The Journal of Experimental Biology 202, 2839–2849 (1999)
Printed in Great Britain © The Company of Biologists Limited 1999
JEB2221

ISOTOPE DILUTION SPACES OF MICE INJECTED SIMULTANEOUSLY WITH DEUTERIUM, TRITIUM AND OXYGEN-18

ELZBIETA KRÓL AND JOHN R. SPEAKMAN*

Aberdeen Centre for Energy Regulation and Obesity, Department of Zoology, University of Aberdeen, Aberdeen AB24 2TZ, UK

*Author for correspondence (e-mail: J.Speakman@abdn.ac.uk)

Accepted 28 July; published on WWW 30 September 1999

Summary

The isotope dilution technique for measuring total body water (TBW), and the doubly labelled water (DLW) method for measuring energy expenditure, are both sensitive to small variations in the ratio of the hydrogen to oxygen-18 dilution space. Since the dilution space ratio varies between individuals, there has been much recent debate over what causes this variability (i.e. physiological differences between individuals or analytical error in the isotope determinations), and thus which values (individual or a population mean dilution space ratio) should be employed for TBW and DLW calculations. To distinguish between physiological and analytical variability, we injected 15 non-reproductive and 12 lactating mice (*Mus musculus*, outbred MF1) simultaneously with deuterium, tritium and oxygen-18. The two hydrogen labels were administered and analysed independently, therefore we expected a strong correlation between dilution space ratios based on deuterium and tritium if most of the variation in dilution spaces was physiological, but only a weak correlation if most of the variation was analytical. Dilution spaces were significantly influenced by reproductive status. Dilution spaces expressed as a percentage of body mass averaged 15.7 % greater in lactating mice than in non-reproductive mice. In addition, the hydrogen tracer employed had a significant effect (deuterium spaces were 2.0 % larger than tritium spaces). Deuterium and tritium dilution spaces, expressed as a percentage of body mass, were highly correlated. Dilution space ratios ranged from 0.952 to 1.146 when using deuterium, and from 0.930 to 1.103 when using tritium. Dilution space ratios based on deuterium and tritium were also highly correlated. Comparison of standard deviations of the dilution space ratio based on deuterium *in vivo* and *in vitro* indicated that only 4.5 % of the variation in the dilution space ratios observed in the mice could be accounted for by analytical variation in the deuterium and oxygen-18 analyses. Although our results include data which were outside the limits previously regarded as biologically possible, the correlations that we detected strongly suggest that variation in the observed dilution space ratio was mostly physiological rather than analytical.

Key words: dilution space ratio, total body water, body composition, energy expenditure, doubly labelled water, breath samples, mouse, *Mus musculus*.

Introduction

The isotopes of hydrogen and oxygen, including deuterium, tritium and oxygen-18, play a key role in studies of the physiology and nutrition of animals and humans. The dilution of all three isotopes in body water has been used for determining total body water (TBW), from which evaluations of body composition can be derived (e.g. Hevesy and Hofer, 1934; Schoeller et al., 1980; Kashiwazaki et al., 1998; Webb et al., 1998; Oritz et al., 1999). In addition, these isotopes are used to determine CO_2 production and thus energy expenditure by the doubly labelled water (DLW) technique (e.g. Lifson et al., 1955; Nagy, 1980; Schoeller et al., 1986; Bryant and Tatner, 1991; Speakman, 1997). Both these isotope-based approaches are non-destructive (with some methodologies being non-invasive) and can be used in the laboratory or for free-living subjects.

It has been frequently observed that the body water content estimated by hydrogen dilution (N_H) exceeds that established by oxygen-18 dilution (N_O) (17 studies of humans and 26 measurements of 24 species of animals reviewed in Speakman, 1997, Table 7.10). Comparisons of oxygen and hydrogen dilution spaces with total body water, measured by desiccation, reveal that N_O provides an accurate measure of TBW (generally overestimating by less than 2 %), whereas N_H overestimates TBW by on average 3-5 % (reviewed by Speakman, 1997, Table 7.4; see also Bowen and Iversen, 1998). These overestimates occur because the hydrogen and oxygen isotopes exchange with other compounds in the body in addition to water. On the basis of theoretical calculations for adult humans, it has been estimated that exchangeable carboxyl

and phosphate oxygen might amount to 0.7 % of the oxygen in body water (Schoeller et al., 1980). In contrast, exchangeable hydrogen on carboxyl, hydroxyl and amino groups could amount to up to 5.2 % of water hydrogen (Culebras and Moore, 1977). Matthews and Gilker (1995) have emphasised that the value of 5.2 %, derived by Culebras and Moore (1977), represents an upper limit for non-aqueous hydrogen exchange, and a more realistic estimate is less than 2.3 %.

It is unfortunate that the greater overestimate of TBW occurs with the hydrogen isotopes, because they cost between one-tenth and one hundredth of the price of oxygen-18. It is therefore considerably cheaper to label animals with hydrogen isotopes, unless very low doses of oxygen-18 are employed (Vache et al., 1995). The overestimate by hydrogen isotopes would not be a serious problem if the extent of overestimate was constant, and a correction factor could then be applied. However, comparisons of hydrogen (N_H) to oxygen (N_O) spaces reveals substantial inter-individual variability in the ratio of the two dilution spaces, called the dilution space ratio (N_H/N_O).

There has been some dispute in the literature over the nature of the inter-individual variability in N_H/N_O. On the one hand some researchers have suggested that the observed variation reflects mostly physiological factors, and is influenced by the availability of exchangeable hydrogen to undergo exchange. This might be related to exercise (Klein et al., 1984), gender (Goran et al., 1992), endurance training (Goran and Poehlman, 1992), body fatness (Westerterp et al., 1995) or age and weight gain in infants (Wells et al., 1998). Others, however, suggest that the variation is mostly due to random analytical error in the isotope analyses. This might be traced to the gas preparation technique, analysis machine, analytical laboratory, contamination effects, isotopic fractionation or matrix effects (e.g. Speakman et al., 1990; Schoeller et al., 1995). Most of this analytical error has been attributed to errors in deuterium rather than oxygen-18 analyses (e.g. Racette et al., 1994; Roberts et al., 1995; Schoeller and Hnilicka, 1996).

The extent to which N_H exceeds the body water content may introduce errors into reconstructions of body composition when these are based on hydrogen isotope dilution. Moreover, variation in the ratio of N_H to N_O may introduce large errors into the calculation of CO_2 production by DLW. Several different models are available for the calculation of CO_2 production when using doubly labelled water (Speakman, 1997). In two-pool models the turnover of each of the isotopes in body water is multiplied by their respective dilution spaces. A question arises, however, as to how this calculation is best achieved. If the variation in the dilution space ratio is mostly analytical then the best approach would be to take a population or group mean for the ratio and substitute it into the equations (Schoeller et al., 1986; Speakman et al., 1993; Racette et al., 1994; Matthews and Gilker, 1995). Conversely, if the variation is mostly physiological then the individual ratios should be employed in the equations (as advocated by Coward and Prentice, 1985; Coward et al., 1985; Prentice, 1990).

There are three lines of evidence to support the notion that the observed variation in dilution space ratios may represent mostly analytical error. First, dilutions *in vitro*, and repeatability measurements in humans, indicate that between 70 % and 100 % of the variation in the ratio is analytical (Speakman et al., 1993). Second, measured ratios frequently include observations that would appear biologically unrealistic because $N_H<N_O$ or $N_H>1.052\times N_O$ (Culebras and Moore, 1977), or $N_H>1.023\times N_O$ (Matthews and Gilker, 1995). Third, in some validation studies of the DLW method, using the individual ratios does not enhance the fit of individual data to the reference methods (e.g. Schoeller et al., 1986, Bevan et al., 1995a; Boyd et al., 1995).

