Tracer Disequilibrium in CO_2 Compartments During $NaH^{14}CO_3$ Infusion

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The failure of labeled CO_2 to equilibrate between extracellular and intracellular CO_2 compartments may influence the accuracy of substrate oxidation measurements during infusion of carbon-labeled tracers because it may generate errors in estimate of fixation of labeled CO_2 derived from control experiments in which labeled bicarbonate is infused. In this study, normal volunteers received a 14-hour overnight primed continuous infusion of $NaH^{14}CO_3$. Over the last 4 hours of the study, steady-state conditions were achieved in the specific activities (SAs) of expired $^{14}CO_2$ and plasma urea, which was used as a probe for hepatic intracellular CO_2 SA. Plasma urea SA was approximately 17% lower than expired CO_2 SA (46.4 ± 5.6 *v* 56.8 ± 3.9 disintegrations per minute · μmol^{-1}, $P < .02$). Fractional $^{14}CO_2$ recovery was 94.8% ± 0.8%; when corrected for failure to equilibrate with intracellular CO_2, fractional recovery was 89.5% ± 1.9%. These data indicate that compartmentalization of CO_2 may occur in humans. The duration of our experiments, required because of the long half-life of plasma urea, may have minimized the apparent magnitude of compartmentalization. Furthermore, it is possible that compartmentalization in extrahepatic tissues could be of either lesser or greater magnitude than that which we observed in liver. Whether this phenomenon contributes to incomplete recovery of $^{14}CO_2$ during $NaH^{14}CO_3$ infusion cannot be determined from our results. Additional studies using different experimental approaches will be required to better measure CO_2 compartmentalization.

THE OXIDATION OF VARIOUS substrates (eg, glucose, amino acids, free fatty acids) is often estimated isotopically in research studies by administering a tracer labeled with ^{14}C or ^{13}C and measuring labeled CO_2 in expired air.[1-4] It has been the practice of most investigators to apply a correction factor to the oxidation calculation to account for incomplete recovery of labeled CO_2. This correction is necessary because less than 100% of infused bicarbonate tracer is recovered from the breath of both animals[5,6] and humans.[4,7,8] In most studies, a correction factor of 0.81 is used based on 81% recovery of $^{14}CO_2$ in breath during $NaH^{14}CO_3$ infusion.[5-7] It is possible that infusion of labeled HCO_3 underestimates incomplete recovery of labeled CO_2 during infusion of labeled substrates, since in the latter instance labeled CO_2 originates from intracellular sites of oxidation, and in the former instance bicarbonate ions are not readily transported from the extracellular space into the intracellular space.[9,10] If the limitation on HCO_3^- transport could be measured, then a better estimate of true CO_2 fixation (ie, fixation of labeled CO_2 originating from the intracellular space) could be made.

Hetenyi et al[11] have proposed that measurement of plasma urea specific activity (SA) during $NaH^{14}CO_3$ infusion could serve as a probe for intracellular CO_2 SA, reporting a significantly lower ^{14}C SA in urea compared with that in expired CO_2 in normal volunteers. However, isotopic equilibrium was not achieved in that study because the infusion was only 90 minutes in duration and was not primed.[10] More recently, Fuller and Elia infused $NaH^{14}CO_3$ in humans for 36 hours and measured ^{14}C SA in breath and urinary urea over the last 12 hours.[12] The results indicated that urea SA was approximately 84% of expired CO_2 SA. However, samples throughout the last 12 hours were taken variously before and after meals and bouts of exercise, factors that may influence $^{14}CO_2$ recovery.[13] Therefore, the degree of CO_2 compartmentalization in humans after an overnight fast remains uncertain. The present study was undertaken to estimate this compartmentalization.

