^-$ concentration of collected fluid was uniformly $<5\%$ of that in the bath.

Since volume absorption in the cTALH was insignificant and since tracer backflux from lumen to bath was trivial (the tracer concentration in the collected fluid was uniformly $<5\%$ of that in the bath), the unidirectional flux of the i th species from bath to lumen ($J_i^{b \rightarrow l}$, $\text{pmol s}^{-1} \text{cm}^{-2}$) was calculated from the rate of appearance of isotope in the collected fluid according to the expression:

$$J_i^{b \rightarrow l} = \frac{\dot{V}_L * [C_{L,i}]}{AX_i^b}, \quad (4)$$

where \dot{V}_L (cm/s) is the collection rate, $*[C_{L,i}]$ (cpm/cm³) is the tracer concentration of the i th species in the collected fluid, and X_i^b (cpm/mol) is the isotopic activity of the i th species in the bathing solution. The ionic permeability coefficients (P_i , cm/s) were computed as described previously (Schafer et al., 1974; Hebert et al., 1981a) from the expression

$$P_i = \frac{J_i^{b \rightarrow l} RT}{V_e Z_i^2 F} \frac{1 - \exp\left(\frac{Z_i F V_e}{RT}\right)}{C_i^b}, \quad (5)$$

where F , R , T , and Z have their usual meaning, C_i^b (cpm/cm³) is the concentration of the i th solute in the bathing solution, and V_e (mV) is the observed spontaneous transepithelial voltage, lumen with respect to bath.

It should be noted that calculation of P_i according to Eq. 5 requires explicit values for V_e as a function of tubule length. But the measured values of V_e indicate the voltage only within one length constant of the perfusion pipette. However, at perfusion rates in excess of 10 nl/min in tubule segments <1 mm in length, we found that the composition of tubular fluid differed only slightly from that of the perfusate. Thus, since the rates of axial volume delivery exceeded the rates of radial solute transport events, it is reasonable to argue that the spontaneous transepithelial voltage was more or less uniform with respect to tubule length, at least for the tracer ion flux experiments.

Furthermore, it can be shown (Schafer et al., 1974; Hebert et al., 1981a), using L'Hospital's rule, that for V_e in the range of 2.5–10 mV, i.e., the V_e values observed in our experiments, the calculated values of P_i computed from Eq. 5 vary by $<10\%$ from those computed using Fick's first law for an uncharged species. Based on these considerations, we argue that variations in V_e with tubule length had a relatively insignificant effect on the P_i values reported in this paper.

Electrical Measurements

The spontaneous transepithelial voltage (V_e , mV, lumen with respect to bath) and zero-current voltages (V) were determined as described previously (Schafer et al., 1974). During perfusion with symmetric external solutions, the perfusate and bath had nearly identical ionic compositions and the protein concentration of the bathing solution, 0.4 g/dl, was negligible. Accordingly, no corrections for liquid junction or Donnan voltages were required.

Sodium:chloride and chloride:bicarbonate electrical permselectivity ratios were determined from the separate measurement of zero-current salt dilution and bi-ionic voltages, respectively, in the same tubule. Low-[NaCl] solutions and low-[HCO₃⁻]/high-[Cl⁻] solutions, separately against the KRB solution (Table I), were used for the

measurement of Na:Cl and Cl:HCO₃ selectivity ratios, respectively. The electrical permeability ratios were calculated as described previously (Schafer et al., 1975; Schafer and Andreoli, 1979), using the Nernst-Planck equations. The zero-current voltages were expressed as:

$$V = \sum_{i=1}^n t_i E_i \quad (6)$$

where t_i is the transference number of the i th ion. E_i , the equilibrium voltage for the i th ion, was computed as:

$$E_i = \frac{RT}{Z_i F} \ln \frac{C_i^b}{C_i^l} \quad (7)$$

where C_i^l and C_i^b are the activities of the i th ion in the luminal perfusate and peritubular bathing solution, respectively. The sum of Na⁺, Cl⁻, and HCO₃⁻ concentrations in the KRB solutions used (Table I) was, at a minimum, an order of magnitude greater than the sum of the other ionic constituents of the external media. Accordingly, for these solutions:

$$t_{\text{Na}} + t_{\text{Cl}} + t_{\text{HCO}_3} \cong 1.0. \quad (8)$$

It should be noted that Eq. 8 is an approximation, since other ions present in the external solutions at low concentrations, e.g., K⁺, might also have contributed to the total electrical conductance. But if the permeability coefficients for these ions are not more than an order of magnitude greater than that for sodium, the contribution to the transepithelial conductance of other ionic constituents will be negligibly small.

The relative permeability coefficients of the i th and j th ions were related to the transference number of the i th and j th ions by the expression (Schafer and Andreoli, 1979):

$$\frac{P_i}{P_j} = \frac{t_i \bar{C}_i}{t_j \bar{C}_j}, \quad (9)$$

where \bar{C}_i and \bar{C}_j are the mean concentrations of the i th and j th ions, respectively, in the two external solutions. It should be noted that we obtained virtually the same electrical permselectivity ratios when the Goldman-Hodgkin-Katz equation was used in place of Eqs. 6-9 (Schafer et al., 1975).

In the zero-current voltage experiments, there was an imposed ionic asymmetry between perfusate and bath, and consequently two liquid junction voltages in series: one between NaCl-agar bridge:perfusate and the other between bath:NaCl-agar bridge. The magnitude of the net liquid junction voltage was calculated as described previously (Hebert et al., 1981a), using the Henderson equation (MacInnes, 1961) modified (Barry and Diamond, 1970) for ionic activities and limiting ionic conductances at 37°C (Robinson and Stokes, 1955). The activity coefficients of Na⁺, Cl⁻, and HCO₃⁻ were calculated from the Debye-Hückel law (Eq. 6 in Salling and Siggard-Andersen, 1971) using the ion-size parameters reported by Kielland (1937). The liquid junction voltage corrections for low-[NaCl] and low-[HCO₃⁻]/high-[Cl⁻] solutions were 6.4 and -1.7 mV, respectively.

Statistical Analyses

Two to four determinations were performed for each experimental condition in a given tubule; the average of these values was the mean value for that tubule. The

results reported in this paper were calculated using average values from a number of tubules to compute a mean \pm standard error of the mean (SEM) for the indicated number (n) of tubules. Statistical significance for mean paired differences was evaluated by Student's t test. Probability values (P) were computed from the t distribution.

Chemicals and Reagents

Acetazolamide was generously provided by H. Eisner, Lederle Laboratories, Pearl River, NY; furosemide was kindly provided by H. Dettlebach, Hoeschst Pharmaceuticals, Somerville, NJ. Ethoxzolamide was purchased from Sigma Chemical Co., St. Louis, MO, and SITS (4-acetamido-4'-isothiocyano-2,2'-disulfonic acid stilbene) was obtained from the Pierce Chemical Co., Rockford IL. All other reagents were of analytical grade.

RESULTS

Effect of CO₂, HCO₃⁻, and Furosemide on V_e and Salt Transport

Fig. 1 illustrates representative experiments on two individual tubules in which the measured spontaneous transepithelial voltage (V_e , mV, lumen to bath) was expressed as a function of elapsed time. At zero time, the tubules were mounted in a 23°C bath. The temperature was gradually raised to 37°C and V_e rose monotonically to an average value of ~ 8 mV. The upper panel (A) shows that neither ADH nor dibutyl cyclic adenosine monophosphate (dBcAMP) addition to the bath had any effect on V_e . The lower panel (B) shows that V_e was reduced by $\sim 50\%$ when (CO₂ + HCO₃⁻) was omitted from the external solutions. Finally, both A and B show clearly that addition of 10⁻⁴ furosemide to the perfusion solution abolished V_e .

These findings, in accord with earlier observations reported by this laboratory (Hebert et al., 1981a, b, c), illustrate certain aspects of the functional heterogeneity of mouse TALH segments. As reported previously (Hebert et al., 1981a), both V_e and net salt absorption in the mouse mTALH are enhanced dramatically either by ADH or by cAMP analogues and are unaffected by (CO₂ + HCO₃⁻) removal. In contrast, the data in Fig. 1 illustrate that, in the mouse cTALH, neither ADH nor a cAMP analogue affected V_e , whereas omission of (CO₂ + HCO₃⁻) from the external solutions produced a 50% inhibition of V_e .

A more quantitative evaluation of the effects of furosemide on V_e , and the simultaneously measured net rate of Cl⁻ absorption ($J_{\text{Cl}}^{\text{net}}$, pmol s⁻¹ cm⁻²), is presented in Table II. The experiments involved paired measurements of V_e and $J_{\text{Cl}}^{\text{net}}$ in the same tubule, either in the presence or absence of luminal furosemide, 10⁻⁴ M. Perfusion rates were maintained at a constant between control and experimental periods and among individual tubules. The data presented in Table II show that, coincident with abolishing V_e , 10⁻⁴ M luminal furosemide also inhibited completely net Cl⁻ absorption.

