Influence of Metabolic Fuel on the $^{13}C/^{12}C$ Ratio of Breath CO_2

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Natural differences in $^{13}C/^{12}C$ ratios of various metabolic fuels can produce systematic changes in the $^{13}C/^{12}C$ ratio of breath CO_2, and therefore introduce errors into $^{13}CO_2$ breath tests. To gain insight into the potential problem, we compared $^{13}C/^{12}C$ ratios of plasma macronutrients to those of breath CO_2 under conditions that should alter the percentages of carbohydrate and lipid being oxidized. In rats, 48 h of starvation decreased the $^{13}C/^{12}C$ ratio of breath CO_2 by 3.5‰. At this time the $^{13}C/^{12}C$ ratio of breath CO_2 was very similar to that of plasma lipids. In humans, 30 min of heavy exercise increased the breath $^{13}CO_2/^{12}CO_2$ ratio by 1.3‰. These changes in breath $^{13}C/^{12}C$ ratios could be predicted from $^{13}C/^{12}C$ ratios of plasma macronutrients and the percentage of carbon dioxide derived from each macronutrient, but only when compared within the same populations. For example, the $^{13}C/^{12}C$ ratios of plasma macronutrients of residents of Chicago, Illinois (USA) and Tokyo (Japan) differed by 1–3‰. An empirical correction of $^{13}CO_2$ breath test data is recommended when breath tests are run under conditions that will change metabolic fuel utilization.

INTRODUCTION

Stable carbon isotope analysis of human breath CO_2 has been used to measure the *in vivo* oxidation of various drugs, metabolic fuels and products of intermediate metabolism. In each of these analyses, excess $^{13}CO_2$ produced by oxidation of labeled substrate must be measured in the presence of a natural background of ^{13}C. This natural ^{13}C comprises about 1.1% of all carbon, but ^{13}C abundance varies slightly depending on the carbon source.[1] In human studies, natural variations in the $^{13}C/^{12}C$ ratio have been noted between geographically distinct populations,[2,3] between individuals within a single population[4] and within a single individual.[5] These natural variations can be sources of error in the breath $^{13}CO_2$ analyses, especially when final breath ^{13}C enrichment is small because the substrate is given in small amounts or because it is only partially oxidized.

Errors resulting from carbon isotope differences between individuals are generally avoided by collecting a pre-dose breath sample from each subject and measuring the $^{13}CO_2/^{12}CO_2$ ratio of the endogenous CO_2. Substrate oxidation is then calculated from the increase in the $^{13}CO_2/^{12}CO_2$ ratio relative to the pre-dose value.

Errors in $^{13}CO_2$ breath analysis resulting from variations in endogenous $^{13}C/^{12}C$ ratios of breath CO_2 within a single individual during the breath test are not eliminated through the use of the pre-dose breath sample. These errors, however, are usually kept as small as possible by using conditions which keep endogenous $^{13}CO_2/^{12}CO_2$ ratios almost constant. This requires the subject to fast overnight before the test and to remain at rest during the test.[5] When fasting is not possible, isotopic variations are kept small by using foods that are isotopically similar to the endogenous CO_2.[4]

We have recently begun studies in subjects during exercise in which we have not been able to keep the endogenous $^{13}CO_2/^{12}CO_2$ ratio constant during the test. Wolfe *et al.*[6] have also demonstrated the effect of exercise on endogenous breath $^{13}CO_2$ ratios and related the changes to the percentage of lipid and carbohydrate being oxidized. In addition, previous investigators have demonstrated in both plants[7] and animals[8] that the $^{13}C/^{12}C$ ratio of respired CO_2 reflects the $^{13}C/^{12}C$ ratio of the metabolic fuel and that the ratio is generally greater in carbohydrates than it is in lipids.

Because exercise-related variations in endogenous $^{13}C/^{12}C$ ratios could reduce the accuracy of our breath test results, we initiated a series of studies aimed at determining the magnitude of the potential error. In this report, we demonstrate the influence of changes in metabolic fuel on the $^{13}C/^{12}C$ ratio in breath CO_2, then relate these results to actual measurements of the $^{13}C/^{12}C$ ratio of plasma macronutrients. Finally, we discuss methods for correcting breath test results for changes in the metabolic fuel being oxidized.

