Technical Note: Labeling of Forages with ^{13}C for Nutrition and Metabolism Studies[1,2,3]

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ABSTRACT: Alfalfa was labeled in the field with 99 atom % $^{13}CO_2$ and cut either the same day (C1) or 30 d after labeling (C30). The C1 alfalfa contained 84% of the ^{13}C label in cell contents, whereas C30 alfalfa contained 47% of the ^{13}C label in cell contents. In two separate trials, C1 and C30 alfalfa were dosed to two or four Suffolk ewes fed natural abundance alfalfa diets. Carbon isotope ratios ($^{13}C/^{12}C$, expressed as $\delta^{13}C‰$[parts per thousand] vs Pee Dee Belemnite standard) were determined for breath, feces, blood, and blood serum from ewes fed C1 alfalfa and blood and feces from ewes fed C30 alfalfa. In the C1 trial, carbon isotope ratios of respired CO_2 peaked 4 h after feeding, then declined to baseline levels by 40 h after the dose. Fecal samples increased in ^{13}C only slightly from 12 to 40 h after the meal. Blood serum values increased by approximately .5‰ from 0 to 4 h after the dose and remained relatively constant thereafter. In both trials, carbon isotope values from whole blood were constant. In the C30 trial, fecal samples peaked in carbon isotope value approximately 30 to 36 h after dosing, then declined; the time of this peak corresponded closely to that from a concurrent study that used a pulse dose of Yb-labeled alfalfa hay. Thus, when incorporated into cell wall material, the excretion pattern of ^{13}C in feces was similar to that of Yb-labeled hay, but little ^{13}C enrichment in feces was found when ^{13}C was primarily in cell contents of the labeled forage. When the soluble cell contents were enriched in ^{13}C, the marker was detected in respired CO_2 soon after feeding, which is consistent with the results of previous marker studies. These results demonstrate the feasibility of using forage labeled with the stable isotope ^{13}C in nutrition and metabolism studies. Carbon-13, not subject to the regulatory constraints associated with ^{14}C, provides a useful alternative when a carbon tracer is desired.

Key Words: Alfalfa, Sheep, Rumen Metabolism, Carbon Dioxide

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Introduction

The study of carbon metabolism and carbon flows is of general interest in both plant and animal systems. Most previous research in this area used the radioactive isotope ^{14}C as a tracer; however, regulatory and cost constraints have limited the use of radioactive isotopes in large animal research. Stable isotope research has expanded in recent years because of reduced safety and health concerns and increased availability of both labeled compounds and analytical facilities (Boutton, 1991b).

The two naturally occurring stable isotopes of carbon are ^{12}C (98.89% abundance) and ^{13}C (1.11% abundance). Cool-season (C_3) species such as alfalfa have lower isotope ratios than do warm-season (C_4) species such as corn and sorghum. Shifting animal diets from C_3 to C_4 species, or vice-versa, and measuring changes in carbon isotope ratios has been used to study digestibility of diet components (Bruckental et al., 1985) and metabolic turnover of carbon in ruminants (Tyrrell et al., 1984; Boutton et al., 1988;

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[3]Mention of trade names, proprietary products, or specific equipment does not constitute a guarantee of warranty of the product by the USDA and does not imply its approval to the exclusion of other products that may also be suitable.

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Wilson et al., 1988; Metges et al., 1990). However, such dietary shifts can change the ruminal microflora and fauna and fermentation processes (Van Soest, 1982).

An alternative approach to shifting between C_3 and C_4 plants is to artificially enrich a plant species in ^{13}C and feed the enriched material to test subjects. Boutton et al. (1987) described a procedure for enriching rice in ^{13}C and using enriched fractions to study nutrient absorption by humans. Others have labeled plants with $^{13}CO_2$ to study carbon translocation (Kouchi and Yoneyama, 1984; Mordacq et al., 1986). We used the procedures outlined by Svejcar et al. (1990) to enrich alfalfa in ^{13}C and fed doses of the labeled hay to sheep to assess the potential of this technique in nutrition and metabolism studies.

