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Research Article

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Investigation of Tubular Handling of Bicarbonate in Man

A NEW APPROACH UTILIZING STABLE CARBON ISOTOPE FRACTIONATION

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ABSTRACT Two alternative mechanisms have been proposed for tubular reabsorption of bicarbonate: (*a*) H^+ secretion and CO_2 reabsorption and (*b*) direct reabsorption of HCO_3^-. In an attempt to differentiate between the two mechanisms, the present study utilized the natural abundance of stable carbon isotopes (^{13}C, ^{12}C) in the urinary total CO_2. This novel methodology used mass spectrometric analysis of $^{13}C/^{12}C$ ratios in urinary total CO_2 under normal conditions and during acetazolamide treatment. Blood and respiratory CO_2 were analyzed to yield reference values.

The results demonstrate that alkaline urine is preferentially enriched with ^{13}C relative to the blood. It is suggested that this fractionation results from reaction out of isotopic equilibrium in which HCO_3^- converts to CO_2 during the reabsorption process in the distal nephron. The presence of carbonic anhydrase in the proximal nephron results in rapid isotopic exchange between CO_2 and HCO_3^- and keeps them in isotopic equilibrium. The ratio of urinary $^{13}C/^{12}C$ increases strikingly after acetazolamide administration and consequent inhibition of carbonic anhydrase in the proximal tubule. Although it is possible that in the latter case high HCO_3^- generates the CO_2 (ampholyte effect), the isotope fractionation indicates that CO_2 rather than HCO_3^- is reabsorbed. In contrast, at low urinary pH and total CO_2 values, the carbon isotope composition approaches that of blood CO_2. This indicates rapid CO_2 exchange between urine and blood, through luminal membrane highly permeable to CO_2. These results could be anticipated by a mathematical model constructed to plot ^{13}C concentration of urinary total CO_2.

It is concluded that the mechanism of HCO_3^- reclamation in man (and, by inference, in other mammals as well) works by conversion of HCO_3^- to CO_2 and reabsorption of CO_2.

INTRODUCTION

More than 99% of the filtered load of bicarbonate is reabsorbed along the nephron under normal conditions. Although the relative contribution of each nephron segment to this process is established, the mechanism of HCO_3^- reabsorption is hotly debated. Pitts and Alexander (1) originally proposed that bicarbonate reabsorption and urinary acidification are mediated by H^+ secretion (1, 2). Accordingly, the secreted protons combine with the filtered bicarbonate in the tubular lumen. The carbonic acid thus formed is then dehydrated to CO_2 and water, with the catalytic aid of the carbonic anhydrase present at the brush border.

This theory was challenged by Brodsky and Schilb (3) and Maren (4, 5), who argued against H^+ ion secretion as the sole mediator of bicarbonate reabsorption. They suggested direct HCO_3^- transport as an important alternative mechanism. The experimental data favoring H^+ secretion are based largely on the demonstration of a negative disequilibrium pH either in the proximal nephron during carbonic anhydrase inhibition (6–9) or spontaneously in the distal nephron (6–8).

The finding of a more acidic pH in situ under these conditions was assumed to indicate an accumulation

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of carbonic acid above its equilibrium concentration, thus favoring H^+ ion secretion (10). Nevertheless, the recent demonstration by several laboratories, that CO_2 tension in the proximal (11–14) as well as in the distal tubule exceeds the systemic arterial P_{CO_2}, casts doubt on the validity of the previously reported disequilibrium pH values. Recently, measurements of disequilibrium pH were performed in vivo by DuBose et al. (9), using a new aspiration pH electrode. This showed no disequilibrium in the distal tubule under normal conditions. In vivo negative disequilibrium pH was also found in the collecting duct (15, 16).

The demonstration of high tubular P_{CO_2} raised the question whether there was a limitation in transepithelial CO_2 diffusion. The presently available experimental data are conflicting. Results compatible with very high permeability of tubular epithelium to CO_2 were obtained by DuBose et al. (12, 17), Warnock and Rector (18), and Schwartz et al. (19). In contrast, studies by Malnic and Mello Aires (20) and Sohtell (21) suggest that a transepithelial diffusion barrier for CO_2 might exist.

Finally, another disputed issue is the interpretation of increased urine minus blood P_{CO_2} gradient during bicarbonate infusion. Originally, it was proposed that H^+ secretion into bicarbonate rich tubular fluid in the distal nephron resulted in the formation and subsequent delayed dehydration of carbonic acid. In fact, urine minus blood P_{CO_2} gradient has been used as a semiquantitative index of H^+ secretion in the distal nephron (22–24), but this assumption has recently been questioned by Arruda and co-workers (25, 26) and Maren (27). Both groups suggest that the increased P_{CO_2} in highly alkaline urine is a result of the physicochemical properties of the bicarbonate solution, the so-called "ampholyte" effect, rather than as a result of distal secretion of H^+ ion.

In spite of an increasing interest in the theoretical aspects of the renal CO_2 system and the latest methodological progress in this field, which has been summarized in two excellent recent reviews by Malnic (28, 29), no consensus on HCO_3^- reabsorption has been reached. In the hope of clearing up this problem, we introduced a new method for studying the renal CO_2 system. The method is based on measurements of stable carbon isotopes ($^{13}C/^{12}C$) ratios in total CO_2 ($TC^1 = CO_3^{2-} + HCO_3^- + H_2CO_3 + CO_2$) of urine samples and interpretation of the results in terms of natural isotopic fractionation during the process of urine formation.

In recent years, although application of stable carbon isotopes as tracers has become a standard technique in various biomedical fields (30–34), natural changes in isotope ratios due to biochemical reactions have not received much attention.

Carbon isotopes resemble one another in their atomic structures, but they differ somewhat in their chemical and biochemical properties (35) and thus the $^{13}C/^{12}C$ ratio varies among natural materials (35, 36). In other words, carbon isotopes are fractionated between chemical species involved in reactions.

The present study reports variations in carbon isotope fractionation of TC between urine (U_{TC}) and blood (B_{TC}) by human kidney, in normal physiological conditions as well as during carbonic anhydrase inhibition.