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EXPERIMENTAL

Animals studies

Eight male Wistar rats were maintained on an *ad libitum* diet of MM-1 rat chow (Funabashi Farm, Chiba, Japan) for four weeks. Four rats fasted for 4 h, then were individually placed in an air-tight cage having a dead volume of 200 ml. The cage was swept with CO_2-free air at 400 ml min^{-1} for 5 min, then sealed. Air samples for $^{13}CO_2$ analysis were taken at 30 s intervals for the next 2 min, and placed in 20 ml evacuated tubes. After breath collection, 6 ml of blood was obtained by decapitation, treated with 1 mg ml^{-1} NaF, and freeze dried. The other four rats fasted for 48 h. Breath and blood were then collected as described above.

Human studies

Samples of blood and breath CO_2 were collected from healthy, 21–38 year old, mostly college educated adults residing either in metropolitan Chicago, Illinois (USA) or in metropolitan Tokyo, Japan. Vegetarians were not included in the study. Subjects fasted for 10–15 h before morning blood and breath collection. Blood, 10 ml, was collected in heparinized tubes and plasma was isolated and frozen. Breath was collected in 3 l syran bags and stored in 20 ml evacuated tubes.[9]

Exercise studies were performed at the University of California, Berkeley. Three male subjects age 23–25 years exercised on a Quinton model 5A electrically braked bicycle ergometer at two separate intensities. Easy exercise required subjects to work at 50% of their maximum oxygen consumption (VO_2 max) for 2 h 20 min, while hard exercise was performed at 75% VO_2 max for 65 min. All tests were given in the morning following an overnight fast and the two exercise tests were performed at least seven days apart. Through the experimental trials, 20 ml aliquots of expired air were collected from a mixing chamber for subsequent $^{13}CO_2$ enrichment analysis.

Respiratory exchange ratios (R) were measured using an open circuit, on-line system employing an Applied Electrochemistry S-3A oxygen analyser and a Beckman LB2 carbon dioxide analyser.

The human studies were approved by the Human Studies Committees of the respective institutions.

Plasma analyses

Neutral lipids, proteins and glucose were isolated from serum. Neutral lipids were extracted with two equal volumes of 2:1 chloroform:methanol, which was washed with aqueous 0.1 M KCl. The solvents were evaporated at 80 °C. Protein was precipitated with an equal volume of saturated $Ba(OH)_2$ and an equal volume of 10% $ZnSO_4$, centrifuged, separated from the supernate and freeze dried. Glucose in the resulting supernate was separated from glucose metabolites and other ionic material in a manner similar to that of Brady *et al.*,[10] by passage through a strong base/strong acid mixed bed ion exchange column (Rexyn™ 300) followed by freeze drying. Because glucose is not actually isolated, the resulting material may contain some other carbon compounds. Previous studies by Wolfe *et al.*,[11] however, have shown that the isotopic enrichment of CO_2 derived from the infusion of ^{13}C-labeled glucose separated from serum in a similar manner is not significantly different from that derived from glucose isolated in a more rigorous manner.

Isotopic analyses

Samples were combusted to CO_2 as previously described.[12] Briefly, 1–4 mg of dry sample was placed in a 150 mm × 6 mm quartz tube with 500 mg of copper oxide, that had been previously heated to 825 °C to remove residual carbon, and a 25 mm × 0.7 mm silver wire. The tube was evacuated, sealed and heated to 825 °C for 1.5 h, then cooled overnight to room temperature. The resulting CO_2 was purified by cryogenic distillation between traps cooled with ethanol slush (*ca.* −100 °C) and liquid nitrogen (−196 °C). The CO_2 was isotopically analysed on a dual inlet, dual collector Nuclide 3-60 isotope ratio mass spectrometer. Carbon isotope ratios were corrected for abundance sensitivity, background and ^{17}O.[1] Results were expressed as the 'per mil' (‰) difference from Pee Dee Belemnite limestone, CO_2 where:

$$\delta^{13}C = \left[\frac{(^{13}C/^{12}C)_{sample}}{(^{13}C/^{12}C)_{PDB}} - 1\right] \times 1000$$

Analyses of duplicate serum samples indicated the precision (1 SD) of the isotopic analyses was 0.2‰. The average from 10 samples of NBS 22 analysed at monthly intervals was −29.4‰ with a SD of 0.3‰.

