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Doubly Labelled Water Validation in the Marsupial *Petauroides volans*

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Abstract

Amounts of carbon dioxide (CO_2) produced by caged greater gliders were measured simultaneously by means of potassium hydroxide absorption, and by the low-level, doubly labelled water method, which incorporated a gas isotope ratio mass spectrometer for determining the low oxygen isotope enrichments. The two methods yielded significantly different values, but doubly labelled water underestimated CO_2 production by only 8·3% on average, and this error is in the range of mean errors found in previous validation studies where no significant differences existed. In addition, we tested procedures designed to reduce the costs of using the doubly labelled water method, by streamlining field and laboratory procedures for sample handling and processing.

Introduction

The doubly labelled water (DLW) method for measuring energy and material balance in free-ranging animals (Lifson & McClintock 1966; Nagy 1975, 1989) has transformed our understanding of how animals operate in their natural habitats. Several recent studies have provided new insights into animal physiological ecology. For example, previously hypothesised devices for conserving energy, such as estivation by a lizard (Nagy & Shoemaker 1975), torpor by desert rodents (Mullen 1971), and 'slothfulness' by sloths (Nagy & Montgomery 1980) have been confirmed in the field. Extraordinarily low energetic costs of living have been found in some desert birds (Goldstein & Nagy 1985) and in desert scorpions (King & Hadley 1979), and costs of flight in small birds that feed on the wing are much lower than expected from laboratory wind tunnel studies of flight costs (Hails 1979; Flint & Nagy 1984). Regarding the food and energy requirements associated with reproduction, the actual costs measured with DLW in the field are in surprising contrast with theoretical expectations in birds (Hails & Bryant 1979) and in lizards (Bennett & Nagy 1977; Nagy 1983*a*; Merker & Nagy 1984). Some desert rodents have lower field metabolic rates in winter than expected on the basis of ambient temperatures because they become gregarious and huddle in burrows (Karasov 1983). The energetic costs, benefits, and profits of the ways animals get their food (foraging mode) have been evaluated in two species of syntopic, congeneric lizards, and the wide forager made a higher profit than did the ambush predator during spring (Nagy, Huey & Bennett 1984). The population ecology of wild animals has also been studied with the aid of DLW. Annual energy flow through a population of desert lizards has been shown to be a much larger fraction of ecosystem

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energy flow than previously thought (Nagy 1983*a*). Penguins off the coast of Africa are competing for food with a local fishing industry that harvests anchovies more than twelve times as fast as do the penguins (Nagy, Siegfried & Wilson 1984). Given the diversity of applications of the DLW method, and the likelihood of exciting discoveries yet to be made, it is not surprising that interest in this technique has increased greatly in the last several years.

Most of the DLW studies to date have involved relatively high enrichments of oxygen-18 (^{18}O), as required for accurate measurement by the proton activation analysis method (Wood *et al.* 1975) and by earlier mass spectrometers (Lifson & McClintock 1966; Mullen 1971). The high cost of enriched oxygen isotope has restricted DLW studies to relatively small animals. Large animals necessarily require large and prohibitively expensive doses of ^{18}O. The recent availability of gas isotope ratio mass spectrometers, which are capable of measuring much lower enrichments of ^{18}O, has made studies on large animals economically feasible (Lifson *et al.* 1975; Nagy 1989). However, many laboratories that have attempted to use or modify the DLW method have had difficulties. Thus, it is imperative that each change in the DLW method be accompanied by tests to check the reliability of the techniques. Validation studies of the 'low-level' DLW method, which utilises an isotope ratio mass spectrometer (IRMS), have been done on humans (Schoeller & van Santen 1982; Schoellwer & Webb 1984; Klein *et al.* 1984; Coward & Prentice 1985; Schoeller *et al.* 1986) and on an ungulate mammal (Fancy *et al.* 1986). The present study was made to test the validity of the low-level DLW method in a marsupial, with isotope analyses being done at both the University of California, Los Angeles, and Global Geochemistry Corporation in Los Angeles. In addition, we evaluated several procedures which may reduce the costs of isotope analysis.