Materials and Methods

Labeling was conducted in a 1.4-m × 3.0-m × 1.1-m chamber constructed of 5-cm × 10-cm dimensional lumber and covered with clear plastic. The chamber was placed in an alfalfa sward, and soil was used to seal around the base. During each of two labeling periods 5 L of 99 atom% $^{13}CO_2$ were released into the chamber at approximately 0700 and circulated with two 15-cm fans. The chamber was removed at approximately 1100. The first labeling was conducted on June 7, 1988, and the alfalfa was cut with a sickle bar mower at 1700 on the same day (**C1**). The second labeling was conducted on September 9, 1988, and forage was cut 30 d after labeling (**C30**). In both cases, the sward height was between 50 and 60 cm, and the growth stage was earlier than one-tenth bloom when the sward was cut. The cut hay was placed on a greenhouse bench and air-dried at 25 to 30°C for 5 d. Dried hay was ground in a hammer mill to pass a 2.5-cm screen. Details of $^{13}CO_2$ labeling were presented by Svejcar et al. (1990).

Two trials were conducted in which labeled alfalfa was fed to two or four ruminally cannulated Suffolk ewes. All surgical procedures were approved by the University of Nevada-Reno Animal Care Committee, and animal care followed procedures outlined in the Consortium (1988). Ewes were fed an alfalfa diet before and during the trial. Isotope ratios of the alfalfa diets averaged −26.2 and −26.0 parts per thousand (‰) vs Pee Dee Belemnite (**PDB**; n = 4) for Trials 1 and 2, respectively. In Trial 1, approximately 200 g (.2% of BW) of the C30 alfalfa were fed to four 2-yr-old ewes (average BW = 92 kg) before feeding the remainder of the diet at 0700 on January 19, 1989. The dose was consumed in approximately 15 min. Ewes had been previously adapted to metabolism stalls (1.25 m × 3 m) for 14 d and were fed 18.6 g of chopped alfalfa hay (2.5-cm screen) per kilogram of BW in two equal portions at 0730 and 1830 daily. Blood and fecal samples were collected at 4-h intervals for 44 h after feeding and then at 60, 72, and 81 h after feeding. Blood samples (approximately 18 mL) were collected via indwelling catheters that had been inserted 24 h earlier. Blood samples were lyophilized and ground with a mortar and pestle, whereas fecal samples were dried at 50°C for 48 h in a forced-air oven and ground to pass a 2-mm screen in a Wiley mill. In a concurrent study, an intraruminal dose of Yb-labeled, chopped alfalfa hay was administered via the ruminal cannula to each ewe, and rectal grab samples were taken at 0, 8, 12, 24, 28, 32, 42, 48, 60, 72, 84, and 96 h after dosing. Details of the Yb procedures can be found in the paper by Gunter et al. (1990).

In the second trial, approximately 500 g (.5% of BW) of C1 alfalfa was fed to each of two 2-yr-old ewes (average BW = 89 kg). Breath, blood, and feces were collected at 2-h intervals from just before dosing to 12 h after dosing and then at 4-h intervals from 12 to 40 h after dosing, with a final sample at 48 h. Blood and fecal samples were collected using procedures similar to those described in Trial 1. Breath samples were collected into a plastic bag using a face mask that covered both the mouth and nose. The mask was fitted with one-way valves that allowed fresh air to enter the mask upon inhalation and respired air to leave the mask upon exhalation. Contents of the plastic bag were sampled using 50-mL evacuated serum bottles. Additional details on breath collection can be found in the report by Boutton (1991b). Serum was separated from whole blood by allowing blood to clot at 0°C and centrifuging at 2,300 × *g* for 15 min at 4°C for analysis. All samples were processed using the same procedures as in the first trial.

Samples of C30 and C1 alfalfa (Trial 1 and Trial 2, respectively) were analyzed nonsequentially for ADF and NDF (Goering and Van Soest, 1970). Approximately 3 g of C1 and C30 alfalfa (ground to pass a 2-mm screen in a Wiley mill) were placed in 10-cm × 20-cm Dacron bags (pore size, 53 ± 10 μm) and suspended in duplicate in the rumen of each of four steers consuming (2% of BW) the same alfalfa hay diet as the ewes. One empty bag identical to filled bags was included with each duplicate set of bags, which were removed at 0, 11, 48, and 72 h. Dacron bags were used to assess in situ disappearance of ground, labeled hay and changes in ^{13}C:^{12}C ratios at each incubation time.