Alternatively, the notion that the variation is physiological is supported by propagating error from the mass spectrometric determinations of hydrogen and oxygen enrichment. Such error propagation suggests that only 20-50 % of the observed variation is analytical (Speakman et al., 1993). Moreover, the DLW validation studies of Coward and Prentice (1985), Wong et al. (1990) and Bevan et al. (1995b) suggested there *was* an improved fit of DLW data to the reference method when individual ratios were used.

As hydrogen occurs with two rare isotopes, there is another approach that can be employed to determine whether variability in the dilution space ratio is mostly analytical or physiological. If both hydrogen isotopes were injected simultaneously, at the same time as the oxygen isotope, the dilution space ratios could be determined with respect to both of the hydrogen isotopes. These dilution space ratios would be strongly correlated if variations in the dilution spaces were physiological, but not correlated if the variation was mostly analytical. Anbar and Lewitus (1958) injected these three isotopes into rabbits but did not present sufficient data to evaluate whether the dilution space ratios were correlated or not. Reilly and Fedak (1990) and Arnould et al. (1996) both injected seals (*Halichoerus grypus* and *Arctocephalus gazella*, respectively) with deuterium and tritium, and then desiccated them. In both cases estimated dilution spaces of the two hydrogen isotopes, relative to body water content, were not correlated, supporting the notion that the variability is mostly analytical. However, the sample size in both these experiments was small (4 and 5, respectively), so the possibility of type 2 error is strong.

In the present study we injected mice with deuterium mixed with oxygen-18, and separately with tritium. This allowed us to establish the dilution space ratios based on both deuterium and tritium. The hydrogen isotopes were analysed using independent methods. The principal aim of the study was to examine whether the dilution space ratios using the two independent hydrogen labels were correlated or not.

Materials and methods

Animals

Experiments were conducted on non-reproductive and lactating female mice (*Mus musculus*: outbred MF1), 10-12 weeks old. Each mouse was housed separately in a plastic 'shoe-box' cage, provided with sawdust and *ad libitum* supplies of water and food (CRM pellets, Special Diet Services, Witham, Essex, UK). The ambient temperature was

regulated at 30±1 °C and the photoperiod was 16 h:8 h L:D. Mice were acclimated to the experimental conditions for at least 3 weeks prior to measurements.

Estimation of dilution spaces in vivo

15 non-reproductive and 12 lactating mice at the peak of lactation (14 days after parturition) were injected with isotopes between 10:00 h and 12:00 h. Individuals were weighed to ±0.01 g using a Sartorius top-balance and labelled with two intraperitoneal injections of approximately 0.2 g water containing: (1) enriched deuterium (4.63 atom %) and oxygen-18 (9.44 atom %) and (2) enriched tritium (767 674.6 disints min^{-1} mg^{-1}; 12.795 MBq g^{-1}). Injections were made within 10 s of each other. Syringes were weighed to ±0.0001 g (using an Ohaus Analytical Plus balance, calibrated daily using standard weights traceable to the international standard) immediately before and after injection. We allowed 60 min for the isotopes to reach equilibrium (Anbar and Lewitus, 1958; Nagy, 1983), giving the animals free access to food and water over this interval. After 60 min, an initial 80-100 µl blood sample was collected (tail tipping). Blood samples were immediately flame-sealed into pre-calibrated pipettes (50 µl; Vitrex, Camlab Ltd) and stored in a fridge at 4 °C until analysis. 24 h after the initial samples were taken, the final samples were collected. Blood samples were taken from each mouse the day before injection to evaluate background isotope enrichments of deuterium, oxygen-18 and tritium,. For each time point, two independent samples of blood were vacuum-distilled into two separate glass Pasteur pipettes (Volac, John Poulten Ltd) (Nagy, 1983). One of these distillates was used for mass spectrometric analysis of stable isotopes (deuterium and oxygen-18), while the other was used for liquid scintillation counting of tritium. We rejected one of the estimates of deuterium dilution space for the non-reproductive individuals, which was dependent on an analysis following a malfunction of the mass spectrometer and was clearly erroneous. We had insufficient sample to reschedule analysis for this animal.

Total body water by desiccation

In a separate group of mice we measured total body water (TBW) by desiccation. Nine non-reproductive and eight lactating (14 d after parturition) females were weighed as described above and immediately killed by cervical dislocation. The carcasses were split open along the midline and transverse cuts across the body were also made so that there was increased exposure of the tissues for drying. The carcasses were dried to constant mass in a convection oven at 60 °C, to avoid loss of short chain-fatty acids (Crum et al., 1985). Desiccation was assumed to be complete when the carcasses lost no further mass (to within ±0.05 g) for three consecutive days. Carcasses required 12-14 days for complete desiccation. The difference between live and dry mass was taken as TBW, and expressed as a percentage of the body mass prior to desiccation.

Dilution experiment in vitro

To assess the analytical variability in the measured dilution space ratio we made a series of five isotope dilutions in vitro. Between 6.7 and 10.2 g of tap water of known isotopic enrichment was added to dry weighed (to ±0.0001 g) glass vessels. A weighed quantity of the deuterium/oxygen-18 injectate (approximately 0.1-0.2±0.0001 g) was added to each vessel (in proportions similar to those expected in the injected mice), and the mixture was vortex-mixed for 1 min to promote equilibration. Five sub-samples of each mixture were analysed in the same way as samples derived from the animals, but they were not distilled prior to the analysis since previous comparisons in our laboratory have shown that the distillation process does not involve fractionation of the sample. The mean enrichment of the five replicate analyses for each mixture was used to generate estimates of the enrichment of the deuterium/oxygen-18 injectate (Prentice, 1990). The mean of these estimates across the five mixtures was then employed to evaluate the isotope dilution spaces and the dilution space ratio for each vessel. The variation in these ratios reflects the analytical variation (i.e. precision) in the dilution space ratio resulting from our analytical procedures. We employed the same derived estimates of the mean injectate enrichments of deuterium and oxygen-18 to establish the dilution spaces of the injected mice.

Time course of isotope equilibration and elimination

For small animals such as mice it is not possible to take blood samples from them repeatedly at frequent intervals during the period of isotope equilibration. To overcome this problem we examined the washout kinetics of oxygen-18 for 5 h following injection, using breath sampling of expired CO_2 (Speakman and Thomson, 1997). Three non-reproductive mice were weighed and labelled with the deuterium/oxygen-18 injectate as described above. Within 10 s of injection, each mouse was placed into a sealed perspex chamber (216 ml volume) with a continuous throughflow of air (approximately 400 ml min^{-1}), dried upstream from the chamber by silica gel. Samples of gas from the chamber, containing respiratory CO_2 from the mouse, were collected via a 19-gauge needle directly into Vacutainers® (10 ml; Becton Dickinson Vacutainer Systems Europe). Single breath samples were collected every minute between 2 and 15 min after injection. Thereafter batches of five breath samples (approximately 5 s apart) were collected every 15 min until 5 h after injection. A final batch of five samples was taken 25 h after injection. During the first 15 min of the experiment, the mice were continuously inside the chamber. After this the mice were removed from the chamber between consecutive measurements and replaced in their cages with free access to food and water. During these intervals any urine and faeces were cleaned from the chamber. The mice were kept in the chamber for 2.5 min before breath sampling. Five breath samples were collected 5 min before injection to evaluate the background enrichment of oxygen-18. Breath samples were analysed by isotope ratio mass spectrometry as described below.

Deuterium and oxygen-18 analyses

Mass spectrometric analysis of deuterium enrichment was performed using H_2 gas. The H_2 was produced by reacting water, distilled from blood, with $LiAlH_4$ (after Bocek et al.,

1973; S. Ward, M. Scantlebury, E. Król, P. J. Thomson, C. Sparling and J. R. Speakman, in preparation). Reactions were performed inside 10 ml Vacutainers®. Approximately 0.05 g of $LiAlH_4$ powder (Aldrich, Cat. No. 19,987–7) was transferred to the Vacutainers® in a N_2 atmosphere. Vacutainers® were then re-evacuated to a pressure of 10 Pa, and left for 12–14 h in normal atmosphere. After this period the Vacutainers® were re-attached to a vacuum manifold, and only those which maintained a vacuum of 10 Pa (approximately 80 % of tubes) were replaced in the N_2 atmosphere, injected with 10 µl of distilled water and vortex-mixed for 30 s.

