On the mechanism of bicarbonate exit from renal proximal tubular cells

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On the mechanism of bicarbonate exit from renal proximal tubular cells. We compare here the results of electrophysiological measurements on proximal tubular cells performed on rat kidney *in vivo* and on isolated rabbit and rat tubules *in vitro*. Based on different effects of carbonic anhydrase inhibitors in the *in vivo* and *in vitro* preparation, we conclude that $NaHCO_3$ cotransport across the basolateral cell membrane functions as Na^+-CO_3^{2-}-HCO_3^- cotransport *in vivo*, but as Na^+-HCO_3^--HCO_3^- cotransport in the classical *in vitro* preparation. The former, but not the latter, transport mode is characterized by generation of local disequilibrium pH/CO_3^{2-} concentrations that oppose fluxes if membrane-bound carbonic anhydrase is inhibited. In support of this conclusion, we find that overall transport functions with a HCO_3^- to Na^+ stoichiometry of 3:1 *in vivo* (since each transported CO_3^{2-} eventually generates 2 HCO_3^- ions), but 2:1 *in vitro*. This has been deduced from various measurements, among them super-Nernstian and reverse Nernstian, potential responses to changing ion concentrations which are characteristic of obligatorily coupled cation-anion cotransporters, but are not known in classical electrochemistry. The different transport modes *in vivo* and *in vitro* suggest that isolated proximal tubules have functional deficits compared to proximal tubules *in vivo*.

In mammalian kidney approximately 85% of the filtered HCO_3^- is reabsorbed along the renal proximal tubule. This absorption proceeds in two steps.

In the first step HCO_3^- is taken up from the lumen into the cells. This process is driven by the secretion of H^+ ions into the tubular lumen via a Na/H countertransporter and/or via a H^+ATPase pump which work in parallel. The secreted H^+ ions react with the filtered HCO_3^- to generate CO_2, which diffuses into the cells and recombines with the OH^- ions that were left behind during H^+ secretion, thus generating HCO_3^-. Both reactions, the disintegration of HCO_3^- in the tubular lumen and the regeneration of HCO_3^- in the tubular cell, are catalyzed by carbonic anhydrase (CA), which is bound to the brush border membrane and is also dissolved in the cytoplasm. Although HCO_3^- ions are not transported as such, the overall process may be characterized as active HCO_3^- absorption, since HCO_3^- is accumulated inside the cell against an adverse electrical potential gradient and—except for the first tubular loop—also against its concentration gradient. The necessary energy for this process is provided by the above-mentioned H^+ secretory mechanisms, which may be classified as primary and secondary active transport

processes. Both transporters, the vacuolar-type H^+ATPase, which consists of several subunits [1], and the Na/H countertransporter (NHE3) [2], have already been purified or even cloned. The same holds for both carbonic anhydrases, the renal cytosolic enzyme (CAII) [3], and the renal membrane bound enzyme (CAIV) [4].

By contrast the second step of HCO_3 absorption, that is, the passive exit across the peritubular cell membrane, is less well characterized. A Na-HCO_3 cotransporter has been identified in the peritubular cell membrane of both amphibian and mammalian renal proximal tubules [5–7]. Depending on its stoichiometry this cotransporter should be capable of exporting both HCO_3^- and Na^+ against their concentration differences from the cell into the peritubular fluid with the electrical cell membrane potential providing the driving force. However, the stoichiometry is not yet firmly established [5, 6, 8, 9], and the molecular structure of the cotransporter is not yet known. In addition, controversial observations have been published regarding its possible inhibition by carbonic anhydrase inhibitors [10, 11]. From the effect of various divalent anions such as sulphite on Na-HCO_3-cotransport, Soleimani and Aronson have postulated that the cotransporter actually transports 1 Na^+ plus 1 CO_3^{2-} plus 1 HCO_3^- per transport cycle [12]; however, the transport of CO_3^{2-} as such has never been directly demonstrated.

How to distinguish between CO_3^{2-} and HCO_3^- transport?

