INVESTIGATION OF APPARENT ISOTOPE EFFECT BETWEEN $^{13}CO_2$ AND $^{14}CO_2$ TRIOCTANOIN BREATH TESTS

by

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ABSTRACT

A decrease in the recovery of $^{13}CO_2$ relative to $^{14}CO_2$ has been previously reported at low oxidation ~~ratio~~ rates of 1-C- labeled trioctanoin. To determine whether or not this phenomenon is due to an isotope effect, and if so, to identify the metabolic step at which it occurs, the in vivo oxidation of ^{13}C and ^{14}C acetate, octanoate and trioctanoin were compared. The ^{13}C and ^{14}C substrates were administered simultaneously to rats whose expired CO_2 was collected in 1N NaOH, and subsequently released by acidification, and cryogenically purified in vacuum. Total CO_2 was measured manometrically, and the ^{14}C and ^{13}C recoveries were determined by scintillation counting and mass spectrometry, respectively. The trioctanoin breath tests did not reproduce the previously reported isotopic discrepancy. Further investigation revealed that the emulsified corn oil used as a pancreatic stimulant significantly depressed the endogenous $^{13}CO_2$ abundance in breath and caused the discrepancy. These studies did reveal a small time delay in the appearance of $^{14}CO_2$ relative to $^{13}CO_2$ during the trioctanoin breath test, which is probably due to a kinetic isotope effect. This difference in oxidation rates, however, is too small to affect the results of clinical breath tests.

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TRIOCTANOIN

During the last 5 years, a series of clinical $^{13}CO_2$ breath tests has been developed as a non-hazardous alternative to ^{14}C breath tests (1). Although the preference for the use of stable isotopes in human studies is generally acknowledged, the two tests must be demonstrated to be equivalent before the $^{13}CO_2$ breath tests can be considered as a replacement for the $^{14}CO_2$ breath test.

Several validations of the $^{13}CO_2$ breath test against its $^{14}CO_2$ analog have been reported, each with a different degree of agreement between the isotopic recoveries of the 2 labels. Almost exact equivalence was demonstrated for ^{13}C and ^{14}C aminopyrine (2), but a discrepancy in recoveries has been reported for the trioctanoin breath test (3). The labeled CO_2 recoveries following oral administration of labeled trioctanoin were in good agreement whenever the trioctanoin oxidation rate was fast enough to yield an excess $^{13}CO_2$ excretion of $30^{o}/oo$ or more above the natural abundance level. However, at an oxidation rate that produced less than a $5^{o}/oo$ increase in $^{13}CO_2$ abundance, the $^{13}CO_2$ recovery was only half that of $^{14}CO_2$ (Figure 1). A similar discrepancy has been reported during the validation of ^{13}C glycocholate breath test (4). Here, the recoveries of $^{13}CO_2$ were only about 60% of $^{14}CO_2$ for a series of simultaneous measurements in which the excess $^{13}CO_2$ abundance ranged from 1 to $20^{o}/oo$.

These discrepancies are quite surprising because one would expect the two isotopes of carbon to behave in the same manner. However, the possibility of an interfering isotope effect should be considered. The labeled carbon must undergo a series of metabolic reactions and pass through numerous metabolic pools, each of which may introduce some isotopic discrimination. It is also possible that these discrepancies are artifacts, arising either from the CO_2 sampling and analysis, or perhaps from some effect of the test protocol itself.

In this study, we have examined the trioctanoin breath test to establish whether the reported isotopic discrepancy was an artifact or whether it was due to some in vivo isotope effect. If it was the latter, we wished to identify the metabolic step at which it occurred. This has been established using simultaneous ^{13}C and ^{14}C breath tests with carboxyl labeled acetate, octanoate, and trioctanoin in animal studies using rats.

