Original articles

Clinical diagnosis with the stable isotope ^{13}C in CO_2 breath tests: methodology and fundamental considerations

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The methodology for measuring in vivo oxidation of substrates labeled with the nonradioactive carbon isotope ^{13}C has been developed with isotope ratio mass spectrometry. The use of ^{13}C offers the possibility of utilizing CO_2 breath tests in infants, children, pregnant women, and all subjects in whom $^{14}CO_2$ breath tests cannot be used. The excretion of 140 nmol/kg-hr of $^{13}CO_2$ produced from the oxidation of the labeled substrate could be detected with 95% confidence during a total CO_2 excretion of 9 mM/kg-hr. The precision of CO_2 breath tests using ^{13}C is limited by the natural fluctuations of the ratio of $^{13}C/^{12}C$ in expired CO_2, which occur with a standard deviation of 0.72‰, or approximately 7 parts $^{13}CO_2$ per 10^6 parts expired CO_2. Larger excursions in the ratio were observed if the subject ate shortly before or during the breath test. Clinically significant diagnostic tests can reasonably be expected to require the excretion of 2 to 20 times as much labeled CO_2, or 0.28 to 1.4 μM/kg-hr.

Breath tests measuring the respiratory excretion of $^{14}CO_2$ following the administration of a ^{14}C-labeled substrate have been shown to be useful for the diagnosis of bile acid malabsorption,[3, 4] fat malabsorption,[5] lactose intolerance,[6] and hepatic microsomal function.[7, 8] Unfortunately, the potential radiation hazard severely restricts the use of ^{14}C in infants, children, and women of child-bearing age. To extend the use of breath tests to

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Portions of this work have appeared in abstract form[1, 2] and have been presented at the Second International Conference on Stable Isotopes, Oct., 1975, Oak Brook, Ill., and the 24th Annual Conference for Mass Spectrometry and Allied Topics, May, 1976, San Diego, Calif.

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these populations, we have developed the basic methodology to perform CO_2 breath tests using the nonradioactive isotope ^{13}C.

Before $^{13}CO_2$ breath tests could undergo clinical evaluation, the basic questions concerning the limitations and possible sources of error of these tests required investigation. The major constraints placed on $^{13}CO_2$ breath tests arise from the large natural abundance of ^{13}C (1.1%). For comparison, if ^{14}C tests were conducted under similar conditions, a 1.1% abundance of ^{14}C would produce a background of 1.5×10^9 dpm/mmol of CO_2. Because of the magnitude of the ^{13}C background, it was necessary to determine the extent of natural fluctuations in the exhaled $^{13}CO_2$ abundance and to establish whether the test protocols produced any further variation. Knowing the extent of the fluctuations, we could estimate the detection limits, precision, and substrate dose requirements. The problem of preserving the isotopic integrity of the CO_2 during sample collection and handling was also investigated.

Materials and subjects

Human subjects. Subjects included healthy adult volunteers employed at Argonne National Laboratory and the University of Chicago, and patients at the University of Chicago, the Boston Children's Hospital, and the Clinical Center of the Biomedical Division of the Institute of Nutrition of Central America and Panama (INCAP). All protocols were approved by the University of Chicago Clinical Investigations Committee and, in addition, the human studies committees of the individual institutions. Written informed consent was given by either the subject or the subject's legal guardian.

Unlabeled substrates. Aminopyrine (dimethylaminoantipyrine, Aldrich Chemical Corp., Milwaukee, Wis.) was dissolved in 50 ml of water and administered orally. The dosage was 2 mg/kg of body weight. The purity of the aminopyrine was 98%. Crystalline sodium glycocholate (Sigma Chemical Corp., St. Louis, Mo.) was dissolved in 11 ml of 1:10 ethanol:water solution and administered orally. The dosage varied between 3 and 10 mg/kg of body weight. Trioctanoin with a purity of 99.9% was administered intraduodenally through a 5 mm silicone rubber tube. The total dose, equivalent to 10 mg/kg of body weight, was followed by oral administration of 90 cc of standard Enfamil (Mead-Johnson, Evansville, Ind.) in order to simulate a standard meal.

Labeled substrates. Aminopyrine labeled to 86.3 atom % excess with ^{13}C on each of the N-methyl carbons was prepared by Merck Sharp and Dohme (Pointe Claire, Canada) IND 11,734). Administration was identical to that used for the unlabeled aminopyrine.

Statistical tests. Student's t test was used to test for significant differences in isotope ratios and the F test was used to test for significant differences in variances.