Even though CO_3^{2-} concentration is very low in physiological solutions and although CO_3^{2-} may be readily converted into HCO_3^-, there is a chance to detect CO_3^{2-} transport if the transport rates are rather high. Indeed, this seems to be the case. From cell pH measurements we know that cell HCO_3^- concentration can change by several mmol/liter per second in response to large changes in peritubular HCO_3^- concentrations [6]. Table 1 shows the approximate equilibrium concentrations of carbonic acid in physiological solutions. If 1 mmol/liter CO_3^{2-} is suddenly transferred from the cell into the peritubular space (basolateral labyrinth), virtually 100% of the added CO_3^{2-} will react with H^+ to generate HCO_3^-. In principle this reaction is nearly instantaneous; however, because of the low concentration of H^+ ions, it can only proceed as rapidly as new H^+ (together with HCO_3^-) are formed from CO_2 and H_2O, and in the absence of CA this reaction is rather slow. Therefore, a transient disequilibrium pH (disequilibrium CO_3^{2-} concentration) will develop (Fig. 1A) if CA is inhibited or absent. By contrast, if the cotransporter

Table 1. Carbonic acid equilibria: Reactions and approximate equilibrium concentrations (in italics) in physiogical solutions

CO_3^{2-}	+	H^+	$\rightleftarrows$	HCO_3^-		
($\approx$20 μM)		*($\approx$0.1 μM)*		*(25 mM)*		
HCO_3^-	+	H^+	$\rightleftarrows$	CO_2	+	H_2O
(25 mM)		*($\approx$0.1 μM)*		*(1.2 mM)*		*(55.5 M)*

transports only HCO_3^- ions approximately 96% of the transported HCO_3^- remains unchanged, and only 4% can be expected to react with H^+ to form H_2O and CO_2. In the absence of CA the latter reaction is also slow, but since it is quantitatively less important there is no significant disequilibrium pH associated with it (Fig. 1B).

Following these considerations, we conclude that it should be possible to distinguish between transport of CO_3^{2-} and HCO_3^- by measuring pH in the peritubular space of the basolateral labyrinth after sudden changes in HCO_3^-/CO_3^{2-} efflux or influx in presence and absence of CA inhibitors. If CO_3^{2-} is transported, an alkaline disequilibrium pH should develop in the basolateral labyrinth in case all CA was inhibited, and this disequilibrium pH should disappear if CA inhibition was removed. Alternatively, if only HCO_3^- is transported, local pH should not change significantly and CA inhibitors should not have an effect. Instead of the pH in the basal labyrinth we have measured the cell membrane potential (V_b) under the same experimental conditions. These experiments should allow essentially the same information to be obtained if we consider that the development of a disequilibrium pH with opposing CO_3^{2-} gradients should act as a counterforce that retards rheogenic $Na^+-CO_3^{2-}-HCO_3^-$ efflux, and thus depresses the V_b response.

Effect of CA inhibitors on the V_b response to step changes in peritubular HCO_3^- concentration

More than ten years ago we reported that suddenly decreasing the concentration of HCO_3^- or Na^+ in the peritubular capillary bed led to a fast transient depolarization of proximal tubular cells that was followed by a slower partial repolarization and terminated in a new, slightly lower but stable V_b level [6, 13]. Upon sudden reintroduction of peritubular HCO_3^- a mirror image-like transient hyperpolarization occurred, after which V_b returned to its control value (Fig. 2). By cell pH measurements we have meanwhile confirmed that the fast initial depolarization reflects the charge flow associated with the sudden increment in HCO_3^- (and/or CO_3^{2-}) efflux across the peritubular cell membrane, while the secondary repolarization reflects a gradual decrease in efflux following the gradual fall of intracellular HCO_3^- concentration towards a new, more acidic steady state.

When testing this V_b response in the presence of CA inhibitors such as acetazolamide or ethoxzolamide, we noticed that the initial fast depolarization was strongly depressed and that the gradual repolarization was retarded, in agreement with what could be expected if the charge transfer (cotransport) was inhibited (Fig. 3). In addition we noticed that even high concentrations of CA inhibitors, unlike high concentrations of stilbenisothiocyanatodisulfonates (DIDS or SITS), did not completely suppress the V_b response to bicarbonate concentration steps [14]. This incomplete blockage, in spite of increasing concentrations, speaks against a direct inhibition of the cotransporter protein.

