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Research Article

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Deficiency of Carbonic Anhydrase in the Vasculature of Rabbit Kidneys

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ABSTRACT The transit of $^{14}CO_2$ and $H^{14}CO_3^-$ through the renal vasculature was studied in rabbit kidneys perfused without erythrocytes and in an in vivo preparation in which erythrocytes were present. In the absence of erythrocytes, the transit of $^{14}CO_2$ from the renal artery to renal vein was much more rapid than that of $H^{14}CO_3^-$. This suggests that (*a*) there is insufficient carbonic anhydrase (c.a.) in the vasculature between the renal artery and the exchange vessels of the kidney to ensure equilibration between CO_2 and HCO_3^- and (*b*) CO_2 can diffuse directly between arterial and venous vessels in the kidney. Following infusions of carbonic anhydrase, the renal venous outflow patterns of $^{14}CO_2$ and $H^{14}CO_3^-$ became the same in the perfused kidneys. Although the initial recovery of $^{14}CO_2$ remained greater than that of $H^{14}CO_3^-$ after infusions of acetazolamide (a c.a. inhibitor), arteriovenous diffusion of $^{14}CO_2$ was diminished by this agent. This is attributed to inhibition of renal tubular c.a. The outflow patterns of $H^{14}CO_3^-$ and $^{14}CO_2$ were nearly the same in the presence of erythrocytes, indicating that erythrocyte c.a. is sufficiently accessible to permit virtual equilibration of these radionuclides during the interval required for transit between the renal artery and exchange vessels. However, addition of carbonic anhydrase to the plasma seemed to accelerate transit of both $^{14}CO_2$ and $H^{14}CO_3^-$ through the kidneys, and a small disequilibrium between CO_2 and HCO_3^- may therefore normally be present in the renal interstitium and capillaries.

INTRODUCTION

Evidence for endothelial carbonic anhydrase (c.a.)[1] in the lung and leg has now been obtained by indicator dilution procedures (1–5) and a variety of other physiological, histochemical, immunocytologic, and cytochemical procedures (6–12). This enzyme is associated with the same membranes that have angiotension converting enzyme activity and is apparently located on the outer surface of the endothelial cell. Endothelial c.a. is presumably more accessible to plasma bicarbonate than the erythrocyte enzyme, which seems to be strictly intracellular. Whitney and Briggle (13) have successfully isolated the endothelial enzyme from the lung and shown that it differs in molecular weight, composition, and activity from erythrocyte c.a. Synthesis of the enzyme by cultures of bovine pulmonary artery endothelial cells has also been documented (12).

Recently, DuBose et al. (14) suggested that there may be insufficient c.a. accessible to HCO_3^- in the renal vessels or interstitium to ensure equilibration with CO_2. Utilizing the same indicator dilution approach that demonstrated the presence of c.a. activity in the vessels of the lung and leg, we have obtained data indicating that there is not enough c.a. associated with the renal endothelium to ensure equilibration between $^{14}CO_2$ and $H^{14}CO_3^-$ in the absence of erythrocytes. Equilibration was promoted by erythrocytes in the intact animal but may nevertheless be incomplete in the renal capillaries and/or interstitium.

METHODS

General procedures

In the initial in vitro studies, comparisons were made between the transit $^{14}CO_2$ and $H^{14}CO_3^-$ from the renal artery to renal vein in kidneys perfused with artificial solutions not containing erythrocytes or c.a. The basic premise was that if the outflow patterns of $H^{14}CO_3^-$ and $^{14}CO_2$ were different, then there is insufficient c.a. on the renal arterial and exchange vessels to ensure equilibration between these indicators. The effects of c.a. and acetazolamide upon transit of these indicators through the kidney were also determined. The studies were then repeated with an in vivo preparation in which the kidneys were perfused with erythrocytes to see

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[1] *Abbreviations used in this paper:* c.a., carbonic anhydrase.

if erythrocyte c.a. is sufficiently accessible to permit equilibration between $^{14}CO_2$ and $H^{14}CO_3^-$. An in vivo preparation was used to minimize hemolysis that might occur in kidneys artificially perfused with erythrocyte suspensions. Additional studies were conducted to determine the effect of acetazolamide upon extravascular pH in the in vitro kidney. The effect of injections and infusions of c.a. upon the exchange of equilibrated mixtures of $^{14}CO_2$ and $H^{14}CO_3^-$ was investigated in the in vivo preparation.

The significance of differences between mean values were determined by analysis of variance with either repeated measures or randomized design (Tukey test).

In vitro kidney studies

Experimental preparations. 16 albino New Zealand rabbits weighing between 2.3 and 3.3 kg were injected with 12 ml of a 25-g/dl mannitol solution and 200 mg of sodium heparin in an ear vein. 5 min later they were sacrificed with 150 mg pentobarbital administered by the same route. The abdominal cavity was then opened and polyethylene catheters (PE 90) were placed in the renal artery and vein of the left or right kidney. A small catheter (PE 50) was inserted in the ureter. The kidney was removed and placed in a dish mounted in a 37°C bath (see Fig. 1). Blood was flushed from the kidney both before and after removal with several hundred milliliters of perfusion fluid. This served to lower c.a. levels in the renal venous outflow to <0.0001 of that in the rabbit blood cells (as determined by the Hodgen and Falk procedure) (15).

Perfusion solution. Each liter of the perfusion fluid contained 50 g bovine serum albumin (Cohn fraction V), 130 meq Na^+, 4 meq K^+, 1.5 mmol Ca^{2+}, 109 meq Cl^-, 25 meq HCO_3^-, 28 meq lactate, 1,500 mg glucose, and 2,500 IU porcine heparin. The pH of this solution was adjusted to 7.4 at a PCO_2 of 40 torr and a temperature of 37°C with small volumes of 2 N NaOH. The kidneys were perfused with this solution at 0.73±0.03 (SD) ml/s (0.061±0.015 ml/s per g of kidney wt). The weight of the kidneys at the end of the experiments averaged 12.2±2.1 (SD) g. Renal artery pressure averaged 139±20 (SD) torr.

In many experiments, some leakage of fluid appeared to occur from small venous branches. This should not have altered the indicator outflow curves. Small amounts of fluid drained from the ureters in most experiments, but no attempt was made to analyze this fluid.

Injection solutions. The passage of $H^{14}CO_3^-$ or $^{14}CO_2$ with each of the other indicators through the kidneys was studied in the following manner.

Simultaneous injections of 0.09 ml of an alkaline solution and 0.09 ml of an acid solution were made through a small T-tube into the renal arterial catheter within a 1-s interval. These solutions were adjusted so that when equal volumes were mixed, the pH of the solution would promptly become 7.4. In half of the runs, $NaH^{14}CO_3$ was included in the alkaline solution to yield $H^{14}CO_3^-$. In the other half, it was placed in the acid solution to produce $^{14}CO_2$. The acid solution contained 12 mM HCl in 0.1 g/dl bovine serum albumin-saline solution and the alkaline solution contained 30 mM of the buffer Hepes in Ringer's lactate at pH 8.4. Hepes buffer was used rather than a CO_2-HCO_3^- system to ensure that a constant pH was rapidly attained. In contrast, equilibration of $^{14}CO_2$ and $H^{14}CO_3^-$ proceeded slowly at the uncatalyzed rate in the absence of carbonic anhydrase. Because of the small dimensions of the injection catheter assembly, only 0.014 ml of each solution remained in the injection tubing after injection and the delay from the point of injection to the renal artery averaged 0.05 s.

