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 CO2 reabsorption and (b) direct reabsorption of HCO3−. In an attempt to differentiate between the two mechanisms, the present study utilized the natural abundance of stable carbon isotopes (13C, 12C) in the urinary total CO2. This novel methodology used mass spectrometric analysis of 13C/12C ratios in urinary total CO2 under normal conditions and during acetazolamide treatment. Blood and respiratory CO2 were analyzed to yield reference values.

The results demonstrate that alkaline urine is preferentially enriched with 13C relative to the blood. It is suggested that this fractionation results from reaction out of isotopic equilibrium in which HCO3− converts to CO2 during the reabsorption process in the distal nephron. The presence of carbonic anhydrase in the proximal nephron results in rapid isotopic exchange between CO2 and HCO3− and keeps them in isotopic equilibrium. The ratio of urinary 13C/12C 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 HCO3− generates the CO2 (ampholyte effect), the isotope fractionation indicates that CO2 rather than HCO3− is reabsorbed. In contrast, at low urinary pH and total CO2 values, the carbon isotope composition approaches that of blood CO2. This indicates rapid CO2 exchange between urine and blood, through luminal membrane highly permeable to CO2. These results could be anticipated by a mathematical model constructed to plot 13C concentration of urinary total CO2.

It is concluded that the mechanism of HCO3− reclamation in man (and, by inference, in other mammals as well) works by conversion of HCO3− to CO2 and reabsorption of CO2.

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 HCO3− 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 CO2 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 HCO3− 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...
of carbonic acid above its equilibrium concentration, thus favoring H+ ion secretion (10). Nevertheless, the recent demonstration by several laboratories, that CO2 tension in the proximal (11–14) as well as in the distal tubule exceeds the systemic arterial PCO2, 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 PCO2 raised the question whether there was a limitation in transepithelial CO2 diffusion. The presently available experimental data are conflicting. Results compatible with very high permeability of tubular epithelium to CO2 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 CO2 might exist.

Finally, another disputed issue is the interpretation of increased urine minus blood PCO2 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 PCO2 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 PCO2 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 CO2 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 HCO3− reabsorption has been reached. In the hope of clearing up this problem, we introduced a new method for studying the renal CO2 system. The method is based on measurements of stable carbon isotopes (13C/12C) ratios in total CO2 (TC1 = CO2−3 + HCO3− + H2CO3 + CO32−) 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 13C/12C 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 CO2 and HCO3−. This fractionation involves transfer of 12C and 13C from CO2 to HCO3−, as expressed in the following isotopic reaction:

\[ {^{13}}\text{CO}_2 + {^{12}}\text{H}^{+} \rightarrow {^{13}}\text{HCO}_3^{-} + {^{12}}\text{CO}_2 \]  

(1)

In general, HCO3− is more enriched in 13C than CO2. This enrichment, however, is rather small; it can be expressed as follows:

\[ \alpha_{(\text{HCO}_3^-/\text{CO}_2)} = \frac{(^{13}C/^{12}C) \text{HCO}_3^-}{(^{13}C/^{12}C) \text{CO}_2}, \]  

(2)

where \( \alpha_{(\text{HCO}_3^-/\text{CO}_2)} \) is the 13C fractionation factor between HCO3− and CO2. The degree of fractionation depends both on whether CO2 and HCO3− 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 CO2 to HCO3− 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 PDB2 standard (δ13C) (39), by the following equation:

1. **Abbreviations used in this paper:** \( \alpha \), fractionation factor; B(TC), blood TC; \( \delta \), deviation in isotopic values; \( f \), fractional excretion of TC; TC, total CO2; U(TC), urinary TC.

2. PDB is a CaCO3 standard prepared from a fossil belamnite.
\[ \delta^{13}C \ (\text{‰}) = \left( \frac{^{13}C/^{12}C_{\text{sample}}}{^{13}C/^{12}C_{\text{PDB}}} - 1 \right) \times 1,000. \]  

Thus, in \( \delta \) 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\(_2\)CO\(_3\) and CO\(_3^\)). 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\(_2\) (\( \delta^{13}C_{\text{TC}} \)):

\[ \delta^{13}C_{\text{TC}} = \delta^{13}C_{\text{CO}_2} + 6 \approx \delta^{13}C_{\text{HCO}_3} \ (\text{at } 37^\circ\text{C}), \]  

\[ \delta^{13}C_{\text{TC}} = \delta^{13}C_{\text{CO}_2} \cdot (\text{CO}_2/\text{TC}) + \delta^{13}C_{\text{HCO}_3} \cdot (\text{HCO}_3/\text{TC}). \]