The above-described experiments had been performed on proximal tubules of rat kidney *in vivo*, perfusing the tubular lumen and the peritubular capillaries with small glass pipettes. When we wanted to re-investigate and further clarify the effect of CA inhibitors, after we had adopted the technique of isolating and perfusing rabbit tubules *in vitro* [15], we could not reproduce these results. The control V_b response to reduction of peritubular HCO_3^- concentration was essentially identical, but CA inhibitors did not reduce the magnitude of the initial V_b change (Fig. 4). Instead, they affected the secondary phase of the V_b response, causing a faster and larger repolarization that was now followed by a further slow depolarization (tertiary phase) [16]. This observation, which agrees with observations on isolated proximal tubules published by others [17], suggested that CA inhibitors do not interfere with the cotransport itself, but reduce the availability of HCO_3^- inside the cell most likely through inhibition of cytosolic CA. In confirmation of this interpretation, we also noticed that the initial rate of acidification associated with the fast spike-like depolarization was unaltered, but that the acidification rate exhibited a breakpoint after which acidification proceeded less rapidly. This coincided with the onset of the repolarization phase (Fig. 4). We also confirmed this interpretation by repeating the latter experiment at elevated cytosolic HCO_3^- concentrations under which conditions the breakpoint in the pH trace and the fast repolarization phase virtually disappeared, presumably because enough HCO_3^- was present in the cell [16].

In conclusion, the above-reported experiments suggest that $NaHCO_3$ cotransport functions differently in both preparations. In rat tubules *in vivo* CA inhibitors reduce the initial rate of CO_3^{2-}/HCO_3^- efflux in response to suddenly lowering peritubular HCO_3^- concentration. This suggests that a local pH/CO_3^{2-} disequilibrium (rise in extracellular and fall in intracellular CO_3^{2-} concentration in the basal labyrinth) develops that counteracts and retards the efflux. In rabbit tubules *in vitro* CA inhibitors have no such effect. This suggests that instead of CO_3^{2-} (and HCO_3^-) only HCO_3^- ions are transported so that no sizable local pH/CO_3^{2-} disequilibrium develops. Hence we tentatively conclude that in rat tubules *in vivo* $NaHCO_3$ cotransport functions as $Na^+-CO_3^{2-}-HCO_3^-$ cotransport, while in rabbit tubules *in vitro* it functions as $Na^+-HCO_3^--HCO_3^-$ cotransport.

Supporting evidence for CO_3^{2-} or HCO_3^- transport from studies of transport stoichiometry

Since upon attaining the steady state for each transported CO_3^{2-} ion two HCO_3^- ions will be present (Fig. 5), the transport model for rat tubules *in vivo* predicts an overall stoichiometry of 3 HCO_3^- to 1 Na^+, while for rabbit tubules *in vitro* it predicts a stoichiometry of 2 HCO_3^- to 1 Na^+. These different stoichiometric ratios have indeed been observed. Using double-barrelled Na^+ selective and pH sensitive microelectrodes we succeeded in measuring and comparing the initial efflux rates of Na^+ and HCO_3^- in response to identical fast step changes of peritubular HCO_3^- concentration. The experiments on rat tubules *in vivo*, which were published 10 years ago, yielded a ratio of 3.1:1 [6], while the more recent experiments on rabbit tubules *in vitro* yielded a ratio of 2.2:1 [19] in confirmation of the above postulated difference in stoichiometry.