Radioisotopes were added to the acid or alkaline injection solutions ~1 h before injection. Each injection contained 0.8 μCi ^{125}I-albumin, and 5 μCi (121 nmol) $NaH^{14}CO_3^-$.

Measurements of extravascular pH were conducted with single injections of 0.09 ml containing ^{125}I-albumin, 0.22 μCi of [2-^{14}C] 5,5-dimethyl-2,4-oxazolidine dione (DMO, 4 nmol) and 4 μCi^3H_2O.

Sample collection. Fluid was pumped at a rate just under that of the venous outflow through a side arm in the venous outflow catheter. The fluid was collected at 0.75-s intervals into syringes mounted on an anaerobic collector (see Fig. 1). The venous outflow was augmented by an equal flow of saline to be sure there was sufficient fluid in each syringe to minimize dilution from the perfusion fluid placed in the dead space of these syringes. This was accomplished by ganging two identical Cole-Parmer peristaltic heads (Cole Parmer, Chicago, IL) on the collection pump. The delay between the renal vein and collection syringes averaged 2.5 s. In the pH studies, the renal venous outflow was collected directly into cuvettes at 0.7-s intervals with an aerobic collector.

Sample measurements and data analysis. Aliquots of 0.2 ml were withdrawn from each syringe and delivered with 10 ml of 15% phenethylamine in Aqueous Counting Scintillant (Amersham Corp., Arlington Heights, IL) into scintillation vials. Pure isotope standards and injection fluid standards were made in the perfusion fluid and processed in the same manner.

Gamma and beta activities were determined in multichannel, multiple sample counters. Corrections were made for crossover between channels and the radioactivities of each sample were divided by the amounts of each isotope injected to yield fractional concentrations (milliliters^{-1}).

The fractional concentrations of each indicator were plotted against time as indicated in Figs. 2 and 3. The areas under each of these curves were calculated from the time of appearance to the peak of the ^{125}I-albumin curves. The calculated areas were divided by those of ^{125}I-albumin to yield the ratios, R_I. Values of R_I were used to compare the initial recovery of each indicator relative to ^{125}I-albumin in the venous outflow (see below). The total recovery of each indicator relative to that of ^{125}I-albumin and the indicator mean transit times, $\bar{t}$, were calculated from the indicator dilution curves extrapolated on semilogarithmic coordinates as described previously (16). Renal blood flow was calculated from the area under the ^{125}I-albumin curve. The mean transit times were corrected by subtracting the calculated delays between the point of injection and the renal artery and the renal vein and collection syringes. In order to compare indicator mean transit times between experiments, values were calculated for the ratios, T:

$$T = (\bar{t}_{^{14}C} - \bar{t}_{^{125}I})/\bar{t}_{^{125}I},$$

where $\bar{t}^{125_I}$ and $\bar{t}_{14C}$ indicate the corrected mean transit times of ^{125}I-albumin and $^{14}CO_2$ or $H^{14}CO_3^-$.

The extravascular pH (pH_e) of the kidney was calculated from the perfusate pH (pH_p) and the mean transit times of ^{125}I-albumin, [^{14}C]DMO and ^3H_2O in the following manner: Values were calculated for r from the mean transit times.

$$r = (\bar{t}_{[^{14}C]DMO} - \bar{t}_{^{125}I})/(\bar{t}_{^3H_2O} - \bar{t}_{^{125}I}).$$

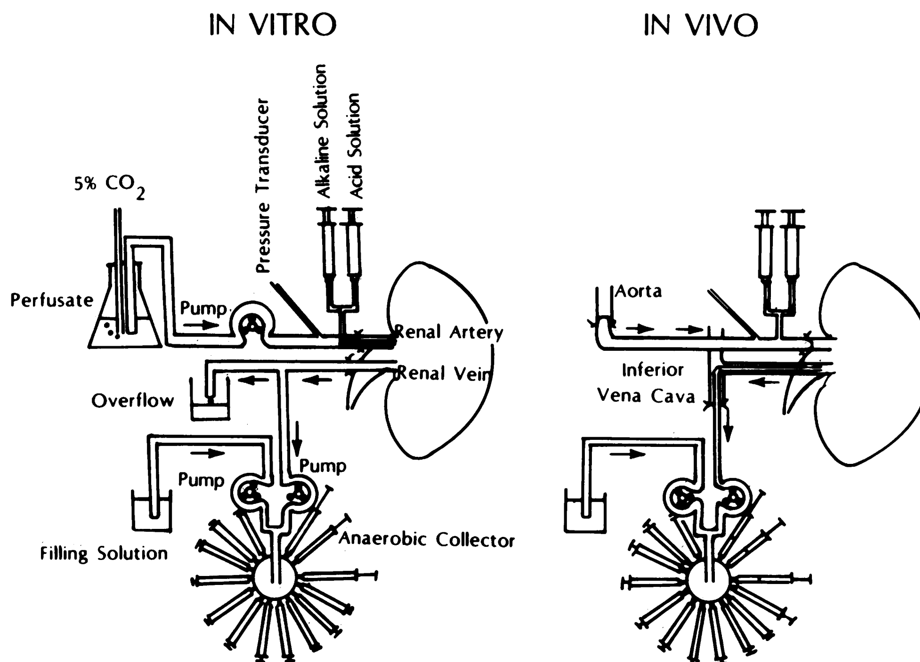

FIGURE 1 Experimental procedures. In vitro studies: An isolated rabbit kidney was perfused at 37° with an erythrocyte-free solution described in the text. Injections of 0.09 ml of an alkaline solution and 0.09 ml of an acid solution were administered simultaneously into a catheter in the renal artery line. By placing $H^{14}CO_3^-$ in the acid solution, $^{14}CO_2$ was generated, whereas $H^{14}CO_3^-$ was the principal ^{14}C indicator in the alkaline solution. ^{125}I-albumin, was included in the solution as a reference vascular indicator. Fluid was collected from the outflow with a second pump into an anaerobic syringe collector at 0.75-s intervals. To ensure that there was sufficient fluid in each syringe, the collected outflow was augmented by an equal flow of saline ("filling solution"). In vivo studies: The kidneys were perfused with blood flowing through a catheter connecting the aorta and renal artery. The indicators were injected through the renal artery catheter. Blood was pumped from a catheter that was inserted in the renal vein but did not obstruct it. This flow was diluted by an alkaline "filling" solution, which increased the volume of fluid in the collection syringes. The syringes were filled at 1-s intervals.

The hydroxyl ion concentration of the extravascular compartment, $[OH^-]_e$ was then calculated by solving the equation

$$r = \frac{K_b' + [OH^-]_e}{K_b' + [OH^-]_p},$$

where K_b' is the alkaline dissociation constant of DMO (0.14×10^{-7} M) and $[OH^-]_p$ is calculated from pH_p. pH_e is then calculated from the equation

$$pH_e = 14 + \log_{10}[OH^-]_e.$$

These relationships are derived elsewhere (16–19). It was assumed that perfusate pH did not change significantly in transit through the kidney. (If any change did occur, it would suffice to assume that the magnitude of this change was unaltered by acetazolamide.)

Experimental protocols. In the first five experiments, the first two injections included alternatively either $^{14}CO_2$ or $H^{14}CO_3^-$, and ^{125}I-albumin. For the third and fourth runs, the kidney was perfused for 5 min before injection and during the subsequent collection period with 200 mg/liter bovine erythrocyte c.a. (2,500 Wilbur-Anderson units/mg, Sigma Chemical Co., St. Louis, MO) in the same perfusion fluid. In the second set of five experiments, either 20 or 100 mg/dl acetazolamide (Diamox, Lederle Laboratories, Pearl River, NY) was added to the perfusate in the third and fourth runs.