Theoretical considerations

Isotopic equilibrium. It is essential to understand the isotopic fractionation between dissolved CO_2 and HCO_3^-. This fractionation involves transfer of ^{12}C and ^{13}C from CO_2 to HCO_3^-, as expressed in the following isotopic reaction:

$$^{13}CO_2 + H^{12}CO_3^- \rightleftarrows {}^{12}CO_2 + H^{13}CO_3^-. \qquad (1)$$

In general, HCO_3^- is more enriched in ^{13}C than CO_2. This enrichment, however, is rather small; it can be expressed as follows:

$$\alpha_{(HCO_3^- - CO_2)} = \frac{(^{13}C/^{12}C)\ HCO_3^-}{(^{13}C/^{12}C)\ CO_2}, \qquad (2)$$

where $\alpha_{(HCO_3^- - CO_2)}$ is the ^{13}C fractionation factor between HCO_3^- and CO_2. The degree of fractionation depends both on whether CO_2 and HCO_3^- are in isotopic equilibrium or not and on temperature. A system in chemical equilibrium is not necessary in isotopic equilibrium. The time needed to approach isotopic equilibrium depends on the rate of transfer from CO_2 to HCO_3^- and according to Mills and Urey (37) is on the order of minutes. We might expect, however, shorter equilibration time in systems in which the transfer is catalyzed by carbonic anhydrase.

The expected equilibrium fractionation factor at body temperature (37°C) can be estimated from Deuser and Degens (38) as $\alpha = 1.006$.

When performing routine measurements, it is easier and more accurate to measure deviations in isotopic ratios (δ) from a known standard, rather than to measure absolute ratios. Thus, the change in isotopic ratio is expressed in per mill (per thousand; ‰) deviation from the international PDB[2] standard ($\delta^{13}C$) (39), by the following equation:

[1] *Abbreviations used in this paper:* α, fractionation factor; B_{TC}, blood TC; δ, deviation in isotopic values; f, fractional excretion of TC; TC, total CO_2; U_{TC}, urinary TC.

[2] PDB is a $CaCO_3$ standard prepared from a fossil belamnite

$$\delta^{13}C\ (‰) = \left[\frac{(^{13}C/^{12}C)_{sample}}{(^{13}C/^{12}C)_{PDB}} - 1\right] \times 1{,}000. \quad (3)$$

Thus, in δ notation, $\delta^{13}C$ of HCO_3^- is 6‰ larger (or heavier) than $\delta^{13}C$ of CO_2 at 37°C.

It is important to note that $\delta^{13}C$ measurements are not performed separately on dissolved CO_2 or HCO_3^-. They are made on the TC gas that is extracted from a urine sample. For this reason, the results are a weighted average of $\delta^{13}C$ of dissolved CO_2 and HCO_3^- (and in fact also H_2CO_3 and CO_3^{2-}). In equilibrium systems that exchange rapidly with an infinite reservoir of CO_2 gas, $\delta^{13}C$ of total dissolved CO_2 is thus a function of pH (and temperature). It becomes lower at low pH (~4), where the dissolved species are dominated by CO_2 and becomes ~6‰ higher at pH of 7, where HCO_3^- is the dominant species. If we assume isotopic equilibrium, $\delta^{13}C$ of both CO_2 and HCO_3^- can be estimated from measurements of $\delta^{13}C$ of total dissolved CO_2 ($\delta^{13}C_{TC}$):

$$\delta^{13}C_{CO_2} + 6 \approx \delta^{13}C\ HCO_3^-\ (\text{at } 37°C), \quad (4)$$

$$\delta^{13}C_{TC} = \delta^{13}C_{CO_2} \cdot (CO_2/TC) + \delta^{13}C_{HCO_3^-} \cdot (HCO_3^-/TC). \quad (5)$$

Relative proportions of CO_2 and HCO_3^- are determined from chemical equilibrium reactions (Appendix I). The same logic is used in calculating $\delta^{13}C$ of arterial blood from $\delta^{13}C$ of venous blood and respiratory CO_2 (Eq. 10 below).

Carbonate ion and H_2CO_3 were neglected in Eq. 5. The former species is relatively rare in most cases, and becomes more significant only at elevated pH values (Appendix I). In addition, isotopic fractionation between CO_3^{2-} and CO_2 is fairly similar to HCO_3^- and CO_2 fractionation. The fractionation factor is $\alpha_{(CO_3^{2-}-CO_2)} = 1.0064$ (39). The isotopic composition of H_2CO_3 is unknown, but this species is also very rare (Appendix I) and does not pose a problem in estimating isotopic compositions.

Isotopic distillation. A major goal of the present study was to understand the isotopic effects of the conversion of bicarbonate to CO_2. Since CO_2 has less ^{13}C than does HCO_3^-, removal of CO_2 from a solution containing both CO_2 and HCO_3^- should enrich the solution in ^{13}C. If this process continues and if CO_2 is removed immediately, and is not allowed to reequilibrate (isotopically), the TC in the system will become progressively more and more enriched in ^{13}C. Similar isotopic distillation is known to occur in natural processes and has been studied in detail, especially in connection with evaporation of water (40, 41).

The degree of ^{13}C enrichment (or $\delta^{13}C$ increase) in the remaining TC expressed as a function of a fraction (f) of the amount at the beginning of the process, can be easily calculated:

$$\delta^{13}C\ \text{final} = (1{,}000 + \delta^{13}C\ \text{initial}) \times f^{(1-\alpha)} - 1{,}000, \quad (6)$$

where α is the fractionation factor between CO_2 and HCO_3 (~1.006 at 37°C). For the derivation of Eq. 6, see Appendix II.

If renal absorption of HCO_3^- involves its transition to CO_2 without isotopic reequilibrium, then U_{TC}, which is a small fraction of the TC filtered, should become very enriched in ^{13}C. For example, if 99% of the filtered load is absorbed ($f = 0.01$) and if the initial $\delta^{13}C$ (that of the glomerular filtrate) is −20‰, the final $\delta^{13}C$ is calculated as 7.5‰. In this case, $\delta^{13}C$ has been enriched by 27.5‰. This process is demonstrated graphically in Fig. 7. In an attempt to find out whether such enrichment takes place in the human kidney, we performed a series of experiments.

We assumed that no isotopic fractionation occurs during glomerular filtration, and hence that $\delta^{13}C$ of arterial B_{TC} represents $\delta^{13}C$ of glomerular filtrate. Previous studies have demonstrated that $\delta^{13}C$ of total CO_2 in blood is rather constant in a certain human population (42, 43) and is determined by the isotopic ratio in the diet (43–45). There is a relatively large reservoir of carbon in the body, which is not easily affected by occasional meals (33, 46).

METHODS

Test group. Six healthy (serum creatinine 0.8–1.1 mg/100 ml) volunteers (age 25–37 yr, from the Department of Geology at the Hebrew University of Jerusalem), provided a total of 44 urine samples. All the volunteers had normal capability for acidifying urine. Each individual gave a morning urine sample, two or three samples of daytime urine, and two or three samples after oral administration of 500 mg acetazolamide in one dose. Three of them gave two samples after oral administration of 80 mg furosemide (Lasix) in one dose.