SUBJECTS AND METHODS

Protocol

After approval by the Mayo Institutional Review Board, 13 subjects (six men and seven women, aged 18 to 35 years) were recruited for study; they were each in good health, at a stable weight, and taking no medications. All subjects were admitted to the Clinical Research Center in the afternoon, where they consumed a standard (10 kcal/kg; 50% carbohydrate, 30% fat, 20% protein) meal at 5:30 PM. Subsequently, two catheters were placed in forearm veins (one in each arm) and kept patent with controlled (30 mL/h each) infusions of 0.45% NaCl. One catheter was used for tracer infusion and the other for blood sampling.

At 10:00 PM, a 14-hour primed continuous infusion of $NaH^{14}CO_3$ (57 mCi/mmol, RPI, Mount Prospect, IL) diluted in 2 mmol/L $NaH^{12}CO_3$ was begun. In six subjects, a priming dose of 150 μCi and an infusion rate of 0.42 $\mu Ci \cdot min^{-1}$ was used; this represents a priming dose:minute infusion rate (P:I) ratio of 357:1. Because steady-state urea SA was not achieved in these subjects (see the Results), another six subjects were studied using a priming dose of 230 μCi and an infusion rate of 0.32 $\mu Ci \cdot min^{-1}$ (P:I = 720:1). A seventh subject received only the 230-μCi priming dose of $NaH^{14}CO_3$. At 7:00 AM the next morning, each subject received an oral dose of ^3H_2O (20 μCi) and 3% NaBr (1 $mL \cdot kg^{-1}$; Sigma Chemical, St Louis, MO) for determination of total body and extracellular water content, respectively (TBW and ECW). All subjects were allowed to sleep between 11:00 PM and 6:00 AM.

Blood samples were obtained hourly for determination of ^{14}C urea SA, beginning with a sample before $NaH^{14}CO_3$ infusion to serve as a blank in the assay. Blood samples were also obtained before administration of ^3H_2O and NaBr and hourly for 4 hours thereafter for measurement of plasma ^3H_2O and Br^- concentrations. Three blood samples were obtained 10 minutes apart beginning at 9:00 AM for measurement of total blood CO_2 concen-

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tration. Finally, hourly breath samples were obtained for measurement of $^{14}CO_2$ excretion rate and SA beginning at 7:00 AM. A sample was also taken the previous night before $NaH^{14}CO_3$ infusion to serve as a blank.

Analytical Procedures

A procedure was developed for determination of plasma urea SA using high-performance liquid chromatography (HPLC). Two milliliters of plasma was passed over a cation-exchange resin (AG 50W-X8, Bio-Rad, Richmond, CA) in a column (Isolab, Akron, OH) with a bed volume of 1.5 mL. Columns were rinsed three times with 3 mL distilled H_2O and eluted with 2.0 mL 5% NH_4OH × 2. The eluate was collected and taken to dryness by lyophilization. The residue was resuspended in 240 μL of 98:2 acetonitrile:H_2O. Two hundred microliters of the sample was injected onto a 10-μm Beckman Ultrasil NH_2 HPLC column (Beckman Instruments, San Ramon, CA) eluted with 98:2 acetonitrile:H_2O at 1.0 mL/min, using UV detection at 214 nm. Urea eluted at approximately 9.5 minutes as a single peak (Fig 1). Samples were injected by a Waters Intelligent Sample Processor (WISP) autosampler (Waters, Milford, MA) at 60-minute intervals (because of late-eluting peaks), and data was collected on an IBM PS-2 computer using Spectra-Physics WINner 386 software (San Jose, CA). The ^{14}C urea peak was collected on an ISCO fraction collector (ISCO, Lincoln, NE) controlled by the data system. Purity of the urea peak was confirmed by isolating the urea fraction from plasma with ion-exchange chromatography as described above and adding urease to the sample. After a 30-minute incubation, samples were passed through an Amicon filter to remove the enzyme, lyophilized, and resuspended as described above for HPLC analysis. This procedure resulted in total elimination of the urea peak. The urea content of the sample was calculated using external standards (urea > 99% pure, Sigma Chemical) ranging from 1.0 to 8.0 μmol. The urea fraction was collected and allowed to dry before resuspension in 1 mL distilled H_2O and 7 mL liquid scintillation cocktail (Opti-fluor, Packard, Meriden, CT). Samples were counted by liquid scintillation spectrometry (Pharmacia LKB Nuclear, Gaithersburg, MD). The standard curve was linear up to 8 μmol, and the coefficient of variation (replicate analysis, n = 15) for urea SA measurement was 2.6%.