A more elegant way to determine the transport stoichiometries of rheogenic anion and cation cotransporters is to exploit membrane diffusion potentials in response to sudden changes in anion
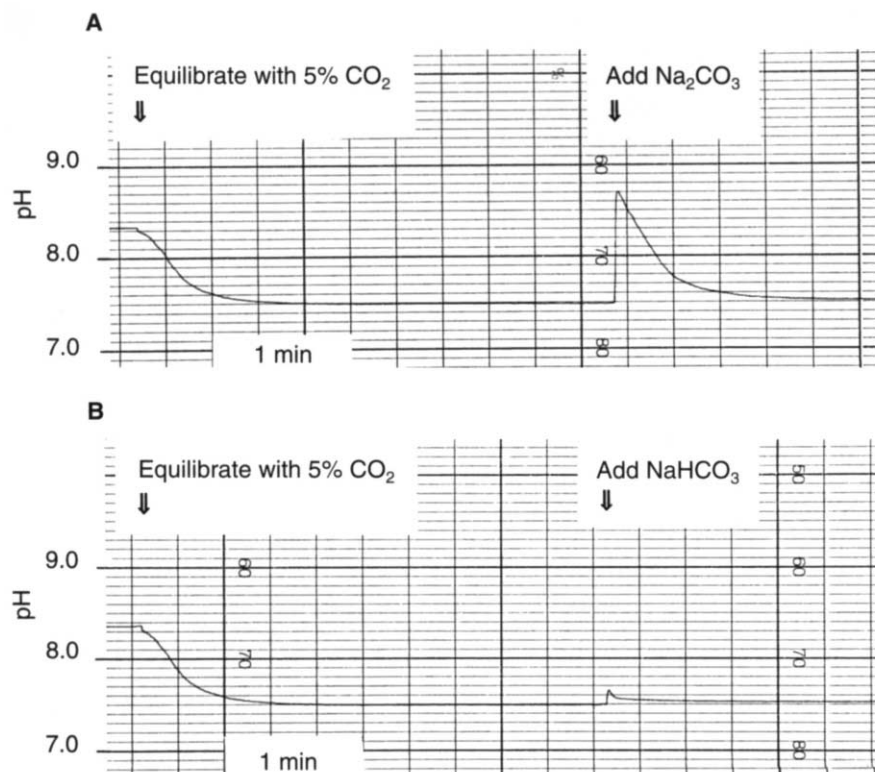
A

B

or cation concentration. Zero-current membrane potentials, $(\Delta V)_{I = 0}$, which arise from concentration differences of one or more permeable ions (i = 1. . . . k) across a membrane are defined as:

$$\Delta V_{I = 0} = -\frac{1}{F} \sum_{i = 1}^{k} \frac{t_i}{z_i} \Delta\mu_i \quad (1)$$

where F is the Faraday constant, z_i denotes valency and sign, $\Delta\mu_i$ is the chemical potential difference of i across the membrane and t_i is the transference number defined as charge carried with the net flux of i (J_i) in relation to total current (I):

$$t_i = \frac{z_i F J_i}{I} \text{ with } \sum_{i = 1}^{k} t_i = 1 \quad (2)$$

In classical electrochemistry transference numbers are known to vary only between 0 and 1 ($0 < t_i < 1.0$). For example, if a membrane is permselective for ion i ($t_i = 1$), equation 1 predicts that $\Delta V_{I = 0}$ changes by 61 mV for a tenfold unilateral concentration change (Nernst slope), or if two ions are equally permeable their transference numbers will be 0.5 each, which results in a potential change of 30 mV in response to a tenfold concentration change of one ion, and so on. However, if obligatorily coupled cotransporters are present transference numbers may become greater than 1.0 or negative. For example, if the membrane contains only coupled transporters for one univalent cation (+) and anion (−), and if the stoichiometry of coupling is q:

$$q = \frac{J_-}{J_+} \text{ eq. 2 yields } t_+ = \frac{1}{1 - q} \text{ and } t_- = \frac{q}{q - 1} \quad (3)$$

From these equations we can predict that the negative ratio of the transference numbers equals the stoichiometric flux ratio:

$$q = -\frac{t_-}{t_+} \quad (4)$$

and we can predict the following interesting relations, which would not have been envisaged in classical electrochemistry:

q = 1 yields electroneutral ion transport ($t_{\pm}$ undefined)

q = 2 yields $t_- = 2$ and $t_+ = -1$ (double-Nernst slope for anion and inverse Nernst slope for cation) and

q = 3 yields $t_- = 1.5$ and $t_+ = -0.5$ (super-Nernst slope for anion and inverse half Nernst slope for cation).