Isotope ratio measurements

Small excesses of exogenous $^{13}CO_2$ arising from the oxidation of the labeled substrate are calculated from the increase in the ratio of $^{13}CO_2$ to $^{12}CO_2$. The ratios are obtained on a dual-inlet, dual-collector, isotope ratio mass spectrometer of the type developed by Nier[9] and McKinney et al.[10] The ratio of the signals from the ionized species $^{13}CO_2^+$ and $^{12}CO_2^+$, each focused on one of the dual collectors, is recorded. The ratio is then compared with that obtained when a standard CO_2 sample of known isotopic abundance is introduced under identical conditions. The results of this differential measurement are expressed as the del per mil (δ‰) difference between the $^{13}CO_2/^{12}CO_2$ ratio of the sample (R_u) and the standard (R_s).[11]

$$\delta^{13}C \equiv \frac{R_u - R_s}{R_s} \times 1{,}000, \text{‰} \qquad (1)$$

For small changes in the isotopic enrichment, the del value is analogous to a molar specific activity as it is proportional to the ^{13}C isotopic content in moles per mole of carbon. Instruments are capable of measuring differences as small as 0.05‰,[12] which corresponds

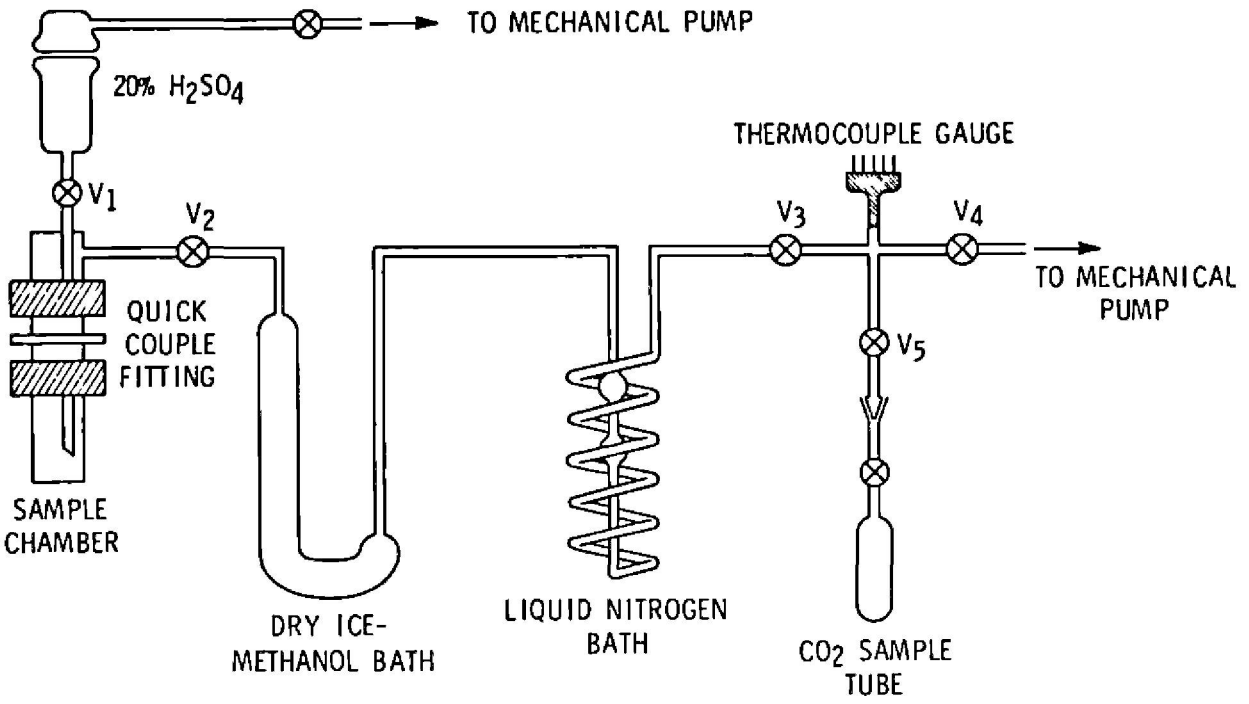

Fig. 1. Vacuum apparatus for the release and purification of CO_2 from NaOH solution.

to a difference of 0.5 ppm of $^{13}CO_2$ in a sample of CO_2. The high precision is possible only because the use of a standard CO_2 sample corrects for instrumental instabilities and mass discrimination. To facilitate interlaboratory comparisons, the results are commonly expressed relative to CO_2 derived from PDB limestone, a belemite limestone of well-defined isotopic abundance ($^{13}C/^{12}C = 0.011237$).[13] Further details of the isotope ratio measurement and the calculations of results are admirably discussed by Mook and Grootes.[14]