For analysis of oxygen-18 enrichment in blood samples, water distilled from blood was equilibrated with CO_2 gas using the small sample equilibration technique (Speakman et al., 1990). Pre-weighed Vacutainers® were injected with 10 µl of distilled water and re-weighed (to ±0.0001 g), to account for differences in the amount of water added. Subsequently, the Vacutainers® with the samples were injected with 0.5 ml CO_2 with a known oxygen isotopic enrichment, and left to equilibrate at 60 °C for 16 h.

An Optima isotope ratio mass spectrometer combined with a dual-inlet and a Multiprep system (Micromass Ltd) was used to measure the ^2H:^1H ratios. For analysis of ^{18}O:^{16}O ratios, equilibrated water or breath samples were admitted to an ISOCHROM µGAS system (Micromass Ltd), which uses a gas chromatograph column to separate nitrogen and CO_2 in a stream of helium gas before analysis by IRMS. We used isotopically characterised gases of H_2 and CO_2 (CP grade gases, BOC Ltd) in the reference channels. Reference gases were characterised every 3 months relative to SMOW and SLAP (Craig, 1961) supplied by the IAEA. Analysis of standards distributed to other laboratories showed good comparability to the mean results across these other laboratories (Speakman et al., 1990). Each batch of samples was run adjacent to triplicates of three laboratory standards to correct for day-to-day differences in mass spectrometer performance. All isotope enrichments were measured in $\Delta\,ml^{-1}$ relative to the working standards and converted to p.p.m., using the established ratios for these reference materials. Most measures of isotope enrichment in blood samples and in vitro water samples were based on independent analysis of two sub-samples; all further calculations were performed on the mean values.

Tritium analysis

The specific activity of tritium was measured using a liquid scintillation counter (Packard, Model 1600TR). Independently distilled 10 µl samples of water from blood were pipetted into pre-weighed standard liquid scintillation vials and re-weighed (to ±0.0001 g), to correct for differences in the amount of water added. After adding 2 ml of scintillation fluid (Ultima Gold™ XR) and vortex-mixing for 1 min, the samples were counted twice for 10 min, and the average of those two readings was used for each vial. All samples were corrected for background level of radioactivity by counting vials containing only scintillation fluid. Specific activity of tritium was expressed as disints min^{-1} mg^{-1} of water. Each measure of specific activity of tritium in the sample was based on the independent analysis of three sub-samples; all further calculations were performed on the average values.

Estimation of the enrichment of the tritium injectate was conducted in the same manner as for the deuterium/oxygen-18 injectate, using five mixtures of tap water and the tritium injectate, and counting five sub-samples for each mixture. For each mouse, background, initial and final samples were analysed on the same day, so there was no need to perform corrections for physical decay of tritium (Nagy and Costa, 1980). Corrections were made only for the injectate to establish the specific activity of the injectate on each day that the samples were analysed.

Calculations

Isotope dilution spaces were calculated by the plateau method (for mice and in vitro samples) and by the intercept approach (mice only). Calculation followed Halliday and Miller (1977) for both methods:

$$N_i = \frac{M_{inj}(E_{peak} - E_{inj})}{(E_{bg} - E_{peak})}, \quad (1)$$

where N_i (mol) is the dilution space for deuterium, oxygen-18 or tritium; M_{inj} is the amount of injectate (mol) injected into the mouse (or the vessel); E_{peak} is the initial isotope enrichment (p.p.m.) of body water (or the isotope enrichment of the mixture in the vessel) in the case of the plateau method, or the isotope enrichment of the body water at the time of injection in the case of intercept method (see equation 2); E_{inj} is the enrichment (p.p.m.) of the deuterium/oxygen-18 or tritium injectate; E_{bg} is the background isotope enrichment (p.p.m.) of body water (or tap water). Calculated molecular masses for the deuterium/oxygen-18 injectate and tritium injectate were 18.282 and 18.010, respectively.

When the initial isotope enrichment of the body water (E_{peak}) was calculated by the intercept method (Coward and Prentice, 1985), we used the following equation:

$$E_{peak} = \text{antilog}_e(\log_e(E_{init} - E_{bg}) + k_i) + E_{bg}, \quad (2)$$

where E_{init} and E_{bg} are the initial and the background isotope enrichments of body water (p.p.m.); k_i is the isotope elimination rate between the initial and final samples (h^{-1}).

Isotope dilution spaces (mol) were converted to g, assuming that the molecular mass of body water and tap water is 18.002. Dilution spaces in vivo were expressed as percentage of body mass before injection.

Statistics

Data are presented as mean ± S.D., N = sample size. Comparison of body mass, dilution spaces, dilution space ratios and desiccation spaces between non-reproductive and lactating mice were made using two-tailed two-sample *t*-tests. The effect of tracer employed (deuterium compared with tritium) on dilution spaces and dilution spaces ratios were determined by two-tailed, paired *t*-tests. Relationships between

deuterium and tritium dilution spaces, dilution space ratios based on deuterium and tritium, and the relationship of the dilution space ratios to the oxygen dilution space were described using Pearson product–moment correlation coefficients. For percentage data (dilution and desiccation spaces expressed as a percentage of body mass), arcsine–square-root transformations were performed prior to analysis (Zar, 1996). All statistical analyses were conducted using Minitab for Windows (version 12, Minitab Inc.) (Ryan et al., 1985). A level of significance $P<0.05$ was used in all tests.

Results

Dilution spaces in vivo

Background enrichments of deuterium for non-reproductive and lactating mice were 149.15±0.97 p.p.m. (N=15) and 149.71±0.98 p.p.m. (N=12) respectively. The background enrichment of oxygen-18 was 2001.6±2.9 p.p.m. (N=15) for non-reproductive mice and 2009.35±4.5 p.p.m. (N=12) for lactating individuals. The background activity of tritium was 4.5±1.55 disints min^{-1} mg^{-1} water (N=15) and 4.08±1.22 disints min^{-1} mg^{-1} water (N=12) for non-reproductive and lactating mice, respectively. Dilution spaces calculated by the plateau and the intercept method are presented in Table 1, and expressed as a percentage of body mass in Table 2. Using the plateau method, deuterium dilution spaces (N_D) ranged from 59.0 % to 88.0 % of body mass. Tritium dilution spaces (N_T) covered a similar range from 55.9 % to 83.7 %. Oxygen-18 dilution spaces (N_O) varied between 53.5 % and 86.1 %. Non-reproductive and lactating mice did not differ significantly in body mass (t_{24}=–0.13, P=0.90). However, for all three isotopes, the absolute dilution spaces and dilution spaces as a percentage of body mass were significantly greater in lactating females than in non-reproductive individuals (absolute dilution spaces for N_D, t_{24}=–5.88; for N_T, t_{24}=–5.80; for N_O, t_{24}=–6.53; percentage dilution spaces for N_D, t_{24}=–9.15; for N_T, t_{24}=–9.33; for N_O,

Table 1. *Dilution spaces of deuterium (N_D), tritium (N_T) and oxygen-18 (N_O), calculated by the plateau and the intercept method*