Fig. 2 presents the results of an analysis of the effects of (CO₂ + HCO₃⁻) omission on the spontaneous V_e in mouse cTALH in 13 paired experiments. Each tubule was allowed to equilibrate for a minimum of 25 min after an

exchange of external media and gas phase. The mean V_e during the initial control period was 8.2 ± 1.0 mV when the external solutions were KRB and the gas phase was 95% O₂-5% CO₂, and fell to 4.0 ± 0.8 mV when the external solutions were KRP and the gas phase was 100% O₂. Finally, the third panel in Fig. 2 indicates that, when (CO₂ + HCO₃⁻) was restored to the

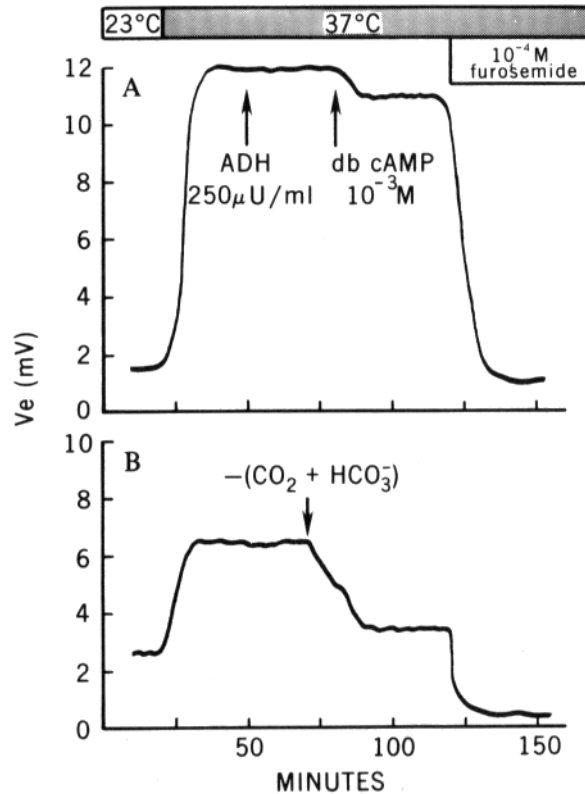


FIGURE 1. Representative measurements of spontaneous transepithelial voltage (V_e) in the cTALH. The tubules were perfused and bathed with KRB solutions and bubbled with 95% O₂-5% CO₂. A: after warming to 37°C, 250 μ U/ml ADH (first arrow) and then 10^{-3} M dibutyryl cyclic adenosine monophosphate (dBcAMP) (second arrow) were added to the bathing solutions. At the time indicated, 10^{-4} M furosemide was added to the luminal perfusate. B: after warming to 37°C, the perfusate and bath were exchanged for bicarbonate-free KRP solutions and the chamber was gassed with 100% O₂ (first arrow). 10^{-4} M furosemide was added to the luminal perfusate at the indicated time.

external solutions, V_e rose to 7.2 ± 1.4 mV, i.e., almost entirely to control values. Thus, one may argue reasonably that the reduction in V_e produced by (CO₂ + HCO₃⁻) removal was not referable to irreversible inhibition of transepithelial transport processes.

It is relevant to inquire whether the net rate of Cl⁻ absorption observed in

TABLE II
EFFECT OF FUROSEMIDE ON J_{Cl}^{net} AND V_e

Condition	\dot{V}_L	J_{Cl}^{net}	V_e
	<i>nl/min</i>	<i>peq s⁻¹ cm⁻²</i>	<i>mV</i>
Control	8.1±0.8	5,178±493	6.8±2.5
10 ⁻⁴ M furosemide (lumen)	8.0±0.4	-18±510	0.1±0.5
Mean paired difference (n = 4)	0.1±0.6 NS	5,195±975 P < 0.02	6.7±2.1 P < 0.05

The external solutions consisted of KRB, gas phase 95% O₂-5% CO₂, 37°C. J_{Cl}^{net} and V_e were measured simultaneously in the same tubules as described in Methods. The control and experimental periods were varied at random among individual tubules. The results are expressed as mean values ± SEM.

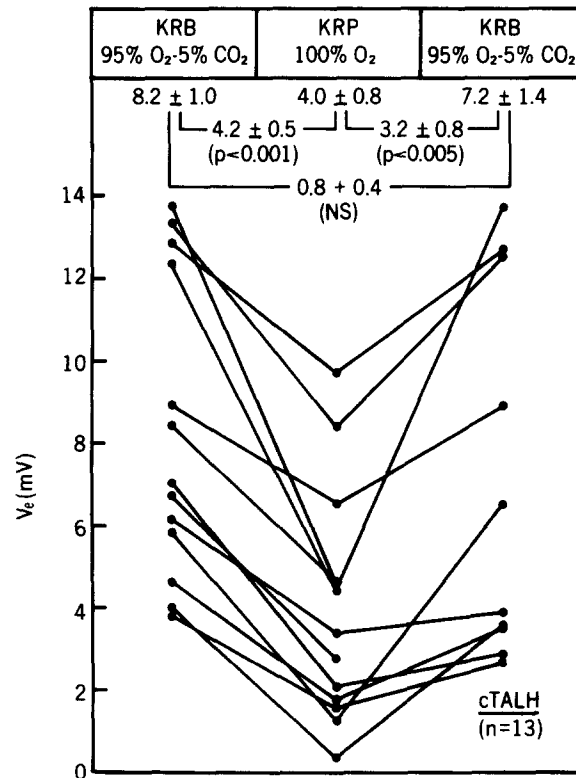


FIGURE 2. The effect of (CO₂ + HCO₃⁻) removal on V_e . During control and recovery periods, the tubules were perfused and bathed with KRB at 37°C using a 95% O₂-5% CO₂ gas phase. During the experimental periods, KRP served as perfusate and bath with a 100% O₂ gas phase. The lines connect data points on individual tubules. The results are expressed as mean values ± SEM.

these cTALH segments (Table II) correlates with the in vivo diluting capacity of the TALH (see Introduction), and, simultaneously, to evaluate the effects of (CO₂ + HCO₃⁻) removal on the net rate of Cl⁻ absorption by the mouse cTALH. The results of a series of experiments intended to address these

questions are presented in Table III. We measured, in the same tubule, V_e , J_{Cl}^{net} , and the osmotic solute flux (J_{osm}^{net}). Paired observations of these variables were made, in each tubule, in the presence and absence of ($CO_2 + HCO_3^-$) in the external solutions. The perfusion rate was held constant between control and experimental periods and among individual tubules.

Several observations shown in Table III are particularly noteworthy. First, at a constant perfusion rate and for either experimental condition, namely, external KRB solutions containing 95% O_2 -5% CO_2 or KRP with 100% O_2 , the mean paired difference [$\Delta(Cl - \frac{1}{2}J_{osm}^{net})$] between J_{Cl}^{net} and one-half of the net osmotic solute flux ($\frac{1}{2}J_{osm}^{net}$) was not significantly different from zero. Thus, for both experimental situations, net Cl^- absorption was accompanied *pari passu* by the net absorption of a monovalent cation, presumably Na^+ ; and since J_v was virtually zero in these experiments (see Methods), this salt

TABLE III
CO₂ EFFECT ON SIMULTANEOUSLY DETERMINED NET Cl^- FLUX (J_{Cl}^{net}),
ONE-HALF NET OSMOTIC SOLUTE FLUX ($\frac{1}{2}J_{osm}^{net}$) AND V_e

External solutions	\dot{V}_L	J_{Cl}^{net}	$\frac{1}{2}J_{osm}^{net}$	$\Delta(Cl - \frac{1}{2}J_{osm}^{net})$	V_e
	<i>nl/min</i>	<i>pmol s⁻¹ cm⁻²</i>	<i>posm s⁻¹ cm⁻²</i>		<i>mV</i>
KRB 95% O_2 ; 5% CO_2	5.9±1.3	3,633±910	3,520±831	111±350 NS	6.8±3.0
KRP 100% O_2	5.6±0.8	1,817±440	1,818±510	0±110 NS	3.9±2.9
$\Delta (\pm CO_2)$	0.3±1.3 NS	1,820±410 $P < 0.02$	1,700±370 $P < 0.02$		2.9±0.4 $P < 0.02$

$n = 4$

J_{Cl}^{net} , J_{osm}^{net} , and V_e were measured simultaneously in the same tubule in paired observations using the two sets of external solutions. The temperature was uniformly 37°C. The difference $\Delta(Cl - \frac{1}{2} osm)$ denotes the mean paired difference between J_{Cl}^{net} and $\frac{1}{2}J_{osm}^{net}$ for a given experimental condition (i.e., data in the same rows). The differences $\Delta \pm CO_2$ denote the mean paired differences for the individual variables \dot{V}_L , J_{Cl}^{net} , $\frac{1}{2}J_{osm}^{net}$, and V_e when CO_2 and HCO_3^- were present or absent from external solutions (i.e., data in the same columns). The results are expressed as mean values \pm SEM.

absorption resulted in dilution of luminal fluid. Moreover, these data indicate that, within experimental error, net Cl^- absorption was not associated with an osmotically silent anion exchange process. Second, the results in Table III indicate that, coincident with the fall in V_e produced by omitting ($CO_2 + HCO_3^-$) from the external solutions, there was an ~50% reduction in both J_{Cl}^{net} and in $\frac{1}{2}J_{osm}^{net}$. Importantly, the mean paired differences for these variables in the presence and absence of ($CO_2 + HCO_3^-$) were nearly the same.