The pH of the perfusate was measured in the reservoir during the runs and did not change significantly in the c.a. runs (pH averaged 7.43±0.01 SD). A small rise in pH (0.03 U) occurred in the acetazolamide studies (control pH = 7.43±0.01 SD, acetazolamide pH = 7.46±0.01 SD). To be sure that neither this difference nor the sequence of studies affected the data, an 11th study was performed in which pH was more closely regulated and acetazolamide was administered in the initial two runs. Following a 10-min flush with perfusate not containing acetazolamide, recontrol studies were obtained. The data closely resembled that found in the other five acetazolamide studies and the data of this experiment were not included in the statistical analysis.

In the five pH studies, control experiments were followed by infusions of acetazolamide.

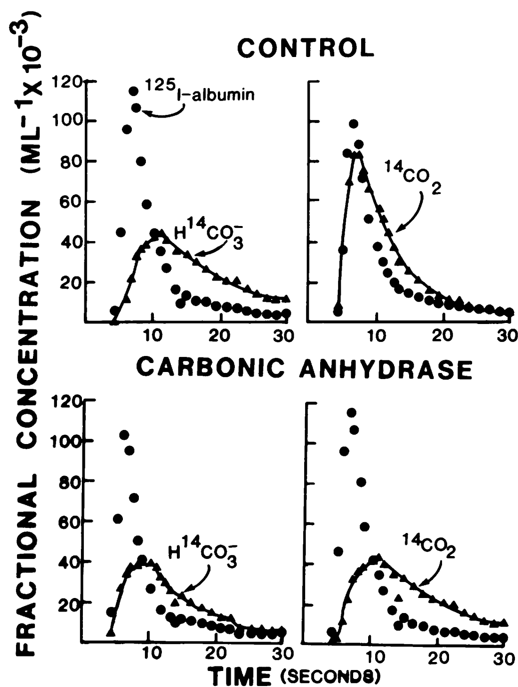

FIGURE 2 In vitro c.a. study. Fractional concentrations of radionuclides are plotted against time. Under control circumstances, the initial recovery of $^{14}CO_2$ compared with ^{125}I-albumin exceeds that of $H^{14}CO_3^-$. The outflow patterns of these indicators become very similar when 200 mg/liter c.a. is added to the perfusion fluid.

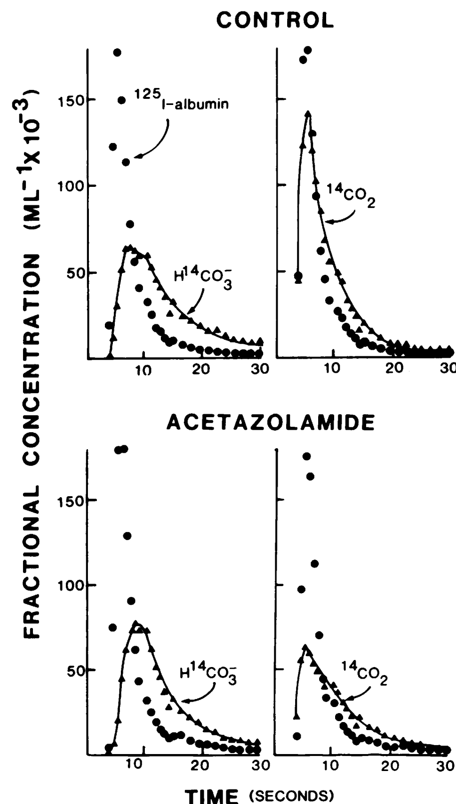

FIGURE 3 In vitro acetazolamide study. Perfusion of the kidney with 100 mg/liter acetazolamide significantly decreases initial recovery of $^{14}CO_2$ but the unslope and peak remains skewed to earlier times than those of $H^{14}CO_3^-$.

In vivo kidney studies

Experimental preparation. 27 albino New Zealand rabbits with average weights of 2.64±0.35 (SD) kg were anesthetized with 8–12 ml of a 10-mg/ml solution of sodium pentobarbital into an ear vein and a cannula was placed in the trachea. The animals were ventilated 30 times per minute with a tidal volume of 15–20 ml of room air. The abdomen was then opened and catheters were placed in the right or left renal vein, renal artery, aorta, and ureter as shown in Fig. 1. Perfusion of the kidney was accomplished by connecting the aortic and renal artery catheters with a polyethylene tube that had a side arm. 60 ml of 25 g/dl mannitol and 2 ml of a 1,000-U/ml solution of sodium heparinate were administered through the ear vein and 0.2 ml of a 3-mg/ml papaverine hydrochloride solution was injected in an ear vein or the renal artery catheter. Urine flow remained low in these studies and was not collected. Kidney weight averaged 9.64±1.25 (SD) g at the end of the study.

Injection solutions. The injection solutions used for the double injection studies in the in vivo studies were the same as those used for the perfused kidneys. ^3H_2O was included in the injection solutions of most of these experiments to provide a second reference curve to which the ^{14}C curves could be compared. In addition, six studies were conducted in which 0.09 ml of a single solution containing an equilibrated mixture of $H^{14}CO_3^-$ and $^{14}CO_2$ at pH 7.4 was injected into the renal artery.

Sample collections. Fluid was pumped from the renal venous catheter at 3.85 ml/min with a peristaltic pump into syringes mounted on an anaerobic collector. Each syringe was filled for 1 s. The outflow (Fig. 1) was diluted with a flow of 13.0 ml/min of an alkaline trapping solution: 0.03 M of the buffer tris (hydroxymethyl) aminomethane (Calbiochem, Behring Corp., Div. American Hoechst Corp., La Jolla, CA) in normal saline at pH 8.4. This was accomplished by ganging together a model 7014 and a model 7013 head on a Cole Parmer peristaltic pump. Augmenting flow in this fashion permitted collection of enough fluid in the syringes to minimize dilution by fluid in the dead space of these syringes. The mean delay between the renal vein and collection syringes was 9.8 s.

Sample measurements and analysis. To minimize quenching, small volumes (0.05 ml) of the collected samples were added to 10 ml of 15% phenethylamine. The ratio of the area under the ^{14}C curve to that of the ^{125}I-curve was calculated as described above. A comparable ratio, R_H, of ^{14}C area to ^3H_2O area (up to the time that the peak of the ^{125}I curve was reached) was also calculated in those experiments in which ^3H_2O was included in the injection bolus. It was reasoned that the outflow patterns of neither ^{125}I-albumin nor ^3H_2O would be influenced by whether $^{14}CO_2$ or $H^{14}CO_3^-$ injections were made because this distinction was