Experimental procedure. Each volunteer voided in a slow stream and completely filled a 100-cm^3 glass bottle and then closed it hermetically. This precaution was taken in order to minimize CO_2 escape. The samples were analyzed from within a few minutes up to 2 h after urination. Immediately after opening the sample, pH was determined (accuracy, ±0.05 pH units), and the bottle sample connected to the CO_2 extraction line (Fig. 1). U_{TC} was determined and $\delta^{13}C$ of the collected CO_2 was measured on a double inlet double collector

(*Belamnitella americana*) from the Pee Dee formation. It is customary to express ^{13}C enrichment or depletion with respect to this international standard. The standard itself is no longer available, but many substandards were prepared and are available. Our calibration is done with respect to PDB-IV from Prof. S. Epstein, California Institute of Technology.

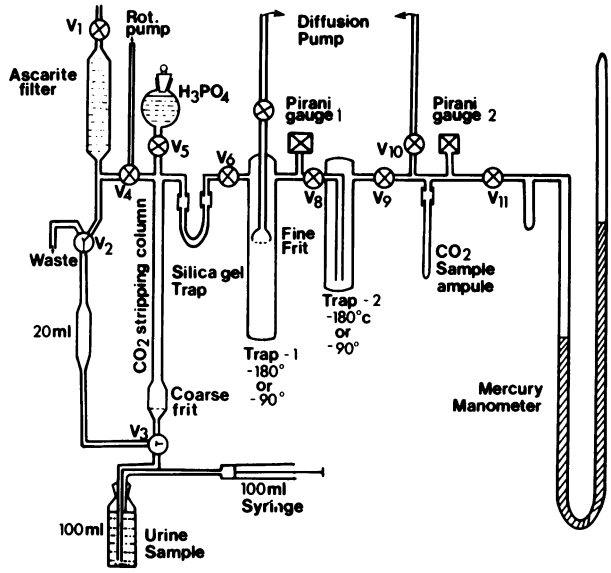

FIGURE 1 Total CO_2 extraction line. V_1-V_{11}-vacuum valves. 20 cm^3 of the sample is transferred to the stripping column. Phosphoric acid is added to convert the TC to gaseous CO_2. Evolved CO_2 stripped with N_2 for 20 min (to assure nearly complete CO_2 extraction). CO_2, water vapor, and nitrogen pass through a silica gel water trap to trap 1, which is kept at −190°C with liquid nitrogen. CO_2 is trapped and nitrogen is pumped out. To eliminate any possible remaining water, cooled isopropranol (−90°C) is applied. This procedure is repeated in trap 2. TC is measured in the manometer and collected in a glass ampule. The ampule is transferred to a mass spectrometer to determine its $\delta^{13}C$. In a similar way, B_{TC} is determined. Respiratory CO_2 is collected in this line by connection of a bulb with exhaled air directly to the silica gel trap.

mass spectrometer. The extraction method is similar to that of Kroopnick (47), whose details are illustrated in Fig. 1. The accuracy of measurements is ±0.1 mmol/liter for total CO_2 and ±0.2‰ PDB for $\delta^{13}C$.

Effects of sample aging and CO_2 loss during exposure of the urinary stream to air were tested in a few duplicate samples. In addition, $\delta^{13}C$ was determined in expiratory CO_2 (metabolic CO_2) of the group and in four samples of total CO_2 of venous blood.

RESULTS

The results are shown in Figs. 2–4. Duplicate analyses demonstrate that the effects of CO_2 escape from sample aging are relatively small if the sample is analyzed within 2 h of voiding (up to 17% of CO_2 loss and 1.88‰ PDB changes in $\delta^{13}C$). The rate of flow during voiding does not appear to be an important factor in determining $\delta^{13}C$ of U_{TC} (Table I).

Fig. 2 demonstrates the chemistry of the urinary CO_2 system. As expected, pH increases with U_{TC} from pH 4.9 in normal samples, up to 8.08 after acetazolamide treatment. pH is approximately constant (~7.8) from U_{TC} of 50 mmol/liter. All morning urine samples are acidic with low U_{TC}. Furosemide treatment does not cause unusually high pH or U_{TC}. It is thus clear that the test group consists of healthy individuals with normal ability to acidify urine.

$\delta^{13}C$ increases with U_{TC} in normal samples (Fig. 3) and reaches maximum values in acetazolamide treatment, but in the latter case a decrease in $\delta^{13}C$ is observed at the highest U_{TC} values. Intermediate $\delta^{13}C$ occurs in samples in which the treatment did not produce maximum pH change (Fig. 3).

Morning samples and furosemide treatment samples fall within the normal range and do not define discrete groups (Figs. 2, 3).

The relationships between pH and $\delta^{13}C$ are shown in Fig. 4. In normal samples $\delta^{13}C$ increases linearly with pH:

$$\delta^{13}C_{TC} = 10.828\ pH - 78.528 \quad (r = 0.90). \qquad (7)$$

The linear relations are expected from the similar trends of pH and $\delta^{13}C$ versus U_{TC} (Figs. 2, 3). Poor correlation is observed in acetazolamide treated samples, where $\delta^{13}C$ increases with no appreciable change of pH.

Fig. 5 demonstrates the relationship between HCO_3^-/TC and TC in normal urines, which is expressed in the following equation:

$$HCO_3^-/TC = 0.00000823(TC)^4 + 0.00071486(TC)^3 - 0.02138231(TC)^2 + 0.26300894(TC) - 0.308861849 \quad (r = 0.97). \qquad (8)$$

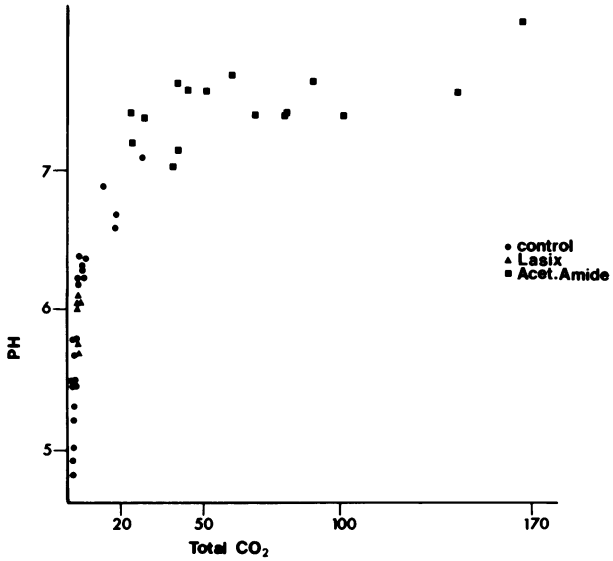

FIGURE 2 pH vs. U_{TC}. A plateau is reached at pH 7.80 in the acetazolamide-treated (Acet. Amide) samples. Total CO_2 is given in millimoles per liter.