Validation Study

Materials and Methods

Five greater gliders, *Petauroides volans*, fed *Eucalyptus radiata* foliage *ad libitum* were used in these experiments. Measurements of carbon dioxide (CO_2) production rate were made simultaneously by the DLW method, and by direct measurement of the amount of CO_2 absorbed by potassium hydroxide (KOH) solutions. The procedures used in the maintenance of the animals in captivity, and the diet of the animals, are described by Foley (1987) and Foley & Hume (1987).

(i) *Carbon dioxide production by potassium hydroxide absorption*

CO_2 production was measured by KOH absorption over two 3-day periods while the animals were in closed-circuit respirometers. Full details of the construction and operation of these respirometers are given by Foley (1987). Briefly, circulating chamber air passed through flasks containing KOH to absorb CO_2, and then through silica gel cartridges to absorb water. Oxygen was bled into the system in response to loss of pressure due to absorption of CO_2. Calculated CO_2 production rates were corrected for differences between the initial and final concentration of CO_2 in the circulating air. The maximum CO_2 concentration at the end of any experiment was $0\cdot25\%$.

Control experiments showed that *E. radiata* foliage alone also produced significant amounts of CO_2. The amount of CO_2 produced was linearly related to the mass of fresh foliage in the chamber (Foley 1987), so recovered CO_2 was also corrected for this factor. The amount of CO_2 attributable to the foliage was $3\cdot6\pm0\cdot4\%$ of the total CO_2 recovered, on average.

(ii) *Carbon dioxide production by doubly labelled water*

At the beginning of the experiment, a blood sample (2 mL) was taken from each animal from a vein on the outer edge of the gliding membrane for measurement of background levels of ^{18}O. The animals were then injected intraperitoneally with $0\cdot501$ mL of water containing 24 MBq of tritium (^3H) and $38\cdot3$ atom % ^{18}O. The exact volume of the injection was determined later by weighing an equivalent volume of distilled water, assuming $1\cdot0$ mL $= 1\cdot0$ g for distilled water. All injections were made by the same operator using the same technique. After allowing 4 h for isotope equilibration, a second 2-mL blood sample was collected and the animal was placed in the respirometer for measurement of CO_2 production by KOH absorption as described above.

At the end of the third day, the animals were removed from the chamber and a clean urine sample was collected directly from the cloaca. The animals were then returned to the respirometers for a further 3 days after which a final blood sample was collected.

(iii) *Sample analyses*

Blood samples were distilled to complete dryness in an evacuated glass container placed in a thermal gradient (Vaughan & Boling 1961). Ten microlitres of the water were counted (in duplicate) for tritium activity using a Beckman LS230 liquid scintillation counter, and toluene-Triton X100-POP scintillation cocktail. Approximately 1 mL of each sample was analysed for ^{18}O content by isotope ratio mass spectrometry at Global Geochemistry Corporation (Canoga Park, California). The pH of these water samples was measured and adjusted to 5 or lower with concentrated phosphoric acid (H_3PO_4) if necessary. The samples were then equilibrated with CO_2 (introduced under vacuum) of known isotopic composition at 25°C for 72 h. The equilibrated CO_2 was isolated and purified under vacuum, and the mass 46 : mass 44 ratio was determined by mass spectroscopy. Results were reported as 'per mil' (parts per thousand, or ‰) relative to standard mean ocean water (SMOW). Rates of CO_2 production and water flux were calculated from isotope data using the equations for linearly changing body water volumes (Nagy 1980; Nagy & Costa 1980). Results were compared statistically using a paired t-statistic (Dixon & Massey 1969).

Results

The low-level DLW method yielded rates of CO_2 production that were lower than the rates yielded by the KOH absorption method for six of the seven measurement intervals (Table 1). (Loss of some blood samples in processing prevented us from obtaining DLW measurements for all ten measurement intervals.) The difference between methods is statistically significant ($P<0\cdot05$), and the DLW method had an average error of $-8\cdot3\%$.