Predicted $^{13}C/^{12}C$ ratio

The $^{13}C/^{12}C$ of breath CO_2 was predicted from the respiratory quotient and the $^{13}C/^{12}C$ ratios of plasma macronutrients. The percentage of carbon from carbohydrate and lipid was calculated according to Kleiber.[13] The percentage of carbon from protein was assumed to be 2%.[14] These percentages were then used as weighting factors for summing the $^{13}C/^{12}C$ ratios of glucose, lipid and protein in plasma.

RESULTS

Table 1 summarizes the rat studies. The isotope ratio of breath CO_2 in rats fasted only 4 h was intermediate between the isotope ratio of blood glucose and lipids. After a 48 h fast, the isotope ratio of breath CO_2 was 3.5‰ ($p < 0.01$) less abundant in ^{13}C (lighter) than that in the 4 h fasted groups, and very similar to the isotope ratio of the blood lipids. The plasma lipid was isotopically lighter in the 48 h fasted rats ($p < 0.01$), but changes in the isotope ratios of the blood protein and glucose during fasting were not statistically significant.

Table 2 summarizes the plasma macronutrient studies in humans. Protein was the most abundant in ^{13}C (heaviest) and lipid was the lightest fraction. This pattern

Table 1. Carbon isotope composition of blood macronutrients and breath CO_2 in 4 and 48 h fasted rats

Sample	4 h fast δ^{13}_{PDB}, (SD, ‰)	48 h fast δ^{13}_{PDB}, (SD, ‰)
Blood protein	−20.1 (0.2)	−20.1 (0.2)
Blood glucose	−17.6 (0.9)	−18.5 (1.4)
Blood lipid	−24.9 (0.1)	−23.9 (0.3)[b]
Breath CO_2	−19.7 (0.7)	−23.2 (0.4)[b]

[a] $n=4$.
[b] $p<0.01$ V. 4 h fast (t test).

was seen in eight of the nine American subjects and all 12 Japanese subjects. The difference (±SD) between the plasma glucose and lipid averaged 1.9 ± 1.3‰ ($p<0.01$, paired t test) in nine American subjects and 2.4 ● 0.8‰ ($p<0.01$) in 12 Japanese subjects. Breath CO_2 was isotopically intermediate between plasma glucose and lipid in both populations. Although the isotopic relationships between macronutrients were very similar in both populations, the $^{13}C/^{12}C$ ratios in American subjects averaged 1–1.5‰ heavier than the respective values in Japanese subjects.

From the respiratory exchange data of Ravussin *et al.*[12] for subjects at rest and in the post-absorptive state, we calculate that 55% of expired CO_2 comes from carbohydrates, 43% from lipids and 2% from proteins. Thus from the measured $^{13}C/^{12}C$ ratios in plasma, we predicted the $^{13}C/^{12}C$ ratios in breath CO_2 to be −20.5‰ for American subjects and −21.7‰ for Japanese subjects. These predicted values were very similar to the mean observed values (Table 2).

Comparison of predicted $^{13}C/^{12}C$ ratios to the measured values was also made on an individual basis for the nine American subjects (Fig. 1). The predicted and measured isotope ratios were correlated ($r=0.81$). The correlation indicated that the isotopic composition accounted for 65% of the interindividual variation in $^{13}C/^{12}C$ ratio of breath CO_2. The residual variation about the regression line was 0.5‰, which was about twice the measurement error and indicates only a small unaccounted for variability. A similar comparison for Japanese subjects could not be made because breath and blood were not collected on the same day. The breath samples collected on the day blood was drawn were lost due to contamination.