Sample collection and handling

Methods. Respiratory CO_2 was collected in three ways: by total collection in a liquid nitrogen trap, by static absorption in NaOH solution, or by dynamic trapping by bubbling through NaOH solutions. Total collection of CO_2 was achieved by having the subject exhale through an 8 mm i.d. spiral trap immersed in liquid nitrogen. The CO_2 was then purified by distillation between a Dry Ice–methanol trap and a liquid nitrogen trap. Static absorption was carried out by having the subject exhale through a Tygon tube into an empty 250 ml round-bottom flask. A 10 ml aliquot of freshly prepared carbonate-free 2N NaOH (Dilut-it, J. T. Baker Chemical Co., Phillipsburg, N. J.) was then added to the flask, and the flask was stoppered and shaken for 30 min. The CO_2, as $CO_3^=$, was transferred to a Teflon- or plastic-lined screw-cap vial for transportation and storage. For dynamic trapping, the patient exhaled through a Tygon tube 1 cm or larger in i.d. into 10 ml of NaOH solution for 5 min. Generally, the NaOH solution was contained in a 250 ml round-bottom flask to maximize surface exposure; in selected studies, samples were collected in a 1 by 15 cm tapered centrifuge tube in place of the flask. In studies involving infants and small children a face mask equipped with a one-way Rudolph valve was used to prevent aspiration of the trapping solution.

The CO_2 was released from the basic solution by acidification, trapped, and then purified cryogenically[12] with the apparatus shown in Fig. 1 as follows. Valves 2 and 5 are closed, 3 and 4 are opened, and the traps are evacuated. A 1 ml NaOH sample containing $CO_3^=$ is pipetted into the sample chamber. Valve 2 is opened and the system is evacuated. After the pressure drops below 200 μm Hg, valve 1 is opened and 1 ml of degassed 20% H_2SO_4 is added to the sample. A period of 1.5 min is allowed for the CO_2 to evolve and be transferred to the −196° C liquid nitrogen trap. The −78° C Dry Ice–methanol trap scrubs most of the water vapor from the evolved CO_2. Valves 2 and 3 are closed, 5 is opened, and the gas sample bulb is evacuated. The bulb is heated in a bushy flame at this time to drive off absorbed water. The liquid nitrogen trap is removed from the CO_2 trap and placed around the gas sample bulb, and a second Dry Ice–methanol bath is placed around the CO_2 trap. Valve 4 is closed, 3 is opened, and the CO_2 is "distilled" into the sample bulb for 1.5 min. The pure CO_2 is then introduced into the dual collector mass spectrometer (a modified consolidated Nier Model 21-201) for isotopic

