

THE DOUBLY LABELED WATER METHOD: INTER LABORATORY COMPARISON OF STABLE ISOTOPE MEASUREMENTS

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Abstract

Doubly labeled water (DLW) method is the most correct method for measuring energy of wild animals, and the most difficult key in the method is the quantitative analysis for isotopes of body water in animals. For testing reliability of isotope measurements in different laboratories, the Chengdu College of Geology (CCG) and the University of California at Los Angeles (UCLA) had cooperated on measuring identical blood samples taken from doubly labeled goats living near Chengdu in May of 1990, and got a successful result. The deuterium values from each laboratory were nearly identical, but the ^{18}O results from UCLA were consistently higher than those from CCG. However, the energy metabolism values determined by each laboratory were very similar. Metabolic rate values differed between laboratories by 8.6% on average, and water influx rates differed by an average of 1.2%, but neither difference is statistically significant.

Because the UCLA laboratory has been calibrated against laboratories in United Kingdom and in the Netherlands, the determined results in the isotope laboratories in CCG and UCLA have been validated for DLW method.

This paper tries to discuss the error source in the different laboratories, and has a reference significance for the similar laboratories.

Key words Doubly labeled water (DLW); Isotope; Energy metabolism

Doubly labeled water is water that contains enriched levels of stable or radioactive isotopes of hydrogen and oxygen. The doubly labeled water method is a technique for measuring energy, water and material balance of animals (Lifson et al., 1966; Nagy, 1975; Nagy, 1980; Nagy et al., 1980) and man (Lifson et al., 1975). This method is based on the washout rates of a hydrogen and an oxygen isotope which have been added to the body water of a subject. The washout rate of the hydrogen isotope (tritium or

ACKNOWLEDGEMENTS. We thank Lu Qixun, Zhang Yachua, Qin Yaosheng, He Jianming and Jitti Parnayakosol for help. This research was funded by the Chengdu College of Geology, and by the International Studies and Overseas Programs (Ms. Sue Fan) at University of California, Los Angeles.

Received 31 March 1992. Accepted 16 November 1992.

deuterium) is a measure of the rates of water gain and loss by the subject, and the oxygen isotope (oxygen-18), because it is in equilibrium with body water and with the CO_2 dissolved in body water, traces the loss of water and CO_2 combined. Thus, the difference between the washout rates of the two isotopes is a measure of CO_2 production alone, and is a measure of the rate of energy metabolism (Lifson et al., 1966). The rate of food consumption needed to achieve energy balance can then be estimated from the metabolic rate (Nagy, 1989). This method has been used to study the food, water and energy requirements of many species of wild animals (Nagy, 1987; Nagy et al., 1988) and humans under a variety of conditions (Schoeller et al., 1982).

The accuracy of the doubly labeled water method is good, with average errors in validation experiments done on captive animals under controlled conditions being usually less than $\pm 8\%$ (Nagy, 1989). Under field conditions, errors may be somewhat higher in certain environmental circumstances (such as high ambient CO_2 or water vapor in the air), but the largest common source of error is analytical error in isotope concentration measurements (Nagy, 1989). Errors as small as 2% in a single isotope measurement can cause errors in calculated metabolic rate values to be larger than 50% in some circumstances likely to occur in the field (Nagy, 1980).

This study was done to establish the accuracy of the Laboratory of isotope Geochemistry in analyzing blood samples from a doubly labeled water study. We did field measurements on five goats living on a farm near Chengdu, we took duplicate blood samples, and one set of samples was analyzed at the Chengdu College of Geology and the other was analyzed at the University of California, Los Angeles. Both sets of isotope results were used to calculate metabolic rates and water fluxes, and these results were examined for statistically significant differences. None were found, indicating that CCG isotope results yield accurate DLW estimates.