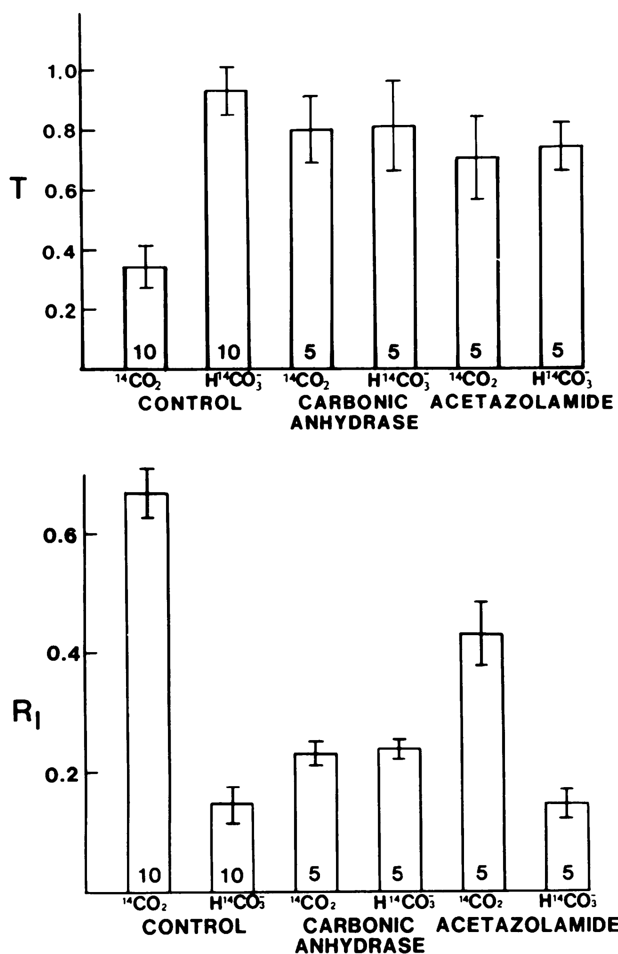

FIGURE 4 Summary of in vitro studies. Mean values of R_I and R_H are indicated with standard errors. Note significant differences of R_I and T values between control $^{14}CO_2$ and $H^{14}CO_3^-$ studies ($P < 0.01$). Following infusions of c.a., the outflow parameters of $^{14}CO_2$ and $H^{14}CO_3^-$ became very similar with a decrease in R_I of $^{14}CO_2$ and an increase in T of $^{14}CO_2$ ($P < 0.01$). After acetazolamide, R_I of $^{14}CO_2$ decreased but remained greater than R_I of $H^{14}CO_3^-$ ($P < 0.01$). T values became the same after acetazolamide.

simply based upon whether the indicators were placed in the acid or alkaline solutions. Experimental interventions such as infusions or injections of c.a. or inhibitors could alter the outflow patterns of ^{125}I-albumin or ^3H_2O by changing renal blood flow. No significant effect upon renal blood flow was observed (see below). One advantage of using the ^{3}H curve as a reference for ^{14}C is the rather close proximity of these curves, which seemed to permit detection of smaller changes in the ^{14}C outflow patterns. Because they are washed out of the organ during comparable intervals, small variations of flow would be expected to have similar effects on the outflow concentrations of each. As indicated below, significant differences in R_H were observed in the control experiments and those with single injections of equilibrated $^{14}CO_2$-$H^{14}CO_3^-$, whereas no significant differences in R_I were observed in these studies. This may indicate that use of R_H provided a more sensitive parameter of change.

Recirculation of indicators in these in vivo experiments obscured the later portions of the outflow curves. This problem was particularly troublesome for the ^{14}C and ^{3}H curves, which are relatively prolonged. Monoexponential extrapolations of the ^{125}I-albumin curves were used for calculations of blood flow. Comparable extrapolations of the ^{14}C and ^{3}H curves were not sufficiently reliable to permit mean transit time calculations.

Experimental protocols. The double injection procedure was used in each of the first two studies. In the first study (Figs. 5 and 6), $^{14}CO_2$ and $H^{14}CO_3$ were injected alternately into the renal artery catheter with ^{125}I-albumin. Thereafter 50 mg/kg acetazolamide (Lederle Laboratories), an inhibitor of c.a., was injected intravenously and the renal artery injections were repeated.

In the second study (Fig. 7), the control studies were followed by intravenous injections of 25 mg/kg of bovine erythrocyte c.a. (2,700 Wilbur-Anderson units/mg, Sigma Chemical Co.) that were given 5 min before each injection of the radioactive solutions.

In a third study (Fig. 9), a single injection containing a mixture of 5% $^{14}CO_2$ and 95% $H^{14}CO_3^-$ (0.03 mM Hepes in normal saline, pH 7.4) was injected into the renal artery and samples were collected in the usual fashion. 1 g/100 ml of the bovine c.a. was included in the radioactive injection solution of the second run and 25 mg/kg i.v. of this enzyme was infused for 5 min before the third run.

RESULTS

In vitro experiments. Under control conditions, the passage of $^{14}CO_2$ through the kidney perfused without erythrocytes was much more rapid than that of $H^{14}CO_3^-$ (see upper panels of Figs. 2 and 3). The initial recovery, R, of ^{14}C after injections of $^{14}CO_2$ exceeded that following injections of $H^{14}CO_3^-$ by a factor of more than four (Fig. 4). Furthermore, the mean transit times of $^{14}CO_2$ were significantly shorter than those of $H^{14}CO_3^-$ ($T_{^{14}CO_2}$ was only one-third of $T_{H^{14}CO_3^-}$). In 9 of 10 control studies, the fractional concentration of $^{14}CO_2$ actually exceeded that of ^{125}I-albumin (by an average factor of 5.6±2.4 SEM) in the first collection tube that contained radioactivity. This never occurred with $H^{14}CO_3^-$.

After perfusion of the kidney with 200 mg/liter c.a., the outflow patterns of $^{14}CO_2$ became very similar to those of $H^{14}CO_3^-$ (lower panels of Fig. 2). This similarity was reflected in terms of both the initial recovery and mean transit times of these indicators (Fig. 4). Changes in $H^{14}CO_3^-$ parameters were not significant.

Perfusion with acetazolamide also slowed $^{14}CO_2$ transport through the kidney but did not have as great an effect on initial recovery of $^{14}CO_2$ as c.a. (lower panels of Fig. 3). The initial recoveries of $^{14}CO_2$ fell significantly from control values ($P < 0.01$), but remained significantly greater than those of $H^{14}CO_3^-$ af-

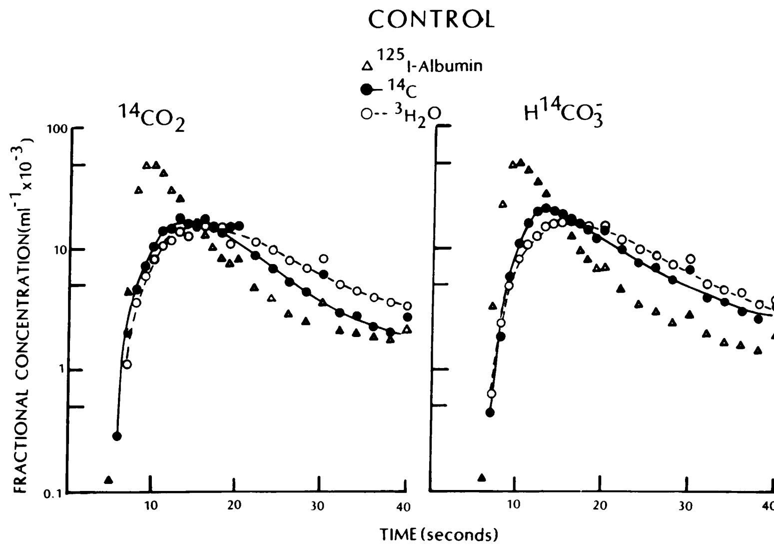

FIGURE 5 In vivo control study. Semilogarithmic coordinates are used to facilitate visual comparison of the initial concentrations of the ^{14}C and ^{3}H indicators. Although the outflow patterns of $^{14}CO_2$ and $H^{14}CO_3^-$ are quite similar, R_H for $^{14}CO_2$ proved to be slightly greater than that of $H^{14}CO_3^-$.

ter infusion of from 20 to 100 mg/liter acetazolamide (Fig. 4, $P < 0.01$). Despite the fact that $R_{^{14}CO_2}$ was greater than $R_{H^{14}CO_3^-}$, T values for these indicators were not significantly different. This reflects the fact that although some of the $^{14}CO_2$ reaches the venous outflow very rapidly, the remainder is released from the renal tissue quite slowly.