All relevant predictions of this theoretical treatment have been confirmed to the expected extent in recent years in measurements on rat tubules *in vivo* and rabbit tubules *in vitro*:

(1.) In both preparations the polarity of the potential change in response to a step change in Na^+ concentration was opposite to that expected for a simple Na^+ permeable membrane. This indicates a negative transference number for Na^+ (inverse slope).

(2.) In rat tubules *in vivo* the potential response to HCO_3^- concentration steps was 2.95 times larger than the response to Na^+ concentration steps [6]. By contrast, in rabbit tubules *in vitro* the ratio was only 2.1:1 [8], confirming the difference in stoichiometries postulated above.

(3.) As shown in Table 2, the absolute slopes of potential responses to HCO_3^- concentration steps were greater in rabbit
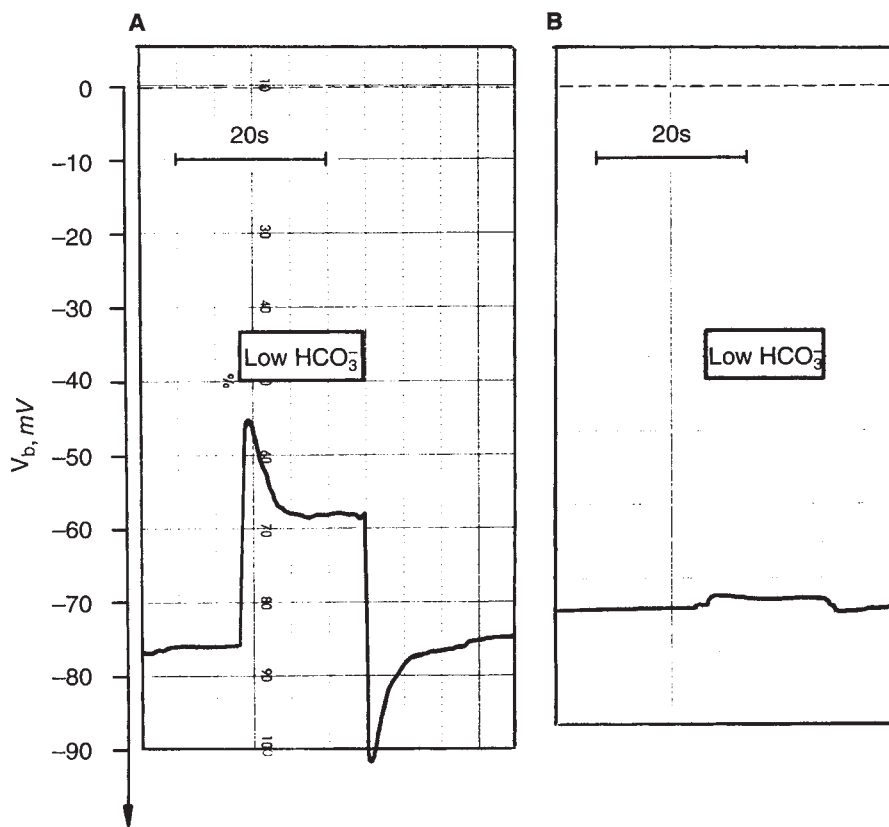
Fig. 2. *Response of basolateral membrane potential (V_b) of a rat proximal tubular cell in vivo to intermittently lowering peritubular HCO_3^- concentration from 30 to 3 mmol/liter at constant pCO_2* (**A**). Solution change was obtained by perfusing peritubular blood capillaries from a double-barrelled glass pipette. Virtually no response is obtained when the same solution change is performed in the tubular lumen (**B**). Used with permission from [19].
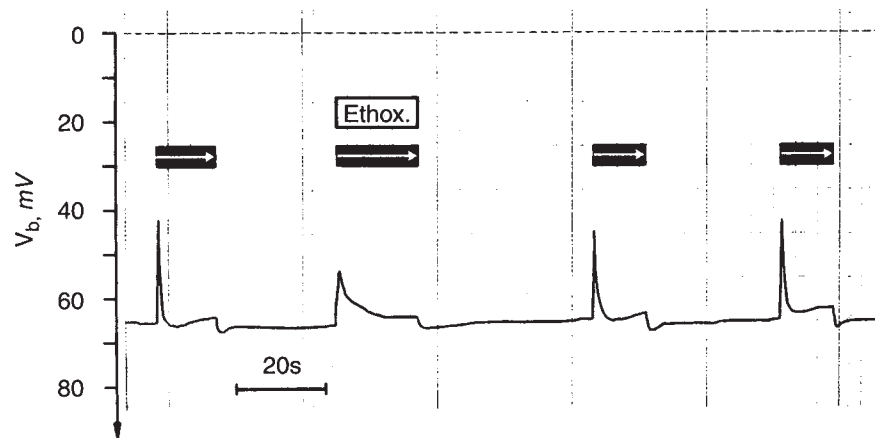