All samples were analyzed using isotope ratio mass spectrometry to determine ^{13}C:^{12}C ratios. Results are reported as parts per thousand (‰) relative to the international PDB standard. The standard notation for this analysis is $\delta^{13}C$ (‰ vs PDB). Details of sample preparation and mass spectrometry have been described by Boutton (1991b).

Table 1. Composition and stable carbon isotope ratios (‰ vs Pee Dee Belemnite [PDB]) for alfalfa that was either cut the day of labeling (C1) or 30 days after labeling (C30)[a]

	C1		C30	
Item	% of DM	$\delta^{13}C_{PDB}$,‰	% of DM	$\delta^{13}C_{PDB}$,‰
Whole plant	100	+15.3[b]	100	+15.2
Cell contents	55	+38.8	56	+10.5
NDF − ADF	9	−4.5	7	+48.7
ADF	36	−16.0	37	+16.0

[a]Prelabel values for C1 and C30 alfalfa were −25.7 and −25.9‰, respectively.
[b]n = 3.

Results

Forage. Distribution of ^{13}C label in detergent fiber fractions depended on date of harvest relative to date of labeling. When alfalfa was harvested on the same day it was labeled, most of the ^{13}C was in the cell contents (Table 1); however, when the alfalfa was harvested 1 mo after labeling, the fiber fraction was enriched to a greater degree. From percentage of C of the fraction (whole plant = 44.5% C and NDF = 46.9% C), and carbon isotope ratios, we calculated the percentage of ^{13}C label in cell contents of C1 and C30 alfalfa as 84 and 47%, respectively. Procedures for calculating fractional abundance and excess ^{13}C are detailed in the report by Svejcar et al. (1990). Values of percentage ^{13}C composition were calculated from ADF and NDF analysis. Carbon isotope ratios of the components were calculated from proportional values and isotope ratios of whole plant tissue, and ADF and NDF residues. Isotope ratios of residue remaining in Dacron bags and percentage of initial ^{13}C label in residue are presented in Table 2.

Trial 1. The peak in $\delta^{13}C$ for fecal samples coincided closely with results from a concurrent study using Yb-labeled hay (Figure 1). In both instances, peak values were obtained approximately 32 h after dosing. There was no change over time in ^{13}C of whole blood. Apparently, the dose of ^{13}C provided by the enriched alfalfa was not sufficient to influence baseline ^{13}C in blood samples. Values of ^{13}C for the blood were approximately 3‰ enriched in ^{13}C relative to the alfalfa diet (~ −23.4 vs −26.2‰). This finding is consistent with the enrichment found in protein and bone collagen in herbivores (Tieszen and Boutton, 1989).

Trial 2. In this trial, 84% of the ^{13}C label was in cell contents of C1 alfalfa (Table 1). Presumably, this accounts for the speed at which the peak in ^{13}C values appeared in the breath (Figure 2). Although breath samples remained enriched in ^{13}C up to 40 h after dosing, there was an average 4.7‰ decline at 6 h after a peak at 4 h. Fecal samples did not exhibit the peak at 32 h after dosing as in Trial 1. The ^{13}C from cell contents apparently was absorbed and metabolized rapidly, and thus did not appear in the feces; the fiber fraction in the C1 alfalfa was not enriched in ^{13}C to the extent it was in C30 alfalfa (Table 1). Fiber fraction enrichment as a percentage of total ^{13}C label for C1 and C30 alfalfa was 16 and 53%, respectively.

There was a slight, but consistent, increase in the ^{13}C of serum after labeling, but the increase was only .5‰ (Figure 2). Serum values were quite constant between 4 and 32 h after dosing. Isotope ratios of whole blood varied by approximately .5‰ during the

Table 2. In situ Dacron bag dry matter disappearance (DIS), and stable isotope ratio in the situ residue (n = 4) of alfalfa cut 30 days after labeling with 99 atom % $^{13}CO_2$

Time, h	DIS, %	Residue, %	$\delta 13_{PDB}$,‰[a]	^{13}C Label remaining in residue, %[b]
0	0	100	15.2 ± .2[c]	100
11	58.0 ± 5.1	42	18.0 ± 1.8	46
24	69.4 ± 2.7	30.6	16.3 ± 2.1	32
48	71.1 ± 1.6	29.9	13.0 ± 1.3	28
72	72.4 ± 1.6	27.3	11.0 ± 1.7	24

[a]PDB = Pee Dee Belemnite.
[b]Values were calculated assuming 45% C in the residue.
[c]Mean ± SD.