MATERIALS AND METHODS

Five domestic goats (*Capra hircus*, an adult male, an adult female, and three juvenile females), living on a farm near Chengdu College of Geology. On 28 August 1990, a preinjection sample of blood (20 milliliters from a jugular vein) was taken from each animal for measurement of natural abundances of deuterium and oxygen-18. Each goat was weighed to an accuracy of 0.1 kg, and its physical markings (coloration, coat pattern) and other characteristics were recorded for purposes of future identification. Then, each goat was given an intravenous injection (using a leg vein) of 0.25 milliliters per kilogram of body mass of sterile water containing 60.8% H_2^{18}O and 36% $^2\text{H}_2\text{O}$. Total dose volumes ranged from 1.8 ml for the smallest goat (6.7kg) to 4.5 ml for the largest (16.8kg). The injection syringes were saved for later calibration. We waited four hours for the injected isotopes to mix completely in the goats' body water. The goats were not allowed to eat or drink during the equilibration period. Then, another 20-ml blood sample was taken from a jugular vein, and the animals were allowed to feed, drink and behave normally.

Four days later, we returned to the farm, weighed the goats, and took another 20 ml blood sample from each one. After another four days, we returned again for the final weighing and blood sampling. Goat number five, who was sick at the beginning

of the experiment, died during the second measurement interval.

Blood samples were collected into heparinized Vacutainer tubes to retard clotting. The samples were centrifuged upon return to CCG, and the plasma fraction was divided between two heparinized 10-ml Vacutainers. One Vacutainer was analyzed at CCG and the other was analyzed at UCLA. The samples were kept refrigerated or frozen before analysis.

In each laboratory, 50 microliters of plasma were distilled under vacuum to obtain pure water for deuterium analysis (Wood et al., 1975). Hydrogen gas was generated from the water by reacting about 10 microliters with zinc reagent at 500°C for at least 8 hours. The mass 3: mass 2 ratio was then determined by isotope ratio mass spectrometry, using Finnigan mass spectrometers at CCG and UCLA, by comparison against standard Mean Ocean Water (SMOW). SMOW was assumed to have a concentration of deuterium of 157.6 ± 0.3 PPM (CCG) or 155.76 PPM (UCLA).

For 0-18 analysis, plasma samples were poisoned with a few milligrams of sodium fluoride (NaF) to inhibit bacterial metabolism. Duplicate subsamples of about 2 milliliter volume from each specimen were placed in clean Vacutainer tubes along with about 8 milliliters (at room temperature and atmospheric pressure) of pure CO₂ gas obtained from a laboratory cylinder of CO₂. Samples were equilibrated overnight in a shaking water bath set at 20°C. Then, the gas was removed, scrubbed in cold traps to remove water vapor, oxygen, nitrogen and other gasses than CO₂, and the mass 46: mass 44 ratio was determined with an isotope ratio mass spectrometer, again using SMOW as the standard in both laboratories. SMOW was assumed to contain 1993.4 ± 2.5 PPM of 0-18 (CCG) or 2001.2 (UCLA).

The DLW solution injected into the goats was calibrated by diluting it (5.0 microliters into 10.00 milliliters of distilled water) and measuring it as above. The distilled water used in the dilution was also measured for deuterium and 0-18 concentrations, and the tank CO₂ was measured for 0-18 in order to correct the isotope values for these factors.

Estimates of the body water volumes of the goats are needed to do the calculations (Nagy, 1983). These were determined from the dilution spaces of injected 0-18 and deuterium (Nagy, 1980). Rates of water influx and efflux, and rates of CO₂ production were determined using the equations for linearly changing body water volumes (Nagy 1980, Nagy et al. 1980). Differences between means were tested for significance ($P < 0.05$) using Student's *t*-test (Dixon et al., 1969).

RESULTS

The oxygen-18 concentration values obtained at UCLA were consistently higher than those measured at CCG (Table 1). The difference was rather constant, it was statistically significant, and averaged 14 ± 2 parts per million (PPM). On the other hand, the deuterium values from both laboratories were nearly identical, and did not differ significantly (Table 1).

Total body water spaces estimated from ¹⁸O dilution averaged 72.4% (standard deviation = $\pm 2.7\%$, sample size = $N = 5$). This is lower than the $78.6 \pm 2.6\%$ estimated from dilution space of injected deuterium. The average difference of 6.2% is statisti-

Table 1 Comparison of isotope analysis results from Chengdu College of Geology (CCG) and University of California at Los Angeles (UCLA) results for identical doubly labeled water samples from five goats.