During exercise, $^{13}C/^{12}C$ ratios of breath CO_2 changed systematically with time. During the first few minutes of exercise, the isotope ratios decreased rapidly, then

Table 2. The $^{13}C/^{12}C$ ratios of human plasma macronutrients and breath CO_2

Sample	United States δ^{13}_{PDB}, (SD, ‰)[a]	Japan δ^{13}_{PDB}, (SD, ‰)[b]
Plasma protein	−18.1 (0.7)	−19.2 (0.3)[c]
Plasma glucose	−19.7 (1.9)	−20.7 (0.7)
Plasma lipid	−21.6 (0.5)	−23.1 (0.5)[c]
Breath CO_2	−20.9 (1.0)	−21.8 (1.2)

[a] $n=9$.
[b] $n=12$.
[c] $p<0.01$ *v.* US (t test).

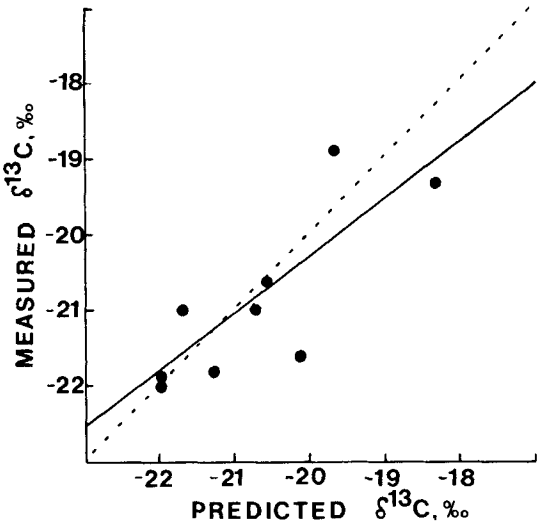

Figure 1. Relationship between the measured $^{13}C/^{12}C$ ratio of breath CO_2 and that predicted from the $^{13}C/^{12}C$ ratios of plasma macronutrients. Solid line from least-squares analysis.

began to increase slowly. The $^{13}C/^{12}C$ ratios were greatest at about 35 min. At easy exercise, the maximum was only 0.3‰ heavier than at rest; while at hard exercise, the maximum was 1.3‰ heavier than at rest.

The change in the isotope ratio of breath CO_2 during exercise predicted from measured respiratory gas exchange ratios and average isotope ratios of plasma macronutrients in Americans (Table 2) is also shown in Fig. 2. The predicted and measured values were very