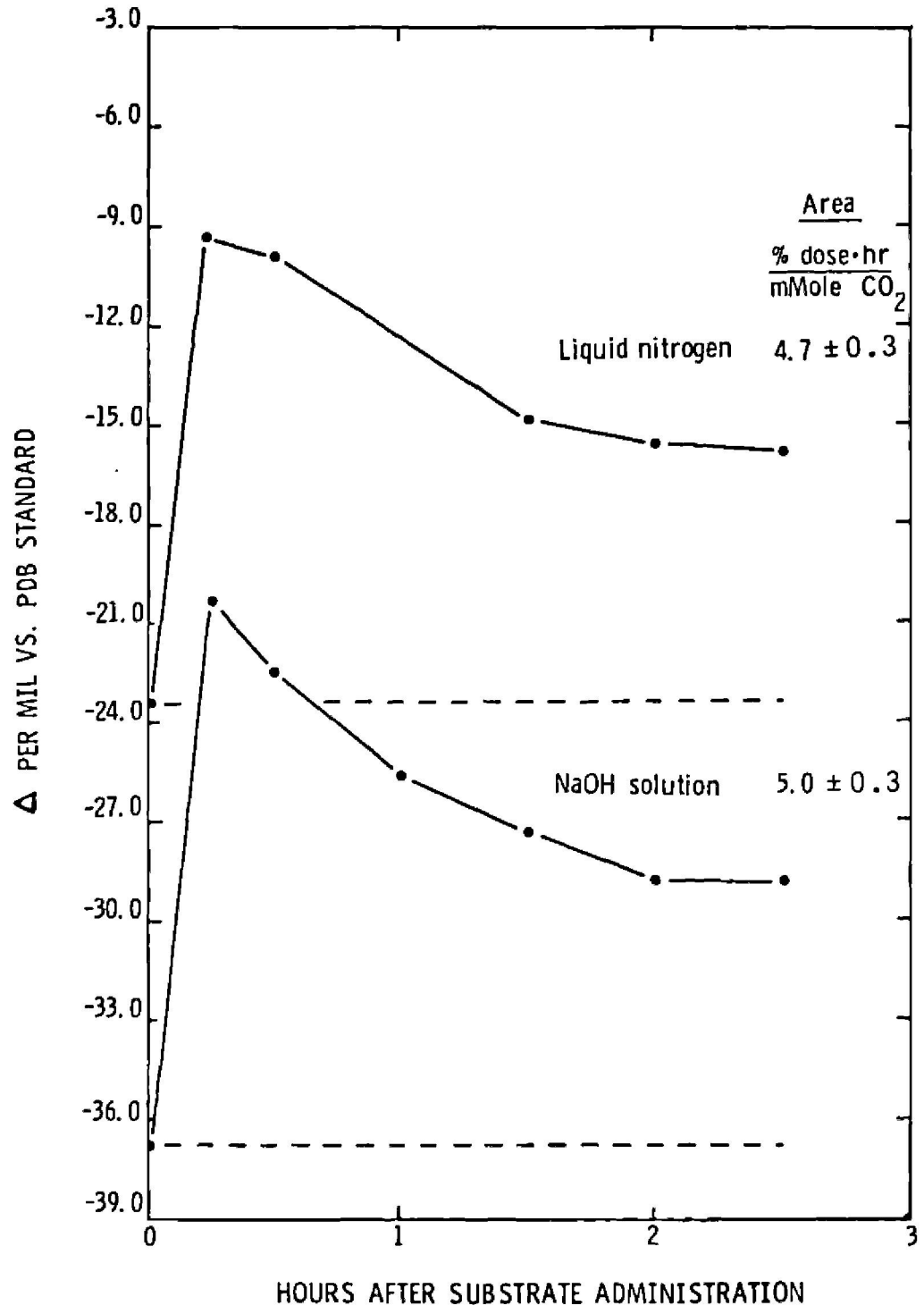

Fig. 2. Isotope fractionation during collection of CO_2 as $CO_3^=$ in 2N NaOH compared to an unfractionated sample collected in a liquid nitrogen–cooled trap. Immediately after collecting the basal CO_2 samples at time 0, 2 mg/kg of aminopyrine were administered.

measurement. The percent of label recovered per millimole of CO_2 was calculated with the following equation:

$$\%\ \text{recovery/mM}\ CO_2 = (\delta^{13}C_a - \delta^{13}C_b)\ R_s \times 10^{-1}/d \qquad (2)$$

where $\delta^{13}C_b$ and $\delta^{13}C_a$ are the per mil isotopic enrichments of $^{13}CO_2$ before and after substrate administration, R_s is the $^{13}C/^{12}C$ ratio of the CO_2 standard, and d is the dose of labeled substrate administered (in milliequivalents). Eq. 2 is accurate to within 2% for enrichments of up to 100‰. At greater enrichments the error increases as a result of several approximations made during the derivation.

Results. Dynamic collection of CO_2 as $CO_3^=$ in NaOH was shown to result in fractionation of the carbon isotopes, i.e., the lighter isotope was trapped in preference to the heavier isotope. The magnitude of this fractionation was established to be -13 ± 0.8‰ (95% confidence limits) by comparing the isotope ratios of samples collected alternately in NaOH and a liquid nitrogen–cooled trap (Fig. 2). Collection in this latter manner was

shown to be nonfractionating, within an experimental error of 0.5‰, by analyzing CO_2 samples of known isotopic abundance. Comparison of the amount of CO_2 collected per unit of time by these two methods indicated that the trapping efficiency of the NaOH was between 10% and 20%.

Attempts to isolate and eliminate the cause of the fractionation included bubbling the breath through a longer column of NaOH solution by using the 1 by 15 cm centrifuge tube in place of the 250 ml flask during collection. Under these conditions, the fractionation became very erratic, varying between −5 and −20‰. Alternatively, collection as $CO_3^=$ under static conditions, in which the NaOH solution was permitted to absorb the CO_2 from exhaled breath over a 30 min period, eliminated the fractionation. Although the static technique eliminated the carbon isotope fractionation, the 30 min of shaking added to the time required to perform the test.

Although the dynamic method of CO_2 collection resulted in low trapping efficiency and isotopic fractionation, the amounts of excess $^{13}CO_2$ excretion calculated from the data in Fig. 2 for the two collection techniques were statistically identical. This results from the high reproducibility of the isotopic fractionation in the NaOH sampling techniques employed and the collection of a basal CO_2 sample before substrate administration to serve as a control or baseline value.