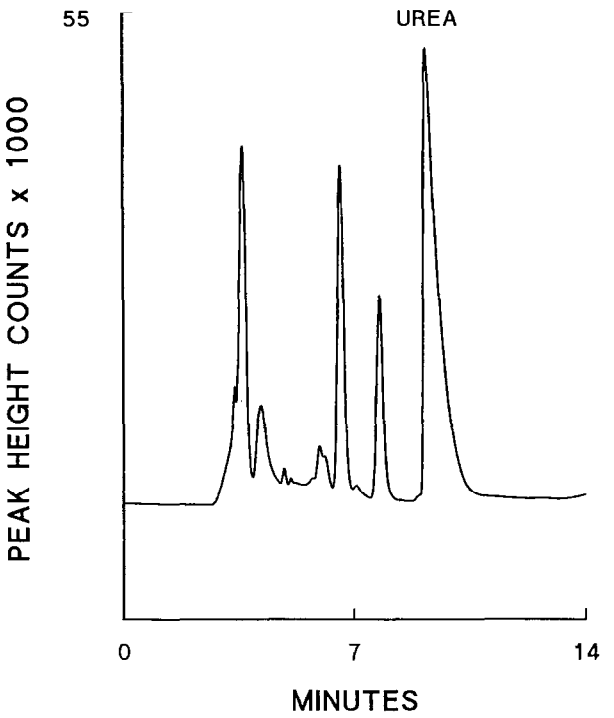

Fig 1. Chromatogram of plasma urea using HPLC. Absorbance (214 nm) is expressed in arbitrary units.

Plasma bromide level was also determined by HPLC using a modification of the method of Wong et al.[14] Briefly, 0.25 mL plasma was diluted in 1.75 mL H_2O, and the sample was then deproteinized by centrifugal ultrafiltration using 10-kd cut-off filter cones (Amicon, Beverly, MA) at 1,000 × *g* for 60 minutes. One hundred microliters of the sample was injected onto a Waters IC-Pak anion column eluted with 0.015 mol/L KH_2PO_4 at 1.7 mL/min. In this system, bromide eluted at approximately 11.5 minutes and was detected at 214 nm.

Plasma ^3H_2O content was determined on perchloric acid extracts (0.5 mol/L, 1:1) and counted using dual-channel liquid scintillation spectrometry. In the present experiments, the ^3H:^{14}C ratio in deproteinized plasma was approximately 2:1.

$^{14}CO_2$ excretion rate and SA in breath were determined using ethanolamine[15] and hyamine[16] traps, respectively. The concentration of hyamine hydroxide (1.0 mol/L) was carefully verified by titration with HCl, and samples were suspended in scintillation cocktail immediately after collection to prevent losses. Total venous blood CO_2 content ($CO_2 + HCO_3^-$) was determined by blood gas analysis (Instrumentation Laboratories, Lexington, MA).