Effect of Carbonic Anhydrase Inhibitors

Since the results presented in Table III indicate that the combination ($CO_2 + HCO_3^-$) enhanced the rate of salt absorption by the cTALH, it is reasonable to surmise that this enhancement might involve tubular carbonic anhydrase

activity. The results of a series of experimental maneuvers intended to evaluate this possibility are shown in Table IV and Figs. 3 and 4.

Table IV shows the effect of adding the lipophilic (Maren, 1967, 1969) carbonic anhydrase inhibitor ethoxzolamide (10^{-4} M) to the lumen on $J_{\text{Cl}}^{\text{net}}$, $\frac{1}{2}J_{\text{Cl}}^{\text{net}}$, and V_e . The external solutions were KRB and the gas phase was 95% O_2 -5% CO_2 ; the format for Table IV is like that used in Table III. The results in Table IV illustrate clearly that, in paired observations on individual tubules, 10^{-4} M luminal ethoxzolamide produced, simultaneously, an $\sim 70\%$ reduction in $J_{\text{Cl}}^{\text{net}}$ and in $\frac{1}{2}J_{\text{osm}}^{\text{net}}$, and a 60% reduction in V_e . It should also be noted from Table IV that, for a given experimental circumstance, the values of $J_{\text{Cl}}^{\text{net}}$ and $\frac{1}{2}J_{\text{osm}}^{\text{net}}$ were indistinguishable, and that the mean paired differences for both $J_{\text{Cl}}^{\text{net}}$ and $\frac{1}{2}J_{\text{osm}}^{\text{net}}$ in the presence and absence of ethoxzolamide were virtually the same. Obviously, these latter data are in close accord with the results shown in Table III.

TABLE IV
EFFECT OF ETHOXZOLAMIDE ON SIMULTANEOUSLY DETERMINED $J_{\text{Cl}}^{\text{net}}$,
 $\frac{1}{2}J_{\text{osm}}^{\text{net}}$, AND V_e

Condition	\dot{V}_L	$J_{\text{Cl}}^{\text{net}}$	$\frac{1}{2}J_{\text{osm}}^{\text{net}}$	$\Delta(\text{Cl} - \frac{1}{2}J_{\text{osm}}^{\text{net}})$	V_e
	nl/min	$\mu\text{mol s}^{-1} \text{cm}^{-2}$	$\mu\text{osm s}^{-1} \text{cm}^{-2}$		mV
Control	5.6 ± 0.8	$5,433 \pm 1,416$	$5,429 \pm 1,480$	5 ± 172 NS	6.0 ± 0.3
Ethoxzolamide, 10^{-4} M lumen	6.1 ± 0.8	$1,673 \pm 778$	$1,527 \pm 677$	146 ± 120 NS	2.3 ± 0.7
Δ (\pm ethoxzolamide)	0.5 ± 0.6 NS	$3,761 \pm 793$ $P < 0.02$	$3,592 \pm 1,153$ $P < 0.03$		3.7 ± 0.6 $P < 0.02$
		$n = 4$			

The format for this table is the same as that for Table III. The perfusate and bath contained KRB solutions at 37°C and the system was gassed with 95% O_2 -5% CO_2 .

In certain instances, the use of carbonic anhydrase inhibitors has resulted in suppression of transport processes in isolated proximal tubule segments for nonspecific reasons, rather than because of selective inhibition of carbonic anhydrase activity (Grantham, 1973). To evaluate this possibility, we adopted a protocol used previously in our laboratory (Schafer and Andreoli, 1976), in which the effect of a carbonic anhydrase inhibitor was evaluated in the absence of ($\text{CO}_2 + \text{HCO}_3^-$). The results, shown in Fig. 3, indicate that 10^{-4} M luminal ethoxzolamide had no effect on V_e when the external solutions were KRP and the gas phase was 100% O_2 . Thus, it is reasonable to conclude that the ethoxzolamide-induced reduction in V_e shown in Table IV depended on interference with a salt transport process mediated in part by ($\text{CO}_2 + \text{HCO}_3^-$).

We also evaluated the sequential effects of acetazolamide and ethoxzolamide on V_e in paired experiments on individual tubules. In the experiments shown in Fig. 4, KRB served as the perfusate and bath, with a 95% O_2 -5% CO_2 gas phase. After control measurements of V_e , 2.2×10^{-4} M acetazolamide was added to the perfusate and bath; this concentration of acetazolamide

inhibits virtually all carbonic anhydrase-mediated NaHCO₃ absorption in isolated rabbit proximal tubule segments (Schafer and Andreoli, 1976; Burg and Green, 1977; McKinney and Burg, 1977). Subsequently, acetazolamide was removed and 10⁻⁴ M ethoxzolamide was added either to the perfusate or to the bath; in four of the tubules listed in Fig. 4, ethoxzolamide was added to the perfusate, and in the remaining four tubules, the agent was added to the bath.

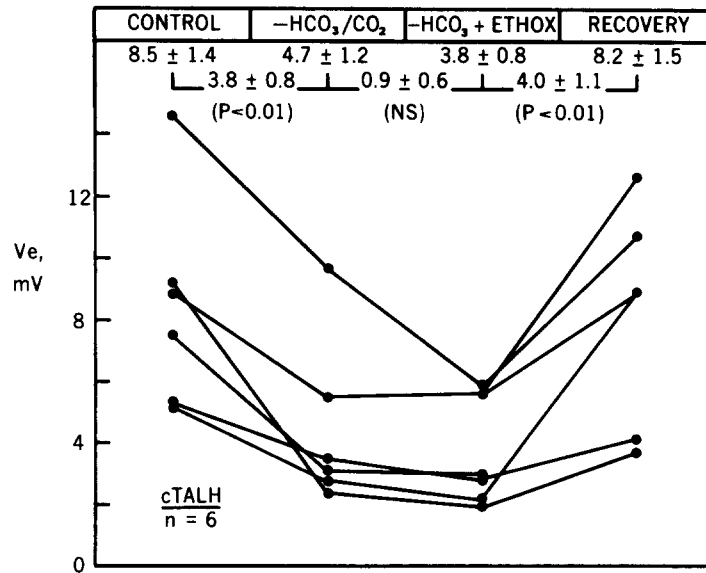


FIGURE 3. The effect of luminal ethoxzolamide (10⁻⁴ M) on V_e when (CO₂ + HCO₃⁻) were omitted from the external solutions. In the first period, the perfusate and bath contained KRB solutions and the gas phase was 95% O₂-5% CO₂. In the second period, the external solutions were KRP and the gas phase was 100% O₂; ethoxzolamide, 10⁻⁴ M, was added to the perfusate in the third period. In the fourth period (indicated as "recovery"), the external solutions were KRB and the gas phase 95% O₂-5% CO₂. The temperature was 37°C in all cases. The lines connect data points on individual tubules. The results are expressed as mean values ± SEM.

It is evident from the data shown in Fig. 4 that 2.2 × 10⁻⁴ M luminal plus peritubular acetazolamide had no effect on V_e , whereas ethoxzolamide, added either to the perfusate or to the bath, reduced V_e by nearly 50%. In the four tubules where ethoxzolamide was added to the bath, V_e fell from 8.6 ± 1.0 mV to 5.2 ± 1.0 mV (P < 0.02); in the other four tubules, the control V_e of 8.8 ± 1.7 mV fell to 4.8 ± 1.8 mV (P < 0.02) when ethoxzolamide was added to the perfusate.

Net CO₂ Transport

The results shown in Tables III and IV indicate virtual equality between J_{Cl}^{net} and $\frac{1}{2}J_{osm}^{net}$; in other words, net Cl⁻ absorption was accompanied by net

absorption of a monovalent cation, presumably Na^+ , rather than by an osmotically silent exchange with a peritubular anion. To evaluate this relation more explicitly, all of the individual data points from the experiments listed in Tables III and IV were plotted in relation to one another (Fig. 5). In addition, $J_{\text{Cl}}^{\text{net}}$ and $\frac{1}{2}J_{\text{osm}}^{\text{net}}$ were measured in three additional tubules exposed to 10^{-4} M luminal furosemide to examine whether 10^{-4} M luminal furosemide inhibited $\frac{1}{2}J_{\text{osm}}^{\text{net}}$ *pari passu* with $J_{\text{Cl}}^{\text{net}}$.