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\(_2\)CO\(_3\) 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\(_2\)\(^{13}C\) and CO\(_2\) is fairly similar to HCO\(_3^\) and CO\(_2\) fractionation. The fractionation factor is \( \alpha_{\text{CO}_2 - \text{CO}_2} = 1.0064 \) (39). The isotopic composition of H\(_2\)CO\(_3\) is unknown, but this species is also very rare (Appendix I) and does not pose a problem in estimating isotopic compositions.

\textit{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:

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

where \( \alpha \) 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 reequilibration, then \( U_{\text{TC}} \), 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 \( B_{\text{TC}} \) 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 (45–45). 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). \( U_{\text{TC}} \) was determined and \( \delta^{13}C \) of the collected CO\(_2\) was measured on a double inlet double collector...
Effects of sample aging and CO₂ loss during exposure of the urinary stream to air were tested in a few duplicate samples. In addition, δ¹³C was determined in expiratory CO₂ (metabolic CO₂) of the group and in four samples of total CO₂ of venous blood.

RESULTS

The results are shown in Figs. 2–4. Duplicate analyses demonstrate that the effects of CO₂ escape from sample aging are relatively small if the sample is analyzed within 2 h of voiding (up to 17% of CO₂ loss and 1.88% PDB changes in δ¹³C). The rate of flow during voiding does not appear to be an important factor in determining δ¹³C of Uₘₐₜ (Table I).

Fig. 2 demonstrates the chemistry of the urinary CO₂ system. As expected, pH increases with Uₘₐₜ from pH 4.9 in normal samples, up to 8.08 after acetazolamide treatment. pH is approximately constant (~7.8) from Uₘₐₜ of 50 mmol/liter. All morning urine samples are acidic with low Uₘₐₜ. Furosemide treatment does not cause unusually high pH or Uₘₐₜ. It is thus clear that the test group consists of healthy individuals with normal ability to acidify urine.

δ¹³C increases with Uₘₐₜ in normal samples (Fig. 3) and reaches maximum values in acetazolamide treatment, but in the latter case a decrease in δ¹³C is observed at the highest Uₘₐₜ values. Intermediate δ¹³C 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 δ¹³C are shown in Fig. 4. In normal samples δ¹³C increases linearly with pH:

\[
δ^{13}C_{i} = 10.828 \times pH - 78.528 \quad (r = 0.90) \quad (7)
\]

The linear relations are expected from the similar trends of pH and δ¹³C versus Uₘₐₜ (Figs. 2, 3). Poor correlation is observed in acetazolamide treated samples, where δ¹³C increases with no appreciable change of pH.

Fig. 5 demonstrates the relationship between HCO₃⁻/TC and TC in normal urines, which is expressed in the following equation:

\[
\text{HCO}_3^-/\text{TC} = 0.0000823(\text{TC})^4 + 0.00071486(\text{TC})^3 - 0.0218231(\text{TC})^2 + 0.26300894(\text{TC}) - 0.308861849 \quad (r = 0.97) \quad (8)
\]
Fig. 6 demonstrates the linear correlation (Eq. 9) between $\delta^{13}$C and HCO$_3^-$/TC:

$$\delta^{13}$C = 25 \cdot (\text{HCO}_3^-/\text{TC}) - 25.2 \quad (r = 0.93). \quad (9)$$

When HCO$_3^-$/TC = 0, $\delta^{13}$C$_{TC} = -25.2\%$, which is similar to $\delta^{13}$C of respiratory CO$_2$ (-24.3±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±0.7%, whereas the average $\delta^{13}$C of respiratory CO$_2$ is -24.3±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:

$$\delta^{13}$C$_{AB} = [\delta^{13}$C$_{vb} - 0.1 \cdot \delta^{13}$C$_{RE}] / 0.9 = -20.1\%, \quad (10)$$

Fig. 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.
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$_2$ ($-24.3\%\pm1.1\%$) (Table II).