Calculations

TBW was determined using the following equation:

$$\text{TBW} = \text{oral } {}^3H_2O \text{ dose} \div \text{plasma } {}^3H_2O \text{ concentration.} \quad \text{Eq 1}$$

ECW was calculated as follows from plasma bromide concentration, correcting for intracellular Br^- distribution, Donnan's equilibrium factor, and the water content of plasma[17]:

$$\text{ECW} = \frac{\text{Br dose} \cdot 0.804}{\text{plasma Br concentration}}. \quad \text{Eq 2}$$

Intracellular water content (ICW) was calculated as the difference between TBW and ECW, as follows:

$$\text{ICW} = \text{TBW} - \text{ECW}. \quad \text{Eq 3}$$

The following equations were used to calculate fractional CO_2 recovery and "corrected" fractional CO_2 recovery, respectively:

$$\text{Fractional } CO_2 \text{ recovery (percent)} = \frac{{}^{14}CO_2 \text{ excretion rate}}{{}^{14}HCO_3^- \text{ infusion rate}} \cdot 100. \quad \text{Eq 4}$$

$$\text{Corrected fractional } CO_2 \text{ recovery (percent)} = 100 \cdot \frac{\dfrac{SA_I[CO_2]_I \cdot ICW + SA_E[CO_2]_E \cdot ECW}{[CO_2]_I \cdot ICW + [CO_2]_E \cdot ECW} \cdot \dfrac{{}^{14}CO_2 \text{ excretion rate}}{SA_E}}{H^{14}CO_3^- \text{ infusion rate}}. \quad \text{Eq 5}$$

where SA_I = intracellular (urea) SA; SA_E = extracellular (expired CO_2) SA; $[CO_2]_I$ = estimated intracellular CO_2 content = 10 mmol · L^{-1};[18,19] and $[CO_2]_E$ = total extracellular CO_2 content (from blood gas data).

This equation represents the expected fractional recovery of $^{14}CO_2$ if the tracer had free access to intracellular CO_2 pools such that intracellular $^{14}CO_2$ SA and $^{14}CO_2$ SA in breath were equal. For the above calculations, mean urea and $^{14}CO_2$ SA values over the last 5 hours of the study were used.

$$^{12}CO_2 \text{ excretion rate (mmol} \cdot \text{min}^{-1}) = \frac{^{14}CO_2 \text{ excretion rate (dpm} \cdot \text{min}^{-1})}{[\text{breath } CO_2 \text{ SA (dpm} \cdot \mu\text{mol}^{-1}) \cdot 1{,}000]} \cdot \quad \text{Eq 6}$$

All statistical comparisons were made using a paired t test. Data are presented as means ± SEM.

RESULTS

Plasma urea SA increased progressively in the first six subjects over the 14-hour sampling period (Fig 2). Because steady-state conditions were not achieved in this group, a second group of subjects was studied using twice the tracer P:I ratio (~ 720:1 v ~ 360:1); those results are shown in Fig 3. As can be seen, plasma urea SA plateaued by 7:00 AM, and mean SA at 8:00 AM (45.8 ± 6.0 dpm · μmol^{-1}) was not different from that at 12:00 PM (47.0 ± 5.4 dpm · μmol^{-1}, P = NS). The slope of urea SA over the last 5 hours of study was not significantly different from zero (0.29 ± 0.18 dpm · μmol^{-1} · h^{-1}, P = NS); urea SA averaged 46.6 ± 5.6 dpm · μmol^{-1} over that interval. $^{14}CO_2$ SA in expired air was 56.8 ± 3.9 dpm · μmol^{-1}; urea SA was 83.0% ± 5.7% of $^{14}CO_2$ SA in expired air (P < .02). There was a gradual decrease in urea SA in the one subject who received only the priming dose.

TBW, ECW, and ICW were determined in each of the subjects in the second study (Table 1). These results were used to calculate corrected fractional recovery of $^{14}CO_2$. Table 2 shows $NaH^{14}CO_3$ infusion rates, $^{14}CO_2$ and $^{12}CO_2$ excretion rates, and fractional recovery of $^{14}CO_2$. Corrected fractional recovery of $^{14}CO_2$, calculated from equation 5 above, was lower than actual fractional recovery by 5.7% ± 1.7% (P < .025).