The individual data points from the latter experiments, together with the individual data points from the experiments listed in Tables III and IV, are

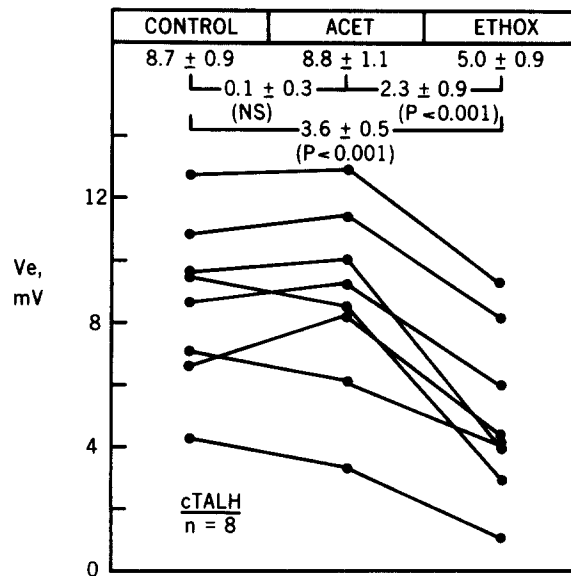


FIGURE 4. A comparison of 2.2×10^{-4} M acetazolamide (ACET) addition to lumen and bath with 10^{-4} M ethoxzolamide (ETHOX) to lumen or bath on V_e . In all instances, the perfusate and bath were KRB solutions, and the gas phase was 95% O_2 -5% CO_2 . In four of the eight tubules, 10^{-4} M ethoxzolamide was added to the perfusate; in the remaining four tubules, 10^{-4} M ethoxzolamide was added to the bath. The lines connect data points on individual tubules. The results are expressed as mean values \pm SEM.

plotted in Fig. 5. Evidently, the relation between $\frac{1}{2}J_{\text{osm}}^{\text{net}}$ and $J_{\text{Cl}}^{\text{net}}$ was linear, with a regression coefficient (r) of 0.99; the least-squares linear regression was virtually coincident with the line of identity. The results in Fig. 5 also show that 10^{-4} M luminal furosemide inhibited $\frac{1}{2}J_{\text{osm}}^{\text{net}}$ to the same degree as $J_{\text{Cl}}^{\text{net}}$. Thus, the data in Fig. 5, in agreement with the results in Tables III and IV, indicate that net Cl^- absorption was accompanied by net absorption of a monovalent cation, presumably Na^+ .

It is also implicit from the data shown in Fig. 5 that there was negligible net transport of HCO_3^- , either in the secretory or absorptive direction. To assess this issue directly, we measured total CO_2 flux ($J_{\text{CO}_2}^{\text{net}}$) simultaneously

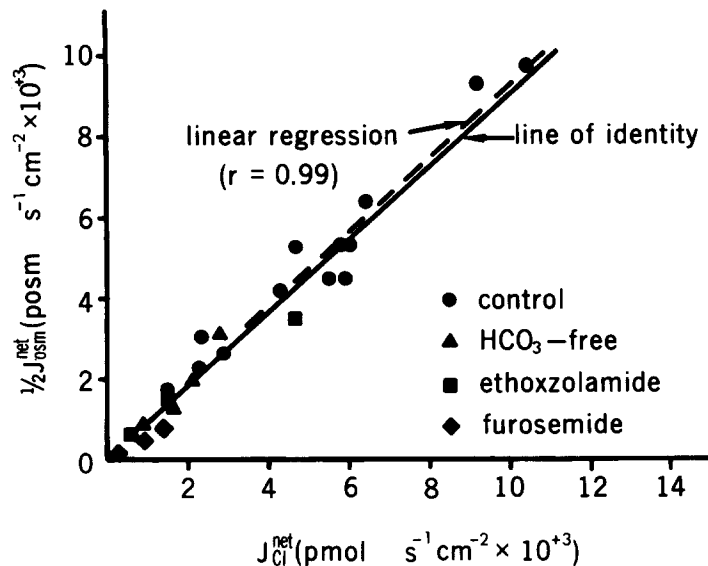


FIGURE 5. The relation between $\frac{1}{2}J_{osm}^{net}$ and J_{Cl}^{net} . The data indicated as “control,” “HCO₃⁻-free,” and “ethoxzolamide” are the data on individual tubules from the experiments reported in Tables III and IV. The data indicated as “furosemide” are from individual tubules in which J_{Cl}^{net} and J_{osm}^{net} were measured while the external solutions consisted of KRB at 37°C, the gas phase was 95% O₂-5% CO₂, and 10⁻⁴ M furosemide was present in the lumen.

TABLE V
SIMULTANEOUS ANALYSIS OF $J_{CO_2}^{net}$, $\frac{1}{2}J_{osm}^{net}$, AND TOTAL CO₂ ($J_{CO_2}^{net}$) FLUXES

\dot{V}_L	Species	Flux	Δ
<i>nl/min</i>			
10.9±2.5	J_{Cl}^{net} pmol s ⁻¹ cm ⁻²	4,799±1,279	
	$\frac{1}{2}J_{osm}^{net}$ posm s ⁻¹ cm ⁻²	4,494±1,163	NS
	$J_{CO_2}^{net}$ pmol s ⁻¹ cm ⁻²	61±62	<i>P</i> < 0.01
	<i>n</i> = 4		

$J_{CO_2}^{net}$ was measured as described in Methods. J_{Cl}^{net} and $\frac{1}{2}J_{osm}^{net}$ were measured simultaneously in each tubule. The perfusate and bath contained KRB. The gas phase was 95% O₂-5% CO₂. The temperature was 37°C. The results are expressed as mean values ± SEM.

with J_{Cl}^{net} and J_{osm}^{net} in individual tubules. The data, shown in Table V, again illustrate virtual identity between J_{Cl}^{net} and $\frac{1}{2}J_{osm}^{net}$, and the fact that the net flux of total CO₂ was indistinguishable from zero. Accordingly, we conclude that (CO₂ + HCO₃⁻)-stimulated net salt absorption in the cTALH (e.g., Table III) occurred in the absence of detectable net HCO₃⁻ transport, either secretory or absorptive.

The Effect of Ion Replacements or Inhibitors on V_e

The data in Tables II–IV indicate that, on the average, there was a close relation between the magnitudes of $J_{\text{Cl}}^{\text{net}}$ and V_e . To evaluate this phenomenon more explicitly, the values of $J_{\text{Cl}}^{\text{net}}$ and V_e from each of the tubules listed in Tables II–IV were plotted in relation to one another. Fig. 6 shows that these $J_{\text{Cl}}^{\text{net}}$ and V_e values were linearly related (regression coefficient = 0.83). We attach no conceptual significance to this relation; nor is it evident from the data in Fig. 6 how net salt absorption in the mouse cTALH produces a spontaneous V_e . Nevertheless, the close relation between the magnitude of V_e and that of $J_{\text{Cl}}^{\text{net}}$, and the comparable effects of various experimental maneuvers on these two variables (i.e., Tables II–IV), indicate that the effects of various

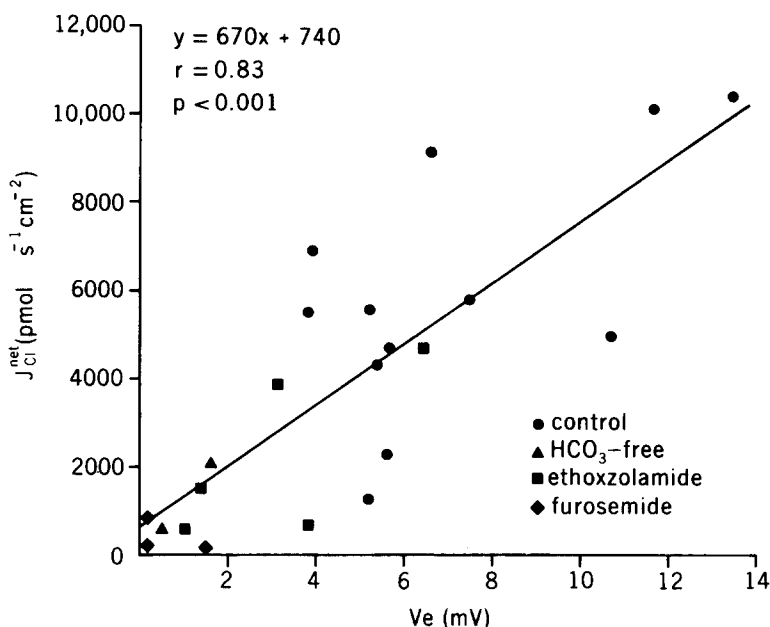


FIGURE 6. The relation between $J_{\text{Cl}}^{\text{net}}$ and V_e . The points represent the data on individual tubules from the experiments reported in Tables II–IV.

ionic substitutions or inhibitors on V_e provide a reasonable index to the actions of such maneuvers on net salt absorption.