From Table III and Fig. 3 it is evident that very significant $\delta^{13}$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 equilibration, 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 $\sim 3$ and 1% of the total. We can apply Eq. 6 to calculate expected $\delta^{13}$C if we express these in fractions rather than permilages. In these cases of U$_{TC}$ of 150 mmol/liter (Fig. 3), observed $\delta^{13}$C is $\sim 2\%$. 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 $\alpha = 1.00$. 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 $\sim 12\%$. 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.
model of bicarbonate reabsorption through CO₂ generation and opposed to direct HCO₃⁻ transfer as suggested by Brodsky and Schilb (3) and Maren (4, 5).

**Table I**

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<th>δ¹³C (‰ PDB)</th>
<th>[CO₂]⁺ (mmol/liter)</th>
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* Values calculated from Appendix I.
† Morning samples.
‡ Sample forcefully injected by syringe.

The case of carbonic anhydrase inhibition calls for special attention. High PCO₂ values have been measured during acetazolamide treatment, as well as during bicarbonate loading (2, 23, 25). The cause of the high PCO₂, however, is controversial. While Maren (27) and Arruda et al. (25, 26) attribute CO₂ formation from HCO₃⁻ to ampholyte effect, Stonebaugh 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 PCO₂ and claim that delayed dehydration and the CO₂ counter-current system play an important role in determining urinary PCO₂. Our data are insufficient to resolve this problem, but they seem to indicate that whatever the source for the high PCO₂, the carbon isotopic enrichment results from CO₂ transfer and not from direct HCO₃⁻ reabsorption. The results shown in Fig. 4 might lend some support for CO₂ formation at high pH due to ampholyte effect. CO₂ reabsorption should result in increased δ¹³C.

**Table II**

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* Donor received acetazolamide.

Stable Carbon Isotope in Studying Renal CO₂ Transport 2131
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* 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 δ\(^{13}\)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 δ\(^{13}\)C is still consistent with our interpretation, since it fits the increase in δ\(^{13}\)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\(_{TC}\), δ\(^{13}\)C is decreased (second category) (Fig. 3). To explain the results, it is necessary to consider variations in acidity. When 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 δ\(^{13}\)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 δ\(^{13}\)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, δ\(^{13}\)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 δ\(^{13}\)C of tubular CO\(_2\) constant (−25.5\%).

We thus conclude that δ\(^{13}\)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 δ\(^{13}\)C\(_{TC}\) changes during urine formation.

The expected δ\(^{13}\)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 δ\(^{13}\)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:

\[
\begin{align*}
\delta^{13}C_{U_{TC}} &= \frac{\text{Urinary HCO}_3^-}{\text{HCO}_3^- \text{ that enters DN}} \\
&= \frac{U_{TC} \cdot V \cdot X}{0.15 \cdot (\text{HCO}_3^- \text{ filtered load})} \\
&= \left(\begin{array}{c}
\frac{X (980.2 \cdot f_{-1.006} - 1.000)}{0.15} \\
+ (1 - X) \cdot (-25.5)
\end{array}\right)
\end{align*}
\]

where \(V\) 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).

\(X = \text{HCO}_3^- / \text{TC}\) and is calculated by Eq. 8. Model values of δ\(^{13}\)C are calculated by the following equation:

\[
\begin{align*}
\delta^{13}C_{TC} &= X (980.2 \cdot f_{-1.006} - 1.000) \\
&+ (1 - X) \cdot (-25.5)
\end{align*}
\]

Expected δ\(^{13}\)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, δ\(^{13}\)C of the remaining HCO\(_3\)- is plotted. Despite its high δ\(^{13}\)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 δ\(^{13}\)C of TC in normal urine samples and the actual data (from Table III) (\(r = 0.96\)). The upper plot is calculated δ\(^{13}\)C of HCO\(_3\)- (increased with the reabsorption). The lower plot is the weighted δ\(^{13}\)C of TC due to contribution of HCO\(_3\)- (as plotted) and CO\(_2\) with δ\(^{13}\)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 isoate distillation with fractionation factor ($\alpha$) 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 $f$ (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 $37^\circ$C and osmolality range of urine are listed below.

\[
\text{CO}_2 + \text{H}_2\text{O} \rightleftharpoons \text{H}_2\text{CO}_3, \quad K = \frac{[\text{H}_2\text{CO}_3]}{[\text{H}_2\text{O}][\text{CO}_2]}, \quad (13)
\]

but since $[\text{H}_2\text{O}] \approx 1$, $K = [\text{H}_2\text{CO}_3]/[\text{CO}_2]$. The constant $K$ is on the order of 1/650.