Fig. 3. *V_b response to lowering peritubular HCO_3^- concentration of rat proximal tubular cell in vivo.* Effect of carbonic anhydrase (CA) inhibitor. Details as in Figure 2 except that peritubular HCO_3^- concentration was intermittently lowered to zero (during black bars) and that the second perfusate contained also ethoxzolamide (0.1 mM). Used with permission from [19].

tubules *in vitro* than in rat tubules *in vivo*, in agreement with the different stoichiometric ratios. In addition, in the latter preparation super-Nernst slopes were observed. When comparing measured slopes with absolute slopes predicted from equation 1, it should be kept in mind, however, that proximal tubule cells, besides $NaHCO_3$ cotransport, also contain a number of other ion transporters that act as a shunt, or in other words have a tranference number unequal zero of their own. The highest individual slope that we have ever recorded in response to a tenfold change in HCO_3^- concentration was indeed close to 120 mV in rabbit tubule *in vitro*, whereas the in rat tubule *in vivo* it was near 55 mV.

Exclusion of species differences and possible reason why tubules *in vitro* behave differently from tubules *in vivo*

As described thus far, the difference in the cotransport function between rat tubules *in vivo* and rabbit tubules *in vitro* could either represent a species difference or could be a consequence of the different technical approaches used. Regarding the first possibility, one might speculate that in rabbit tubules *in vitro* the missing effect of CA inhibitors on the initial V_b response to peritubular HCO_3^- reduction might be due to an absence of membrane bound CA in the basolateral cell membrane of this species. However, this is not true. Unpublished immunohistochemical
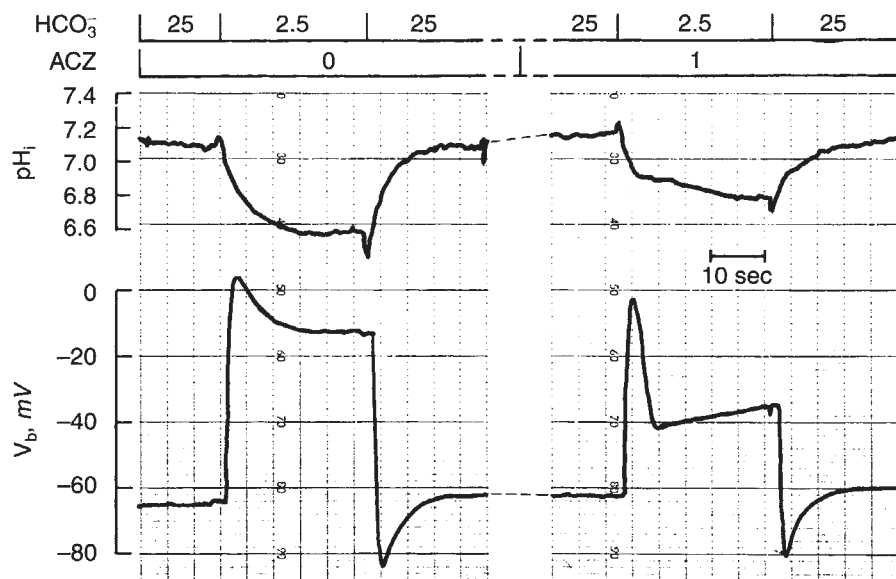
Fig. 4. *Cell pH and V_b response to lowering peritubular HCO_3^- concentration of a rabbit proximal tubular cell in vitro.* Effect of CA inhibitor. Change of HCO_3^- concentration is indicated in mM on top. Left panel: measurements in control state; right panel: measurements in presence of acetazolamide (1 mM). Note different responses in presence of CA inhibitor in Figures 3 and 4. Used with permission from [16].
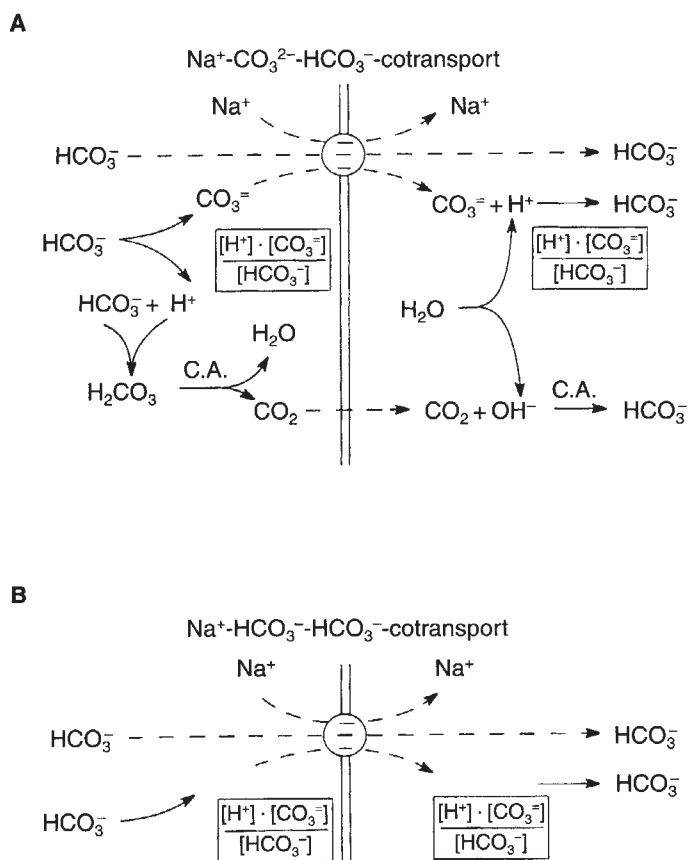
A