The ionic dependence of the spontaneous transepithelial voltage is summarized in Fig. 7. Symmetrical replacement of chloride with isethionate resulted in near-total abolition of V_e . Chloride substitution for bicarbonate, with exclusion of CO_2 from the gas phase, was associated with a reduction of the spontaneous V_e by 55%. When sodium was removed and replaced by tetraethylammonium (TEA), V_e fell by 67%. We note in this regard that, in similar experiments on the rabbit cTALH, Greger and Frömter (1980) found that the use of Na^+ -free glass micropipettes in Na^+ substitution experiments produced a nearly 100% reduction in V_e ; we did not test this possibility

directly in the present studies. Finally, omission of potassium from the peritubular bathing solution caused a 95% reduction of the spontaneous transepithelial voltage.

Fig. 8 summarizes the effects of other agents or maneuvers on the spontaneous transepithelial voltage. The amino-reactive anion exchange inhibitor SITS (Knauf and Rothstein, 1971; Cabantchick et al., 1978) induced a dose-dependent inhibition of V_e : addition of 10^{-5} M SITS to the luminal perfusate was associated with a 25% reduction of V_e , whereas 10^{-3} M SITS reduced V_e by 52%. In contrast, when added to the peritubular bathing solution, 10^{-3} M

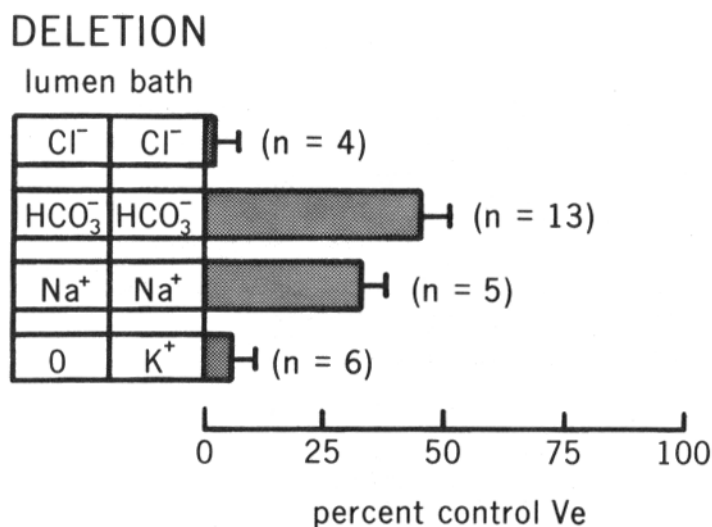


FIGURE 7. A summary of some ionic requirements for the spontaneous transepithelial voltage (V_e) in the cTALH. In the initial (control) and recovery periods, the external solutions consisted of KRB with a 95% O_2 -5% CO_2 gas phase, 37°C . Chloride was replaced by isethionate; bicarbonate by chloride (simultaneously, 100% O_2 replaced 95% O_2 -5% CO_2 in the gas phase); sodium by tetraethylammonium; and potassium by sodium. The composition of these replacement solutions is given in Table I. Each bar indicates the mean and the SEM for each measurement of the indicated number (n) of tubules. In all instances, experimental measurements were bracketed by control measurements with KRB solutions and 95% O_2 -5% CO_2 gas phase.

SITS had no effect on V_e . The cardiac glycoside ouabain, a known ($\text{Na}^+ + \text{K}^+$)-ATPase inhibitor, also caused a dose-dependent inhibition of V_e : at 10^{-4} M ouabain, V_e fell to 40% of control values, and at 10^{-3} M ouabain, V_e fell to ~10% of control values. Finally, Fig. 8 shows that cooling the system to 16°C reduced V_e by ~80%.

Passive Transport Properties

Table VI presents the results of the unidirectional tracer fluxes for $^{22}\text{Na}^+$ and $^{36}\text{Cl}^-$ and the values of P_{Na} and P_{Cl} computed from these fluxes. The values of

both P_{Na} and P_{Cl} are two- to threefold greater than the comparable values for tracer permeability coefficients in the mouse mTALH (Hebert et al., 1981a). The $P_{\text{Na}}/P_{\text{Cl}}$ ratio computed from the data in Table VI is 1.23, which is qualitatively similar to the $P_{\text{Na}}/P_{\text{Cl}}$ ratio observed in the mouse mTALH (Hebert et al., 1981a) and the rabbit cTALH (Burg and Green, 1973).

The conductance of the epithelium can be estimated from the data in Table VI using the expression (Ussing, 1952):

$$g_i = P_i \frac{Z^2 F^2}{RT} [\bar{C}_i] \quad (10)$$

where g_i (mS/cm^2) is the electrical conductance of the i th ion computed from the tracer permeabilities (P_i) and $[\bar{C}_i]$ is the arithmetic mean concentration of

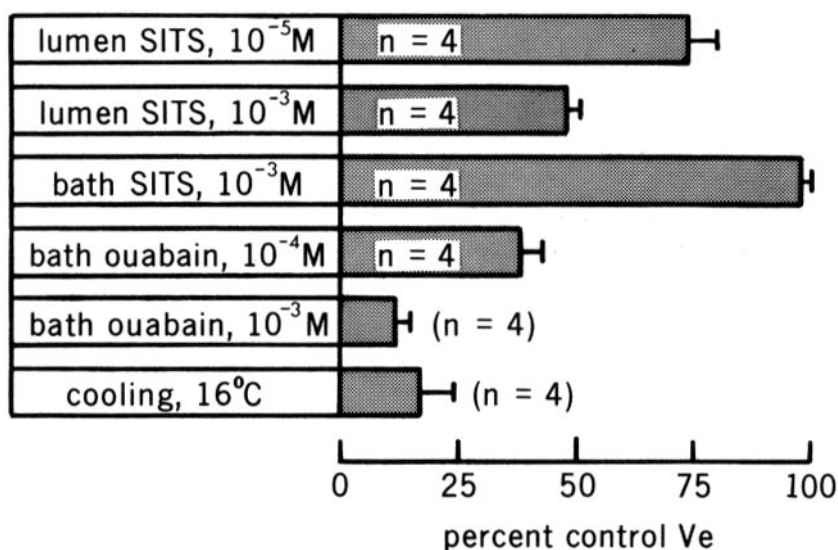


FIGURE 8. The effect of various inhibitors on V_e . The external solutions were KRB with a gas phase 95% O_2 -5% CO_2 . Except where indicated, the temperature was 37°C . Each bar indicates the mean and the SEM for the indicated number of tubules. Control measurements without inhibitor-bracketed experimental measurements.

the i th ion in the external solutions. Using the P_{Na} and P_{Cl} data listed in Table VI, Eq. 10 yields $(g_{\text{Na}} + g_{\text{Cl}}) = 57 \text{ mS}/\text{cm}^2$ or a resistance of $18 \Omega \text{ cm}^2$. This conductance is three times that calculated from tracer permeability data in the mouse mTALH (Hebert et al., 1981a).

Table VII presents the electrical ionic selectivity characteristics of these tubules. In the five tubules indicated as lumen dilution or lumen change, the selectivity ratios were determined from zero-current voltages observed when the perfusate was either a low- $[\text{NaCl}]$ solution, for $\text{Na}:\text{Cl}$ selectivity, or a low- $[\text{HCO}_3^-]/\text{high-}[\text{Cl}^-]$ solution, for $\text{HCO}_3:\text{Cl}$ selectivity, and the bath contained a KRB solution. In the eight tubules denoted as bath dilution or bath change,

the luminal perfusate contained KRB and the bath either a low-[NaCl] solution or a low-[HCO₃⁻]/high-[Cl⁻] solution. For a given ionic selectivity determination, we observed similar voltage displacements either in the presence of a spontaneous voltage or when the spontaneous voltage was abolished with 10⁻⁴ M luminal furosemide. Thus, for convenience, the latter two sets of data are reported as single groups in Table VII.