Table 1. Comparison of simultaneous measurements of CO_2 production by KOH absorption and by doubly labelled water (DLW) in greater gliders *Petauroides volans*

% error = 100 [(DLW − KOH)/KOH]

Animal number	Measurement interval (days)	CO_2 production (litres) KOH	DLW	% error
1	6·08	124·0	108·1	−12·9
2	3·03	53·0	46·3	−12·8
3	2·99	54·2	51·7	−4·5
3	3·03	57·3	51·5	−10·1
4	2·97	60·8	56·1	−7·8
4	3·07	62·1	54·8	−11·9
5	3·07	65·8	67·2	+2·1
Mean	3·46	68·2[A]	62·2[A]	−8·3
s.d.	1·15	25·0	21·2	5·5

[A] Significantly different via paired t-test ($t=2\cdot95$, $P<0\cdot05$).

Sample Preparation

We conducted a series of experiments designed to reduce the costs and complexity of ^{18}O analysis by isotope ratio mass spectrometry. Currently used procedures require large sample volumes (5–10 mL), and pure water (not blood or urine) is needed. This restricts low-level DLW studies to animals larger than about 2 kg, because taking 5 mL of blood repeatedly from smaller animals may be harmful.

The 5 mL of water distilled from blood or urine samples are used to equilibrate with CO_2 gas introduced over the water. After about 24 h in a shaking water bath at 25°C, the ^{18}O has reached isotopic equilibrium between water and CO_2 via the reversible reaction $CO_2+H_2O \leftrightarrow H_2CO_3$ (carbonic acid). The ^{18}O concentrations in CO_2 and H_2O will differ slightly at equilibrium because of temperature-dependent isotopic fractionation effects, but the fractionation factor at 25°C is known and results are corrected appropriately. A relatively large volume of water is used so that the molar ratio of oxygen in H_2O to oxygen in CO_2 is very large, so the ^{18}O contributed by the introduced CO_2 is very small and will not affect the equilibrium exchange ratio. The equilibrated CO_2 is removed from the equilibration

vessel, other gases such as N_2, O_2, and H_2O vapour are removed by condensation in cold traps, and the clean CO_2 is introduced into an isotope ratio mass spectrometer (IRMS). Water vapour itself is not used to measure ^{18}O by most laboratories, because it is 'sticky', adhering to inner surfaces of the mass spectrometer, and coming off slowly to cause 'memory' effects that either require extensive and difficult mathematical and operational procedures to correct, or require long delays between samples while the memory effects disappear.

We evaluated the possibility of collecting the CO_2 that is already in blood for the analysis. This would eliminate the need for distilling blood. Moreover, this could be done in the field. Alternatively, if exogenous CO_2 must be added to blood samples, perhaps the carbonic anhydrase that is present in red blood can be used to reduce the necessary equilibration time to minutes, so that the equilibrations can be done in the field or in the biologist's own laboratory. Finally, we evaluated procedures to reduce the volume of blood required so that smaller animals can be studied with low-level DLW.

Can Blood Carbon Dioxide be Used?

A minimum of 30 micromoles of CO_2 are required to make a relatively accurate measurement of the ^{18}O content of a sample, but 60 micromoles or more are preferred. Mammalian blood can carry about 20 micromoles of CO_2 (mostly as bicarbonate) per millilitre of whole blood (Altman & Dittmer 1974), so 2 mL of blood, if saturated with CO_2, should provide just enough CO_2 for ^{18}O analysis. Two millilitres of blood could be removed from animals as small as 330 g without harming them (based on human blood-donor criteria of 8% of blood volume, or 0·6% of body mass).