Methodology:

Animal Studies: The comparison of the recoveries of $^{13}CO_2$ and $^{14}CO_2$ in breath following the administration of ^{13}C and ^{14}C labeled substrates were carried out using the rats. The rats, each weighing approximately 400 g were fasted overnight. The rats were placed in individual 2.5ℓ sealed metabolic cages for collection of baseline CO_2 before substrate administration. Laboratory air was drawn through each cage at 2.1ℓ/min. Ambiant CO_2 was scrubbed from the air before it entered the cage by drawing the air through a tube filled with wet NaOH. Air leaving the cage was split to provide a flow of 0.8ℓ/min through the CO_2 collection tower. The remainder was discarded into a hood. The collection tower, to which 5 ml of 1N NaOH had been added, consisted of two vertical, concentric glass tubes separated by a spiral of 4 mm glass rod. Because of the spiral path, air entering the base of the tower would remain in contact with the NaOH solution for a distance of 75 cm in a vertical height of 10 cm. Complete CO_2 adsorption has been demonstrated for this adsorption tower by monitoring the effluent for any residual CO_2 by infra red spectrometry.

After collecting two 15 minute baseline CO_2 samples, the rat was removed from the cage and the substrates were administered intragastricly in a volume of 0.5 to 1 ml of fluid. The rat was returned to the metabolic cage and breath sampling was continued.

Substrates:

Acetate: 1 ^{13}C-acetate (90 atom % excess) was purchased from Merck, Sharp and Dohm (Montreal, Canada) and 1 ^{14}C acetate (0.99 m Ci/mg) was purchased

from Amersham Searle (Arlington Heights, Ill.). In order to obtain a wide range of labeling in the five studies that were performed, the ^{13}C acetate was dosed at either 0.2, 0.5, or 1 mg. The ^{13}C acetate was mixed with 0.5 uCi of ^{14}C acetate in 1 ml of distilled water. Between 0 and 2.5 mg of natural abundance acetate was added as a carrier. Following oral administration, breath samples were taken continuously in 15 min intervals for 2 hrs.

Octanoate: 1 ^{13}C-octanoate (90 atom % excess) was obtained from the Stable Isotope Resource at Los Alamos (New Mexico) and 1 ^{14}C-octanoate was (0.026m Ci/mg) was purchased from New England Nuclear (Boston, Mass.). Either 1, 2.5 or 5 mg of ^{13}C octanoate was mixed in 1 ml of water with 0.5 uCi of ^{14}C octanoate for administration to the rats. Between 0 and 5 mg of natural abundance octanoate was used as a carrier. A total of 6 studies were performed. Breath CO_2 was collected continuously in 30 min fractions for 5 hrs after administration.

Trioctanoin: Tri-1 ^{13}C octanoin was synthesized from the octanoate by Nu Chek Prep (Elysian, Minn.) and the tri-1 ^{14}C octanoin (0.017 mCi/mg) was obtained from New England Nuclear. Between 2 and 5 mg of ^{13}C trioctanoin was mixed with 0.5 m Ci ^{14}C trioctanoin in 200 mg of Lipomul (Upjohn Co., Kalamazoo, Mich.) an emulsified corn oil. In four of the six studies, 8 to 10 mg of natural abundance trioctanoin was added as a carrier.

Analytical Procedures: The CO_2 was released from 2 ml of basic solution in vacuum by the addition of 2 ml of degassed 20% H_2SO_4 solution. The CO_2 was then cryogenically purified using a -94°C bath to trap water and a -196° bath to trap CO_2. Details are given elsewhere (5). Following purification, the CO_2 was measured manometrically and divided in half. One aliquot was transferred to an evacuated rubber septum capped, 50 ml tube (Vacutainer TM, Becton and Dickenson, Rutherford, New Jersey) and 5 ml of 50% methanol/ethanolamine (New England Nuclear) was added and allowed to adsorb the CO_2 for 30 min. The ethanolamine was then transferred to a scintilation vial, 10 ml of Sintisol (Isolab Inc., Akron, Ohio) was added and the ^{14}C was measured by scintillation counting. The $^{13}CO_2$ abundance was measured on the other aliquot of the CO_2 sample on a Nuclide 3-60 Sectorr mass spectrometer (State College, Pa.) as previously described (5).