		Dilution spaces (mol)									
		Plateau method					Intercept method				
Mouse	Status	N_D	N_T	N_O	N_D/N_O	N_T/N_O	N_D	N_T	N_O	N_D/N_O	N_T/N_O
1	Nr	1.358	1.365	1.269	1.070	1.076	1.343	1.353	1.245	1.078	1.086
2	Nr	1.392	1.378	1.446	0.963	0.953	1.376	1.363	1.421	0.968	0.959
3	Nr	1.460	1.377	1.481	0.986	0.930	1.450	1.359	1.466	0.989	0.927
4	Nr	1.464	1.456	1.462	1.001	0.996	1.451	1.444	1.439	1.008	1.004
5	Nr	1.203	1.188	1.216	0.990	0.977	1.194	1.179	1.198	0.997	0.984
6	Nr	1.574	1.486	1.422	1.107	1.045	1.563	1.475	1.402	1.114	1.052
7	Nr	1.339	1.323	1.269	1.055	1.042	1.327	1.311	1.247	1.064	1.051
8	Nr	1.469	1.482	1.343	1.094	1.103	1.454	1.468	1.323	1.099	1.110
9	Nr	1.258	1.239	1.203	1.046	1.029	1.250	1.230	1.186	1.054	1.037
10	Nr	1.251	1.246	1.200	1.043	1.038	1.241	1.235	1.185	1.048	1.043
11	Nr	1.595	1.511	1.391	1.146	1.086	1.580	1.497	1.367	1.155	1.095
13	Nr	1.217	1.148	1.176	1.035	0.976	1.208	1.140	1.160	1.041	0.983
14	Nr	1.228	1.193	1.161	1.058	1.028	1.219	1.184	1.142	1.067	1.037
15	Nr	1.426	1.380	1.451	0.982	0.951	1.415	1.367	1.429	0.990	0.957
Mean		**1.374**	**1.341**	**1.321**	**1.041**	**1.017**	**1.362**	**1.329**	**1.301**	**1.048**	**1.023**
S.D.		**0.130**	**0.121**	**0.119**	**0.053**	**0.054**	**0.129**	**0.119**	**0.118**	**0.054**	**0.056**
16	Lact	1.823	1.734	1.784	1.022	0.972	1.758	1.668	1.703	1.032	0.979
17	Lact	1.933	1.905	1.908	1.013	0.999	1.866	1.840	1.822	1.024	1.010
18	Lact	1.711	1.648	1.646	1.040	1.001	1.651	1.584	1.568	1.053	1.010
19	Lact	1.685	1.632	1.680	1.003	0.972	1.633	1.585	1.611	1.014	0.984
20	Lact	1.545	1.451	1.555	0.994	0.933	1.495	1.403	1.488	1.004	0.942
21	Lact	2.015	1.919	1.858	1.084	1.033	1.953	1.852	1.785	1.094	1.037
22	Lact	1.677	1.681	1.672	1.003	1.005	1.605	1.612	1.588	1.011	1.015
23	Lact	1.747	1.694	1.665	1.049	1.017	1.696	1.642	1.604	1.057	1.024
24	Lact	1.910	1.878	2.006	0.952	0.936	1.825	1.795	1.889	0.966	0.950
25	Lact	1.507	1.504	1.559	0.967	0.964	1.465	1.461	1.496	0.979	0.977
26	Lact	1.633	1.598	1.584	1.031	1.009	1.581	1.547	1.517	1.042	1.020
27	Lact	1.481	1.413	1.399	1.058	1.010	1.450	1.387	1.354	1.071	1.024
Mean		**1.722**	**1.672**	**1.693**	**1.018**	**0.988**	**1.665**	**1.615**	**1.619**	**1.029**	**0.998**
S.D.		**0.171**	**0.169**	**0.170**	**0.038**	**0.032**	**0.161**	**0.157**	**0.155**	**0.037**	**0.031**

Nr, non-reproductive female; Lact, lactating female.
N=14 (non-reproductive mice); N=12 (lactating mice).

Table 2. *Dilution spaces of deuterium (N_D), tritium (N_T) and oxygen-18 (N_O), calculated by the plateau and the intercept method*

			Dilution spaces (% of BM)					
			Plateu method			Intercept method		
Mouse	Status	Bm (g)	N_D	N_T	N_O	N_D	N_T	N_O
1	Nr	37.90	64.5	64.8	60.3	63.8	64.3	59.1
2	Nr	39.02	64.2	63.6	66.7	63.5	62.9	65.5
3	Nr	41.03	64.1	60.4	65.0	63.6	59.6	64.3
4	Nr	42.11	62.6	62.2	62.5	62.0	61.7	61.5
5	Nr	36.72	59.0	58.2	59.6	58.6	57.8	58.7
6	Nr	47.86	59.2	55.9	53.5	58.8	55.5	52.7
7	Nr	34.07	70.8	69.9	67.1	70.1	69.3	65.9
8	Nr	40.37	65.5	66.1	59.9	64.9	65.5	59.0
9	Nr	34.65	65.4	64.3	62.5	65.0	63.9	61.6
10	Nr	32.68	68.9	68.6	66.1	68.4	68.0	65.3
11	Nr	45.53	63.1	59.7	55.0	62.5	59.2	54.1
13	Nr	29.11	75.2	71.0	72.7	74.7	70.5	71.7
14	Nr	29.73	74.4	72.3	70.3	73.8	71.7	69.2
15	Nr	35.56	72.2	69.9	73.5	71.6	69.2	72.4
Mean		**37.60**	**66.4**	**64.8**	**63.9**	**65.8**	**64.2**	**62.9**
S.D.		**5.51**	**5.2**	**5.1**	**6.0**	**5.2**	**5.1**	**6.0**
16	Lact	37.28	88.0	83.7	86.1	84.9	80.6	82.2
17	Lact	41.77	83.3	82.1	82.2	80.4	79.3	78.5
18	Lact	35.56	86.6	83.4	83.3	83.6	80.2	79.4
19	Lact	35.66	85.1	82.4	84.8	82.4	80.0	81.3
20	Lact	33.58	82.8	77.8	83.4	80.1	75.2	79.8
21	Lact	44.46	81.6	77.7	75.3	79.1	75.0	72.3
22	Lact	38.18	79.1	79.3	78.8	75.7	76.0	74.9
23	Lact	39.38	79.8	77.5	76.1	77.5	75.0	73.3
24	Lact	43.67	78.7	77.4	82.7	75.3	74.0	77.9
25	Lact	34.86	77.8	77.7	80.5	75.6	75.5	77.3
26	Lact	36.66	80.2	78.5	77.8	77.6	76.0	74.5
27	Lact	33.06	80.6	77.0	76.2	79.0	75.5	73.7
Mean		**37.84**	**82.0**	**79.5**	**80.6**	**79.3**	**76.9**	**77.1**
S.D.		**3.78**	**3.2**	**2.6**	**3.7**	**3.2**	**2.4**	**3.3**

BM, body mass before the isotope injection; Nr, non-reproductive female; Lact, lactating female.

Values are expressed as a percentage of body mass, for 14 non-reproductive and 12 lactating mice.

t_{24}=–8.54; in all cases P<0.0001). On average, across all three isotopes, the dilution spaces as a percentage of body mass were 15.7±1.0 % larger in the lactating mice.

Across all the individuals, there was a significant effect of the hydrogen isotope on the estimated hydrogen dilution space as a percentage of body mass (paired t_{25}=5.93, P<0.0001), with the deuterium space larger than the tritium space by on average 2.0±1.6 % (N=26). Despite this overall difference, deuterium and tritium spaces, as a percentage of body mass, were highly correlated with each other (Fig. 1; N=26, r=0.984, P<0.001), strongly suggesting that the observed variability was physiological rather than analytical. All these trends were mirrored in the dilution spaces calculated by the intercept method (Tables 1, 2).