The results in Table VII indicate that the electrical ionic selectivity ratios in the mouse cTALH were symmetrical, that is, approximately the same value for each ionic selectivity ratio was obtained whether solution changes were made in the lumen or in the bath. Thus, these data are in accord with the

TABLE VI
UNIDIRECTIONAL BATH-TO-LUMEN ²²Na⁺ AND ³⁶Cl⁻ FLUXES AND IONIC PERMEABILITY COEFFICIENTS IN THE CORTICAL THICK ASCENDING LIMB

$J_{Na}^{b \rightarrow l}$	$J_{Cl}^{b \rightarrow l}$	P_{Na}	P_{Cl}
$mol\ s^{-1}\ cm^{-2} \times 10^9$		$cm/s \times 10^4$	
8.59±1.68	5.82±0.33	0.63±0.13	0.51±0.03
<i>n</i> = 3	<i>n</i> = 3	<i>n</i> = 3	<i>n</i> = 3

The perfusate and bath contained KRB solutions at 37°C, and the gas phase was 95% O₂-5% CO₂. The unidirectional tracer fluxes $J_i^{b \rightarrow l}$, for ²²Na⁺ and ³⁶Cl⁻ were measured as described in Methods, and P_{Na} and P_{Cl} were computed according to Eqs. 4 and 5. The results are expressed as mean values ± SEM.

TABLE VII
IONIC PERMEABILITY RATIOS DETERMINED FROM ZERO-CURRENT SALT DILUTION OR BI-IONIC VOLTAGES

P_{Na}/P_{Cl}		P_{HCO_3}/P_{Cl}	
Lumen dilution	Bath dilution	Lumen change	Bath change
1.41±0.14	1.16±0.16	0.13±0.02	0.11±0.01
<i>n</i> = 5	<i>n</i> = 8	<i>n</i> = 5	<i>n</i> = 8

The Na/Cl dilution voltages were measured between KRB solutions and low-[NaCl] solutions. The data indicate values obtained from lumen dilutions or bath dilutions. The HCO₃:Cl bi-ionic voltages were determined between KRB solutions and low-[HCO₃⁻]/high-[Cl⁻] solutions. The data indicate values obtained when low-[HCO₃⁻]/high-[Cl⁻] solutions were placed in the lumen or bath. Table I lists the composition of different solutions. The P_{Na}/P_{Cl} and P_{HCO_3}/P_{Cl} ratios were computed from Eqs. 6-9 as described in Methods. The results are expressed as mean values ± SEM.

symmetrical characteristics of transepithelial electrical ionic selectivity ratios first noted by Ussing and Windhager (1964) in anuran epithelia and subsequently observed by numerous workers in virtually all epithelia studied. Following Ussing and Windhager (1964), it is generally accepted, but not established, that such symmetry indicates that the salt dilution voltage is expressed across a single barrier, presumably the tight junction, and thus that the paracellular route is the primary pathway for passive transepithelial ion permeation. Moreover, a comparison of the data in Tables VI and VII indicates that the P_{Na}/P_{Cl} ratio determined from tracer fluxes was in close

agreement with those determined electrically. Finally, the results in Table VII indicate that the mouse cTALH was relatively impermeable to HCO_3^- , in accord with earlier *in vivo* deductions (Wallin et al., 1973*a, b*) of the HCO_3^- impermeability of the mammalian TALH.

DISCUSSION

The purpose of the present set of experiments was to evaluate the transport properties of *in vitro* mouse cTALH segments, with particular emphasis on net solute absorption, the effects of ($\text{CO}_2 + \text{HCO}_3^-$) on net solute absorption and the passive permeability characteristics of this epithelium. One set of conclusions consistent with the present experimental data affirms the view (Hebert et al., 1981*a*) that the cTALH and mTALH of mouse kidney exhibit significant functional heterogeneity, despite the fact that these two nephron segments also share a number of common transport characteristics.

In the mTALH, NaCl is the principal solute absorbed during luminal fluid dilution (Hebert et al., 1981*a*). In the cTALH, net Cl^- absorption was accompanied by equiosmolar net absorption of a monovalent cation in each circumstance examined (Tables II and IV; Fig. 5), whereas net total CO_2 transport was undetectable (Table V). Thus, it is reasonable to conclude that, in the mouse cTALH, tubular fluid dilution was effected principally by NaCl absorption. Furthermore, the mouse cTALH, like the mouse mTALH (Hebert et al., 1981*a*), was more permeable to Na^+ than to Cl^- , and rather impermeable to HCO_3^- (Tables VI and VII). The relative impermeability of the mouse cTALH to HCO_3^- noted in the present studies is consistent with the earlier deductions of Wallin et al. (1973*a, b*) concerning the *in vivo* HCO_3^- impermeability of the mammalian diluting segment.

The major functional differences in the transport characteristics of these two nephron segments noted thus far relate to the factors modulating net salt absorption. In the mouse mTALH, the combination ($\text{CO}_2 + \text{HCO}_3^-$) has no effect on either net salt absorption or the spontaneous transepithelial voltage (Hebert et al., 1981*a*); but both of these variables are enhanced significantly by ADH or by cAMP analogues (Hall and Varney, 1980; Hebert et al., 1981*a*), and ADH stimulates selectively adenylyl cyclase activity in the mTALH (Morel, 1981). Thus, as argued previously (Hebert et al., 1981*a, b, c*), ADH-mediated enhancement of net salt absorption in the mTALH may be a significant factor in determining the degree of hypertonicity of the *in vivo* renal medullary interstitium, and hence in regulating the degree to which urine is concentrated.

No such role in enriching renal medullary hypertonicity can be assigned, however, to the topographically distinct cTALH. In keeping with this view, Morel (1981) has noted that ADH has minimal stimulatory action on adenylyl cyclase activity in the mouse cTALH, and the present data (Fig. 1), as well as earlier studies (Hebert et al., 1981*a*) indicate that, in the mouse cTALH, ADH and cAMP analogues had no detectable effect on V_e or net salt transport. But the present experimental data (Fig. 2, Table III) indicate clearly that, in the isolated mouse cTALH, the combination ($\text{CO}_2 + \text{HCO}_3^-$)

consistently enhanced both V_e and net salt absorption. Accordingly, we now consider in further detail the modes of salt absorption by the mouse cTALH, and the effects of $(\text{CO}_2 + \text{HCO}_3^-)$ on these processes.

(CO₂ + HCO₃⁻)-stimulated Salt Transport

The combination $(\text{CO}_2 + \text{HCO}_3^-)$ has been reported to enhance the rate of NaCl absorption in a number of epithelia, including the rat renal proximal tubule (Ullrich et al., 1971; Green and Giebisch, 1975), the toad urinary bladder (Chen and Walser, 1977), the rabbit ileum (Field et al., 1971), the rat jejunum (Podesta and Mettrick, 1977), the frog choroid plexus (Wright, 1977), and the rabbit gallbladder (Diamond et al., 1964). Heintze et al. (1981; Petersen et al., 1981) also noted that $(\text{CO}_2 + \text{HCO}_3^-)$ enhanced NaCl absorption in the guinea pig gallbladder, a tissue in which there is net HCO_3^- secretion and only limited HCO_3^- absorption (Wheeler, 1963). Heintze et al. (1979, 1981) have proposed that parallel Na^+/H^+ and $\text{Cl}^-/\text{HCO}_3^-$ exchangers in guinea pig gallbladder apical plasma membranes account for the enhancement of NaCl absorption by $(\text{CO}_2 + \text{HCO}_3^-)$ in that tissue.

With respect to the present studies, the results in Fig. 2 and Table III indicate that net Cl^- absorption was accompanied by equiosmolar absorption of a monovalent cation, presumably Na^+ , and that under all circumstances examined (Fig. 5), there was a virtual identity between $J_{\text{Cl}^-}^{\text{net}}$ and $\frac{1}{2}J_{\text{osm}}^{\text{net}}$. This salt transport process was inhibited by furosemide (Table II, Fig. 5) and by ethoxzolamide, but not by acetazolamide (Table IV, Figs. 3 and 4). Moreover, V_e , and by inference salt transport (Fig. 6), were inhibited by Na^+ or Cl^- removal from external solutions, by K^+ omission from peritubular solutions (Fig. 7), and by cooling or peritubular ouabain (10^{-3} M) (Fig. 8); luminal but not peritubular SITS, 10^{-3} M, also inhibited the process partially. Finally, although $(\text{CO}_2 + \text{HCO}_3^-)$ enhanced net salt absorption, there was no detectable net transport of CO_2 (Table V).

The fact that V_e , and by inference NaCl absorption, were inhibited markedly by Na^+ or Cl^- removal from external solutions contrasts strikingly with classical rheogenic epithelial salt transport (Ussing and Zerahn, 1951; Koefoed-Johnsen and Ussing, 1958), in which replacement of the passively driven ion by an impermeant species increases the spontaneous transepithelial voltage. The fact that luminal furosemide abolished both $J_{\text{Cl}^-}^{\text{net}}$ and V_e is consistent with the possibility that Na^+ transport across apical membranes was coupled to Cl^- movement; it is reasonable to argue that this linking of Na^+ and Cl^- transport occurred in such a way that $(\text{CO}_2 + \text{HCO}_3^-)$ enhanced salt absorption (Table III) without yielding net total CO_2 transport (Table V).