表 1 成都地质学院和加利福尼亚大学洛杉矶分校有关五只山羊的同一双标水样品的同位素分析结果对比

Sample	oxygen-18 (PPM)		deuterium (PPM)	
	CCG	UCLA	CCG	UCLA
1A (preinjection)	1987	1999	152	150
1B (postinjection)	2177	2189	248	248
1C (after 4 days)	2071	2085	201	201
1D (after 8 days)	2025	2038	179	177
2A	1986	1999	151	149
2B	2188	2200	255	255
2C	2079	2090	204	204
2D	2033	2047	184	182
3A	1987	2000	152	150
3B	2184	2199	254	254
3C	2084	2098	207	207
3D	2035	2049	185	183
4A	1987	2003	152	150
4B	2191	2211	258	257
4C	2084	2101	208	208
4D	2038	2052	186	184
5A	1986	1986	149	147
5B	2184	2201	255	254
5C	2123	2135	229	227

Table 2 Comparison of calculated metabolic rate and water influx rate values using isotope data from Chengdu College of Geology (CCG) and the University of California at Los Angeles (UCLA)

表 2 应用成都地质学院和加利福尼亚大学洛杉矶分校的同位素数据计算所得的代谢速率和水循环结果对比

Animal and measurement period	Metabolic rate (L CO ₂ /day)				Water influx rate (ml H ₂ O/day)			
	CCG	UCLA	Diff.	% diff.	CCG	UCLA	Diff.	% diff.
1 BC	177	192	-15	-7.8	1511	1444	67	4.8
1 CD	292	208	84	40.4	1238	1373	-135	-9.8
2 BC	225	233	-8	-20.5	2219	2189	30	1.4
2 CD	397	282	135	61.5	1540	1673	-133	-7.9
3 BC	82	114	-32	-28.1	900	858	57	4.3
3 CD	157	108	45	45.4	709	753	-44	-5.8
4 BC	95	120	-25	-20.8	892	845	47	5.8
4 CD	148	127	19	15.0	555	687	-32	-4.7
6 BC	88	78	12	-15.4	379	372	7	1.9
Mean			16*	8.8			-17*	-1.2
Standard deviation			82	31.8			75	5.9

* Difference is not statistically significant ($P > 0.05$) by a paired t-test.

cally significant, and it is about as expected for a ruminant mammal (Nagy, 1980; Nagy et al., 1980). The overestimate in the deuterium value apparently is due to disassociation of some deuterium atoms from water molecules and their reassociation with organic molecules. The rather large differences observed in ruminants apparently are due to

the large number of disassociable hydrogen sites on cellulose, which occupies a large portion of their guts. Body water space estimates from oxygen-18 data from CCG and UCLA were essentially the same, because the data sets from each laboratory were internally consistent (samples and standards analyzed together).

The isotope data from UCLA yielded values for metabolic rates of the goats that were slightly higher on average than those based on the data from CCG, but the difference was not significant (Table 2). The UCLA results averaged about 7% higher than the CCG results, but the variation in the difference between these results was large, as indicated by the standard deviation of 32%. There was much better agreement between laboratories in the results for water influx rate, where the difference averaged only 1.2%, and the variation averaged 6% (Table 2).

DISCUSSION

The results show that the laboratories at Chengdu College of Geology and at the University of California, Los Angeles produce doubly labeled water results that are the same. The UCLA laboratory's mass spectrometer has been calibrated against mass spectrometers in the United Kingdom and the Netherlands (Speakman *et al.*, 1990), and against the machine at Global Geochemistry Corporation in Los Angeles (Nagy K unpublished results). Cross-validation studies, in which DLW measurements and direct measurements have been done at the same time on individual animals, have shown that these yield DLW results that are accurate (Nagy *et al.*, 1990; Nagy, 1990). We conclude that the laboratory at CCG should also yield accurate DLW results, although that laboratory has not yet done a cross-validation study.