The dilution space ratios (N_D/N_O and N_T/N_O) derived from the plateau method ranged from 0.952 to 1.146 when using deuterium and from 0.930 to 1.103 when using tritium (Table 1). The mean dilution space ratio did not differ between non-reproductive and lactating females (for N_D/N_O: t_{24}=1.26, P=0.22; for N_T/N_O: t_{24}=1.63, P=0.12). Across all the individuals, there was a significant effect of the hydrogen isotope on the dilution space ratio (paired t_{25}=6.09, P<0.0001), with dilution space ratios based on deuterium (N_D/N_O) exceeding the ratios based on tritium (N_T/N_O) by on average 0.027±0.023 (N=26). Dilution space ratios based on deuterium and tritium were highly correlated with each other (Fig. 2; N=26, r=0.882, P<0.001). Pooling data across all the mice

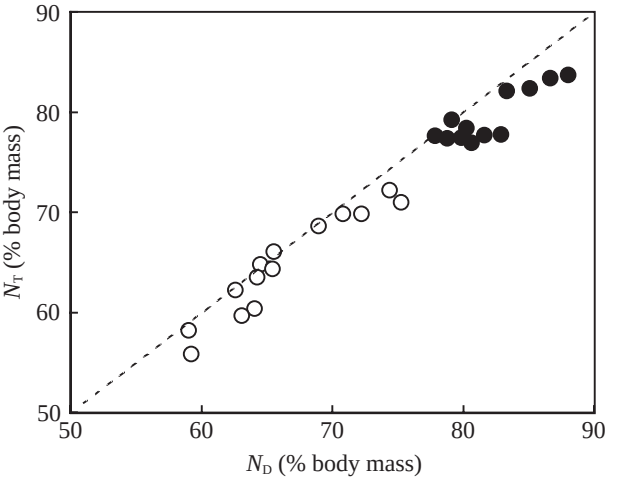

Fig. 1. Correlation between tritium (N_T) and deuterium (N_D) dilution spaces, expressed as a percentage of body mass, for 14 non-reproductive (open circles) and 12 lactating (filled circles) mice (N=26, r=0.984, P<0.001). Dilution spaces were calculated by the plateau method. The line of equality is shown.

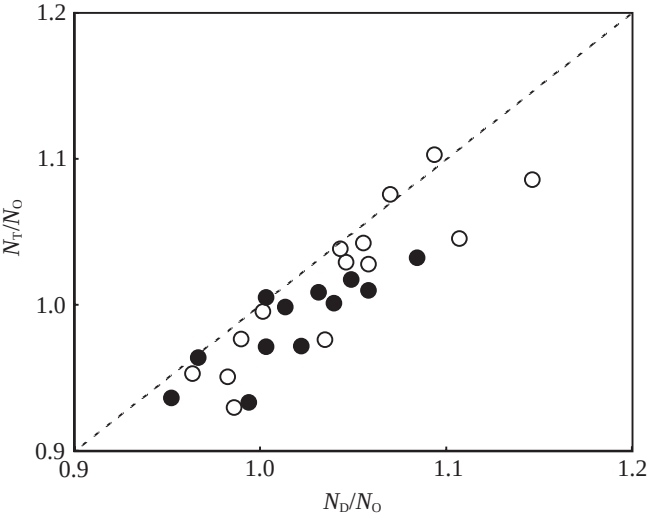

Fig. 2. Correlation between dilution space ratios based on tritium (N_T/N_O) and deuterium (N_D/N_O) for 14 non-reproductive (open circles) and 12 lactating (filled circles) mice (N=26, r=0.882, P<0.001). Dilution spaces were derived by the plateau method. The line of equality is shown.

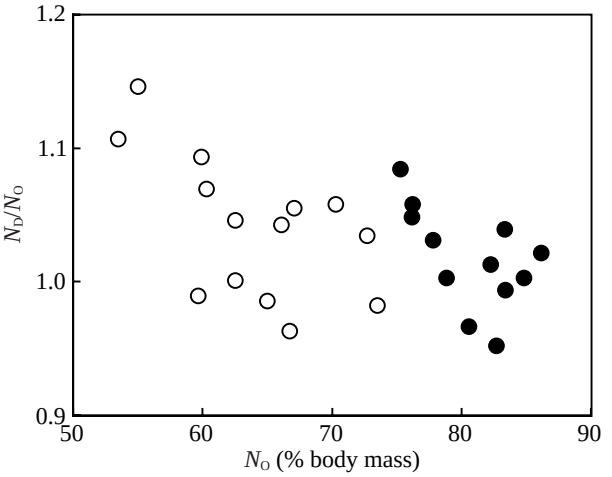

Fig. 3. Correlation between dilution space ratio based on deuterium (N_D/N_O) and oxygen dilution space (N_O), expressed as a percentage of body mass, for 14 non-reproductive (open circles) and 12 lactating (filled circles) mice (N=26, r=–0.498, P=0.010). Dilution spaces were calculated by the plateau method.

(N=26), the standard deviation in the dilution space ratio for both deuterium and tritium was 0.047. Almost identical trends were apparent using dilution spaces derived from the intercept approach.

The dilution space ratio based on deuterium (N_D/N_O), calculated by the plateau method, was negatively related to the oxygen dilution space (N_O), expressed as a percentage of body mass (Fig. 3; N=26, r=–0.498, P=0.010). A similar relationship was observed for the dilution space ratio based on tritium (N_T/N_O) (N=26, r=–0.558, P=0.003).

Total body water by desiccation

Total body water measured by desiccation averaged 63.4±3.2 % of body mass (range 57.0–67.2 %, N=9) for non-reproductive females, and 73.4±1.1 % of body mass (range 71.9–75.0 %, N=8) for lactating females. The difference between the two groups was significant (t_{15}=–8.68, P<0.0001).

Dilution spaces in vitro

Estimates of water content of the vessels based on the dilution of deuterium and oxygen-18 are presented in Table 3. Across all five vessels, the deuterium dilution spaces (N_D) underestimated the actual water content by on average 0.011±0.699 %, while the oxygen-18 dilution spaces (N_O) differed from the actual amount of water by on average –0.0001±1.618 %. The standard deviation of the dilution space ratios was 0.010. Comparing this variation (S.D.=0.010) with that observed *in vivo* (S.D.=0.047), suggests that only 4.5 % of variation in the dilution space ratios observed in the mice ($0.010^2/0.047^2\times100$) could be accounted for by analytical variation in the deuterium and oxygen-18 analyses, and thus by implication 95.5 % of the variation was physiological.

Table 3. *Comparison of water content estimated by dilution spaces of deuterium (N_D) and oxygen-18 (N_O), to actual water content of five vessels*

	Water content (g)			% difference to actual water content		
Vessel	N_D	N_O	Actual	for N_D	for N_O	N_D/N_O
1	6.8663	6.8359	6.8821	–0.230	–0.672	1.004
2	10.2614	10.3790	10.2054	0.548	1.701	0.989
3	9.0709	9.0293	9.1596	–0.968	–1.422	1.005
4	6.7784	6.8451	6.7252	0.791	1.782	0.990
5	9.2612	9.1503	9.2793	–0.195	–1.390	1.012
Mean				–0.011	0	1.0
S.D.				0.699	1.618	0.010

% difference was calculated as [(N_i − actual water content)/actual water content]×100, where N_i is the estimated isotope dilution space for deuterium or oxygen-18.