While the use of Eq. 2 does not require a knowledge of the absolute abundance of ^{13}C in the sample, an awareness of the possible sources of isotope fractionation and their effects is essential in performing and interpreting $^{13}CO_2$ breath tests. This awareness is more important in dealing with the ^{13}C isotope than the ^{14}C isotope because enrichments near natural abundance are involved. For example, an isotope effect that favors $^{12}CO_2$ collection over $^{14}CO_2$ by 1% would introduce an error of 1% in ^{14}C measurements, but would be difficult to observe because of the counting statistics involved in such a measurement. However, a 1% isotope effect in the collection of $^{12}CO_2$ and $^{13}CO_2$ would result in a 10‰ change in the $^{13}CO_2$ abundance ratio, as much as might result from the peak appearance of labeled substrate $^{13}CO_2$. Fortunately, it is not the presence of fractionation per se that causes difficulty; rather it is the variations in the degree of isotope fractionation from one sample to another. Consider the isotope fractionation that occurred during the collection of CO_2 in NaOH, which presumably results from a kinetic isotope effect of the absorption boundary.[15] When the CO_2 collection was performed by bubbling exhaled air through the NaOH solution in a centrifuge tube, there was a wide variation in the degree of isotope fractionation. This variation was greater than the endogenous variations in $^{13}CO_2$ abundance and would have reduced the sensitivity of the test by a factor of 5 to 10. On the other hand, the use of the 250 ml collection flask, containing 10 ml of NaOH, reduced the variability of the isotope fractionation to an insignificant level (SD = 0.17‰) even though the absolute depletion of $^{13}CO_2$ was still −13‰ in all samples. Future developments may make it possible to eliminate this isotope fractionation. Preliminary results in our laboratory have shown that collection of whole breath in a 50 ml evacuated tube and subsequent cryogenic isolation of CO_2 from a 5 ml aliquot eliminates isotope fractionation completely.

Sample storage. CO_2 samples that were stored in the plastic-lined screw-capped vials were found to be unaltered during storage. Aliquots from a single sample were released from the NaOH solution and analyzed at 7, 41, 50, and 59 days after collection. The respective isotope ratios were −34.0, −33.9, −34.1, and 33.9‰. Between analyses the sample was stored at room temperature. This stability is important since most laborato-