The differences between the oxygen-18 results measured by the two laboratories is so consistent (Table 1) that it seems likely that the primary reason for this error is an error in the standard solutions used to calibrate the mass spectrometers. There was a small difference in the PPM value assigned to 0-18 in SMOW between laboratories (1993.4 ± 2.5 at Chengdu, and 2001.2 at UCLA). The more likely problem was probably an error in the actual or assigned 0-18 value of the working standard at UCLA or CCG or both. The reason that this error did not cause a significant difference between laboratories in total body water space based on 0-18 dilution is that both isotope data sets were internally consistent, so any errors would have been present in all samples and standards for a given animal, and they cancel out in the calculations. Similarly, the metabolic rate calculations are based on the relative decline in the two isotopes, and this is not changed by consistent, systematic errors in isotope measurements (Nagy, 1980).

Examination of the metabolic rate results for individual animals (Table 2) reveals that the values are higher than the CCG values in the first 4-day period for each animal, and the UCLA values are lower than the CCG values in the second 4-day period. Moreover, the opposite trend appears in the water influx data, with the UCLA values for the first period being lower than the CCG values, but higher than the CCG values during the second period. Further inspection of these results reveals that the UCLA values are more consistent between first and second measurement intervals. Because it is most likely that goats were doing the same things in both periods, they should have

had similar metabolic rates and water influx rates in both periods. This suggests that the CCG results should be examined first for problems. Interestingly, the problem of suspiciously high water influx coupled with suspiciously low CO_2 production, and vice versa, is caused by small errors in deuterium measurements (Nagy, 1980). In fact, some of the pre-injection samples and some of the final blood samples were reanalyzed at CCG due to problems with the vacuum system. These reanalyzed values may have been very slightly different, due either to slight contamination of the samples in storage, or to minor differences in spectrometer calibration between the time samples were first analyzed and when some of them were reanalyzed. These uncertainties illustrate the need for measuring all the blood samples from one animal together, along with the associated background, injection and distilled water samples, so that any methodological errors will occur in all samples, and should cancel out in the calculations. The water influx rates we measured in goats are within 10% of those expected for a captive mammal of their size (Nagy et al., 1988), and are very similar to other measured influx rates in goats (see summary of results in Nagy and Peterson 1988). The metabolic rates we measured, however, are only about 2/3 of those predicted for wild, free-living mammals of their size (Nagy, 1987). No DWL measurements on goats have been made to date, so we cannot compare our results with published data. However, a metabolic rate that is somewhat lower than that of a wild goat seems reasonable for our subjects, because they were tame, and were hand-fed during our study. Thus, we feel that both the CCG and UCLA laboratories produced results that are reliable.

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中文摘要

双标水法：实验室之间的稳定同位素测量对比

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双标水(doubly labeled water, DLW)法是研究动物野外能量代谢最准确的方法, 其中最关键、最困难的环节是同位素的定量分析。为了检验不同同位素实验室测定动物血样氢氧同位素丰度的准确性及其对估算能量代谢的影响程度, 成都地质学院(CCG)和美国加利福尼亚大学洛杉矶分校(UCLA)的同位素实验室于1990年5月开展了合作, 以成都附近农村的5只山羊为研究对象进行了双标水法的实验。将采集的动物血样分别在两个实验室进行了D和 ^{18}O 含量测定, 取得了成功的结果: 两个实验室所测得的 ^{18}O 值近于相同, 而UCLA同位素实验室测得的 ^{18}O 值略高于CCG的测定值。由此所计算出的能量代谢值却非常接近, 其差值平均为6.6%, 水循环平均相差1.2%, 差异均不显著($P>0.05$)。

UCLA实验室的质谱仪已为英国和荷兰的同类实验室所校正, 这确认了我们的合作测定成果的可信度和有效性, 证明CCG和UCLA实验室的测定结果均可以满足双标法同位素定量测定的需要。测定结果是可信的。

本文还就两地实验室所测结果产生误差的原因进行了初步分析, 对于进行同类研究的其它实验室也有借鉴意义。

关键词 双标水; 同位素; 能量代谢

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