The label recoveries were calculated as follows:

$$^{13}C \text{ \% dose/mmol } CO_2 = k \times \frac{\Delta\ ^{13}C}{\text{mmol administered}} \times 100 \qquad (1)$$

$$^{14}C \text{ \% dose/mmol } CO_2 = \frac{\text{dpm/mmol CO}}{\text{dpm administered}} \times 100 \qquad (2)$$

$$\Delta\ ^{13}C = \delta^{13}C_{\text{time t}} - \delta^{13}C_{\text{time 0}} \qquad (3)$$

$$\delta^{13}C = \left[\frac{(^{13}C/^{12}C) \text{ sample}}{(^{13}C/^{12}C) \text{ reference}} - 1 \right] \times 10^3 \qquad (4)$$

$$K = \frac{R_{std}}{10^3} \times \frac{M}{(P)(n)} \times 100 \qquad (5)$$

where R_{std} is the $^{13}C/^{12}C$ ratio of the standard CO_2 gas, M is the molecular weight of the substrate, P is the isotopic purity in atom percent excess,

and n is the number of labeled carbons per molecule.

The total dose recoveries were calculated by multiplying the percent dose/mmol values by the total CO_2 production. This was obtained from the concentration of CO_2 per ml of collection fluid as measured manometrically, the total volume of collection fluid, and the known split of air flow between the collection tower and the bypass.

Calculation of Relative Reaction Rates:

The overall intermolecular kinetic isotope efrect (k^{14}/k^{13}) for the catabolism of the labeled fats can be calculated from the change in the enrichment of the isotopes in breath CO_2 relative to the enrichment in the starting materials (r) as a function of the completion of the reaction (f). From Raaen (6), the relation of these factors is:

$$k^{14}/k^{13} = \frac{\ln (1-rf)}{\ln (1-f)} \qquad (6)$$

Now, since thre results are expressed as the cumulative percent of administered dose, the relative activity of ^{14}C to ^{13}C in the starting materials is 1, and r is therefore given by:

$$r = \frac{\Sigma \text{ \% dose } {}^{13}C}{\Sigma \text{ \% dose } {}^{14}C} \qquad (7)$$

The completion of the reaction of fat to CO_2 is

$$f = 1-\Sigma\% \text{ dose } {}^{13}C/100 \qquad (8)$$

Substituting into equation 6 gives

$$k^{14}/k^{13} = \frac{\ln (1- \ \Sigma\% \text{ dose } {}^{14}C/100)}{\ln (1- \ \Sigma\% \text{ dose } {}^{13}C/100)} \qquad (9)$$

Thus a plot of ln (1- Σ% dose $^{14}C/100$) vs. ln (1- Σ% dose $^{13}C/100$) will yield a line with a slope of k^{14}/k^{13}.

Results and Discussion:

Three methods of data reduction were employed in analyzing the results from the simultaneous $^{13}C/^{14}C$ breath tests. They were employed in order to detect either a constant proportional difference in isotope recoveries, an absolute difference between the two isotopes, or a difference in the rates of conversion of the two isotopes to CO_2.

To detect a proportional difference, the $^{13}CO_2$ recoveries were graphed against the $^{14}CO_2$ recoveries and a regression of the ^{13}C recovery against ^{14}C was performed. The regression constants for the three substrates are shown in Table 1. The slopes of the regression lines are not significantly different from unity ($p>.05$) nor are the intercepts significantly different from zero ($p>.05$). Thus there is no systematic discrepancy of greater than 5%, the limits of precision, between the recoveries of $^{13}CO_2$ and $^{14}CO_2$ following oral administration of acetate, octanoate or trioctanoin.

A second comparison similar to Figure 1 was made by plotting the ratio of the ^{13}C to ^{14}C recovery as a function of the per mil excess $^{13}CO_2$. This method of comparison is very sensitive to perturbations at low rates of label recoveries. Again, no difference between ^{13}C and

^{14}C recoveries was observed. The plot for the averaged recoveries after trioctanoin administration is shown as an example (Figure 2). This is in disagreement with the previously reported comparison of the ^{13}C and ^{14}C trioctanoin breath test, where the recovery of $^{13}CO_2$ decreased relative to $^{14}CO_2$ as the per mil excess ^{13}C decreased.