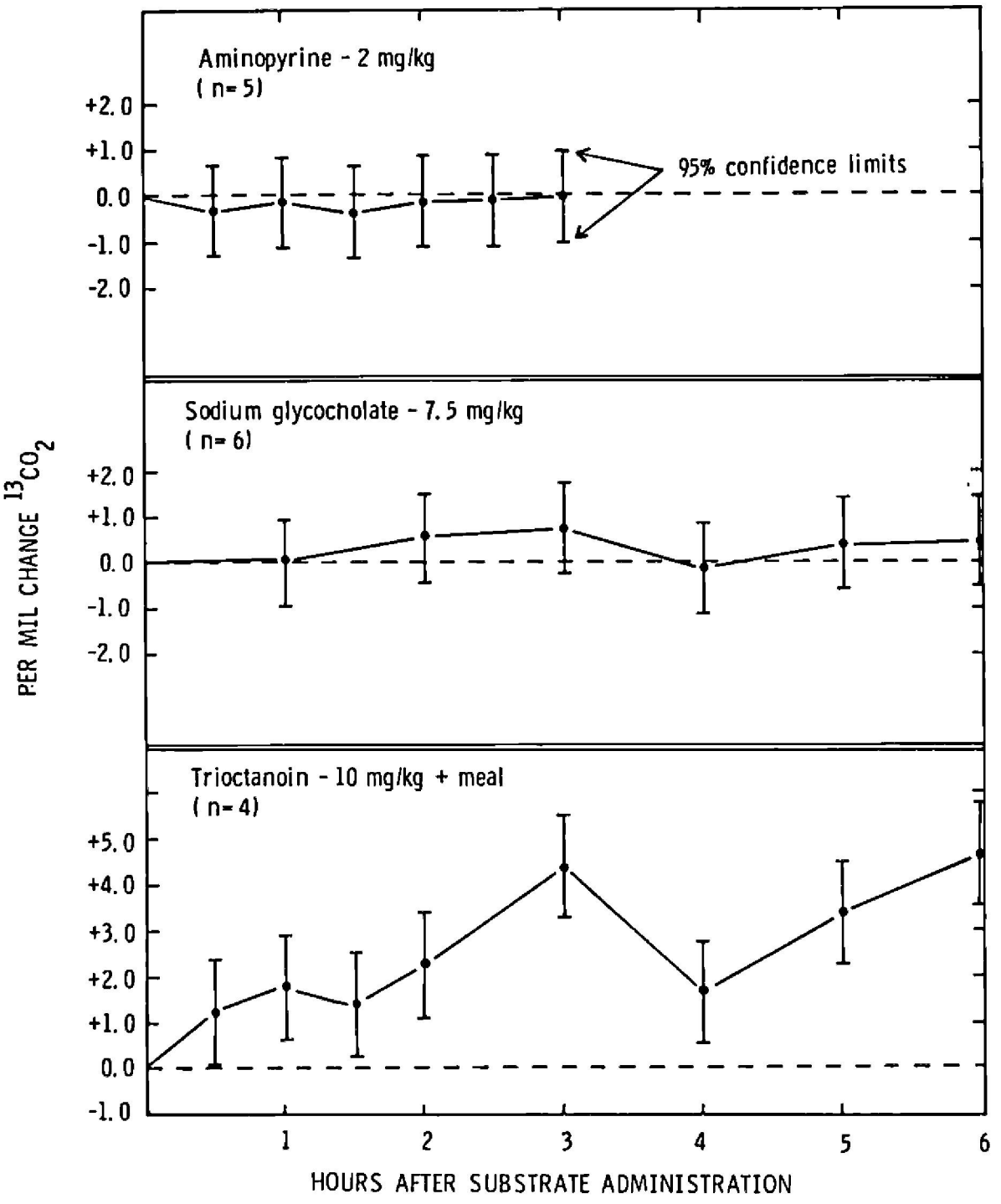

Fig. 3. Effect of unlabeled (natural abundance) substrate on the $^{13}CO_2$ abundance in breath.

ries lack the equipment necessary to measure the $^{13}C/^{12}C$ ratio in expired CO_2 and therefore must send the samples to a central facility for analysis.

Variations in $^{13}CO_2/^{12}CO_2$ ratios

Fasting subjects. The natural variations in the isotope ratios of breath CO_2 were determined by analyzing CO_2 samples collected at 1 hr intervals over 6 hr from two fasting adult subjects. For each subject the ratios varied about a central value with an average standard deviation of 0.79‰ (n = 12). The influence of the unlabeled substrates on the ratios was determined by determining the ratio before and after substrate administration. Sodium glycocholate and aminopyrine administration did not alter the isotope ratios (Fig. 3). The observed standard deviations in the baseline isotope ratios were 0.90‰ for glycocholate (six subjects, 46 samples) and 0.72‰ (five subjects, 35 samples) for aminopyrine, which are not significantly different ($p > 0.5$) from the standard deviation observed in the absence of substrate administration.

An analysis of variance was performed to isolate the source of the random fluctuations

Table I. Variance associated with each step of the $^{13}CO_2$ analysis

Source	*Variance (‰²)*	*Analyses*
Total variance	0.518	39
Ratio determination	0.002	10
CO_2 release	0.017	9
CO_2 collection	0.029	14
Residual (patient endogenous fluctuation)	0.470	

in the isotope ratio of the expired CO_2. Sample collection, CO_2 release and purification, and ratio measurement were performed in replicate. The results are shown in Table I. The variance introduced during these three steps was found to be small, collectively accounting for less than 10% of the total variance. The remaining 90% of the variance (0.470 [‰]²) reflected the actual changes in the $^{13}CO_2/^{12}CO_2$ ratio of the expired CO_2.

Influence of food. The addition of gram quantities of nutrients to the substrate produced significant increases in the ^{13}C abundance of expired CO_2. Examples of this are shown in Fig. 3 and 4. The 90 cc of standard Enfamil that was administered with the unlabeled trioctanoin caused a 4 to 5‰ rise in the baseline. This presumably resulted from the oxidation of the carbohydrates from the Enfamil. Similarly, the addition of 10 gm of sucrose to sweeten the bitter-tasting sodium glycocholate solution caused a significant ($p < 0.002$) rise of 5‰ in the $^{13}CO_2$ abundance (Fig. 4) and replacement of the sucrose with 5 drops of saccharin entirely eliminated this effect.

The increase in the amplitude of the fluctuations in the $^{13}CO_2 : {}^{12}CO_2$ ratios after eating was attributed to differences in the isotope ratio of the carbon being oxidized to CO_2. The subject who had fasted overnight (about 12 hr) before the breath test should have been producing CO_2 mostly from fatty acid oxidation,[16] whereas the subject who had ingested a meal containing carbohydrates and proteins would have an increased percentage of CO_2 arising from these two sources[17] and, since they both tend to be more abundant in ^{13}C than fatty acids,* the relative abundance of $^{13}CO_2$ in breath would increase, reflecting the increase in the ^{13}C content of the carbon being oxidized to CO_2.