Na⁺-CO₃²⁻-HCO₃⁻-cotransport

B

Na⁺-HCO₃⁻-HCO₃⁻-cotransport

Fig. 5. *Schematic diagram representing chemical reactions and overall stoichiometry of Na^+-CO_3^{2-}-HCO_3^--cotransport (A) and Na^+-HCO_3^--HCO_3^--cotransport (B).* (Modified from [18].)

experiments by Sly and Brown (personal communication) have demonstrated that membrane bound CA is present in the baso-lateral labyrinth of proximal tubules of both species.

Table 2. Cell potential response to 10:1 reduction of peritubular HCO_3^- concentration during inhibition of K^+ conductances by Ba^{2+} (1 to 3 mM)

	Rat *in vivo*[a]	Rabbit *in vitro*[b]
$(\Delta V_b)_{HCO_3}$ at constant pCO_2	+35.3 ± 12.7 (mV) ($N = 4$)	+79.9 ± 4.6 (mV) ($N = 12$)
$(\Delta V_b)_{HCO_3}$ at constant pH		+77.9 ± 4.0 (mV) ($N = 4$)

Data are mean values ± SD of measurements on number of tubules.
[a] From [19]
[b] Extrapolated from measurements under 2:1 reduction of bath HCO_3^- concentration (from [8])

Since micropuncture experiments on rabbit kidneys are not feasible we have tried to investigate rat tubules with the *in vitro* technique. This proved exceedingly difficult, but eventually we succeeded in isolating a few tubules and impaling the cells with microelectrodes. As shown in Figure 6, during inhibition of CA the isolated rat tubules responded to HCO_3^- concentration steps essentially in the same way as isolated rabbit tubules. This speaks against a species difference and suggests that the transport function of mammalian proximal tubule is altered under *in vitro* conditions.