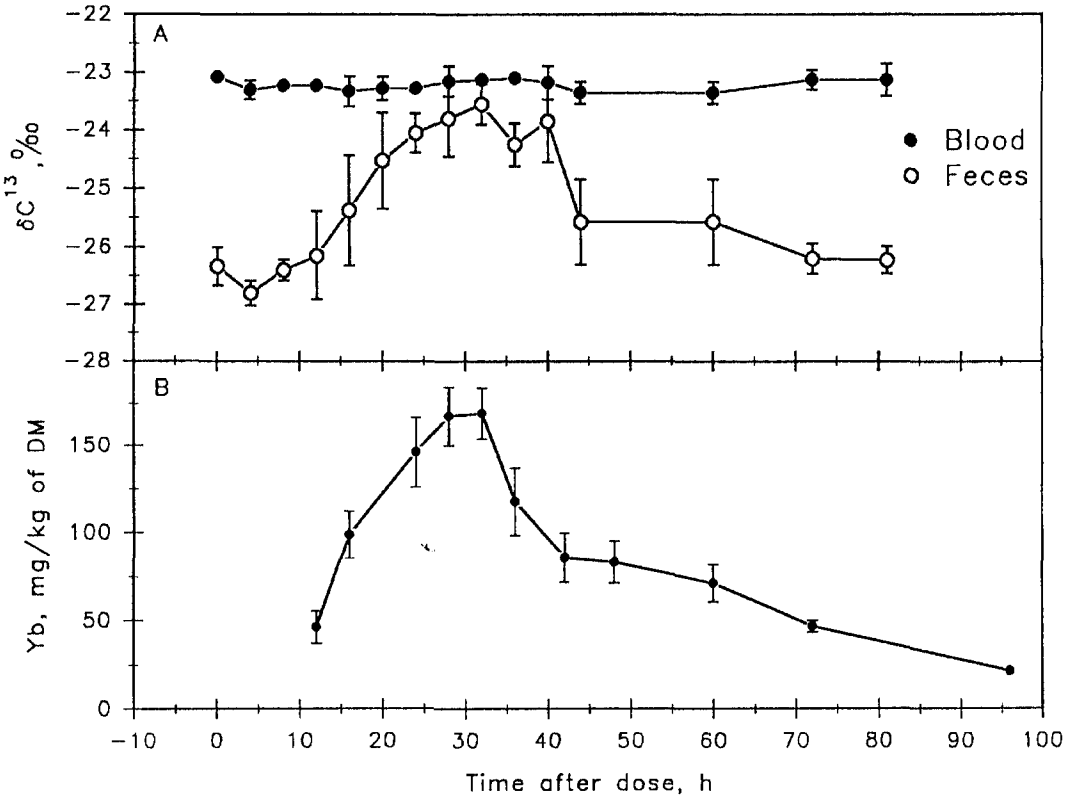

Figure 1. (A) δC_{PDB} values in whole blood and feces of sheep fed one dose of ^{13}C-labeled alfalfa (n = 4). The alfalfa was labeled and harvested 30 d later to ensure that the fiber fractions were enriched in ^{13}C. The standard diet had a $\delta^{13}C$ value of −26.2‰ and the labeled alfalfa a value of +15.2‰. (B) Yb concentration (milligrams/kilogram of DM) of sheep feces. Ytterbium-labeled alfalfa was fed concurrently with ^{13}C-labeled alfalfa. Bars represent standard errors.

entire sampling period, but there was no consistent trend. Whole blood values were approximately 1.5‰ more negative than those from Trial 1, but in both cases the ^{13}C label was not apparent in whole blood.