DISCUSSION

The present study was undertaken to determine whether tracer disequilibrium exists between intracellular and extracellular compartments of CO_2 during infusion of $NaH^{14}CO_3$. Tracer disequilibrium refers to a situation in which two or more pools are not interchanging so rapidly that they behave as a single joint pool; in this situation the SA of the noninfused pool(s) remains lower than that of the infused pool, even after steady-state conditions have been achieved

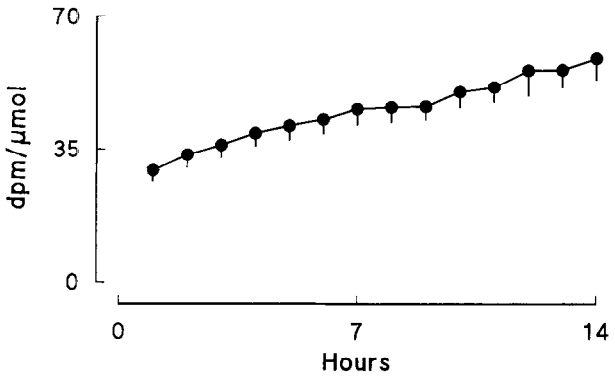

Fig 2. Plasma urea SA in normal subjects (n = 6) during a 14-hour primed continuous infusion (P:I = 357:1) of $NaH^{14}CO_3$.

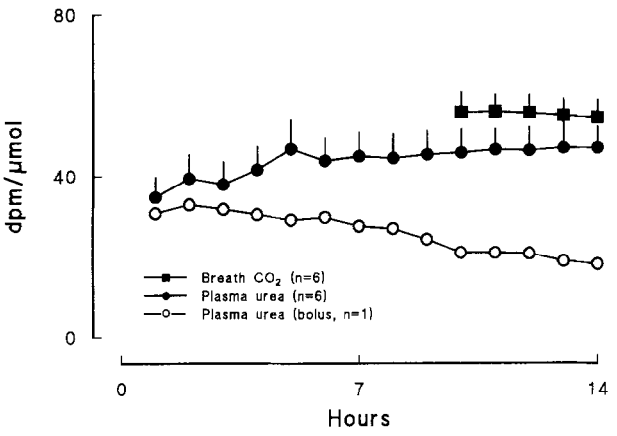

Fig 3. ^{14}C SAs in urea and expired CO_2 in normal subjects (n = 6) during a 14-hour primed continuous infusion (P:I = 720:1) of $NaH^{14}CO_3$. Plasma urea SA in one subject who received only the priming dose of $NaH^{14}CO_3$ is also shown.

with respect to SA in each pool.[20] This issue is of potential importance in the case of labeled bicarbonate infusion, since it could influence the accuracy of substrate oxidation rates during infusion of carbon-labeled tracers. An infusion of labeled bicarbonate has been used by numerous investigators to correct for incomplete recovery of labeled CO_2 in breath.[5-8] This approach assumes that complete equilibration between intracellular and extracellular CO_2 pools is achieved; however, this assumption may not be valid since transport of the HCO_3^- ion into cells is limited.[9,10] Although CO_2 itself does enter cells readily, 95% of extracellular CO_2 is in the HCO_3^- form.[21] Ultimately, the extent of intracellular CO_2 labeling will be determined by the relationship between rate of entry of labeled CO_2 and rate of entry of newly generated unlabeled CO_2.

The results of our study indicate that hepatic intracellular CO_2 SA is approximately 17% lower than extracellular CO_2 SA, using plasma urea SA as a probe for intracellular CO_2 SA and $^{14}CO_2$ SA in breath as a reflection of extracellular CO_2 SA. Plasma urea is derived from hepatic ureagenesis, which in turn requires fixation of equimolar CO_2 of intracellular origin. Our results suggest that disequilibrium does exist between intracellular and extracellular compartments of CO_2, an interpretation consistent with previous in vivo reports. Fuller and Elia infused $NaH^{14}CO_3$ without a priming dose for 36 hours in normal subjects.[12] Unlabeled $NaHCO_3$ was also infused at 2.8 mmol · h^{-1}, a rate that would be expected to have a significant alkalinizing effect. In addition, subjects alternately consumed meals and engaged in aerobic exercise, maneuvers that may influence $^{14}CO_2$ recovery.[13] Despite these differences in experimental design, urinary urea SA was 16.5% lower than breath CO_2