\[
\text{H}_2\text{CO}_3 \rightleftharpoons \text{H}^+ + \text{HCO}_3^-, \quad K_1 = \frac{a_H[HCO_3^-]}{[H_2CO_3]}, \quad (14)
\]

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

The sum $[\text{CO}_2^+] = [\text{CO}_2] + [\text{H}_2\text{CO}_3]$ is more readily measured than either $[\text{CO}_2]$ or $[\text{H}_2\text{CO}_3]$, but $[\text{CO}_2]$ is fairly close to $[\text{CO}_2^+]$. For this reason another, constant ($K_2$) is defined:

\[
K_i = \frac{a_H[HCO_3^-]}{[CO_2]}, \quad (15)
\]

but $[\text{CO}_2^+] = [\text{H}_2\text{CO}_3] \cdot (K + 1)/K$, and

\[
K_2 = \frac{a_H[HCO_3^-]}{[H_2CO_3]} \cdot \frac{(K + 1)}{K} = \frac{K_1 \cdot K}{K + 1}.
\]

\[
\text{HCO}_3^- \rightleftharpoons \text{H}^+ + \text{CO}_2^-, \quad K_2 = \frac{a_H[\text{CO}_2^-]}{[\text{HCO}_3^-]} = 10^{-a_2}(37^\circ\text{C}). \quad (16)
\]

It is evident from Eq. 16 that at pH below 7.2 (the range of all our normal samples), concentration of CO$_2^-$ 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-
reagents 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 (\(^{12}\text{CO}_3^3\) and \(^{13}\text{CO}_3^3\), for example). The reaction rate of each species is proportional to its abundance, and the rate of reaction of each species is different (\(^{13}\text{CO}_3^3\) has a slower rate).

\[
dA = -K_A \cdot A, \\
\frac{dA}{dB} = -K_A \cdot B, \\
\alpha = K_A / K_B, \\
\frac{dA}{dB} = \frac{A}{B}.
\]

Rewriting in integral form, we get

\[
\int_{B_0}^{B} \frac{dB}{B} = \frac{1}{\alpha} \int_{A_0}^{A} \frac{dA}{A}.
\]

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

\[
\ln \left( \frac{B}{B_0} \right) = \left( \frac{1}{\alpha} \right) \ln \left( \frac{A}{A_0} \right) \\
\text{or} \quad \frac{B}{B_0} = \left( \frac{A}{A_0} \right)^{1/\alpha}.
\]

Dividing both sides by \( A/A_0 \), we obtain

\[
\frac{(B/A)/(B_0/A_0)} = \frac{B/A}{B_0/A_0} = \left( \frac{A}{A_0} \right)^{1/\alpha-1}.
\]

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

\[
\frac{(B/A)/(B_0/A_0)} = f^{1-\alpha}.
\]

We consider the reaction \( \text{HCO}_3^- + H^+ \rightarrow \text{CO}_2 + \text{H}_2\text{O} \). Eq. 25 takes the following form:

\[
\frac{\left( ^{13}\text{C} / ^{12}\text{C} \right)_{\text{final}}}{\left( ^{13}\text{C} / ^{12}\text{C} \right)_{\text{initial}}} = f^{1-\alpha}.
\]

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

\[
\left( ^{13}\text{C} / ^{12}\text{C} \right)_{\text{final}} = 10^{-3} \left( \delta^{13}\text{C} \text{ final} \right) + 1 \left( ^{13}\text{C} / ^{12}\text{C} \right) \text{ PDB}.
\]

\[
\left( ^{13}\text{C} / ^{12}\text{C} \right)_{\text{initial}} \text{ is expressed in a similar way:} \\
\left( ^{13}\text{C} / ^{12}\text{C} \right)_{\text{initial}} = 10^{-3} \left( \delta^{13}\text{C} \text{ initial} \right) - 1.
\]

By arrangement, we obtain Eq. 6:

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

The \( \text{CO}_2 \) produced in the reaction is 6% depleted in \( \delta^{13}\text{C} \) with respect to \( \text{HCO}_3^- \) (at \( 37^\circ \text{C} \)). Thus, the fractionation factor \( \alpha \) equals 1.006.

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**REFERENCES**


**Stable Carbon Isotope in Studying Renal CO\(_3\) Transport**