In order to explain the disagreement between this study and the previously reported trioctanoin study a series of substrate baseline studies were performed using Lipomul alone without the addition of ^{13}C labeled trioctanoin. Although the Lipomul did not alter the isotopic content of the CO_2 produced by the rats; a small, but significant decrease in $^{13}CO_2$ abundance of expired CO_2 was observed in human studies. This decrease was about 2°/₀₀ and it would result in an underestimation of the $^{13}CO_2$ recovery from ^{13}C labeled trioctanoin. Correction of the ^{13}C recovery for this depression of the baseline in the earlier reported validation of $^{13}CO_2$ trioctanoin breath test eliminates the decrease in $^{13}CO_2$ recovery shown in Figure 1 and brings the relative recoveries of $^{13}CO_2$ and $^{14}CO_2$ into good agreement.

The previously reported discrepancy between ^{13}C and ^{14}C recoveries during the trioctanoin breath test is therefore an artifact resulting from a shift in the baseline $^{13}CO_2$ abundance. This result again demonstrates the importance of performing unlabeled substrate baseline studies during the development of $^{13}CO_2$ breath tests, and more importantly, in performing them under the exact conditions used in the breath test with subjects who will closely match the subjects in which the breath test is to be performed.

While the first two comparisons did not demonstrate any difference between the two isotopes, the third comparison did reveal a small time dependent discrepancy. The shapes of the curves describing the excretion of the labels were nearly identical, but there was a slight delay in the rise of the $^{14}CO_2$ relative to $^{13}CO_2$ excretion and the rate of decay of the $^{14}CO_2$ excretion after the peak value tended to be slightly slower than the $^{13}CO_2$ (Figure 2). This effect was most evident for trioctanoin which had the slowest rate of metabolism and was negligible for acetate which was the most rapidly metabolized substrate. It should be noted that the label excretion following acetate administration peaks during the first breath collection interval and thus the acetate data does not lend itself to this time course analysis.

The difference in excretion rates for the two isotopes was most evident when the ratio of the cumulative recovery of ^{13}C to ^{14}C was plotted against time (Figure 3). In the early portion of the test when the label excretion rate was increasing, the cumulative recoveries differed by 5 to 10%. The difference then decayed toward zero as the label excretion rate peaked and began decreasing. This observation is consistant with a kinetic isotope effect which would discriminate against the heavier isotope and thus decrease the rate of ^{14}C metabolism and excretion.

The overall isotope effect was calculated for the three substrates (Table 3). Unfortunately the 5% random variation in the label recoveries between individual breath tests was large relative to the isotope effect and did not permit an accurate value for k^{14}/k^{13} to be calculated.

Further analysis is difficult because the isotope effect as calculated is the sum of any isotope effects that occur during hydrolysis, adsorption, transport, catabolism, and excretion as well as any isotope effects that occur during lipid storage.

Comparison of ^{13}C and ^{14}C breath tests following the administration of labeled acetate, octanoate and trioctanoin demonstrates the near equivalence of the stable isotope and radioactive isotope tests. Only a small decrease in the rate of $^{14}CO_2$ excretion relative to $^{13}CO_2$ excretion was observed. This difference was only significant during the rising portion of the excretion curve, and was negligible when the total areas under the excretion curve were compared. This small isotope effect would not have a significant influence on the clinical application of a CO_2 breath test because it is small relative to the patient-to-patient variations that are normally encountered.

SUBSTRATE	SLOPE	INTERCEPT % dose/mMole CO_2	CORR. COEF.
Trioctanoin	0.93 (0.05)	6×10^{-5} (4×10^{-5})	0.970
Octanoate	0.98 (.03)	7×10^{-6} (3×10^{-5})	0.987
Acetate	1.03 (0.04)	-7×10^{-5} (1×10^{-4})	0.984

Table 1. The regression of the individual time point recoveries of $^{14}CO_2$ onto $^{13}CO_2$. Standard deviations are given in parenthesis. None of the regression lines are significantly different from the theoretical line of unity through the origin.

SUBSTRATE	RELATIVE ISOTOPE EFFECT k^{14}/k^{13}
Trioctanoin	0.93 (0.04)
Octanoate	1.00 (0.05)
Acetate	1.01 (0.05)

Table 2. The relative ~~isotope effects~~ reaction rates for the excretion of labeled CO_2 following labeled substrate administration.