Time course of isotope equilibration and elimination

The time course of the enrichment of oxygen-18 above background in breath samples, averaged across the three mice, is presented on Fig. 4. The label reached a peak enrichment between 2 and 6 min after injection at 1159±102 p.p.m. Between 6 and 15 min after injection the enrichment declined rapidly to 1028±34 p.p.m. Thereafter, between 30 and 300 min after injection the enrichment declined much more slowly from 1008±96 to 887±41 p.p.m. The isotope elimination rate (k_O) evaluated over the period from 30 min to 300 min was

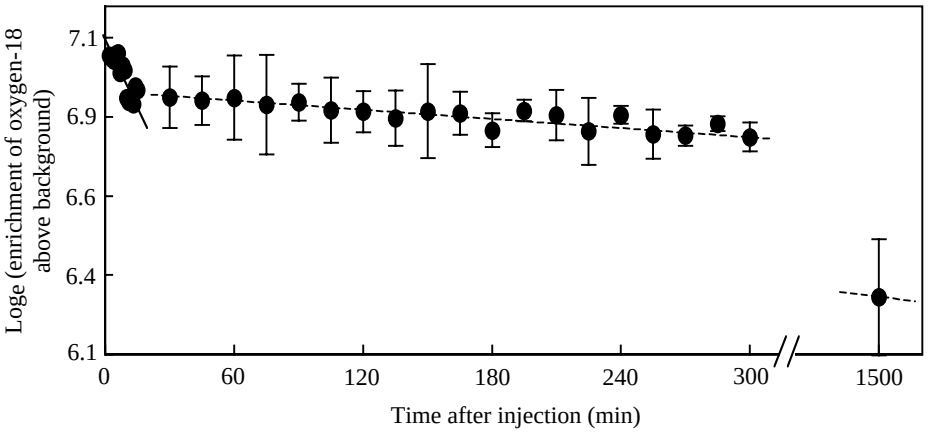

Fig. 4. Time course of $\log_e$ converted enrichment of oxygen-18 above background in mouse breath samples following injection at time 0. Values (p.p.m.) are means for three non-reproductive females; values between 30 and 1500 min values are means ± S.D. The solid line fitted over the first 15 min is based on a linear regression for analyses between 2 min and 15 min post-injection. The dotted line is the fitted elimination curve for samples collected between 30 min and 300 min post injection. This line was extrapolated to 1500 min and is shown with the actual observed enrichment at that time.

$0.028\pm0.013\,h^{-1}$. This gradient, extrapolated to 25 h (504±132 p.p.m.), matched closely the enrichment measured after 25 h (540±94 p.p.m.) (Fig. 4). This pattern suggests that the label rapidly entered the blood and then spread throughout the inter- and intracellular water in the rest of the body, reaching complete equilibrium in around 15 min.

Discussion

Dilution spaces

Background variation in all three isotopes between individuals was low, as might be expected for animals in constant conditions feeding on a constant diet with the same source of water. There were no significant differences in the background enrichments of all three isotopes between non-reproductive and lactating individuals. The large difference in isotope dilution spaces between non-reproductive and lactating individuals (Fig. 1, Table 2) was unexpected, as previous studies of dilution spaces in lactating and non-reproducing humans have not reported such a difference (e.g. Goldberg et al., 1991; Forsum et al., 1992). The mean oxygen-18 dilution space for non-reproductive individuals (plateau approach) was almost identical to the mean TBW by desiccation (0.5 % different), while the mean estimated oxygen-18 dilution space for lactating individuals was 7.2 % greater than the desiccation space, and paralleled by increased hydrogen spaces. One possible explanation for these data is that early in the equilibrium period some body water, containing isotopes at high enrichment (Fig. 4), was transferred into milk water, and this water was then partitioned from further equilibration (i.e. functionally eliminated). This seems unlikely, however, as previous comparisons of isotope enrichments in water derived from body fluids and milk (e.g. Butte et al., 1988) are generally in close agreement, suggesting the water in milk remains in isotope equilibration with body water until elimination from the body.

The implication from these data was that lactating mice contained additional non-aqueous sites that exchange both oxygen and hydrogen in the same ratio as water, which were not present in non-reproductive individuals. The nature of these sites, however, remains obscure. Perhaps some label was incorporated both reversibly and irreversibly into milk solids (Goldberg et al., 1991) during the 1 h equilibration period. However, estimates of milk production by these mice (approximately $8–12\,ml\,d^{-1}$; E. Król, unpublished) suggests this is unlikely to account for all the observed discrepancy.

The significant difference in the dilution spaces derived from the different hydrogen isotopes (Tables 1, 2) was also unexpected. This difference was not found during earlier simultaneous injections of deuterium and tritium (Reilly and Fedak, 1990; Arnould et al., 1996), but in both these previous cases the sample sizes involved were small, and perhaps insufficient to detect a difference. Tritium is a much larger atom than deuterium (the mass ratio of tritium to deuterium is 1.498). The larger dilution spaces reported here for deuterium may thus reflect greater mobility of the smaller molecules containing deuterium, or an increased propensity for the smaller atom to exchange at exchangeable sites in the body tissues. Consistent with our observations, previous studies have reported greater uptake of deuterium than tritium during sequestration into lipids during *de novo* synthesis (Glascock and Duncombe, 1952; Eidinof et al., 1953; Jungas, 1968). The observed difference between deuterium and tritium dilution spaces has potential implications for the calculation of CO_2 production if DLW studies were to employ tritium rather than deuterium in the two-pool methodology. This is because current equations based on the two-pool models have been derived using the population estimate of the dilution space ratio based on deuterium as the tracer (e.g. Coward et al., 1985; Schoeller et al., 1986; Speakman, 1993).

Dilution space ratios

It seems very improbable that the dilution space ratios derived from use of deuterium and tritium were highly correlated because of an interaction between the two hydrogen isotopes, causing them to partition in the same manner. This is because the proportional contributions of both isotopes to the total amount of hydrogen in the body were relatively small, and the chances of the interaction between individual deuterium and tritium atoms were negligible. The procedures for determination of deuterium and tritium enrichment were completely independent. These included independent distillations, which eliminated the possibility of correlated fractionation errors during distillation, and separate dosing, which eliminated potential correlated weighing errors. Another possible explanation for the correlated dilution space ratios, however, was that there was substantial analytical variability in our estimates of the oxygen dilution spaces. This variation might lead to correlated errors in the ratios based on deuterium and tritium. If this explained the effect, however, we would also expect analytical variability in the estimated oxygen enrichments to precipitate a similar level of variation in the dilution space ratios observed in the *in vitro* experiment. Yet this was not the case (Table 3). In addition, deuterium and tritium dilution spaces expressed as a percentage of body mass were also highly correlated (Fig. 1), and these measurements were independent of the oxygen-18 spaces.

In contrast to almost all the previous work on the dilution space ratio, our data strongly suggest that variation in the observed ratio was mostly physiological rather than analytical. We suggest three potential reasons why variation in our study may reflect mostly physiological variation. First, the correlation of the ratios derived from deuterium and tritium spaces (Fig. 2) may reflect other errors in the method of deriving dilution spaces from isotope distributions, which were not traceable to analytical variation in our isotope analyses. In particular, the estimated dilution space may have been affected by individual variation in the process of isotope equilibration (Kirkwood and Robertson, 1997), allied with variations in the extent to which labile hydrogen in body tissues becomes exposed to the circulating labels (Klein et al., 1984). For example, consider an animal that was active, or had a high

resting metabolic rate, during the equilibrium period. The activity or high metabolism might cause isotopes rapidly to permeate all the exchangeable pools, and in addition might cause the isotopic labels to flood through muscles exposing them to exchangeable hydrogen on amino groups (Klein et al., 1984). This would cause the individual to have relatively large dilution spaces for all isotopes, combined with much greater deuterium and tritium spaces than the oxygen space. Alternatively, an inactive animal or one with a low resting metabolism, might have relatively poor mixing of the isotopes, which would not fully permeate all the exchangeable pools during the equilibration period. This could also be linked with lack of exposure of the deuterium and tritium to labile hydrogen in muscles. These 'mixing problems' would precipitate a correlation between dilution space ratios based on deuterium and tritium that would not be traceable to the analytical variation in our isotope analyses.

The suggestion that the variation in dilution space ratios reflects mostly 'mixing problems' is supported by the fact the total body water estimated by dilution of oxygen-18 was substantially more variable than TBW measured by desiccation, and also more variable than anticipated from the analytical variation in oxygen-18 analyses. If individual variation in isotope mixing during the equilibrium period was an important factor, however, we might predict that the discrepancy between the variances in the oxygen dilution space measured *in vivo* and *in vitro* would be removed by using the intercept approach to derive the dilution spaces. This is because the intercept method involves extrapolation back to the point of injection, and should therefore be independent of the mixing processes during the equilibrium period. Yet the variances in the isotope dilution spaces, and the dilution space ratios, calculated by the intercept method were not reduced relative to those derived by the plateau approach (Tables 1, 2). As anticipated, however, the intercept approach did generally result in greater estimates of the dilution space than the plateau approach (Tables 1, 2), and this discrepancy between the two methods was greater in the lactating animals because of their higher water turnovers and thus steeper elimination curves.