Table 1. Characteristics of Subjects

Weight (kg)	70.7 ± 4.9
TBW (L)	40.2 ± 2.3
ECW (L)	15.9 ± 1.1
ICW (L)	24.3 ± 2.0

Table 2. $NaH^{14}CO_3^-$ Infusion Rate, $^{14}CO_2$ and $^{12}CO_2$ Excretion Rates, and Fractional and Corrected Fractional Recoveries

$NaH^{14}CO_3^-$ Infusion rate (mCi · min^{-1})	310 ± 8
$^{14}CO_2$ Excretion rate (mCi · min^{-1})	294 ± 8
$^{12}CO_2$ Excretion rate (mmol · min^{-1})	11.8 ± 0.8
Fractional recovery (%)	94.8 ± 0.8
Corrected fractional recovery (%)	89.5 ± 1.9

SA.[12] Although the investigators concluded that there is little or no compartmentalization of CO_2 in humans, this difference is almost identical to the results of the present study.

The limitations of the use of plasma urea SA in our study should be acknowledged. One limitation is that this approach assesses CO_2 SA only in the liver, and not in other tissues. This is of importance for at least two reasons. First, it is not known whether liver is the chief site of $^{14}CO_2$ fixation during $NaH^{14}CO_3$ infusion. If one assumes that such hepatic fixation is represented quantitatively by the combined processes of gluconeogenesis and ureagenesis and that the rates of the two processes are approximately 5 μmol · kg^{-1} · min^{-1} (roughly one half the rate of hepatic glucose production after an overnight fast) and 3.8 μmol · kg^{-1} · min^{-1},[22] respectively, then such fixation would represent approximately 5% of total CO_2 production in our patients. Whereas this would account for the loss of tracer (ie, ~95% recovery in breath) in our studies, it would not fully account for the higher loss of tracer observed with short-term infusions of labeled bicarbonate[5-7,23] that are relevant to most substrate oxidation studies. Second, it is not known whether the disequilibrium in $^{14}CO_2$ SA is similar in all tissues; in fact, the presence of extensive capillary fenestrations in the liver[24] makes it conceivable that $^{14}CO_2$ disequilibrium would be greater in nonhepatic tissues.

An additional flaw exists in the use of plasma urea to measure intracellular CO_2 SA. Arginine may be taken up by the liver from the circulation and converted to urea, thus decreasing the SA of circulating urea by a mechanism unrelated to isotope disequilibrium in CO_2. Moreover, portal venous CO_2 SA may be lower than arterial CO_2 SA due to release of unlabeled CO_2 by the gut; this would also decrease urea SA independent of CO_2 partitioning.

A final limitation of the urea SA method relates to the extremely long half-life of urea, which requires a large priming dose of labeled urea to achieve steady-state SA or stable isotopic enrichment.[25] In this study, we achieved steady-state conditions in plasma urea by labeling a precursor pool for synthesis (CO_2) after 5 to 7 hours of infusion using a tracer P:I ratio of 720:1. This is similar to the ratio recommended by Matthews and Downey for infusion of ^{13}C urea.[25] It is possible that the large priming dose and long infusion time required in the present study resulted in progressive labeling of intracellular sources of CO_2 (glycogen, nascent proteins, etc.), which might underestimate the $^{14}CO_2$ disequilibrium that would prevail during a shorter experiment.