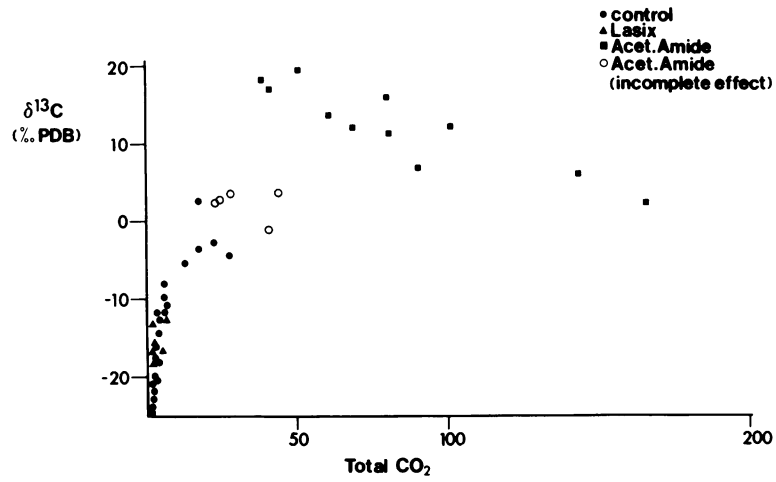

FIGURE 3 $\delta^{13}C_{TC}$ vs. U_{TC} in all urine samples (see Table III). Total CO_2 is given in millimoles per liter.

Fig. 6 demonstrates the linear correlation (Eq. 9) between $\delta^{13}C_{TC}$ and HCO_3^-/TC:

$$\delta^{13}C_{TC} = 25 \cdot (HCO_3^-/TC) - 25.2 \quad (r = 0.93). \quad (9)$$

When $HCO_3^-/TC = 0$, $\delta^{13}C_{TC} = -25.2‰$, which is similar to $\delta^{13}C$ of respiratory CO_2 ($-24.3 \pm 1.1‰$; Table II).

Analyses of respiratory CO_2 and blood TC are reported in Table II. The average blood TC has $\delta^{13}C = -20.6 \pm 0.7‰$, whereas the average $\delta^{13}C$ of respiratory CO_2 is $-24.3 \pm 1.1‰$. Very similar fractionation levels of $-19‰$ and $-23‰$ in B_{TC} and metabolic CO_2, respectively, have been reported previously (42). Since most of the B_{TC} consists of HCO_3^-, these results are consistent with the experimental data that demonstrate depletion in ^{13}C in dissolved CO_2 with respect to coexisting HCO_3^- (38, 48).

In the discussion that follows, we deal with isotopic fractionation between arterial B_{TC} and U_{TC}. But since $\delta^{13}C$ of arterial B_{TC} is not available, we estimate it from $\delta^{13}C$ of respiratory CO_2 and $\delta^{13}C_{TC}$ of venous blood, assuming that 10% of venous TC is expired when blood passes from the lungs. We then derive an estimate using similar mass balance consideration as in Eq. 5:

$$\delta^{13}C_{AB} = [\delta^{13}C_{VB} - 0.1 \cdot \delta^{13}C_{RE}]/0.9 = -20.1‰, \quad (10)$$

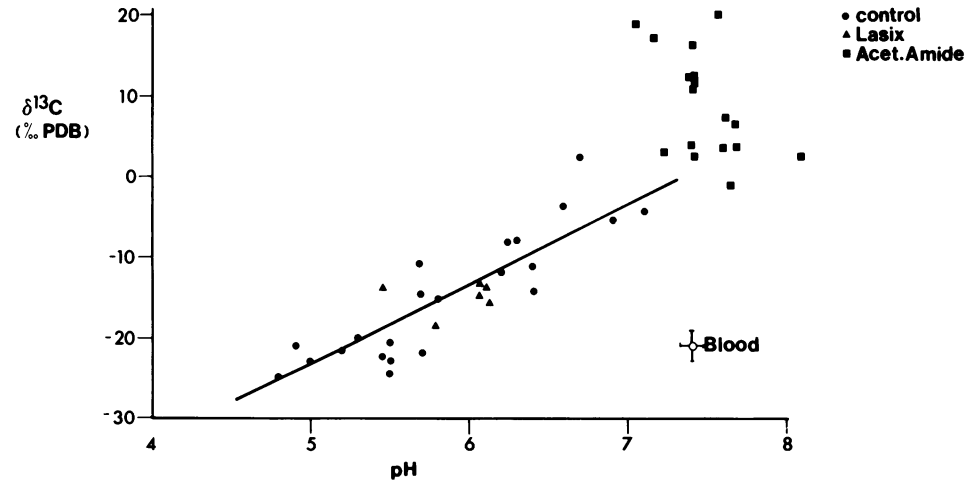

FIGURE 4 $\delta^{13}C_{TC}$ vs. pH. Note the linear correlation ($r = 0.90$) in normal samples and lack of correspondence in the case of acetazolamide samples.

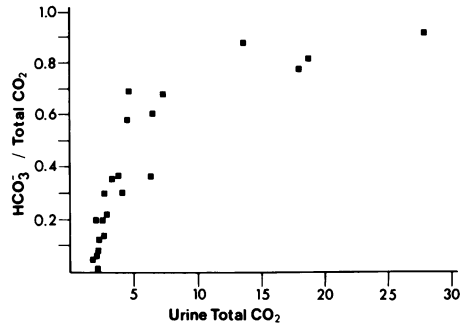

FIGURE 5 The HCO_3^- fraction of TC against TC (r = 0.97). Urine total CO_2 is given in millimoles per liter.

where $\delta^{13}C_{AB}$, $\delta^{13}C_{VB}$, $\delta^{13}C_{RE}$ refer to venous blood, arterial blood, and respiratory CO_2, respectively.

If we take 1.5/25 for the molar ratios of CO_2 and HCO_3^- of arterial blood, we can use Eq. 5 to calculate $\delta^{13}C$ of dissolved CO_2 gas in arterial blood. The calculated $\delta^{13}C$ (−25.8‰) is fairly similar to $\delta^{13}C$ of respiratory CO_2 (−24.3±1.1‰) (Table II).

From Table III and Fig. 3 it is evident that very significant ^{13}C enrichment in U_{TC} with respect to arterial B_{TC} (−20.1‰) occurs in the HCO_3^- reabsorption process (up to 20‰ in the normal samples and up to 40‰ in the acetazolamide-treated samples).