When taken together, these results permit the conclusion that $(\text{CO}_2 + \text{HCO}_3^-)$ -stimulated salt absorption in the cTALH involved the parallel, synchronous, or near-synchronous operation of two antiport processes in apical plasma membranes, at least one of which was furosemide-sensitive: one secreting H^+ into luminal fluid in exchange for Na^+ , and one secreting HCO_3^- , or OH^- , into luminal fluid in exchange for Cl^- . The near-synchronous operation of these parallel exchange processes could account for apical mem-

brane NaCl entry and for the virtual equivalency of $\frac{1}{2}J_{\text{osm}}^{\text{net}}$ with $J_{\text{Cl}}^{\text{net}}$ in all circumstances examined (Tables III and IV; Fig. 4). The luminal H_2CO_3 formed coincident with apical membrane NaCl entry into cells might dehydrate rapidly to $\text{CO}_2 + \text{H}_2\text{O}$, with CO_2 presumably reaching diffusion equilibrium with the entire system, thus resulting in no net transport of total CO_2 (Table V).

According to this hypothesis, Na^+/H^+ exchange across apical membranes was driven by the electrochemical Na^+ gradient between extracellular fluid and cytoplasm, which was, in turn, maintained by active Na^+ extrusion across basolateral membranes mediated by $(\text{Na}^+ + \text{K}^+)\text{-ATPase}$. And in keeping with this possibility, the fact that cooling to 16°C 10^{-3} M peritubular ouabain or peritubular K^+ removal virtually abolished V_e implies that the major energetic source for $(\text{CO}_2 + \text{HCO}_3^-)$ -stimulated salt absorption was basolateral $(\text{Na}^+ + \text{K}^+)\text{-ATPase}$.

$\text{Cl}^-/\text{HCO}_3^-$ (OR Cl^-/OH^-) EXCHANGE: A TERTIARY ACTIVE TRANSPORT PROCESS It is also plausible to argue that the driving force for $\text{Cl}^-/\text{HCO}_3^-$, or Cl^-/OH^- , exchange may have been derived indirectly from Na^+/H^+ exchange. More specifically, apical membrane Na^+/H^+ exchange may have resulted in relative cytoplasmic alkalinity, and therefore a dissipative HCO_3^- , or OH^- , gradient between cytoplasm and extracellular fluid. This dissipative HCO_3^- , or OH^- , gradient may have provided the driving force for cell-to-lumen HCO_3^- , or OH^- secretion which was coupled to lumen-to-cell Cl^- entry.

Thus in the steady state, the Na^+ gradient provided the driving force for coupled Na^+/H^+ exchange, and relative cytoplasmic alkalinity produced by this cation antiport process provided the driving force for coupled $\text{Cl}^-/\text{HCO}_3^-$, or Cl^-/OH^- , exchange. Since conservative transport processes coupled directly to an Na^+ gradient are commonly referred to as secondary active transport processes (Frömter, 1978), it is reasonable, following Stein (1967), to term the proposed $\text{Cl}^-/\text{HCO}_3^-$, or Cl^-/OH^- , antiport system as a tertiary active transport process. This terminology denotes that the Na^+ gradient provided the driving force for $\text{Cl}^-/\text{HCO}_3^-$, or Cl^-/OH^- , exchange indirectly through relative cytoplasmic alkalinity, rather than by a direct coupling of anion flux to apical Na^+ entry; and that, as a consequence, the energy for $\text{Cl}^-/\text{HCO}_3^-$, or Cl^-/OH^- , exchange was derived ultimately from $(\text{Na}^+ + \text{K}^+)\text{-ATPase}$.

THE ROLE OF CARBONIC ANHYDRASE Ethoxzolamide reduced $(\text{CO}_2 + \text{HCO}_3^-)$ -stimulated salt absorption, but not $(\text{CO}_2 + \text{HCO}_3^-)$ -independent salt absorption (Table IV; Fig. 3), whereas acetazolamide had no effect on the $(\text{CO}_2 + \text{HCO}_3^-)$ -stimulated V_e (Fig. 4). A number of pharmacodynamic factors (Maren, 1967, 1969), operating either singly or in unison, may account for the disparity between the actions of ethoxzolamide and acetazolamide on $(\text{CO}_2 + \text{HCO}_3^-)$ -stimulated NaCl absorption. One might argue that $(\text{CO}_2 + \text{HCO}_3^-)$ -stimulated salt absorption required intracellular, or cytoplasmic, carbonic anhydrase activity which was susceptible to inhibition by the lipophilic species ethoxzolamide, but not by the hydrophilic species acetazolamide. Second, the concentrations of acetazolamide used in the present studies may

not have been adequate to provide inhibition of cytoplasmic carbonic anhydrase activity. In a number of different tissues, including stomach mucosal epithelium (Hogben, 1955, 1967), the isolated cornea (Kitahara et al., 1967), and the shark rectal gland (Maren et al., 1979), concentrations considerably in excess of 10^{-4} M are required to inhibit carbonic anhydrase activity. Finally, the inhibition of $(\text{CO}_2 + \text{HCO}_3^-)$ -sensitive salt absorption by ethoxzolamide may have occurred independently of cellular carbonic anhydrase activity: in red blood cells, for example, Cousin et al. (1975) have found that ethoxzolamide, but not acetazolamide, inhibited $\text{Cl}^-/\text{HCO}_3^-$ exchange. In this regard it should also be noted that the complete abolition of the trans-epithelial voltage and net chloride absorption (Table II) after application of 10^{-4} M furosemide may be referable to its action both as an inhibitor of NaCl cotransport (Frizzell et al., 1979) and anion exchange (Brazy and Gunn, 1976; Cousin and Motais, 1976).

Consequently, the present data with ethoxzolamide and acetazolamide do not affirm unambiguously that $(\text{CO}_2 + \text{HCO}_3^-)$ -stimulated NaCl absorption required cytoplasmic carbonic anhydrase activity. But certain quantitative considerations, indicated below, are consistent with such a view. The external and internal diameters of the mouse cTALH used in the present experiments were $\sim 18 \times 10^{-4}$ cm and 12×10^{-4} cm, respectively. Thus, there are:

$$3.77 \times 10^{-4} \text{ cm}^2 \text{ luminal surface area/mm tubule length} \quad (10a)$$

and

$$3.75 \times 10^{-4} \text{ cm}^3 \text{ cell volume/cm}^2 \text{ luminal surface area.} \quad (10b)$$

The uncatalyzed rate of intracellular CO_2 hydration, $\frac{d[\text{H}_2\text{CO}_3]^c}{dt}$ (mol H_2CO_3 formed/cm³ cell volume·s), can be computed from the expression:

$$\frac{d[\text{H}_2\text{CO}_3]^c}{dt} = k_1 \cdot \alpha \cdot p\text{CO}_2, \quad (11)$$

where k_1 is the uncatalyzed hydration rate constant and α is the CO_2 solubility product (0.031 at 37°C). Using a $p\text{CO}_2$ of 40 torr corresponding to the 5% CO_2 gas mixture used in our studies, and a k_1 of 0.08 s^{-1} (Maren, 1978), we obtain:

$$\frac{d[\text{H}_2\text{CO}_3]^c}{dt} = 9.6 \times 10^4 \frac{\text{pmol H}_2\text{CO}_3 \text{ formed}}{\text{cm}^3 \text{ cell volume} \cdot \text{s}}$$

and by using Eq. 10b, the intracellular rate of uncatalyzed H_2CO_3 formation is:

$$\frac{36 \text{ pmol H}_2\text{CO}_3 \text{ formed}}{\text{cm}^2 \text{ luminal surface area} \cdot \text{s}}$$

Clearly, the latter value is two orders of magnitude less than $3,600 \text{ pmol s}^{-1} \text{ cm}^{-2}$, the $(\text{CO}_2 + \text{HCO}_3^-)$ -stimulated value for $J_{\text{Cl}}^{\text{net}}$ or $\frac{1}{2}J_{\text{osm}}^{\text{net}}$ (Table III). These calculations are therefore consistent with the possibility that intracel-

lular carbonic anhydrase activity was required to permit cellular H_2CO_3 generation at a rate sufficient to provide H^+ and HCO_3^- for near-synchronous apical Na^+/H^+ exchange and $\text{Cl}^-/\text{HCO}_3^-$ exchange, respectively.