Discussion

Labeling forages with enriched ^{13}C allows a wider range of research approaches than using natural abundance differences between C_3 and C_4 forage species. The location of the isotope marker within detergent fiber fractions can be controlled to some degree by manipulating the labeling regimen. We were able to enrich preferentially the cell contents of alfalfa by cutting the sward the same day it was labeled vs cutting 30 d after labeling (Table 1). Conversely, when an alfalfa sward was cut 1 mo after labeling, the fiber fractions were relatively more enriched than the cell contents. Enriched fiber fractions isolated using detergent washes could be fed to ruminants to determine the fate of carbon from the various fiber fractions. Smith (1989) reviewed the use of ^{14}C-labeled NDF and listed the following advantages of ^{14}C relative to other markers: 1) simultaneous measurements of particle breakdown, digestion, and passage rates can be made and 2) microbial attachment, VFA, CO_2, and CH_4 production, and rate of incorporation of labeled material into tissues can be studied. However, Holden (1990) suggested that ^{14}C-labeled forages cannot be used to measure particle size reduction. Advantages and limitations for ^{14}C should apply to ^{13}C-labeled material. The degree of enrichment can be controlled by the labeling procedures (Boutton et al., 1987). These authors labeled rice plants with several levels of $^{13}CO_2$ and obtained white rice with $\delta^{13}C$ levels > 1,000‰ vs PDB. The amount of label, nature of labeling, and postlabeling harvest schedule all can be varied depending on specific research objectives. For examples, continuous labeling procedures should provide more uniformly labeled forage than the pulse label used in our study.

Changes over time in isotope ratios of Dacron bag samples reflected the differential labeling of plant fractions (Table 2). During the early phases of digestion (11 and 24 h), slightly less enriched cell contents (Table 1) were removed, and $\delta^{13}C$ values of the residue increased to levels comparable to the fiber fraction. However, at 48 and 72 h, $\delta^{13}C$ of the residue decreased to values less than that of the cellulose +

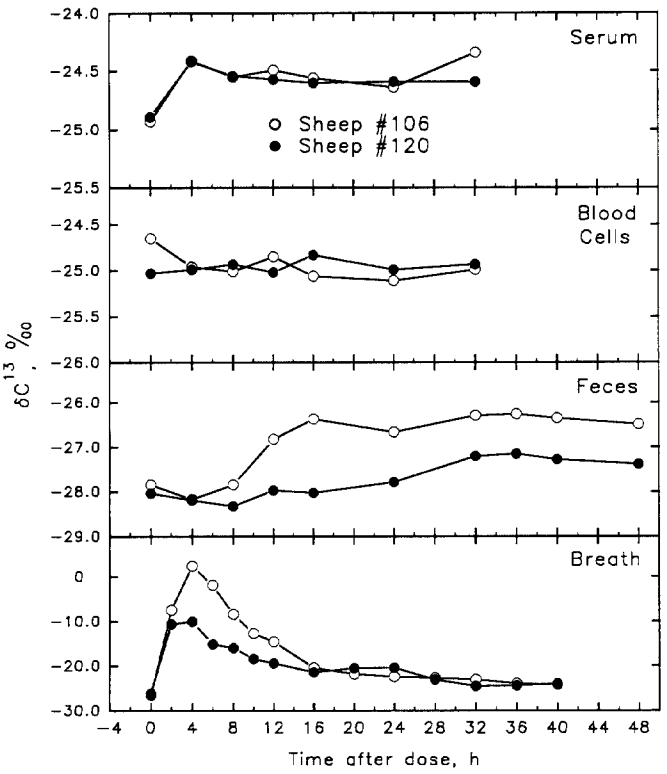

Figure 2. $\delta^{13}C_{PDB}$ values for serum, whole blood, feces, and breath from sheep fed one dose of ^{13}C-labeled alfalfa. The alfalfa was labeled and harvested the same day so that most of the ^{13}C was in the cell contents. The standard diet had a $\delta^{13}C_{PDB}$ value of −26.0‰ and the labeled alfalfa a value of +15.3‰.

lignin fraction. Further fractionation and isotope analyses would be necessary to explain the $\delta^{13}C$ values of the Dacron bag residue. Although the variability suggests that differences in $\delta^{13}C$ among the last three sampling times were not large, there was a consistent decrease with time in $\delta^{13}C$ of the residue from all four steers.

In sheep fed C30 alfalfa, peak $\delta^{13}C$ values in the feces corresponded to the peak in Yb concentration (Figure 1). In a similar study, Alexander et al. (1969) fed ^{14}C-labeled hay to one dairy cow and found a peak in ^{14}C activity of fecal material 32 h after feeding labeled hay. Although peak concentration of marker in the feces after pulse dosing is only one factor involved in calculation of passage rates from compartmental models (Krysl et al., 1988), the concurrence of the Yb peak and the $\delta^{13}C$ peak suggests relatively similar movement of the two markers in the digestive tract. Thus, it seems that forage with fiber fractions enriched in ^{13}C could provide an estimate of rate of passage, provided that the label is primarily in the indigestible components and does not translocate to other compounds. However, when the majority of label was in the soluble fraction, as was the case with C1 alfalfa, no clear trends in fecal $\delta^{13}C$ were evident (Figure 2). The ^{13}C apparently was incorporated into metabolic pools and(or) respired; thus, only a minor but persistent enrichment of fecal $\delta^{13}C$ was noted.