DISCUSSION

The changes in urine $\delta^{13}C$ and U_{TC} seem to fall into two categories (Fig. 3): The first one includes normal samples with high U_{TC} and acetazolamide-treated samples. These alkaline samples (Fig. 2) are highly enriched in ^{13}C with respect to arterial blood, and, presumably, with respect to glomerular filtrate. The second category includes normal samples with decreasing U_{TC} and furosemide-treated samples (Fig. 3). Here, $^{13}C/^{12}C$ decreases as reabsorption proceeds and seems to approach $\delta^{13}C$ of −25.2‰ (Figs. 3 and 6; Eq. 9). The question is, therefore, what are the dominant factors controlling urinary $\delta^{13}C$ in the high TC and alkaline pH range (first category) and in the low TC range and low urinary pH (secondary category).

Isotopic distillation through HCO_3^- − CO_2 chemical reaction. The process of ^{13}C enrichment in the first category indicates that carbon isotopes are removed preferentially from the tubular fluid and the reabsorbed HCO_3^- is depleted in ^{13}C while the remaining HCO_3^- is enriched. This preferential removal can be readily explained by a simple isotope distillation process, if reabsorption requires the transition of HCO_3^- to CO_2. In this chemical reaction, CO_2 has a lower $\delta^{13}C$, and if it is removed from the tubular fluid without requilibration, the remaining fraction of tubular TC becomes enriched in ^{13}C. Whether this is the case can be tested quantitatively by using Eq. 6.

If we assume in all cases of acetazolamide treatments a daily filtered load of total CO_2 of 5,000 mmol, then 150 and 50 mmol/liter are ~3 and 1% of the total. We can apply Eq. 6 to calculate expected $\delta^{13}C$ if we express these in fractions rather than permillages. In these cases of U_{TC} of 150 mmol/liter (Fig. 3), observed $\delta^{13}C$ is ~2‰. We calculate now the expected $\delta^{13}C$ if isotopic distillation took place.

Expected $\delta^{13}C = (1{,}000 + \delta^{13}C_{AB})\, f^{1-\alpha} - 1{,}000 = 0.7‰$, where $\delta^{13}C$ of arterial blood ($\delta^{13}C_{AB}$) is −20.1‰, f = 0.03 (3%), and α = 1.006. In the same way, we calculated expected $\delta^{13}C$ for the case of U_{TC} = 50 mmol/liter (f = 0.01) as 7.4‰. We compared this with an observed value of ~12‰. Recalling that fairly crude assumptions have been made with regard to daily filtered load, the results were rather encouraging, and seemed to support CO_2 reabsorption and not direct HCO_3^- reabsorption at least in the case of acetazolamide treatment.

Effective carbonic anhydrase inhibition resulted in greater $\delta^{13}C$ than in normal samples at the same U_{TC} (Fig. 3). This indicated that in the presence of carbonic anhydrase, CO_2 and HCO_3^- exchange carbon isotopes vary rapidly, and that isotopic equilibrium is maintained. Clearly, isotopic distillation can take place only if the separated species do not exchange with each other. This latter condition may exist normally in the distal nephron and during complete carbonic anhydrase inhibition in the proximal nephron. Thus, our findings are consistent with the classical

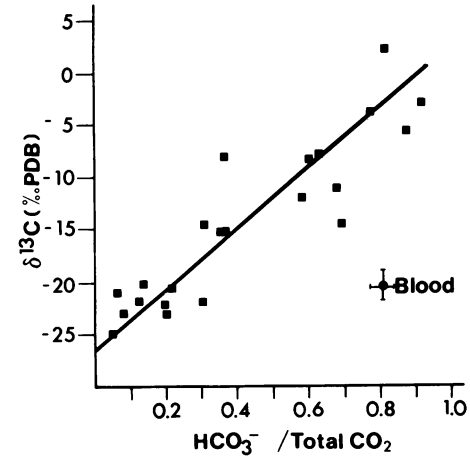

FIGURE 6 $\delta^{13}C_{TC}$ against HCO_3^- fraction of TC (r = 0.98). Note that the results of this figure do not correspond with Eq. 5, because both TC and pH vary with the abscissa, whereas TC is constant in Eq. 5.

TABLE I

Duplicates of Aging Samples and Injected Sample

Duplicate No.	Donor	Aging time	pH	U_{TC}	$\delta^{13}C$	$[CO_2]^*$	$[HCO_3^-]^*$
		h		*mmol/liter*	*‰ PDB*	*mmol/liter*	
1	A	0	6.20	4.25	−11.99	1.88	2.37
	A	1	6.20	3.87	−11.99	1.71	2.16
2	A	0	7.10	27.52	−4.73	2.50	25.02
	A	1	7.10	22.73	−2.85	2.07	20.66
3	C	0	5.20	2.03	−21.78	1.80	0.23
	C	3	5.20	1.56	−24.20	1.38	0.18
4	D	0	5.50	1.84	−23.00	1.47	0.37
	D	1.5	5.50	2.22	−22.96	1.77	0.45
5	B	0	6.90	13.35	−5.58	1.83	11.52
	B	1	6.90	13.30	−5.39	1.82	11.48
6	E‡	0	5.00	1.98	−22.94	1.84	0.15
	E‡	4	5.00	1.79	−24.08	1.66	0.13
7	F	0	6.70	18.49	2.31	3.71	14.78
	F	3	6.70	18.21	2.53	3.66	14.55
8	C	0	5.80	3.54	−15.16	2.36	1.18
	C	1	5.80	3.11	−14.54	2.07	1.04
9	A	0	5.70	3.87	−14.53	2.77	1.10
	A	7	5.70	3.35	−13.38	1.87	1.48
10	A§	—	6.30	6.13	−8.05	2.37	3.76
	A	—	6.30	6.10	−8.00	2.36	3.74

* Values calculated from Appendix I.

‡ Morning samples.

§ Sample forcefully injected by syringe.

model of bicarbonate reabsorption through CO_2 generation and opposed to direct HCO_3^- transfer as suggested by Brodsky and Schilb (3) and Maren (4, 5).

TABLE II

$\delta^{13}C$ of Venous B_{TC} and Respiratory CO_2

Donor	$\delta^{13}C$	
	B_{TC}	Respiratory CO_2
	(‰ PDB)	
A	−21.64	−24.45
B	−20.06	−25.04
B*	−20.34	—
C	−20.54	−25.87
D	—	−22.93
E	—	−23.83
F	—	−23.56
±SD	−20.6±0.7	−24.3±1.1

* Donor received acetazolamide.