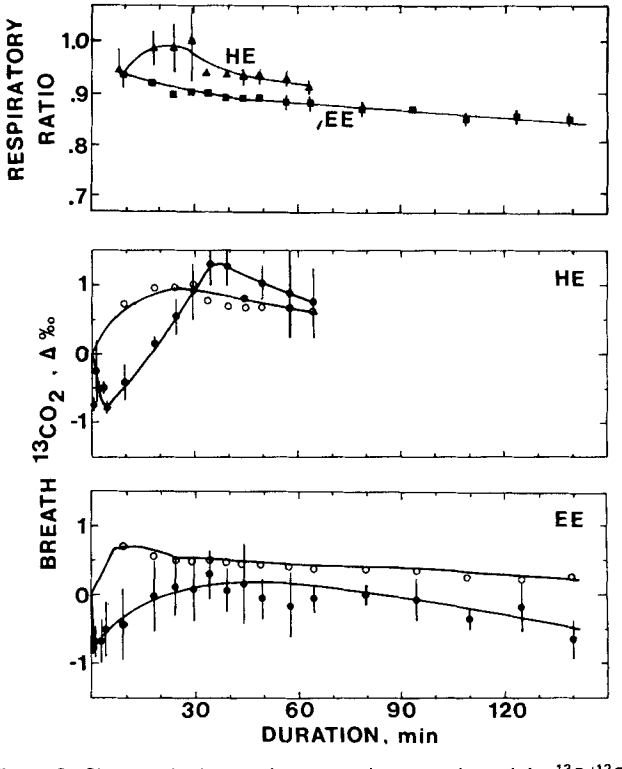

Figure 2. Changes in the respiratory exchange ratio and the $^{13}C/^{12}C$ ratio of breath CO_2 during hard exercise (HE) and easy exercise (EE) relative to a baseline breath sample collected at rest. Open circles are the changes predicted from the respiratory quotients and the $^{13}C/^{12}C$ ratios of the macronutrients in plasma. Closed circles and bars are the measured changes with their standard errors. Lines are visual fits presented for clarity.

dissimilar during the first 20–30 min of exercise, but were similar for the remainder of the exercise periods.

DISCUSSION

The stable carbon isotope ratio of endogenous CO_2 changed in response to physiological changes. As expected from previous studies in humans,[6] animals[7] and plants,[8] these changes generally reflected differences in the $^{13}C/^{12}C$ ratio in the two major metabolic fuels, carbohydrate and lipid. Fasting, which increases lipid oxidation,[15] caused the isotope ratio of breath CO_2 from rats to decrease because the lipids were isotopically lighter than the carbohydrates. Conversely, exercise in humans, which increases carbohydrate utilization,[14,15] was reflected by an increase in the $^{13}C/^{12}C$ ratio of breath CO_2 after the first 30 min of exercise.

The potential for changes in the $^{13}C/^{12}C$ ratio of endogenous breath CO_2 depends on the difference of the $^{13}C/^{12}C$ ratio of carbohydrates and lipids. The larger the difference, the greater the potential change. In rats, where fuels differed by 7‰, we could induce a 3.5‰ change in breath CO_2. In humans, where the difference was less than 3‰, we could only induce a 1.3‰ change. For the most part, the isotopic difference between the two metabolic fuels will reflect diet[8] and will usually be least in mixed diets and potentially greatest in elemental diets where lipids and carbohydrates can differ by 15‰.[4]

In general, we could predict $^{13}C/^{12}C$ ratios of breath CO_2 from the $^{13}C/^{12}C$ rates of plasma macronutrients. The only exception was the first 30 min of the exercise study. The initial decrease in the isotope ratios would indicate increased lipid oxidation, but respiratory gas ratios increased indicating increased carbohydrate oxidation. The area between the predicted and measured $^{13}C/^{12}C$ ratios, normalized for CO_2 production, corresponds to an additional 8 or 9 mmol kg^{-1} of lipid-derived CO_2.

The discrepancy between the measured and predicted $^{13}C/^{12}C$ ratios may have reflected a change in bicarbonate kinetics. At rest, bicarbonate in skeletal muscle comprises about 60% of the bicarbonate pool and thus equals 8 or 9 mmol kg^{-1}.[16] Because the preferred substrate for resting skeletal muscle is lipid,[17] the bicarbonate in this pool should be isotopically light. During transition from rest to exercise, increased blood flow to the skeletal muscle will increase the fractional elimination rate of this pool, presumably at the expense of the internal organs that prefer carbohydrates as their fuel.[15] This change in the relative elimination rates from the two pools could cause the $^{13}C/^{12}C$ ratio of breath CO_2 to decrease temporarily.

Alternatively, the observed decrease in the $^{13}C/^{12}C$ ratio of expired CO_2 could respresent increased lipid utilization during the transition state that was not reflected in the respiratory exchange due to nonequilibrium of gas exchange in the transition state; or the decrease could have been due to a kinetic isotope effect that was expressed during the transition period when bicarbonate kinetics were not at equilibrium.

The amount of error introduced into a $^{13}CO_2$ breath test by the changes in the endogenous $^{13}CO_2/^{12}CO_2$ ratio varies as a function of substrate and dose. For example, large doses of rapidly oxidized substrates will produce $^{13}CO_2$ signals that are much larger than the noise from changes in the endogenous $^{13}C/^{12}CO_2$ ratio, and the error will not be very large. Other breath tests using less oxidized substrates, such as essential amino acids, may only produce signals of 3–5‰ and thus are subject to significant errors if the endogenous $^{13}CO_2/^{12}CO_2$ ratio changes during the test, and some form of correction is needed.