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Figure Legends

Figure 1. A decrease in the relative recovery of ^{13}C to ^{14}C at low oxidation rates of trioctanoin has been observed in an earlier study (3).

Figure 2. Reinvestigation of the simultaneous $^{13}C/^{14}C$ trioctanoin breath test failed to reproduce the effect shown in Figure 1. The effect in Figure 1 was shown to result from a 2 to 3°/oo decrease in the endog nous $^{13}CO_2$ production following ingestion of Lipomul. Data from 6 breath tests were averaged in 5°/oo increments.

Figure 3. A typical simultaneous ^{13}C (solid line) and ^{14}C (dashed line) breath test shows the trend for the $^{14}CO_2$ to lag behind the $^{13}CO_2$ excretion.

Figure 4. Average of 6 trioctanoin breath tests performed in the rat model. The cummulative recovery of ^{13}C is 10% greater than ^{14}C during the first hour and approached equivalence after the labeled CO_2 excretion begins to decrease after reaching its maximum at 2 hrs.

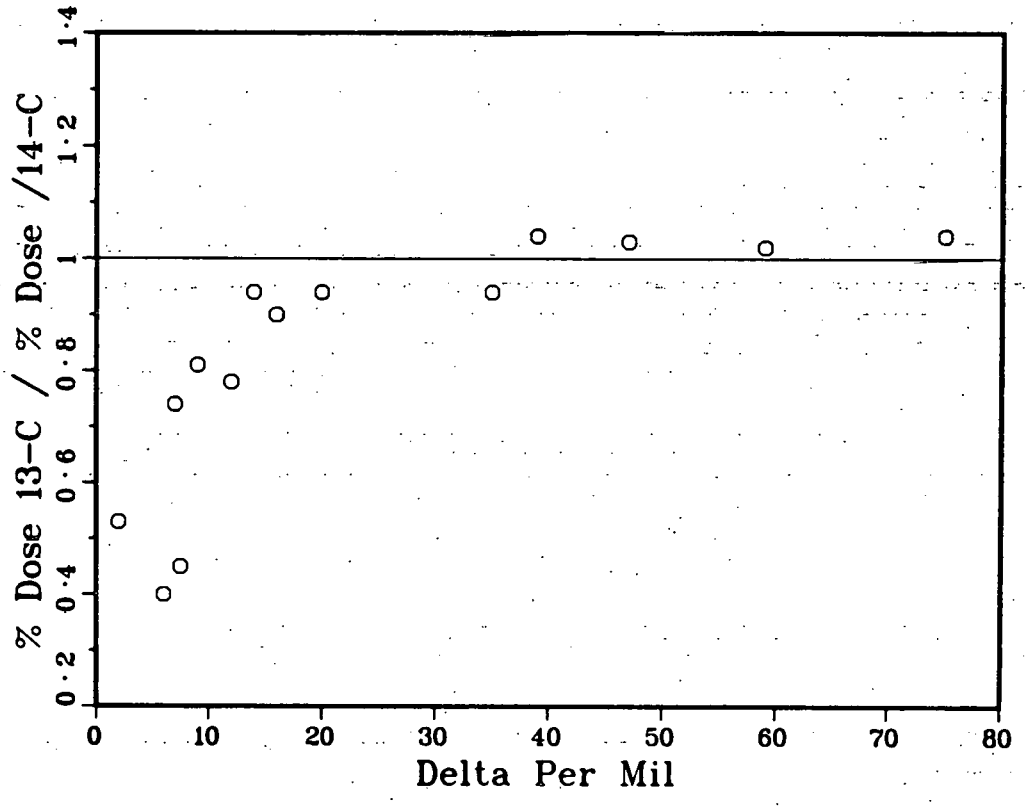
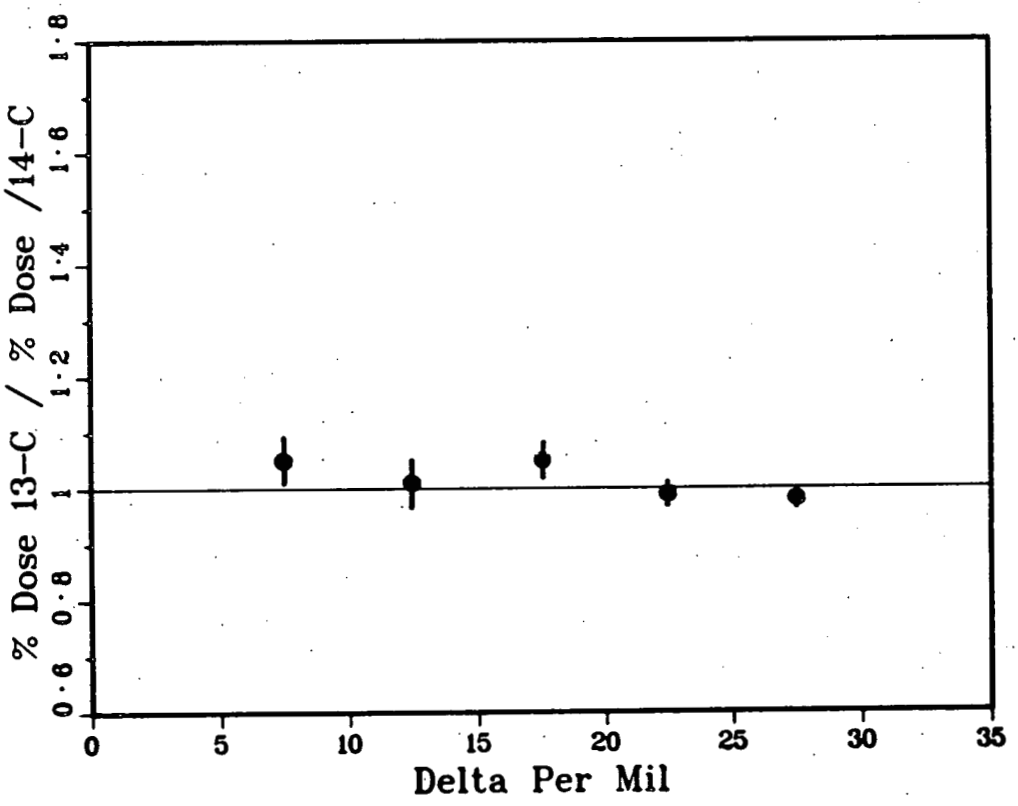