The latter conclusion is both surprising and not surprising. On the one hand, after 30 years of studying isolated tubules, it has been taken for granted that these tubules function properly in every respect. On the other hand, a number of differences has been known for years between the results from experiments *in vivo* and *in vitro*. However, this was usually taken to reflect species differences, since the *in vivo* studies were largely restricted to rat kidney and the *in vitro* studies were largely restricted to rabbit kidney. There is only one report in the literature [20] that compares net volume absorption of rabbit tubules *in vivo* and *in vitro* and finds that the former is considerably larger than the latter (1.9 as compared to 1.3 nl/mm min). Since the lower cell
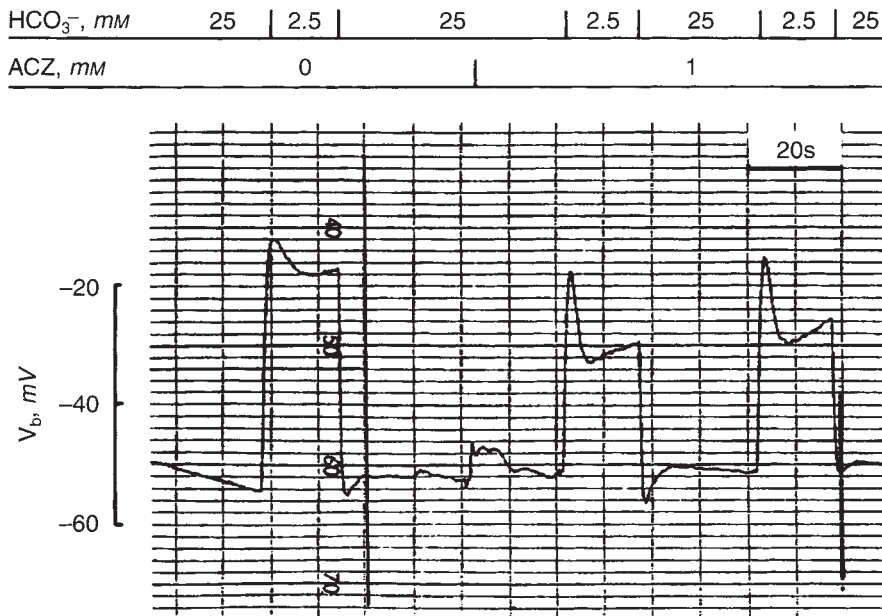
HCO₃⁻, mM	25	2.5	25	2.5	25	2.5	25

ACZ, mM   0   1

Fig. 6. V_b response of isolated rat kidney proximal tubule in vitro to lowering peritubular HCO_3^- concentration. Effect of CA inhibitor (acetazolamide 1 mM). Details as in Figure 4. Note that the response of rat tubule *in vitro* resembles that of rabbit tubule *in vitro* (Fig. 4) rather than that of rat tubule *in vivo* (Fig. 3).

potentials (−40 to −50 mV in rabbit *in vitro* [8, 21] as compared to −73 mV in rat *in vivo* [22] and the higher intracellular Na^+ concentration (57 and 62 mM in rabbit *in vitro* [8, 21] as compared to 17 mM in rat *in vivo* [22]) suggested that the isolated tubules are probably in a state of relative metabolic insufficiency, we recently tried to improve the metabolic situation by bathing the tubule in tissue culture media or adding high concentrations of various metabolic substrates to our regular HCO_3^- Ringers solution that normally contained only 5 mmol/liter D-glucose. This indeed increased the cell potential and lowered intracellular Na^+ concentration. In addition, preliminary experiments indicate that these maneuvers may revert the V_b response to reduction of bath HCO_3^- concentration under CA inhibition from the typical *in vitro* response to the *in vivo* response possibly by ATP(?)-dependent preferential binding of CO_3^{2-} over HCO_3^- at the transporter molecule. These experiments are rather tedious, however, and the *in vivo* type response is not yet always reproducible *in vitro*. As unfortunate as this situation is, it is not really surprising if one tries to figure out what happens to the tubule before and during the process of isolation and in what artificial condition it is later maintained. While proceeding from *in vivo* to *in vitro* was a difficult, but of course very important step in renal physiology, it appears that returning an isolated tubule from *in vitro* function to *in vivo* function may be even more difficult, and whether we will ever fully succeed remains to be seen.

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