Total recovery of ^{14}C relative to ^{125}I-albumin averaged 1.02±0.10 (n = 40, SD) and was not significantly changed by experimental protocols.

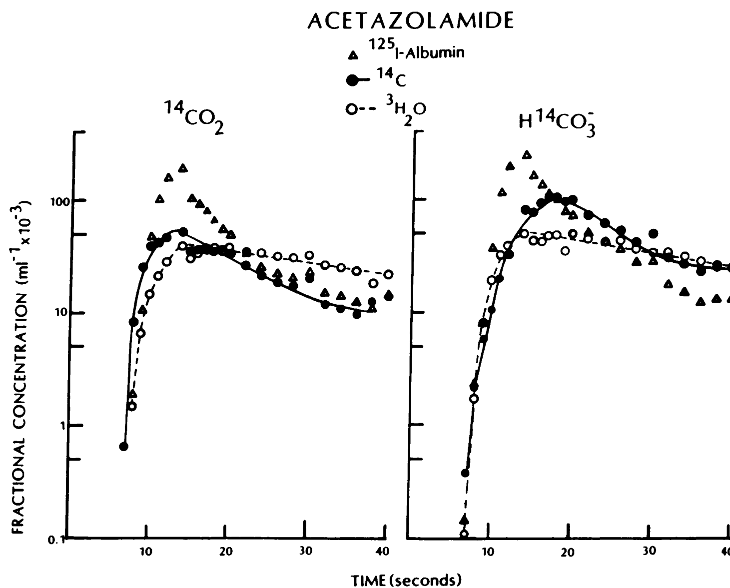

FIGURE 6 In vivo acetazolamide study. Intravenous injection of this inhibitor of c.a. has shifted the $^{14}CO_2$ curve to earlier times than that of $H^{14}CO_3^-$.

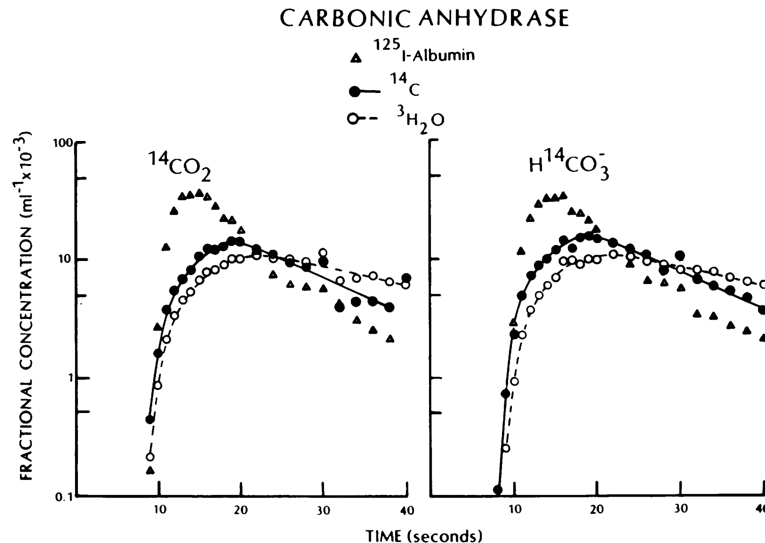

FIGURE 7 In vivo c.a. study. Intravenous injection of this enyzme tended to increase R values of $H^{14}CO_3^-$ somewhat more than those of $^{14}CO_2$.

Infusions of acetazolamide had no effect on the mean extravascular pH, which was 7.30±0.06 (SEM, n = 5) in the control studies and 7.32±0.03 in the acetazolamide studies. Arterial pH averaged 7.42±0.01 in the control experiments and 7.41±0.01 in the acetazolamide experiments.

In vivo experiments. The following average values were obtained (with the exception of hematocrit, values of individual runs in each experiment were averaged and the average values of all the experiments were then averaged): arterial pressure, 82/49; renal blood flow, 0.52±0.36 ml/s (SD); arterial pH, 7.40±0.08; arterial P_{CO_2}, 33±6 torr; arterial P_{O_2}, 91±22 torr; initial hematocrit, 0.32±0.06. There was a tendency for hematocrit to decline between runs (by an average of 0.04) but no consistent changes were found in the other parameters.

Under in vivo conditions, the outflow patterns of $^{14}CO_2$ and $H^{14}CO_3^-$ were much more similar than in the in vitro studies (compare Figs. 2 and 5): the transit of $^{14}CO_2$ through the kidneys of these anesthetized rabbits was only slightly faster than that of $H^{14}CO_3^-$. Although R_H of $^{14}CO_2$ was significantly greater than that of $H^{14}CO_3^-$ ($P < 0.01$), R_I values for these radionuclides were not significantly different (Figs. 5 and 8). This observation suggests that equilibration between $^{14}CO_2$ and$H^{14}CO_3^-$ is not quite complete by the time they reach the exchange vessels of the kidney.

Equilibration between $^{14}CO_2$ and $H^{14}CO_3^-$ was slowed by inhibiting c.a. with acetazolamide. This served to accelerate transit of $^{14}CO_2$ through the renal vasculature (R_I increased 70% from control studies, $P < 0.01$), but had no measurable effect on $H^{14}CO_3^-$ transit (Figs. 6 and 8). (^3H_2O was not used in some of these experiments and only R_I was calculated.)

Addition of bovine c.a. to the plasma phase seemed to facilitate the movement of both $^{14}CO_2$ and $H^{14}CO_3^-$ (and paradoxically made both R_I and R_H of $H^{14}CO_3^-$ slightly greater than the corresponding values of $^{14}CO_2$, $P < 0.01$, Figs. 7 and 8).

These observations concerning the effects of c.a. on R_H were corroborated by the experiments in which a single injection of an equilibrated mixture of 95% $H^{14}CO_3^-$ and 5% $^{14}CO_2$ was injected into the renal artery (Fig. 9). Incorporation of c.a. in the injection solution increased R_H and when the enzyme was infused intravenously, R_H rose further ($P < 0.01$). That these changes were not simply due to a decline in hematocrit was determined in two similar studies in which no c.a. was used: values of R_I and R_H actually declined slightly as hematocrits fell by 10%. Nor did the order of the control studies with $^{14}CO_2$ and $H^{14}CO_3^-$ injections (randomized in 12 experiments) affect the outflow patterns.

The concentration of c.a. in the plasma was less than that contained in a 1:50,000 dilution of hemolyzed erythrocytes in four experiments and less than a 1:20,000 dilution in one experiment.