The case of carbonic anhydrase inhibition calls for special attention. High P_{CO_2} values have been measured during acetazolamide treatment, as well as during bicarbonate loading (2, 23, 25). The cause of the high P_{CO_2}, however, is controversial. While Maren (27) and Arruda et al. (25, 26) attribute CO_2 formation from HCO_3^- to ampholyte effect, Stinebaugh et al. (23, 24) claim that this effect alone cannot account for the phenomenon. Recently, DuBose and associates (16) reviewed the theories for the high urinary P_{CO_2} and claim that delayed dehydration and the CO_2 counter-current system play an important role in determining urinary P_{CO_2}. Our data are insufficient to resolve this problem, but they seem to indicate that whatever the source for the high P_{CO_2}, the carbon isotopic enrichment results from CO_2 transfer and not from direct HCO_3^- reabsorption. The results shown in Fig. 4 might lend some support for CO_2 formation at high pH due to ampholyte effect. CO_2 reabsorption should result in increased $\delta^{13}C$,

TABLE III
Presentation of All the Analyses

Donor	Treatment	Time after administration	pH	U_{TC}	$\delta^{13}C$	$[CO_2]^*$	$[HCO_3^-]^*$
		h		*mmol/liter*	*‰ pDB*	*mmol/liter*	
A	—	—	6.30	6.13	−8.05	2.37	3.76
A	—	—	6.20	4.25	−11.99	1.88	2.37
A	—	—	7.10	27.52	−4.73	2.50	25.02
A	—	—	5.70	3.87	−14.53	2.77	1.10
A‡	—	—	5.80	3.58	−15.25	2.39	1.20
B	—	—	6.60	17.74	−3.76	4.26	13.47
B	—	—	6.90	13.35	−5.58	1.83	11.52
B	—	—	5.80	3.07	−15.30	2.04	1.02
B	—	—	6.38	7.08	−11.02	2.44	4.64
B‡	—	—	6.24	6.27	−8.26	2.62	3.63
C	—	—	5.20	2.03	−21.78	1.80	0.23
C	—	—	5.80	3.54	−15.16	2.36	1.18
C‡	—	—	5.45	2.31	−22.08	1.89	0.42
D	—	—	5.50	1.84	−23.00	1.47	0.37
D	—	—	6.40	4.39	−14.44	1.46	2.93
D‡	—	—	5.70	2.45	−21.75	1.75	0.70
E	—	—	4.80	1.56	−24.90	1.48	0.08
E	—	—	5.30	2.36	−20.06	2.04	00.32
E‡	—	—	5.00	1.98	−22.94	1.84	0.14
F	—	—	6.70	18.49	2.31	3.71	14.78
F	—	—	5.50	2.59	−20.47	2.07	0.52
F‡	—	—	4.90	1.84	−20.91	1.73	0.11
A	Acetazolamide (500 mg)	1.5	8.08	165.59	32.06	1.59	151.63
A	Acetazolamide (500 mg)	2	7.69	66.54	6.92	1.62	62.94
A§	Acetazolamide (500 mg)	3	7.59	44.10	3.47	1.35	38.57
B	Acetazolamide (500 mg)	1	7.17	40.95	16.92	3.18	37.44
B	Acetazolamide (500 mg)	1.5	7.42	80.31	11.30	3.57	74.63
B	Acetazolamide (500 mg)	2	7.58	50.12	19.45	1.57	47.42
C	Acetazolamide (500 mg)	2	7.05	38.55	18.35	3.79	33.81
D	Acetazolamide (500 mg)	1.5	7.66	91.01	6.73	2.37	86.15
D§	Acetazolamide (500 mg)	2.5	7.64	40.09	−1.30	1.09	37.96
E	Acetazolamide (500 mg)	1	7.41	79.39	15.95	3.65	74.53
E	Acetazolamide (500 mg)	1.5	7.41	68.56	12.04	3.15	64.37
E	Acetazolamide (500 mg)	2	7.42	100.64	12.02	4.52	94.53
F	Acetazolamide (500 mg)	1	7.58	142.74	6.07	4.47	135.05
F§	Acetazolamide (500 mg)	2.5	7.39	27.96	3.57	1.34	26.21
F§	Acetazolamide (500 mg)	3	7.42	23.07	2.36	1.03	21.68
F§	Acetazolamide (500 mg)	5	7.22	24.10	2.64	1.68	22.20
A	Furosemide (80 mg)	1.5	6.06	3.82	−14.75	2.00	1.82
A	Furosemide (80 mg)	2	6.10	1.79	−14.00	0.89	0.90
B	Furosemide (80 mg)	1.5	5.78	3.54	−18.32	2.39	1.15
B	Furosemide (80 mg)	2	5.48	3.02	−13.80	2.47	0.55
C	Furosemide (80 mg)	1.5	6.12	3.58	−16.32	1.75	1.83
C	Furosemide (80 mg)	2	6.12	3.87	−15.85	1.89	1.97

* Values calculated from Appendix I.

‡ Morning samples.

§ Ineffective acetazolamide treatment indicated by time after treatment, and a lower pH compared with an earlier sample.

whereas the pH should remain high and constant. This effect might explain the lack of correspondence between pH and $\delta^{13}C$ at high pH values.

Water abstraction might play a role in creating a high tubular HCO_3^- concentration, but it would not change the isotopic ratio. The change in ratio is consistent with the interpretation that reabsorption of HCO_3^- is through its transition to CO_2. Similarly, the possibility of HCO_3^- excretion that might enrich the total CO_2 with $\delta^{13}C$ is still consistent with our interpretation, since it fits the increase in $\delta^{13}C$ with the increase of HCO_3^- concentration.

CO_2 permeability. From the ongoing discussion it might appear that ^{13}C enrichment should proceed until completion of urine formation. However, our data show that in low U_{TC}, $\delta^{13}C$ is decreased (second category) (Fig. 3). To explain the results, it is necessary to consider variations in acidity. While pH is maintained above 7 in high U_{TC} (30 mmol/liter), it decreases rapidly when U_{TC} falls (Fig. 2). Consequently, the fraction of HCO_3^- in U_{TC} becomes smaller and smaller while the CO_2 fraction is increased (Fig. 2). Since only the HCO_3^- can become enriched in ^{13}C in isotopic distillation (Eq. 6), it is not surprising that $\delta^{13}C_{TC}$ which represents the contribution of both CO_2 and HCO_3^-, does not increase with the decline in pH and U_{TC}. There are linear relationships between $\delta^{13}C_{TC}$ and HCO_3^-/TC (Eq. 9); at extremely low pH (<4.5), when HCO_3^- is practically zero and the total CO_2 consists of CO_2 only, $\delta^{13}C$ approaches $-25.2‰$ (Fig. 6). This value seems to fit rather closely the value of blood CO_2 ($-25.76‰$), indicating that, as previously suggested (12, 16–19), CO_2 diffuses freely in both directions of the nephron membrane. As the blood reservoir of CO_2 is far greater than the amount in the distal nephron, the CO_2 passage renders $\delta^{13}C$ of tubular CO_2 constant ($\sim -25.5‰$).