To test this, we separated blood collected from an anaesthetised laboratory white rabbit into 2-mL aliquots in 12 10-mL 'Vacutainer' collecting tubes. Six were quickly evacuated by inserting a 20-gauge hypodermic needle connected to a vacuum line through the stopper for about 3 s. After waiting 10 min for the CO_2 in solution to enter the gas phase down its partial pressure gradient, the gas was drawn into a 20-mL evacuated 'Vacutainer' via a double-ended needle (Vacutainer Single-draw needle) inserted through both stoppers. The remaining six replicates were evacuated quickly as above, but then 0·1 mL of phosphoric acid (H_3PO_4) was added to drive off more CO_2. This CO_2 was collected as above. The 20-mL Vacutainers were then emptied into a vacuum line where the CO_2 was purified and the amount of CO_2 was measured as a pressure increase registered by a Pirani gauge in the vacuum line.

The results indicate that neither method yielded enough CO_2 for reliable mass spectrometer analysis. The vacuum extraction method collected only 2·4 micromoles (s.d. $=0{\cdot}6$ micromoles; $N=6$), whereas the acid treatment yielded $13{\cdot}9\pm2{\cdot}7$ micromoles. This result is unfortunate because it means that exogenous CO_2 must be added, and the attendent requirements for a source of CO_2, and a constant-temperature water bath for the equilibration reaction, make this technique more difficult to use in the field.

Rapid Carbon Dioxide Equilibration

We tested the validity of equilibrating exogenous CO_2 with blood samples as follows. Two millilitres of blood were added to an evacuated, 10-mL Vacutainer, and 2·0 mL of CO_2 gas was also added. Twelve replicates were prepared. They were incubated for 1 h in a shaking water bath at 25°C, after which six were evacuated into 20-mL Vacutainers as above, and the remaining six were acidified with 0·1 mL of H_3PO_4, then transferred. (It was necessary to transfer the gas samples to another container to stop the equilibration process while it was still at the correct temperature.) In addition, six evacuated 20-mL Vacutainers were injected with 2·0 mL of CO_2. To serve as controls, six 2·0-mL aliquots of rabbit blood were distilled (Vaughan & Boling 1961 method) to obtain pure water for routine equilibration with laboratory-standardised CO_2 at the University of California, Los Angeles.

The results indicate that addition of 2 mL of CO_2 yields enough CO_2 from this equilibration system for IRMS analysis. Evacuation of the equilibration tubes alone yielded $58{\cdot}2\pm0{\cdot}8$ micromoles of CO_2, acid addition yielded $68{\cdot}1\pm2{\cdot}0$ micromoles, and direct

collection of 2·0 mL of dry-ice CO_2 into the 20-mL Vacutainers yielded 76·4±7·9 micromoles. However, there were problems with the delta ^{18}O (per mil) values. Water distilled from blood had a suspiciously low delta ^{18}O value of −11·6±1·0 (after correction for ^{18}O in added CO_2). A correspondingly low delta ^{13}C value for those samples strongly suggested that the isotope equilibration between H_2O and CO_2 was incomplete, probably because of a thin layer of silicone stopcock grease (from the distillation device) on top of the water. Thus, we do not known the 'true' delta ^{18}O in the blood, with which to evaluate our new techniques. Even so, a problem exists in the CO_2 extraction method, because the delta CO_2 results differ ($P<0.05$ via a *t*-test): evacuated samples averaged −6·7±0·2‰, and acidified, evacuated samples averaged 5·8±0·3‰. We repeated the equilibration experiment to recheck these results as well as to evaluate equilibration time.

Equilibration Time Required

In the presence of carbonic anhydrase, CO_2 in air should equilibrate with H_2CO_3 in solution very quickly if mixing is thorough. To determine the minimum time required for isotope equilibration in our system, as well as to check for a temperature effect on equilibration time, we set up twenty 10-mL Vacutainers, each with 2·0 mL of blood and 2·0 mL of CO_2 gas from dry ice. Ten were incubated at 25°C and ten at 37°C, with two tubes from each temperature being sampled after 0·5, 1·0, 2·0, 4·0, and 24 h. CO_2 was extracted by evacuation into 20-mL Vacutainers.