DISCUSSION

Under control conditions, ^{14}C label appears in the renal venous outflow at an earlier time following arterial

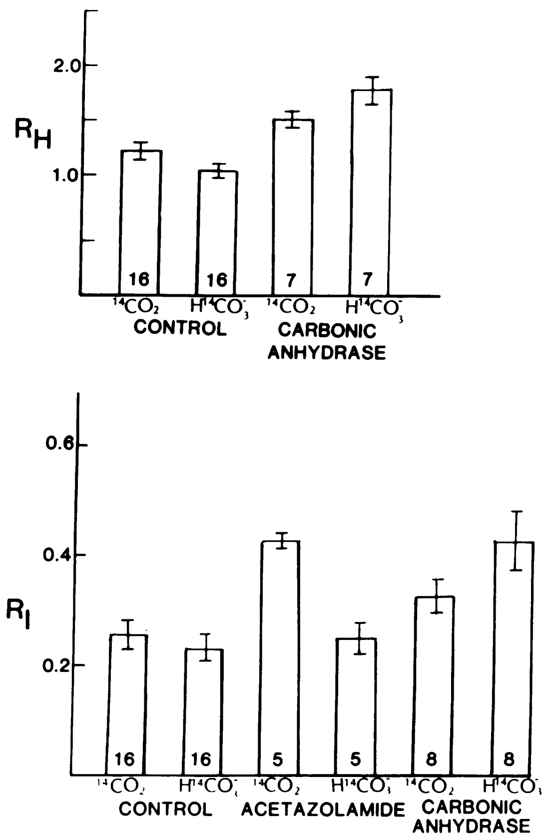

FIGURE 8 Summary of in vivo studies. Mean values of R_I and R_H are indicated with standard errors. Control R_H values of $^{14}CO_2$ were slightly but significantly greater than those of $H^{14}CO_3^-$ ($P < 0.01$). Acetazolamide significantly increased R_I of $^{14}CO_2$ but had no effect on R_I of $H^{14}CO_3^-$. (Values of R_H were not obtained in some of the acetazolamide studies and a mean value is not shown). c.a. increased R_I and R_H of both $^{14}CO_2$ and $H^{14}CO_3^-$. In vivo R_I data cannot be directly compared with in vitro data because of differences in buffering capacity of blood and perfusate.

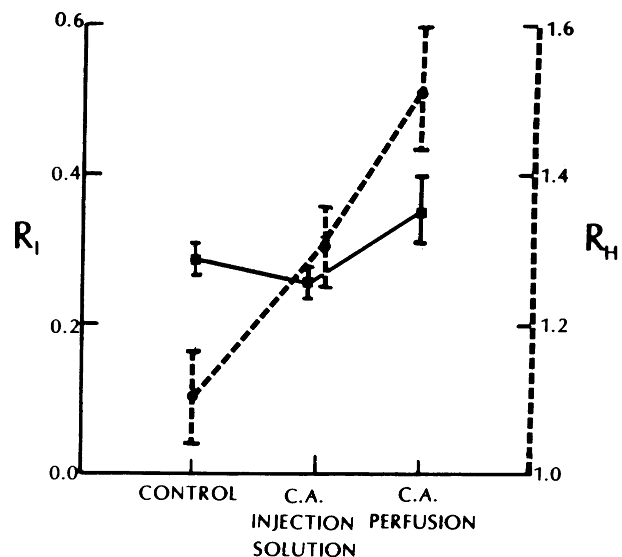

FIGURE 9 Single injection studies (in vivo). The injection solution contained an equilibrated mixture of 5% $^{14}CO_2$ and 95% $H^{14}CO_3^-$. Incorporation of c.a. in the injection solution increased R_H of this mixture and infusion of c.a. further increased R_H. The changes in R_I were not significant.

injections of $^{14}CO_2$ than after similar injections of $H^{14}CO_3^-$. Indeed, at the earliest times, fractional concentrations of $^{14}CO_2$ generally exceeded those of the vascular indicator, ^{125}I-albumin. It can be predicted that some of the injected $^{14}CO_2$ will be converted to $H^{14}CO_3^-$ at the uncatalyzed rate ($t_{1/2}$ = 5.5 s) (3) before reaching the renal microcirculation, but much of it apparently remains as $^{14}CO_2$. These observations suggest that $^{14}CO_2$, like oxygen, tritium gas, 85-krypton, heat, and alcohols, diffuses directly from arterial sources to venous sinks within the kidney (20–24). Shunting results in the relatively early appearance of these indicators in the renal venous blood and skewing of the indicator dilution curves towards earlier times. This change is most easily evaluated by dividing the initial concentrations of the diffusible indicator dilution curve by vascular indicator concentrations to yield R, a procedure analogous to those used by Crone and others (25, 26) for measuring capillary permeability in a variety of organs.

In theory, the mean transit time of each indicator should be determined by its virtual volume of distribution within the kidney rather than the manner in which it traverses the organ (27). The observation that the mean transit time of $^{14}CO_2$ was shorter than that of $H^{14}CO_3^-$ probably reflects the fact that the collection periods were limited to a 40-s interval and the late efflux of $^{14}CO_2$ from more remote compartments of the kidney was not given sufficient weight. The fact that recovery of $^{14}CO_2$ seemed complete in comparison to ^{125}I-albumin does not ensure that the monoexponential outflow pattern assumed in these studies is correct or that the calculated mean transit times are appropriate. The late emergence of a small amount of indicator could significantly lengthen the mean transit time of $^{14}CO_2$.

In contrast to $^{14}CO_2$, diffusional shunting of $H^{14}CO_3^-$ from arterial to venous vessels seems relatively limited. The renal vessels, like those of the lung and leg, appear to be much less permeable to this ion than to $^{14}CO_2$. Alternatively, structures that separate arterial and venous vessels may be impermeable to these ions. $H^{14}CO_3^-$ is presumably delivered with ^{125}I-albumin to the nephrons by the peritubular capillaries.

Unlike ^{125}I-albumin, it then diffuses into the extravascular compartment and consequently arrives in the renal venous blood at a later time than either ^{125}I-albumin or $^{14}CO_2$.

The site at which $^{14}CO_2$ or other lipophilic solutes diffuse from arterial to venous vessels cannot be determined from outflow studies. Since 90% of the renal blood flow is cortical (28), it is likely that much of the shunting found in outflow experiments occurs in the renal cortex. A close proximity is found between arteries and veins in the interlobar, arcuate, and interlobular vessels and some exchange could occur between these adjacent flows. Even more shunting would be expected between adjacent capillaries. The interlobular arteries extend into the cortex in a radial fashion, giving rise to successive generations of afferent arterioles, which in turn supply the glomeruli. The efferent arterioles drain the glomeruli and form a complex, but continuous network of capillaries that surrounds the tubules. In more than half of these capillaries, flow is in the opposite direction to that of tubular fluid in the neighboring nephron (29). It is quite likely that diffusion of lipophilic solutes injected into the renal artery could diffuse between adjacent capillary segments in a manner that would hasten their emergence from the kidney.

Diffusion of CO_2 between adjacent vessels may also contribute to high P_{CO_2} and HCO_3^- concentrations found in the efferent arteriolar blood on the kidney surface (14). CO_2 generated within the superficial cortex might diffuse from venous to arterial vessels in a countercurrent fashion that would tend to keep P_{CO_2} elevated in this region. Equilibration with HCO_3^- would be approached in the blood, thereby elevating regional blood HCO_3^- concentrations. Such a diffusional shunt of CO_2 produced within the cortex might occur between interlobular vessels or adjacent capillary segments. Since less energy need be expended to maintain a P_{CO_2} gradient over a greater distance (e.g., between the superficial and deep cortex) than over a shorter distance (e.g., between different portions of a nephron), the hypothesis of macroscopic countercurrent mechanism is appealing. Shunting of heat between arterial and venous vessels has been reported and is thought to be responsible for slow clearance of this extremely "diffusible" indicator from the superficial cortex (23, 24). Similarly, it has been suggested that diffusion of [^{125}I]iodoantipyrine between deeper cortical vessels may be responsible for the observation that blood flow estimated in the more superficial regions is less than that obtained with microspheres (30). The observation that P_{CO_2} is not elevated in tubular fluid of the medulla is also consistent with countercurrent diffusion in the cortex (31).