We thus conclude that $\delta^{13}C$ of U_{TC} is the weighted average between HCO_3^-, which is enriched in ^{13}C according to Eq. 6, and CO_2 with ^{13}C of $-25.5‰$.

Quantitative model. Keeping in mind the issues of isotopic distillation in HCO_3^- and free passage of CO_2, we can now take a more quantitative approach to the process of $\delta^{13}C_{TC}$ changes during urine formation.

The expected $\delta^{13}C$ in U_{TC} for normal conditions can be calculated according to the following assumptions:

(*a*) In the transition of HCO_3^- to CO_2, the remaining HCO_3^- is enriched in ^{13}C through an isotopic distillation process (Eq. 6). (*b*) The tubular reabsorption of HCO_3^- is coupled with the above mentioned reaction. (*c*) CO_2 diffuses freely across the cell membrane and $\delta^{13}C$ of urinary CO_2 is constant at $-25.5‰$. (*d*) Luminal carbonic anhydrase in the proximal tubule abolishes isotopic distillation because of rapid exchange between HCO_3^- and CO_2, and distillation takes place only in the distal nephron. Thus, only 15% of the total reabsorbed HCO_3^- is subjected to isotopic distillation. (*e*) The remaining urinary HCO_3^- fraction from the HCO_3^- that enters the distal nephron (DN), which is the only part that is affected by isotopic distillation, is estimated as follows:

$$f = \frac{\text{Urinary } HCO_3^-}{HCO_3^- \text{ that enters DN}} = \frac{U_{TC} \cdot \dot{V} \cdot X}{0.15 \cdot (HCO_3^- \text{ filtered load})}, \quad (11)$$

where $\dot{V}$ is urine flow rate (1 ml/min), HCO_3^- is filtered load (3.25 mmol/min), and 0.15 is the fraction of HCO_3^- filtered load that enters the distal nephron (the rest is reabsorbed in the proximal nephron).

$X = HCO_3^-/TC$ and is calculated by Eq. 8. Model values of $\delta^{13}C$ are calculated by the following equation:

$$\delta^{13}C_{TC} = \underbrace{X\,(980.2\,f^{1-1.006} - 1{,}000)}_{HCO_3^- \text{ part}} + \underbrace{(1 - X)\cdot(-25.5)}_{CO_2 \text{ part}}. \quad (12)$$

Expected $\delta^{13}C$ values are plotted in Fig. 7 along with the actual data. Despite the simplicity of the model, it is capable of predicting the major trends of changes in isotopic ratio during urine formation ($r = 0.96$). In the upper part of Fig. 7, $\delta^{13}C$ of the remaining HCO_3^- is plotted. Despite its high $\delta^{13}C$ at low U_{TC}, the HCO_3^- contribution to the total CO_2 composition becomes smaller and smaller with the reduction of HCO_3^- because of decreasing pH.

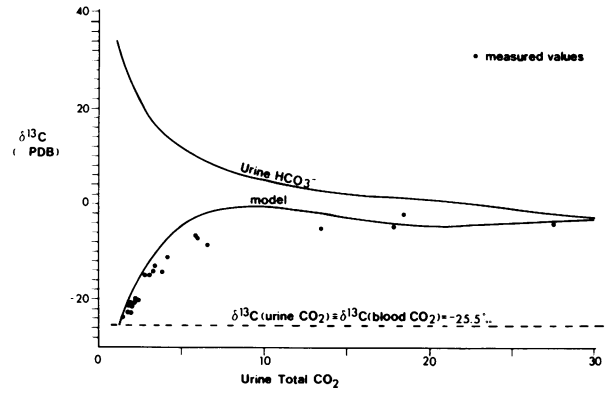

FIGURE 7 A mathematical model for $\delta^{13}C$ of TC in normal urine samples and the actual data (from Table III) ($r = 0.96$). The upper plot is calculated $\delta^{13}C$ of HCO_3^- (increased with the reabsorption). The lower plot is the weighted $\delta^{13}C$ of TC due to contribution of HCO_3^- (as plotted) and CO_2 with $\delta^{13}C$ of $-25.5‰$. Urine total CO_2 is given in millimoles per liter.

The model $\delta^{13}C$ is somewhat higher than the observed values. This greater enrichment can be explained by certain isotopic exchanges between HCO_3^- and CO_2 in the distal tubule which are not taken into account by the model.

Kinetic effects. Finally, it is necessary to consider kinetic effects on isotopic fractionation. It has been demonstrated that where a nonreversible reaction occurs, the heavier isotopes are concentrated in the remaining reactant. Thus, it might be proposed that ^{13}C enrichment in U_{TC} is the result of kinetic fractionation that occurs during direct HCO_3^- transfer through the luminal membrane. This mechanism is unlikely, for it cannot explain the higher ^{13}C enrichment that occurs during acetazolamide treatment. In turn, $\delta^{13}C$ of acetazolamide-treated samples seems to fit isotope distillation with fractionation factor (α) of magnitude similar to that derived experimentally for the system $CO_2 - HCO_3^-$ (38). In addition, high CO_2 permeability is indicated by decreasing $\delta^{13}C$ of low pH samples.

The schema in Fig. 8 summarizes the main ideas discussed above. In normal physiological conditions there is no change in $\delta^{13}C$ of luminal TC relative to B_{TC} in the proximal nephron. This is because of rapid exchange between HCO_3^- and CO_2 in the presence of carbonic anhydrase. ^{13}C enrichment is possible only in the distal nephron, where HCO_3^- and CO_2 coexist, although not in isotopic equilibrium. In this case, HCO_3^- (but not CO_2) becomes enriched in ^{13}C as reabsorption proceeds. The decrease in $\delta^{13}C$ at low