The delta ^{18}O values for the 25°C run remained constant through time, indicating that isotope equilibration was complete within 30 min. This was about as fast as one person could process the ten samples in sequence. Equilibration may occur even more rapidly than this. At 37°C, delta ^{18}O values drifted more negative through time: the regression of delta ^{18}O on log time (h) is statistically significant ($F_{(1,8)}=11.0$, $P<0.025$). This, coupled with the gradual increase in delta ^{13}C values, is consistent with the interpretation that bacterial growth was occurring in the blood during the experiment.

We repeated the experiment at 25°C with fresh blood, which we assumed had less bacterial contamination than the older blood we used above, and we added a sampling point at 15 min to check for rapid equilibration. The results indicate that ^{18}O equilibrium had occurred during the 15 min, and the progressive change through time in delta ^{13}C values revealed that bacterial growth was important even at 25°C, and even in freshly drawn blood, after 1 h had elapsed.

Carbonic Anhydrase Addition

If blood samples must be stored for some time before they are analysed, it is possible that they will have lost their carbonic anhydrase activity, and may yield spurious ^{18}O data. We wondered if addition of commercially available carbonic anhydrase to old blood, and perhaps even to urine or distilled water standards, would permit rapid ^{18}O equilibration between fluids and CO_2. To test this idea, we added 5 mg of dialysed, lyophilised carbonic anhydrase (Sigma Chemical Co., EC 4.2.1.1) to 2-mL replicates of old blood (rabbit), urine from greater gliders and distilled water standards, and then processed them as above to measure delta ^{18}O.

Dry carbonic anhydrase would not dissolve completely in glider urine or in distilled water, so we conclude that this method will not work well with urine or pure water samples. However, carbonic anhydrase addition worked well in old blood. There was no significant difference ($P>0.10$ via a *t*-test) between delta ^{18}O values of enzyme-treated blood (−4·52±0·29‰, $N=3$) and untreated blood (−4·84±0·23‰, $N=4$; Table 2) when blood sample volume was 2·0 mL. However, when only 0·10 mL of blood was used (see below), carbonic anhydrase addition was associated with a significant increase in delta ^{18}O (Table 2). This may be due to contamination of the sample, perhaps by ^{18}O in the enzyme added to the blood.

Minimum Sample Volume

The smaller the volume of blood required for isotope analysis, the smaller the animal that can be studied by this method. We tried exogenous CO_2 equilibration using only

0·10 mL of rabbit blood with 2·0 mL of dry-ice CO_2 in 4-mL Vacutainers (untreated) for 40 min at 25°C. The results (Table 2) were about twice as variable as with larger blood volumes (s.d. of 0·6 versus about 0·3 above), but the mean value of −4·06‰ does not differ significantly from the mean value of −4·70‰ for 2·0-mL sample volumes ($P = 0{\cdot}07$ via Mann–Whitney U-test). The correction for ^{18}O in the added CO_2 accounts for a much larger fraction of the final, corrected value when the blood volume is 0·10 mL (Table 2) because the amount of ^{18}O in exogenous CO_2 relative to ^{18}O in the fluid is larger when blood volumes are smaller. In this situation, precise measurements of the volumes of added CO_2 and blood samples become critical, and these measurements thus become additional sources of error. The DLW method is very sensitive to small errors in isotope measurements (Nagy 1980), but we believe that this greater variability should not cause major problems as long as ^{18}O concentrations differ substantially between initial and final samples, and the final sample is substantially above natural abundance levels.