The distinctly different outflow patterns of $^{14}CO_2$ and $H^{14}CO_3^-$ indicate that there is insufficient c.a. on the luminal surface of the renal arteries or exchange vessels initially encountered by the perfusion fluid to ensure equilibration between these radionuclides. This hypothesis is confirmed by the observation that the outflow patterns of $^{14}CO_2$ and $H^{14}CO_3^-$ become the same when c.a. is added to the perfusate. Histochemical studies have shown that there is abundant c.a. associated with the cytoplasm and membranes of many of the tubular epithelial cells (32–36). Relatively little attention has been given to renal vascular c.a. However, the enzyme is not detectable in glomerular capillaries of mammals, an observation that is consistent with our observation that there is a deficiency of c.a. activity on the luminal surfaces of at least some vessels of the rabbit kidney. The histochemical studies have detected activity in the vasa rectae of man but not in those of rats.

Following administration of acetazolamide, the initial appearance of $^{14}CO_2$ remained earlier than that of $H^{14}CO_3^-$ but $^{14}CO_2$ transit through the kidney was clearly slower than under control conditions. This observation was unexpected and suggested that a more complete model of CO_2 exchange was needed. There are three ways in which the acetazolamide effect upon $^{14}CO_2$ could be explained.

(*a*) If acetazolamide increases the average pH of the extravascular compartments of the kidney, a prolongation and lowering of the outflow pattern of $^{14}CO_2$ would be expected. This effect of increasing the relative pH of the extravascular space has been observed in indicator dilution studies of the lungs and myocardium (16, 18, 19). Since no significant changes in mean extravascular pH were detected in this preparation with transient studies using the pH indicator, [^{14}C]DMO, this hypothesis seems unlikely.

(*b*) If c.a. is located specifically on the luminal surface of the more venous vessels of the kidney, conversion of $^{14}CO_2$ diffusing into these vessels to $H^{14}CO_3^-$ would lower local concentrations of $^{14}CO_2$ and encourage diffusional shunting from arterial to venous vessels. Inhibition of c.a. at this site would make this process less efficient. As indicated above, the enzyme has been detected on the postglomerular capillaries of some species (primates not rats) (32–36). However, there is also abundant enzyme activity associated with the tubular epithelium and it is likely that much of the $^{14}CO_2$ that escapes from the renal vessels is converted to $H^{14}CO_3^-$ in the extravascular compartments of the kidney. There is consequently little reason to believe that such a mechanism would effectively direct $^{14}CO_2$ into the renal venous outflow.

(*c*) A more plausible explanation for the effect of

acetazolamide is based upon the possible role of tissue c.a. in accelerating the conversion of $H^{14}CO_3^-$ to $^{14}CO_2$ at the interface between aqueous and lipid phases at the cell surfaces. This hypothesis is based upon three assumptions:

(*i*) The ^{14}C label is carried across the cell membranes most rapidly when it is in the form of $^{14}CO_2$. Much of the $^{14}CO_2$ injected in the control experiments apparently remains in this form because there is no c.a. in the plasma or associated with the arterial and initial exchange vessels of the kidney. (*ii*) ^{14}C moves more slowly across cell membranes when it is in the form of $H^{14}CO_3^-$ and $^{14}CO_2$ at physiological pH in the presence of c.a. This is presumably the situation in the renal vasculature when c.a. is added to the perfusate. Such infusions of this enzyme inhibit early diffusion of the ^{14}C label out of the initial vessels by reducing the concentration of $^{14}CO_2$ at the surface of the endothelial cells facing the plasma. A similar situation presumably prevails near many of the lipid membranes in the extravascular space of the kidney since there are large amounts of enzymes at most of these sites. (*iii*) ^{14}C label is carried across the cell membranes least rapidly when it is mostly in the form of $H^{14}CO_3^-$ in the absence of c.a. This is responsible for the delay in emergence of $H^{14}CO_3^-$ in control studies because it is not rapidly converted to $^{14}CO_2$ by c.a. and is consequently retained in the vasculature compartment and does not diffuse directly into the venous vessels.

These assumptions have been shown to be valid in the pulmonary and leg circulations (1–5) and similar data have been reported by Gutknecht et al. (37) for artificial lipid bilayers. Indeed, it was possible to show that $H^{14}CO_3^-$ exchanges across the alveolar capillary barrier at least 600 times more slowly than $^{14}CO_2$ (5).

The effect of acetazolamide can be explained with the aid of Fig. 10. During both control and acetazolamide infusions, $^{14}CO_2$ readily crosses the initial capillary walls (B). Under control conditions, much of the $^{14}CO_2$ that has left the circulation (C) is hydrated at the uncatalyzed rate ($t_{1/2}$ = 5.5s). Nearly complete equilibration between $^{14}CO_2$ and $H^{14}CO_3^-$ occurs at the cell membranes and in the cytoplasm of many of the tubular cells that contain c.a. Although much of the ^{14}C label is in the form of $H^{14}CO_3^-$ at these sites, the presence of enzyme ensures that conversion of $H^{14}CO_3^-$ to $^{14}CO_2$ is rapid and movement of the ^{14}C label across cellular membranes to the renal outflow is prompt. The movement of the ^{14}C label in the aqueous compartments may be accelerated by the parallel movement of $H^{14}CO_3^-$ and $^{14}CO_2$ as suggested by Enns (38). Furthermore it is also possible that enzyme located on cell membranes could transport $H^{14}CO_3^-$

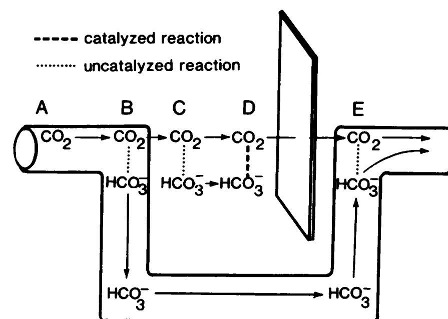

FIGURE 10 Schematic diagram of $^{14}CO_2$ and $H^{14}CO_3^-$ transit through the kidney in control studies after an injection of $^{14}CO_2$ (isotope numbers deleted for clarity). The bent cylinder represents the exchange vessels of the renal cortex, whereas the plane indicates a lipid cell membrane that is assumed to be associated with c.a. The movement of $^{14}CO_2$ is by direct diffusion from the arterial to venous portions of the microcirculation (B to E). Some $^{14}CO_2$ is converted to $H^{14}CO_3^-$ within the vessels at the uncatalyzed rate. The $H^{14}CO_3^-$ is then carried by blood flow through the vessels from B to E, a process that requires more time than direct $^{14}CO_2$ diffusion between these points. Much of the $^{14}CO_2$ that escapes from the vessel is converted to $H^{14}CO_3^-$ at the uncatalyzed rate in the interstitium (C) or at the catalyzed rate on the cell membrane surface (D).

across the lipid barrier. When renal c.a. is inhibited by acetazolamide, whatever $^{14}CO_2$ that has been converted to $H^{14}CO_3^-$ at the uncatalyzed rate (C) will no longer be rapidly reconverted to $^{14}CO_2$ at the cellular borders (D). That this is the correct explanation for the acetazolamide data is suggested by the observation that although initial outflow concentrations of $^{14}CO_2$ are higher than those of $H^{14}CO_3^-$ after infusions of acetazolamide, subsequent washout of ^{14}C is relatively slower. Presumably with the passage of time, more of the $^{14}CO_2$ that remains outside of the vessels is converted to $H^{14}CO_3^-$ at the uncatalyzed rate.