Using the intercept approach did not reduce the variation in the estimated dilution space ratios, which might be because the mixing process was not complete in 60 min, hence both the plateau and the back-calculated intercept would be affected by the same problem. This interpretation, however, is not supported by the detailed time course of changes in the oxygen isotope enrichment following injection, which clearly indicated that equilibration occurred over the first 15 min following injection. Thereafter the elimination process dominated the time course of changes in enrichment (Fig. 4).

In addition, if differential mixing between individuals was causing the correlated variation in the dilution spaces and dilution space ratios, we would anticipate finding a significant positive correlation between the size of the oxygen dilution space relative to body mass, and the dilution space ratio (based on either deuterium or tritium). This is because the oxygen space expressed as a percentage of body mass would give an indication of the extent to which the labels were perfusing the body, while the dilution space ratio would reflect exposure of the hydrogen label to labile non-aqueous hydrogen. A significant relationship between these parameters was found, but the direction of the relationship was opposite to that expected from the 'mixing problems' explanation (Fig. 3).

Finally, some studies have made sequential measurements of dilution space ratios throughout the equilibrium period (Schoeller et al., 1980; Wong et al., 1988), and these do not point to greatly fluctuating ratios over time. However, in these latter studies the subjects (humans) were probably inactive during the equilibrium phase, and the dosing (orally) was different to that used on our mice (intraperitoneal injections), which may affect the mixing dynamics.

A second explanation for our results, which suggest that most variation in the dilution space ratio was physiological, may be undue reliance on theoretical stoichiometric models of where the hydrogen label might be exchanging (Culebras and Moore, 1977; Matthews and Gilker, 1995). These stoichiometries have generally suggested that there is only a maximum of between 2.3% and 5.2% more exchangeable hydrogen in the body tissues than in body water. Deviations from these expectations are then regarded as representing analytical error in isotope analyses. These estimates depend heavily, however, on our predictions of which hydrogen is exchangeable and which is not. The underlying assumption is that our knowledge of hydrogen chemistry is so great that the only reason for a value outside the limits defined by the stoichiometry must be analytical error elsewhere. Our dilution space ratio estimates can be reconciled with the stoichiometric predictions by suggesting that such predictions of which hydrogens are labile are at best imprecise, thus true dilution space ratios can vary over much wider ranges than are currently believed possible. Both Culebras and Morre (1977) and Mathews and Gilker (1995) emphasised the potential exchange of isotopic label on to labile hydrogen in amino groups. The negative correlation between the dilution oxygen spaces as a percentage of body mass and the dilution space ratio observed here, however, might indicate that labile hydrogen is perhaps also to be found in fat tissue. This is because fat tissue is relatively anhydrous and larger fat deposits are generally linked to low total body water contents. Supporting this interpretation, Westerterp et al. (1995) found dilution space ratios of adult humans were also linked to fat content, and in infants changes in the ratio over time were correlated to changing body water content, and by inference body fatness (Wells et al., 1998). Several other studies, however, have failed to detect links of dilution space ratio to body composition (e.g. Ravussin et al., 1991; Goran et al., 1992; Racette et al., 1994).

Third, previous suggestions that the variation in the dilution space ratio is mostly analytical have hinged upon observations that including individual spaces into calculations of CO_2 production by DLW do not generally improve the fit of DLW estimates to those generated by reference methods (e.g. Schoeller et al., 1986; Bevan et al., 1995a; Boyd et al., 1995;

but see Coward and Prentice, 1985; Wong et al., 1990; Bevan et al., 1995b). The interpretation that failure of inclusion of the individual dilution spaces to improve the fit of DLW studies to reference data reflects analytical imprecision is based on several preconceptions. In particular, it assumes that the two-pool model provides the best possible description of the behaviour of the hydrogen and oxygen isotopes in the body and how these relate to CO_2 production. Given this preconception, the deviations between the calculations based on this model and the reference method can only be interpreted as analytical error. Consequently, when using the individual dilution spaces fails to improve the fit, it is easiest to interpret this failure as a result of analytical errors in the dilution space estimates, rather than inferring some fundamental problem in the ability of the model to describe the behaviour of the isotopes in the body. Our data indicate that observed variability in the dilution space ratio may reflect mostly physiological variation. This may also be the case in other studies of dilution space. Including individual values into the model in these previous studies may not have improved the fit of DLW data to the reference methods, however, because there are other more serious deficiencies in the ability of the model to measure CO_2 production. These more serious inadequacies may underpin the individual variability in how closely DLW and reference methods agree (validation studies reviewed by Speakman, 1997, Table 8.3).

The current study is an important advance because it suggests two things. First, isotope dilution spaces, which may appear biologically unrealistic on the basis of our current knowledge of the chemistry of hydrogen, reveal as much about inadequacies in our knowledge of *in vivo* hydrogen chemistry as problems in the analytical instrumentation designed to measure isotope enrichments. Second, even though the individual dilution space ratios may reflect physiological variability, there may be fundamental problems in the DLW model which mean that there is little advantage in using the individual data for calculation.

We thank Sally Ward and Peter Thomson for assistance with isotope analysis and discussions about this work. E.K. was supported by a Royal Society Postdoctoral Fellowship.

References

Anbar, M. and Lewitus, Z. (1958). Rate of body-water distribution studied with triple labelled water. *Nature* **181**, 344.

Arnould, J. P. Y., Boyd, I. L. and Speakman, J. R. (1996). Measuring the body composition of Antarctic fur seals (*Arctocephalus gazella*): validation of hydrogen isotope dilution. *Physiol. Zool.* **69**, 93–116.

Bevan R. M., Speakman, J. R. and Butler, P. J. (1995a). Daily energy expenditure of tufted ducks: a comparison between indirect calorimetry, doubly labelled water and heart rate. *Funct. Ecol.* **9**, 40–47.

Bevan R. M., Woakes, A. J., Butler, P. J. and Croxall, J. P. (1995b). Heart rate and oxygen consumption of exercising gentoo penguins. *Physiol. Zool.* **68**, 855–877.

Bocek, P., Deml, M. and Tesarik, K. (1973). The determination of ^2H_2O in water and biological fluids by gas chromatography. *J. Chrom.* **87**, 246–249.

Bowen, W. D. and Iverson, S. J. (1998). Estimation of total body water in pinnipeds using hydrogen-isotope dilution. *Physiol. Zool.* **71**, 329–332.

Boyd, I. L., Woakes, A. J., Butler, P. J., Davis, R. W. and Williams, T. M. (1995). Validation of heart rate and doubly labelled water as measures of metabolic rate during swimming in California Sea Lions. *Funct. Ecol.* **9**, 151–160.

Butte, N. F., Wong, W. W., Patterson, B. W., Garza, C. and Klein, P. D. (1988). Human milk intake measured by administration of deuterium oxide to the mother: a comparison with the test-weighing technique. *Am. J. Clin. Nut.* **47**, 815–821.

Bryant, D. M. and Tatner, P. (1991). Intraspecific variation in avian energy expenditure: correlates and constraints. *Ibis* **133**, 236–245.

Coward, W. A. and Prentice, A. M. (1985). Isotope method for the measurement of carbon dioxide production rate in man. *Am. J. Clin. Nutr.* **41**, 659–661 (Letter).