Table 2. Comparison of delta ^{18}O (‰), relative to standard mean ocean water measurements for blood sample volumes of 2 mL and 0·1 mL

Both the observed (Obs.) values and the values corrected (Corr.) for ^{18}O in the CO_2 added to the blood are shown. Means with common superscripts differ significantly ($P \leqslant 0{\cdot}02$ via Mann–Whitney U-test for enzyme treatments, and paired t-test for observed versus corrected groups)

	2·0 mL volume		0·10 mL volume	
	Obs. ‰	Corr. ‰	Obs. ‰	Corr. ‰
	No carbonic anhydrase added			
	−5·12	−5·08	−5·36	−4·62
	−4·92	−4·88	−5·40	−4·66
	−4·92	−4·88	−4·01	−3·23
	−4·57	−4·53	−4·42	−3·65
			−5·24	−4·13
Mean	−4·88	−4·84	−4·89^{A}	−4·06^{B}
s.d.	0·23	0·23	0·63	0·62
	Carbonic anhydrase added			
	−4·89	−4·85	−4·01	−3·23
	−4·35	−4·31	−3·96	−3·18
	−4·44	−4·40	−3·90	−3·11
			−3·88	−3·09
			−4·07	−3·29
Mean	−4·56	−4·52	−3·96^{A}	−3·18^{B}
s.d.	0·29	0·29	0·08	0·08
Overall mean	−4·74	−4·70	−4·43^{C}	−3·62^{C}
s.d.	0·29	0·29	0·65	0·62

Field Sample Containers

We used Vacutainers for collecting blood and urine samples in the field because Vacutainers are readily available, inexpensive (compared to sealed glass containers), and are easy to use. However, we discovered that samples stored in Vacutainers longer than a week or so had markedly lower delta ^{18}O values, probably because of diffusional exchange of CO_2 through the rubber stopper. This was probably an unrecognised source of error in the earlier portions of this study. If equilibrated CO_2 samples will not be measured in an IRMS within a few days, we recommend that they be stored in all-glass, sealed containers (such as flame-sealable ampules).

Discussion

Although the validation study indicated a statistically significant difference existed between DLW and KOH absorption results, the mean error of 8% is not large, and is the same as the ±8% average error expected from results of other validation studies where no significant errors were found (Nagy 1980; Nagy 1989). The KOH absorption method undoubtedly has some (unquantified) error associated with it, as do other CO_2 measurement methods (Williams & Nagy 1984). The KOH absorption technique as a method of measuring CO_2 production has been reliable in validations for the respirometer system used in these experiments (Farrell 1972). One source of error in estimating CO_2 output by greater gliders in this study was the contribution of CO_2 from the foliage on which the animals fed. This was greater than anticipated at the beginning of the study. Although we corrected for this error, variations in the amount of leaf relative to stem in each bunch of leaves, as well as variation in the total amount of leaf present in the chamber as the animals ate it (we based our calculation on the mid-point), may still have made a contribution to the significant error between DLW amd KOH estimates of greater glider CO_2 production. In view of these results, we feel that field DLW measurements of CO_2 production by greater gliders are probably accurate to within ±10% [given the additional potential errors in field situations (Nagy 1980)], but such measurements should be applied with caution.

Our results concerning new ways to prepare samples for ^{18}O analysis are encouraging. A major problem yet to be solved is identification of a suitable gas sample container for field use. Vacutainers do not have gas-proof seals, but may be used for periods of hours or a few days. Once an adequate container is found, then CO_2 samples for IRMS analysis can be collected in the field simply by acidifying blood and collecting the gas evolved. At least 4 mL of blood must be used in this procedure, so it is best suited for larger animals. Smaller samples must be equilibrated with exogenous CO_2, and this requires a constant-temperature water bath. The carbonic anhydrase present in fresh, red blood will cause the added CO_2 to reach isotopic equilibrium with blood water within 15 min, with shaking.

Blood samples as small as 0·10 mL can be used, although measurement precision and accuracy suffer somewhat. Another way to apply this method to small animals is to inject them with larger amounts of ^{18}O, but then dilute the blood sample with unenriched blood to make its volume up to a convenient amount. The drawbacks of this approach are that the cost of the isotope needed for such a study will be higher, and the results will be less accurate due to the additional sources of error accompanying the dilution process.

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