The increased isotope ratio of expired CO_2 following the ingestion of naturally enriched carbon need not always be an interference. Lacroix and co-workers[22] have used it to their advantage in a $^{13}CO_2$ breath test for glucose utilization by using naturally enriched glucose derived from corn starch in place of the more expensive ^{13}C glucose obtained by organic synthesis.

Effects of mild exercise. Mild exercise was found to increase the magnitude of the fluctuations in the ^{13}C isotope abundance of exhaled CO_2. During an experiment in which the respiratory CO_2 was collected at hourly intervals for 6 hr after the administration of unlabeled glycocholate, the average standard deviation in the $^{13}CO_2$ abundance for the two

*As classes, lipids, carbohydrates, and proteins are known to have different average isotope ratios. Degens[12] reported the median ^{13}C isotopic abundances of protein, carbohydrate, polar lipids, and nonpolar lipids of marine plankton to be −17‰, −19‰, −21‰, and −29‰, respectively. Moreover, within each group, the isotope ratios reflect the isotope enrichment or depletion that occurred during the synthesis of the compound, and therefore the ratios will vary depending on the specific compound and source. For example, Meinschein et al [18] reported a 4‰ difference between commercial glacial acetic acid and acetic acid from cider vinegar and as much as a 20 to 30‰ difference between the individual carbons in the acetic acid. The enrichment in ^{13}C is most pronounced when the carbohydrate is produced from carbon that was fixed by the Hatch-Slack or C_4 photosynthetic pathway,[19] as this pathway yields carbon that is about 15‰ more abundant in ^{13}C[20] than carbon fixed by the more commonly utilized Calvin or C_3 pathway.[21]