Labeled forage also can be used to assess the metabolic fate of carbon. In both trials, whole blood was not enriched relative to initial or final values. However, whole blood was enriched in ^{13}C relative to the baseline diet. Previous studies with ruminants also have shown an enrichment of whole blood $\delta^{13}C$ values relative to $\delta^{13}C$ of the diet when animals were fed a C_3-based diet (Sutoh et al., 1987; Metges et al., 1990). There also was a slight (.5‰) but consistent enrichment of blood serum in Trial 2 (Figure 2). Sutoh et al. (1987) shifted dairy cattle from a C_3- to a C_4-based diet and measured changes in $\delta^{13}C$ values of whole blood, red blood cells, and plasma. Their results were similar to ours, in that $\delta^{13}C$ of red blood cells did not change after the diet shift, but $\delta^{13}C$ of plasma did. They suggested that the relatively long life span of red blood cells might explain the lack of change during their trial. In addition, size of the blood carbon pool relative to the amount of tracer fed must be considered. In instances in which carbon pools have relatively long residence times, repeated feeding of labeled material and longer sampling periods may be required to detect changes in isotope ratios.

A substantial body of information has been gathered using natural isotope variation in C_3- and C_4-based diets. Generally, animals are fed either a C_3 or C_4 diet, the diet is then shifted, and $\delta^{13}C$ of milk, blood, feces, and so on, is monitored. However, the use of natural abundance diets has several limitations. Metges et al. (1990) cited two primary problems: 1) equilibration time between the diet shift and sample collection is short and 2) C_3 and C_4 diets tend to differ in composition (fiber, cellulose, starch, etc.), and thus in digestibility. In addition, some individual plant species may be fermented preferentially in the rumen. Tyrrell et al. (1984) suggested that carbon from corn silage contributed proportionately more to respired CO_2 than did noncorn ingredients in the diet. Preferential fermentation of specific dietary ingredients may further complicate interpretation of results from C_3 to C_4 diet shifts. Labeling of individual plant species or species mixtures with ^{13}C could resolve some of these concerns.

A wide range of nutrition and metabolism questions can be addressed using forages labeled with ^{13}C, although labeling of any specific component may not be uniform. The labeling procedures can be flexible to meet the needs of a particular line of research. In our study, dimensional lumber and clear plastic were used to build large, inexpensive labeling chambers; chamber size and shape are almost unlimited. Labeling can be conducted in the field, greenhouse, or growth chamber depending on the objectives of a particular research program. At the time of this study, the cost of 5 L of 99 atom % $^{13}CO_2$ (including the cylinder) was <

$500. The total cost of labeling will depend on the amount of forage and degree of enrichment required. Availability of isotope ratio mass spectrometers has increased in recent years (Boutton, 1991b), and many laboratories currently conduct $^{13}C;^{12}C$ analyses. Cost of analysis generally varies from $10 to $30 per sample depending on the degree of precision required. Further discussion of $^{13}CO_2$ labeling, isotope analysis, and calculation of total ^{13}C in plant tissue can be found in the papers by Svejcar et al. (1990) and Boutton (1991a). The primary advantage of using ^{13}C rather than ^{14}C as a marker is that no health and regulatory constraints are associated with the stable isotope ^{13}C. Thus, animals need not be destroyed after a stable isotope study, as often is the case when a radioactive tracer is used (Boutton, 1991b).

Implications

Regulatory and cost constraints associated with radioactive markers have limited our knowledge of carbon partitioning in both plant and animal systems. Labeling of harvested forages or small areas of grazed pastures with the stable isotope ^{13}C will allow the study of digestion kinetics, metabolism, carbon cycling at the plant/animal interface, and so on, without the use of radioactive ^{14}C. Stable isotope labeling is a tool that would seem to be especially appealing to interdisciplinary research teams.

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