Although a disequilibrium of $^{14}CO_2$ and $H^{14}CO_3^-$ occurs in some of the renal vessels in these perfused kidneys, it cannot be concluded that a similar disequilibrium would also be observed in vivo. There is considerable enzyme within erythrocytes that might promote equilibration between $^{14}CO_2$ and $H^{14}CO_3^-$ in the vascular compartment. Indeed, the studies with the in vivo kidney preparation indicate that in the presence of erythrocytes, the outflow patterns of these radionuclides are nearly the same. Infusions of acetazolamide inhibited c.a. in the erythrocytes as well as the kidney and the outflow patterns of $^{14}CO_2$ and $H^{14}CO_3^-$ again become dissimilar. However, the fact that equilibration is almost reached by the time the indicators are delivered from the renal artery to the capillaries does not prove that equilibrium is sustained within the capillaries. If the movement of $^{14}CO_2$ from

the capillaries is sufficiently rapid and the dehydration of $H^{14}CO_3^-$ is sufficiently slow, a disequilibrium between these radionuclides will be generated in the capillaries, which could limit tissue exchange. That this might indeed be the case is suggested by the experiments with c.a., which seemed to accelerate the movement of both $^{14}CO_2$ and $H^{14}CO_3^-$ through the kidney. The observation that the effect upon $H^{14}CO_3^-$ transport was slightly greater than that upon $^{14}CO_2$ is difficult to explain and may have been due to an artifact (such as some $^{14}CO_2$ loss from the acidic injection syringes). In order to confirm that c.a. does accelerate ^{14}C exchange, single injections containing 5% $^{14}CO_2$ and 95% $H^{14}CO_3^-$ were given into the renal artery. Addition of c.a. to the injection solution did increase R_H and this effect was further increased by infusions of the enzyme. The greater effect of the infusions could reflect diffusion of the enzyme to some extravascular sites that are relatively deficient in c.a. activity. It has been proposed by Roughton (39) and Forster and Crandall (40) that because the erythrocyte enzyme is strictly intracellular, delays in equilibration are inevitable. The absence of carbonic anhydrase bound to the surface of endothelial cells might be responsible for some degree of disequilibrium within the renal vessels in vivo.

It is by no means obvious why the renal endothelium, unlike that of the leg or lung, is deficient in c.a. activity. In contrast to the pulmonary epithelium, which seems devoid of c.a. (4, 10), and the muscle cells, which contain a very inactive form of the enzyme (41–43), there is abundant c.a. activity associated with both the cytoplasm and membranes of many renal tubular cells (32–36). There may, consequently, be little need for additional c.a. in the kidney vasculature. Because the renal tubular cells secrete hydrogen ions into the tubular lumen, the cells themselves are alkaline relative to the plasma and presumably the interstitium as well (44, 45). Although there may well be enough c.a. within the cells to promote full equilibration between HCO_3^- and CO_2 intracellularly, the transport of HCO_3^- into the more acid environment of the interstitium might result in the maintenance of a small

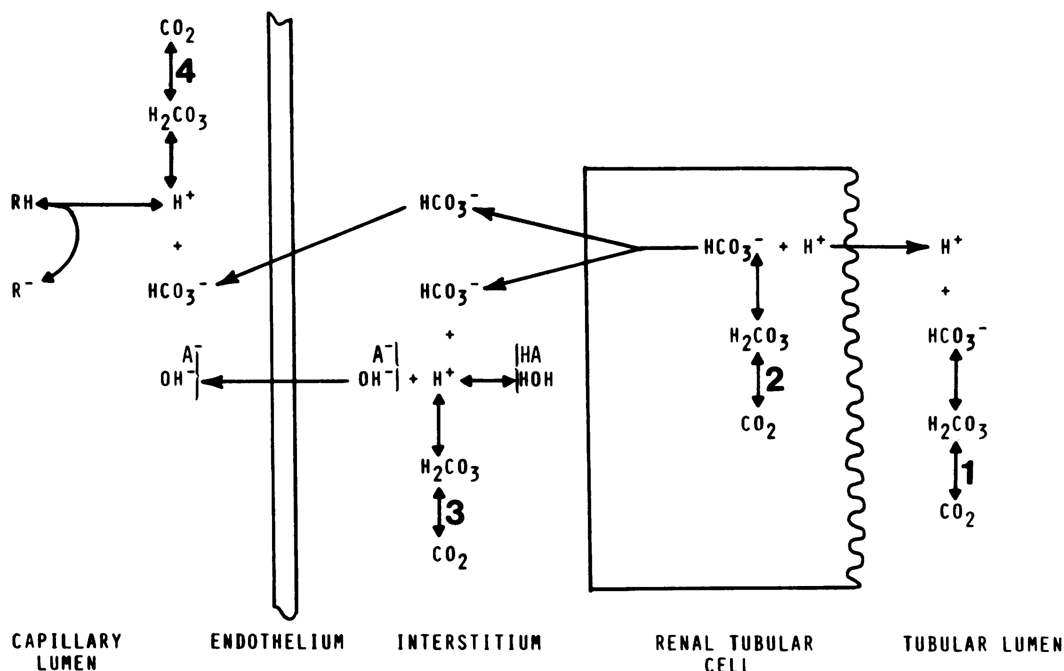

FIGURE 11 CO_2-HCO_3^- equilibration in renal compartments. Site 1 indicates the reaction in the tubular lumen: c.a. bound to the brush border of proximal tubular cells may catalyze this reaction. Site 2 represents the same reaction in the tubular cellular compartment: c.a. is present in many of the tubular cells. Secretion of H^+ is responsible for the fact that cellular pH is relatively alkaline compared to plasma. The interstitial reaction is shown at site 3. It is not clear whether there is c.a. on the surface of the renal tubular cells facing the interstitium or whether any enzyme at this site would be accessible to all the fluid in the interstitium. Since there is a deficiency of carbonic anhydrase on the blood side of the endothelial cells, there may be none facing the interstitium. A disequilibrium in this compartment would favor return of HCO_3^- rather than OH^- or buffer anions (A^-) to the vascular lumen. Equilibration may be incomplete in the plasma (site 4) if erythrocyte c.a. is sufficiently inaccessible.

disequilibrium in this compartment if c.a. is deficient in the interstitium (see Fig. 11). Since the average renal cellular pH is only 0.1 U above that of the plasma (44, 45), it seems unlikely that a disequilibrium greater than this would be sustained in the interstitium. As indicated in Fig. 11, the presence of c.a. in the interstitium would act to decrease HCO_3^- concentrations and increase buffer and hydroxyl anions. Although not shown in the figure, hydrogen ion might also be secured from the plasma and/or from cationic acids (such as NH_4^+). Whether the presence of c.a. in the interstitium would tend to make the return of base equivalents to the blood more or less efficient would depend upon the availability of these alternative ions and the relative permeability of the endothelium to HCO_3^- and the alternative ions. Until information of this nature is available, the significance of a c.a. deficiency on the renal endothelial wall and its role in the formation of CO_2 gradients within the kidney will remain uncertain.

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