However, $(\text{CO}_2 + \text{HCO}_3^-)$ -stimulated NaCl absorption in the cTALH may have occurred without a requirement for luminal carbonic anhydrase activity. Thus, if $(\text{CO}_2 + \text{HCO}_3^-)$ -stimulated NaCl absorption was due to parallel, near-synchronous Na^+/H^+ and $\text{Cl}^-/\text{HCO}_3^-$ antiport processes, the rate of H_2CO_3 addition to luminal fluid in the steady state was equal to the rate of $(\text{CO}_2 + \text{HCO}_3^-)$ -stimulated net salt absorption. If luminal carbonic anhydrase activity were not required for this process, it could be argued that the uncatalyzed rate of luminal H_2CO_3 dehydration was sufficiently rapid, with respect to net NaCl absorption, that the steady state H_2CO_3 concentration in the lumen was small. The following calculations illustrate this argument.

In a typical tubule 1 mm in length, $J_{\text{Cl}}^{\text{net}}$ may be expressed as:

$$J_{\text{Cl}}^{\text{net}} \cong k_{-1} \Delta[\text{H}_2\text{CO}_3]^1 v^1 + \dot{V}_O \Delta[\text{H}_2\text{CO}_3]^1 \quad (12)$$

where $J_{\text{Cl}}^{\text{net}}$, 3,600 $\text{pmol s}^{-1} \text{cm}^{-2}$ (Table III), is expressed by using Eq. 10a as 1.36 $\text{pmol s}^{-1} \text{mm}^{-1}$ (tubule length); k_{-1} is the rate constant for uncatalyzed carbonic acid dehydration (32 s^{-1} ; Maren, 1978); v^1 is luminal volume (1.13 $\times 10^{-7} \text{cm}^3/\text{mm}$ tubule); \dot{V}_O is the perfusion rate (Table III; 6 $\text{nl}/\text{min} \cdot \text{mm}$); and $\Delta[\text{H}_2\text{CO}_3]^1$ is the increment in luminal carbonic acid concentration. Accordingly, we obtain:

$$\Delta[\text{H}_2\text{CO}_3]^1 = 0.36 \text{ mM.}$$

This latter value represents an upper limit for $\Delta[\text{H}_2\text{CO}_3]^1$, since Eq. 12 does not include a term for H_2CO_3 removal by radial transepithelial diffusion and assumes that all NaCl uptake proceeded through Na^+/H^+ and $\text{Cl}^-/\text{HCO}_3^-$ exchange. Nevertheless, this calculation indicates that the steady state H_2CO_3 concentration in luminal fluid was small, with respect to total luminal osmolality, even if luminal carbonic anhydrase activity was absent.

$(\text{CO}_2 + \text{HCO}_3^-)$ -independent Salt Transport

At least three explanations might account for the fact that ~50% of salt absorption remained when $(\text{CO}_2 + \text{HCO}_3^-)$ was omitted from external solutions. There may have been sufficient CO_2 produced metabolically by tubular cells to permit HCO_3^- formation by cytoplasmic carbonic anhydrase activity. Second, Cl^-/OH^- exchange may have occurred when the combination $(\text{CO}_2 + \text{HCO}_3^-)$ was absent. Thus, Cl^- antiport may have involved an exchange process that used either OH^- , in the absence of $(\text{CO}_2 + \text{HCO}_3^-)$, or HCO_3^- , when $(\text{CO}_2 + \text{HCO}_3^-)$ were present. Evidently, additional data are required to evaluate these two possibilities.

Alternatively, apical membrane Cl^- entry may have been coupled directly to apical Na^+ entry. Nearly all NaCl absorption was inhibited by 10^{-4} M luminal furosemide (Table II). Similarly, V_e , and by inference salt absorption, were largely inhibited by Na^+ or Cl^- omission from external solutions (Fig. 7),

by K^+ omission from peritubular solutions (Fig. 7), and by cooling or 10^{-3} M peritubular ouabain (Fig. 8). It is therefore likely that these maneuvers affected salt transport occurring in the absence of $(CO_2 + HCO_3^-)$.

These characteristics of $(CO_2 + HCO_3^-)$ -independent NaCl absorption in the mouse cTALH resemble closely similar properties of NaCl absorption in the mouse mTALH (Hall and Varney, 1980; Hebert et al., 1981*a, b*) and in the rabbit cTALH or mTALH (Burg and Green, 1973; Rocha and Kokko, 1973; Burg and Iino, 1979; Greger and Frömter, 1981; Greger, 1981*a, b*; Greger and Schlatter, 1981). Thus, as in the latter nephron segments $(CO_2 + HCO_3^-)$ -independent NaCl absorption in the mouse cTALH may have involved furosemide-sensitive coupled NaCl entry across luminal plasma membranes, in which Cl^- entry was coupled directly to Na^+ entry, and the

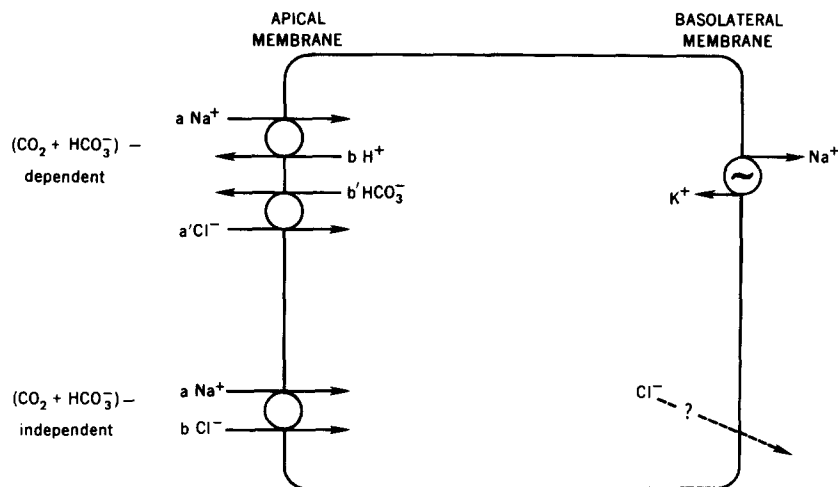


FIGURE 9. Tentative model for $(CO_2 + HCO_3^-)$ -stimulated NaCl transport in the mouse cTALH. $(CO_2 + HCO_3^-)$ -dependent NaCl uptake mediated by parallel Na^+/H^+ and Cl^-/HCO_3^- exchanges is depicted in the upper portion of the apical membrane. $(CO_2 + HCO_3^-)$ -independent NaCl uptake mediated by Na^+ cotransport is indicated in the lower portion of the apical membrane.

electrochemical Na^+ gradient between extracellular fluid and cytoplasm provided the driving force for dissipative Cl^- entry.

Tentative Model and Uncertainties

Fig. 9 presents a tentative model that might account for $(CO_2 + HCO_3^-)$ -stimulated and $(CO_2 + HCO_3^-)$ -independent salt absorption in the mouse cTALH. In $(CO_2 + HCO_3^-)$ -stimulated salt transport, apical membrane NaCl entry is pictured as involving the synchronous or near-synchronous operation of two antiport processes having the indeterminate stoichiometries aNa^+/bH^+ and $a'Cl^-/b'HCO_3^-$ (or OH^-). In $(CO_2 + HCO_3^-)$ -independent salt transport, apical membrane is envisioned as occurring through an ionic symport process having the indeterminate stoichiometry aNa^+/bCl^- . In both circumstances,

basolateral membrane ($\text{Na}^+ + \text{K}^+$)-ATPase is presumed to maintain the Na^+ gradient between cytoplasm and extracellular fluid.

In the mouse mTALH, ADH-stimulated but ($\text{CO}_2 + \text{HCO}_3^-$)-independent NaCl transport appears to involve apical membrane NaCl entry through a symport process having the indeterminate stoichiometry $a\text{Na}^+/b\text{Cl}^-$ (Hebert et al., 1981a). In that nephron segment, apical membrane NaCl uptake may also be associated with K^+ uptake; the lumen-positive transepithelial V_e may be determined in part by a K^+ diffusion voltage between lumen and cells expressed across K^+ -conductive apical membrane channels; and transcellular Cl^- transport may involve a basolateral membrane Cl^- conductive pathway (Hebert et al., 1982). Similarly, Greger and Schlatter (1981) have argued that, in the rabbit cTALH, apical membrane NaCl entry involves the coupled transport of $\text{Na}^+:\text{K}^+:2\text{Cl}^-$, and that the lumen-positive V_e accompanying salt absorption depends in part on a K^+ diffusion voltage between cytoplasm and luminal fluid.

With respect to ($\text{CO}_2 + \text{HCO}_3^-$)-stimulated and ($\text{CO}_2 + \text{HCO}_3^-$)-independent salt transport in the mouse cTALH, the present experiments provide no information about the Na^+ -to- Cl^- stoichiometry of apical membrane salt entry; about the possibility of coupling of K^+ entry to apical membrane NaCl entry; about the origin of the lumen-positive voltage; or about the fraction of total Na^+ absorption that may have been driven through the paracellular pathway by the lumen-positive voltage. Evidently, additional data will be required to evaluate these questions.

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