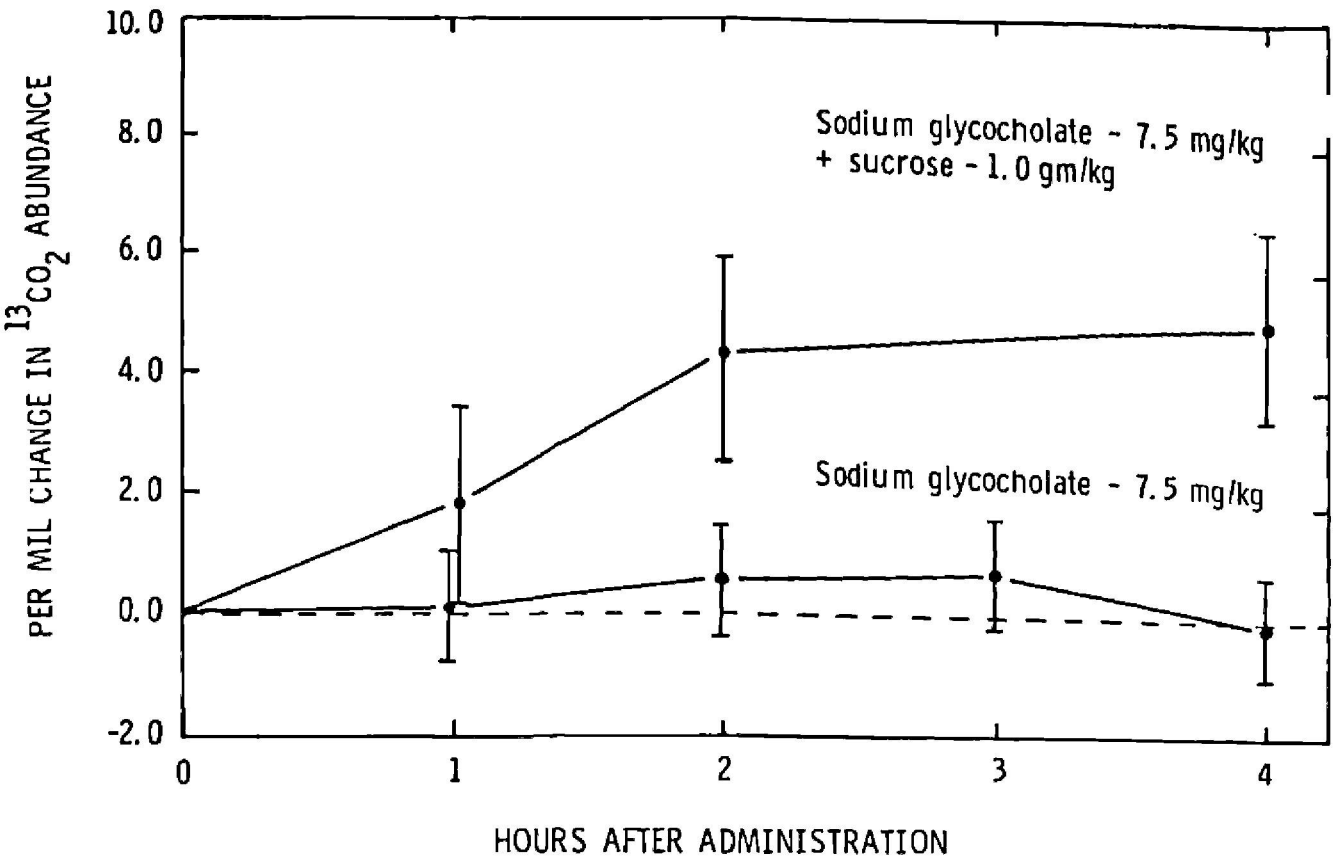

Fig. 4. Effect of the ingestion of 10 gm of sucrose on the sodium glycocholate substrate baseline.

subjects who were restricted to their rooms was 0.51‰. The subjects who were permitted to perform their normal, nonstrenuous duties about the hospital exhibited a significantly greater ($p < 0.005$) average standard deviation of 1.82‰.

Exercise increases both the percentage of CO_2 arising in the skeletal muscle mass[23] and the mobilization of stored nutrients; again changing the percentage of carbohydrate, protein, and lipid being oxidized to CO_2, and altering the $^{13}CO_2/^{12}CO_2$ ratio of expired CO_2 as previously discussed. In addition, the CO_2 produced in the muscle mass during exercise dilutes the labeled $^{13}CO_2$, whose production by hepatic or intestinal enzyme activity is minimally affected by exercise.[24] This dilution reduces the sensitivity of the test and should be avoided by keeping the patient at rest.

Even in the resting state, the determination of excess $^{13}CO_2$ production from a labeled substrate is affected by total CO_2 production in the patient. Strictly speaking, in order to make quantitative comparisons of label oxidation between subjects, it is necessary to know the individual CO_2 production rates so that the exact excretion can be calculated. In the absence of this measurement, the comparison is not rigorously quantitative[25] because of the variation in CO_2 production rates between patients. In actual practice, the relative standard deviation of these production rates is less than 25% for resting adults[23] and children (J. B. W., unpublished data), and omission of this correction will not significantly reduce the diagnostic value of most breath tests in which the responses of normal and diseased patients differ by a factor of 2 or more.

Calculation of minimum dose

Minimum detectable change. As indicated earlier, the most significant factor affecting the sensitivity and precision of the $^{13}CO_2$ breath test is the normal variability of the baseline $^{13}CO_2:^{12}CO_2$ ratio of exhaled CO_2. The fluctuations occurred with a standard deviation of 0.72‰. Thus, the minimum detectable change (2 S.D. definition) is 1.4‰. At a CO_2 production rate of 9 mM/kg-hr, a 1.4‰ increase corresponds to the oxidation

and excretion of 140 nM/kg-hr of ^{13}C label. For a 6 hr breath test performed with sample collections at each hour, the detection limit for label excretion is 870 nM/kg. This minimum can be further reduced to 670 nM/kg by collecting two basal CO_2 samples at 30 min before and immediately before substrate administration, and averaging their $^{13}CO_2$ relative abundances to obtain a better estimate of the basal $^{13}CO_2 : ^{12}CO_2$ ratio. The increased sensitivity results from the increased precision with which the basal $^{13}CO_2 : ^{12}CO_2$ ratio is known.

Calculation of dose. The use of the test to discriminate between healthy and diseased patients would typically require a dose that is 2 to 20 times the minimum detectable dose. For example, if the detectable excretion must be 5% of the dose, the dose must be 20 times the minimum dose or 14 μM/kg.

Recommendations

The methodology that we have developed for performing $^{13}CO_2$ breath tests makes it possible to extend the benefits of this safe and sensitive test to subjects in whom radioactive carbon cannot be used. To obtain maximum sensitivity from breath test with ^{13}C labeled compounds, the following precautions should be observed.

1. The subject should fast overnight before the test and throughout the test. If this is not feasible, as in the case of infants, the meals should be as low as practical in carbohydrate and, secondarily, protein.

2. The test protocol should not produce a change in the endogenous $^{13}CO_2$ abundance of the expired CO_2 other than that resulting from label oxidation. Test protocols can be investigated by conducting "substrate baseline" breath tests with unlabeled substrate. These are required during initial development of the test only and do not have to be conducted as a part of each patient study.

3. Serial respiratory CO_2 samples should be collected prior to substrate administration to provide increased precision in the estimate of initial isotopic abundance.

4. Endogenous CO_2 production in the patient should be kept at a minimum by restricing activity and maintaining the patient at rest throughout the test.

5. CO_2 collection and release procedures should be carefully standardized to avoid variation in isotope content due to fractionation effects.

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