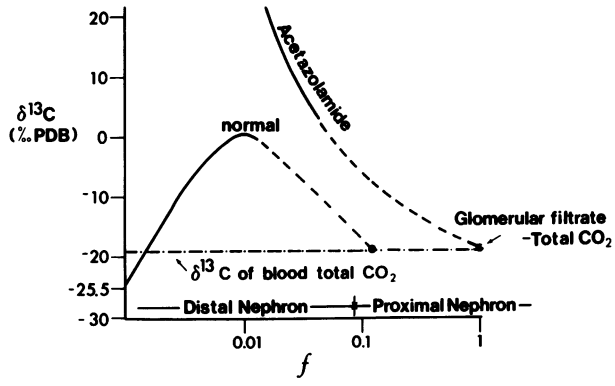

FIGURE 8 A qualitative model of $\delta^{13}C$ changes along different nephron segments as a function of fractional excretion (f) of the TC filtered load (f in logarithmic scale). In the case of acetazolamide, $\delta^{13}C$ starts to increase in the proximal tubule (because of its luminal CA inhibition). (——), measured values; (- - -), predicted values; (-·-·-), $\delta^{13}C$ of arterial B_{TC} (−20.1‰). Note that direct absorption of HCO_3^- would not change the urine $\delta^{13}C$ with respect to blood $\delta^{13}C$ and that it is expected to remain constant (−20.1‰) throughout urine formation.

f (fractional excretion of TC) is explained by the small proportion of bicarbonate in U_{TC} due to low pH. The situation is quite different during acetazolamide treatment. The inhibition of carbonic anhydrase in the proximal nephron results in the development of a disequilibrium isotopic ratio between HCO_3^- and CO_2 and significant ^{13}C enrichment of the bicarbonate. The small proportion of CO_2 in these samples cannot affect this enrichment.

In conclusion, our data substantiate three important hypotheses: (*a*) Bicarbonate is reabsorbed by conversion to CO_2, (*b*) CO_2 diffuses freely in both directions through the nephron membrane, and (*c*) rapid isotope exchange occurs in the proximal nephron because of the presence of luminal carbonic anhydrase.

APPENDIX I

Dissolved CO_2 equilibrium. In deriving concentrations of (CO_2) and (HCO_3^-), we have used equations that are somewhat different from those usually in use in connection with studies of urinary and tubular fluids. We think that in this more rigorous way we derive more realistic values. The relevant equilibrium equations and constants from Stumm and Morgan (49) for 37°C and osmolality range of urine are listed below.

$$CO_2 + H_2O \rightleftarrows H_2CO_3, \quad K = \frac{[H_2CO_3]}{[H_2O][CO_2]}, \tag{13}$$

but since $[H_2O] \approx 1$, $K = [H_2CO_3]/[CO_2]$. The constant K is on the order of 1/650.

$$H_2CO_3 \rightleftarrows H^+ + HCO_3^-, \quad K_1 = \frac{a_H\,[HCO_3^-]}{[H_2CO_3]}, \tag{14}$$

where $a_H = 10^{-pH}$.

The sum $[CO_2^*] = [CO_2] + [H_2CO_3]$ is more readily measured than either $[CO_2]$ or $[H_2CO_3]$, but $[CO_2]$ is fairly close to $[CO_2^*]$. For this reason another, constant (K_1^1) is defined:

$$K_1^1 = \frac{a_H\,[HCO_3^-]}{[CO_2^*]} = 10^{-6.1}\ (37°C), \tag{15}$$

but $[CO_2^*] = [H_2CO_3]\cdot(K + 1)/K$, and

$$K_1^1 = \frac{a_H\,[HCO_3^-]}{[H_2CO_3]\cdot\dfrac{(K+1)}{K}} = \frac{K_1 \cdot K}{K+1}.$$

$$HCO_3^- \rightleftarrows H^+ + CO_3^{2-}, \quad K_2 = \frac{a_H \cdot [CO_3^{2-}]}{[HCO_3^-]} = 10^{-9.2}\ (37°C). \tag{16}$$

It is evident from Eq. 16 that at pH below 7.2 (the range of all our normal samples), concentration of CO_3^{2-} is negligible. This, however, is not the case in acetazolamide treatment at high pH values.

APPENDIX II

Isotopic distillation. All isotope reactions that proceed so that the products are isolated immediately from the reac-

tants will progressively change the isotopic compositions of the reactants (40, 41).

Below, we derive the Rayleigh distillation equation, which is useful for isotopic distillation processes.

We let A and B designate the amount of the species containing the abundant and rare isotopes, respectively ($H^{12}CO_3^-$ and $H^{13}CO_3^-$, for example). The reaction rate of each species is proportional to its abundance, and the rate of reaction of each species is different ($H^{12}CO_3^-$ has a slower rate).

$$dA = -K_A \cdot A, \tag{17}$$

$$dB = -K_B \cdot B, \tag{18}$$

$$\alpha = K_A/K_B, \tag{19}$$

$$\frac{dA}{dB} = \alpha \frac{A}{B}. \tag{20}$$

Rewriting in integral form, we get

$$\int_{B_0}^{B} \frac{dB}{B} = \frac{1}{\alpha} \int_{A_0}^{A} \frac{dA}{A}, \tag{21}$$

where A_0 and B_0 are the initial amounts, and A and B the final amounts of reactants. By integration, we obtain either

$$\ln (B/B_0) = (1/\alpha) \ln (A/A_0) \tag{22}$$

or

$$(B/B_0) = (A/A_0)^{1/\alpha}. \tag{23}$$

Dividing both sides by A/A_0, we obtain

$$(B/A)/(B_0/A_0) = B/A/B_0/A_0 = (A/A_0)^{1/\alpha - 1}. \tag{24}$$

Since B is only a trace of $A + B$, the fraction (f) of the remaining reactants is equal to A/A_0. In addition, α is only slightly different from 1, and $1 - \alpha$ is a close approximation of $1/\alpha - 1$. Thus,

$$(B/A)/(B_0/A_0) = f^{1-\alpha}. \tag{25}$$

We consider the reaction $HCO_3^- + H^+ \rightarrow CO_2 + H_2O$, Eq. 25 takes the following form:

$$\frac{(^{13}C/^{12}C) \text{ final}}{(^{13}C/^{12}C) \text{ initial}} = f^{1-\alpha}. \tag{26}$$

We now change from ratios to the δ notation (Eq. 3), as follows:

$$(^{13}C/^{12}C) \text{ final} = [10^{-3} (\delta^{13}C \text{ final}) + 1] (^{13}C/^{12}C) \text{ PDB}. \tag{27}$$

$(^{13}C/^{12}C)$ initial is expressed in a similar way:

$$\frac{10^{-3} (^{13}C \text{ final}) - 1}{10^{-3} (^{13}C \text{ initial}) - 1} = f^{1-\alpha}. \tag{28}$$

By arrangement, we obtain Eq. 6:

$$\delta^{13}C \text{ final} = (1{,}000 + \delta^{13}C \text{ initial}) \cdot f^{1-\alpha} - 1{,}000.$$

The CO_2 produced in the reaction is 6‰ depleted in $\delta^{13}C$ with respect to HCO_3 (at 37°C). Thus, the fractionation factor α equals 1.006.

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