Breath test results can be corrected for changes in endogenous $^{13}CO_2/^{12}CO_2$ rates resulting from changes in metabolic fuel utilization in at least two ways. Theoretically, it is possible to measure the respiratory gas exchange ratio and the $^{13}C/^{12}C$ ratios of the metabolic fuels for each individual and to calculate the appropriate isotopic correction. This method, however, is laborious and may be subject to some error because of delays between the change in the respiratory ratio and the isotope ratio (Fig. 2).

Alternatively, results can be corrected empirically by measuring the change in endogenous breath $^{13}CO_2/^{12}CO_2$ in the absence of labeled substrate.[4,6] For this correction, the subjects, or a selected subset of the subjects, perform the breath test following the prescribed protocol except that label is not administered. The measured changes in the $^{13}CO_2/^{12}CO_2$ ratio relative to the baseline sample either in 'per mil' or atom percent excess are then subtracted from the results of the breath test performed after administration of the label for each point in time. Obviously, use of this correction requires that intra- and interindividual variation in changes in the $^{13}C/^{12}C$ ratio of breath CO_2 do not exceed the precision required for the study. When the changes in the $^{13}C/^{12}C$ ratio are reproducible, errors in $^{13}CO_2$ breath analysis can be minimized even in the presence of systematic changes in the $^{13}C/^{12}C$ ratio of breath CO_2 resulting from changes in metabolic fuel utilization.

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REFERENCES

1. H. Craig, *Geochim. Cosmochim. Acta* **3**, 53 (1953).
2. K. Nakamura, D. A. Schoeller, F. J. Winkler and H. -L. Schmidt, *Biomed. Mass Spectrom.* **9**, 390 (1982).
3. T. D. B. Lyon and M. S. Baxter, *Nature* **73**, 750 (1978).
4. D. A. Schoeller, P. D. Klein, J. B. Watkins, T. Heim and W. C. MacLean Jr, *Am. J. Clin. Nutr.* **33**, 2375 (1980).
5. D. A. Schoeller, J. F. Schneider, N. W. Solomons, J. B. Watkins and P. D. Klein, *J. Lab. Clin. Med.* **90**, 412 (1977).
6. R. R. Wolfe, J. H. F. Shaw, E. R. Nadel and J. F. Burke, *J. Appl. Physiol.: Respirat. Environ. Exercise Physiol.* **56**, 230 (1984).

7. B. S. Jacobson, B. N. Smith, E. Estein and G. G. Laties, *J. Gen. Physiol.* **55**, 1 (1970).
8. M. J. DeNiro and S. Epstein, *Geochim. Cosmochim. Acta* **42**, 495 (1978).
9. D. A. Schoeller and P. D. Klein, *Biomed. Mass Spectrom.* **5**, 29 (1978).
10. L. J. Brady, D. R. Romos and G. A. Leveille, *Comp. Biochem. Physiol.* **56B**, 421 (1977).
11. R. R. Wolfe, J. R. Allsop and J. F. Burke, *Metabolism* **28**, 210 (1979).
12. D. A. Schoeller, P. D. Klein, W. C. MacLean Jr, J. B. Watkins and E. van Santen, *J. Lab. Clin. Med.* **97**, 439 (1981).
13. M. Kleiber, in *Handbook of Physiology*: *Respiration* Vol. VII, edited by W. O. Fenn and H. Rahn, p. 927. American Physiology Society, Washington, DC (1965).
14. E. Ravussin, B. Burnand, Y. Schutz and E. Jéquier, *Am. J. Clin. Nutr.* **35**, 566 (1982).
15. E. A. Newsholme, *Diabetes* **28**, 1 (1979).
16. D. R. Drury, A. N. Wick and M. C. Almen, *Am. J. Physiol.* **186**, 361 (1956).
17. K. L. Zierler, *Circulation Res.* **38**, 459 (1976).

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