% Dose 13–C / % Dose /14–C
0·2
0·4
0·6
0·8
1
1·2
1·4
0
10
20
30
40
50
60
70
80
Delta Per Mil

FIGURE 1.


% Dose 13-C / % Dose /14-C
0·6
0·8
1
1·2
1·4
1·6
1·8
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5
10
15
20
25
30
35
Delta Per Mil

FIGURE 2.

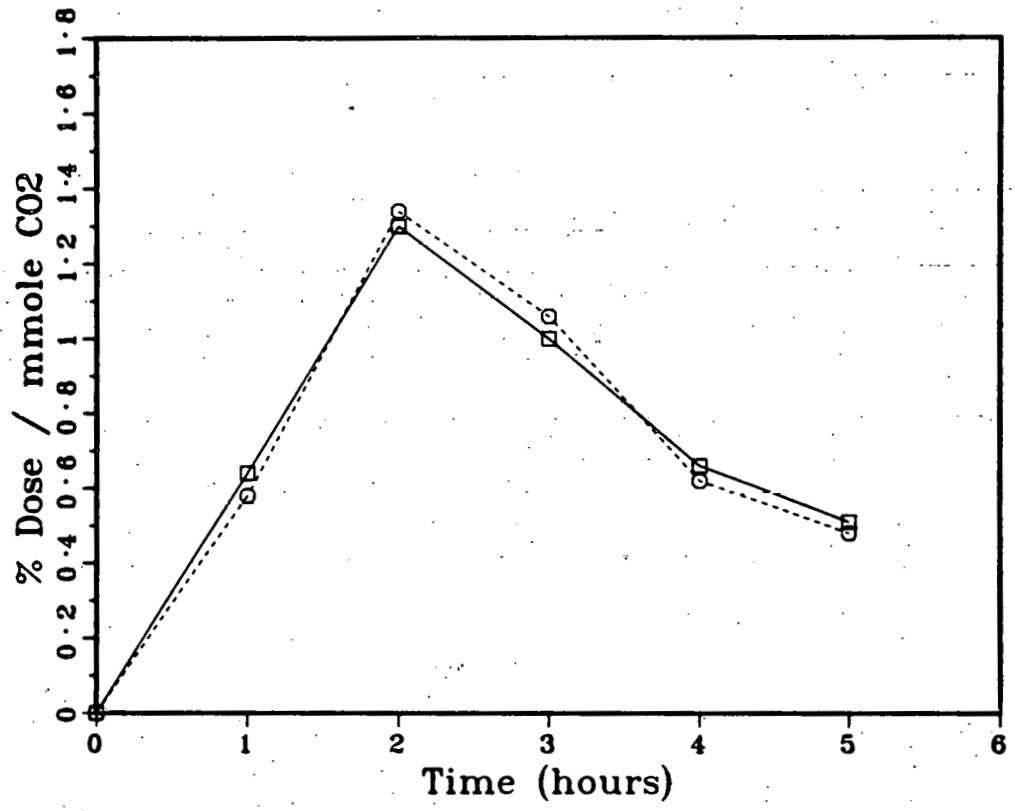

FIGURE 3.

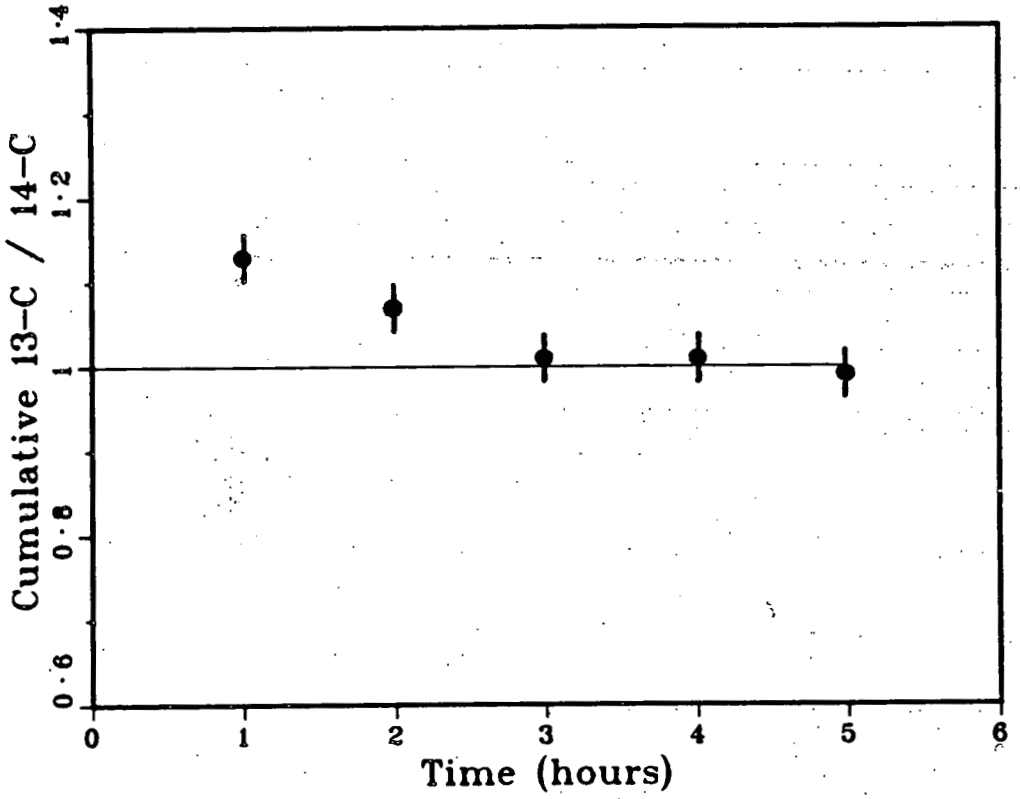

FIGURE 4.