We have attempted to correct for tracer disequilibrium (equation 5) by generating a "whole-body" $^{14}CO_2$ SA (intracellular + extracellular $^{14}CO_2$ ÷ intracellular + extracellular $^{12}CO_2$). Because of the numerous shortcomings of the urea SA method outlined above, this calculation does not provide a useful estimate of the potential impact of tracer disequilibrium on systemic tracer recovery. However, it could be of value if a technique was available that could estimate whole-body intracellular $^{14}CO_2$ SA. Although the difference between actual and corrected fractional recovery in our study is relatively small, larger differences might be seen during a shorter experiment, in which a lower actual fractional recovery would be expected.[5-7,23,26] Unfortunately, a shorter experiment is not possible when plasma urea is used as a probe for intracellular $^{14}CO_2$ SA.

The presence of tracer disequilibrium during infusion of labeled bicarbonate would affect the accuracy of substrate oxidation studies only if two conditions were simultaneously satisfied. First, there would have to be irreversible loss of tracer by nonrespiratory routes. If the only route of irreversible loss of $^{14}CO_2$ is via breath, then failure of two (or more) pools to equilibrate would not influence $^{14}CO_2$ excretion rate. In this circumstance, the rate of tracer entry into the intracellular pool(s) from the extracellular pool would equal the rate of tracer exit from the intracellular to extracellular pool. Few investigators have directly attempted to measure possible routes of nonrespiratory irreversible tracer loss during labeled bicarbonate infusion. In fact, when bicarbonate kinetics have been mathematically modelled, a three-compartment system best describes the data, with respiratory excretion fully accounting for irreversible loss of tracer.[8,13] The second condition that must prevail for bicarbonate tracer disequilibrium to influence the accuracy of substrate oxidation studies is that nonrespiratory tracer loss must occur from the underlabeled noninfused compartment. If irreversible nonrespiratory tracer loss was to occur directly from the extracellular pool without dilution by intracellular $^{12}CO_2$ before exit, then recovery of labeled CO_2 during infusion of labeled bicarbonate would accurately represent labeled CO_2 recovery during infusion of a labeled substrate.

It is not entirely clear to what extent irreversible nonrespiratory loss of labeled CO_2 occurs during infusion of labeled bicarbonate. The sum of ureagenesis and gluconeogenesis accounts for only about 5% of tracer loss, as previously outlined. However, the demonstration by Poyart et al[27] of significant uptake of labeled bicarbonate by bone raises the possibility that intracellular tracer may be lost via another route. In that study, approximately 10% of labeled bicarbonate was taken up by the skeleton of the rat during a constant infusion of $NaH^{14}CO_3$.[27] Considering that pulmonary clearance of CO_2 is much higher (and thus the residence time in blood is much shorter) in rats than in humans,[28] it seems probable that a higher relative rate of skeletal exchange of $^{12}CO_2$ for $^{14}CO_2$ would prevail in humans. If one assumes that a 70-kg adult possesses 3.0 kg bone mineral, of which 6% is $CaCO_3$,[29] then bone mineral contains approximately 1,800 mmol carbonate. It is not known whether all of this carbonate is readily exchangeable; in fact, the observation that fractional recovery of $^{14}CO_2$ increases with duration of infusion of $NaH^{14}CO_3$[12]

suggests that the exchangeable pool is saturable and may reside in a smaller subcompartment of bone mineral. Although the exact nature of CO_2 exchange between skeletal and soluble CO_2 pools is not known, the ultrastructural relationship between bone cells and bone mineral[30] suggests that the exchange occurs between bone carbonate and intracellular CO_2.

In summary, our study indicates that disequilibrium exists between intracellular and extracellular CO_2 pools in liver. Whether this phenomenon contributes to incomplete recovery of $^{14}CO_2$ during $NaH^{14}CO_3$ infusion cannot be determined from our data. The measurement of urea SA has so many limitations that newer methods for indirect measurement of intracellular CO_2 will need to be developed to advance our understanding in this area. Further studies investigating the role of skeletal uptake of labeled CO_2 in humans would be especially useful.

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