Coward, W. A., Prentice, A. M., Murgatroyd, P. R., Davies, H. L., Cole, T. J., Sawyer, M., Goldberg, G. R., Halliday, D. and MacNamara, J. P. (1985). Measurement of CO_2 and water production rates in man using ^2H, ^{18}O-labelled H_2O; comparison between calorimetric and isotope values. In *European Nutrition Reports 5: Human Energy Metabolism: physical activity and energy expenditure measurements in epidemiological research based upon direct and indirect respirometry* (ed. A. J. H. van Es), pp. 126–128. CIP-gegevegens Koninklijke Bibliotheek, The Hague.

Craig, H. (1961). Standard for reporting concentrations of deuterium and oxygen-18 in natural waters. *Science* **133**, 1833–1834.

Crum, B. G., Williams, J. B. and Nagy, K. A. (1985). Can tritiated water-dilution space accurately predict total body water in chukar partridges? *J. Appl. Physiol.* **59**, 1383–1388.

Culebras, J. M. and Moore, F. D. (1977). Total body water and the exchangeable hydrogen I. Theoretical calculation of nonaqueous exchangeable hydrogen in man. *Am. J. Physiol*. **232**, R54-R59.

Eidinof, M. L, Perri, G. C., Knoll, J. E., Marano, B. J. and Arnheim, J. (1953). The fractionation of hydrogen isotopes in biological systems. *J. Am. Chem. Soc*. **75**, 248–249.

Forsum, E., Kabir, N., Sadurskis, A. and Westerterp, K. (1992). Total energy expenditure of healthy Swedish women during pregnancy and lactation. *Am. J. Clin. Nutr*. **56**, 334–342.

Glascock, R. F. and Duncombe, W. G. (1952). Biological fractionation of hydrogen isotopes in mammary gland and other tissues. *Biochem. J*. **51**, xl.

Goldberg, G. R., Prentice, A. M., Coward, W. A., Davies, H. L., Murgatroyd, P. R., Sawyer, M. B., Ashford, J. and Black, A. E. (1991). Longitudinal assessment of the components of energy balance in well-nourished lactating women. *Am. J. Clin. Nutr*. **54**, 788–798.

Goran, M. I. and Poehlman, E. T. (1992). Endurance training does not enhance total energy expenditure in healthy elderly persons. *Am. J. Physiol*. **263**, E950-E957.

Goran, M. I., Poehlman, E. T., Nair, K. S. and Danforth, E. J. R. (1992). Effect of gender, body composition, and equilibration time on the ^2H-to-^{18}O dilution space ratio. *Am. J. Physiol*. **263**, E1119-E1124.

Halliday, D. and Miller, A. G. (1977). Precise measurement of total body water using trace quantities of deuterium oxide. *Biomed. Mass Spectrom.* **4**, 82–87.

Hevesy, G. and Hofer, E. (1934). Elimination of water from the human body. *Nature* **134**, 879.

Jungas, R. L. (1968). Fatty acid synthesis in adipose tissue incubated in tritiated water. *Biochemistry* **7**, 3708–3717.

Kashiwazaki, H., Dejima, Y., Orias-Rivera, J. and Coward, W. A. (1998). Validity of published prediction equations for body composition in high altitude Bolivian Aymara as evaluated by doubly labeled water. *Am. J. Hum. Biol*. **10**, 371–384.

Kirkwood, R. and Robertson, G. (1997). The energy assimilation efficiency of emperor penguins, *Aptenodytes forsteri*, fed a diet of Antarctic krill, *Euphausia superba*. *Physiol. Zool*. **70**, 27–32.

Klein, P. D., James, W. P. T., Wong, W. W., Irving, C. S., Murgatroyd, P. R., Cabrera, M., Dallosso, H. M., Klein, E. R. and Nichols, B. L. (1984). Calorimetric validation of the doubly-labelled water method for determination of energy expenditure in man. *Hum. Nutr. Clin. Nutr*. **38C**, 95–106.

Lifson, N., Gordon, G. B. and McClintock, R. (1955). Measurements of total carbon dioxide production by means of $D_2{}^{18}O$. *J. Appl. Physiol.* **7**, 704–710.

Matthews, D. E. and Gilker, C. D. (1995). Impact of ^2H and ^{18}O pool size determinations on the calculation of total energy expenditure. *Obes. Res*. **3**, 21–29.

Nagy, K. A. (1980). CO_2 production in animals: analysis of potential errors in the doubly labeled water method. *Am. J. Physiol*. **238**, R466-R473.

Nagy, K. A. (1983). *The Doubly Labeled Water (3HH18O) Method: A guide to its use*. UCLA Publication no. 12–1417, University of California, Los Angeles, California, USA.

Nagy, K. A. and Costa, D. P. (1980). Water flux in animals: analysis of potential errors in the tritiated water method. *Am. J. Physiol*. **238**, R454-R465.

Oritz, R. M., Worthy, G. A. J. and Byers, F. M. (1999). Estimation of water turnover rates of captive West Indian manatees (*Trichechus manatus*) held in fresh and salt water. *J. Exp. Biol*. **202**, 33–38.

Prentice, A. M. (ed.) (1990). *The Doubly-Labelled Water Method for Measuring Energy Expenditure: Technical recommendations for use in humans*. A consensus report by the IDECG Working Group, International Atomic Energy Agency, NAHRES–4, Vienna.

Racette, S. B., Schoeller, D. A., Luke, A. H., Shay, K., Hnilicka, J. and Kushner, R. F. (1994). Relative dilution spaces of ^2H- and ^{18}O-labeled water in humans. *Am. J. Physiol*. **267**, E585-E590.

Ravussin, E., Harper, I. T., Rising, R., and Bogardus, C. (1991). Energy expenditure by doubly labeled water: validation in lean and obese subjects. *Am. J. Physiol*. **261**, E402-E409.

Reilly, J. J. and Fedak, M. A. (1990). Measurement of the body composition of living gray seals by hydrogen isotope dilution. *J. Appl. Physiol.* **69**, 885–891.

Roberts, S. B., Dietz, W., Sharp, T., Dallal, G. E. and Hill, J. O. (1995). Multiple laboratory comparison of the doubly labeled water technique. *Obes. Res*. **3**, 3–13.

Ryan, B. F., Joiner, B. L. and Ryan, T. A., Jr. (1985). *Minitab Handbook* (Second edition). PWS-Kent, Boston, Mass.

Schoeller, D. A. and Hnilicka, J. M. (1996). Reliability of the doubly labeled water method for the measurement of total energy expenditure in free-living subjects. *J. Nutr*. **126**, 348S–354S.

Schoeller, D. A., Ravussin, E., Schutz, Y., Acheson, K. J., Baertschi, P. and Jéquier, E. (1986). Energy expenditure by doubly labeled water: validation in humans and proposed calculation. *Am. J. Physiol*. **250**, R823-R830.

Schoeller, D. A., van Santen, E., Peterson, D. W., Dietz, W., Jaspan, J. and Klein, P. D. (1980). Total body water measurement in humans with ^{18}O and ^2H labeled water. *Am. J. Clin. Nutr*. **33**, 2686–2693.

Schoeller, D. A., Taylor, P. B. and Shay, K. (1995). Analytic requirements for the doubly labeled water method. *Obes. Res*. **3**, 15–20.

Speakman, J. R. (1993). How should we calculate CO_2 production in doubly labelled water studies of animals? *Funct. Ecol.* **7**, 746–750.

Speakman, J. R. (1997). *Doubly Labelled Water: Theory and Practice*. London: Chapman & Hall.

Speakman, J. R., Nagy, K. A., Masman, D., Mook, W. G., Poppitt, S. D., Strathearn, G. E. and Racey, P. A. (1990). Interlaboratory comparison of different analytical techniques for the determination of oxygen-18 abundance. *Anal. Chem*. **62**, 703–708.

Speakman, J. R., Nair, K. S. and Goran, M. I. (1993). Revised equations for calculating CO_2 production from doubly labeled water in humans. *Am. J. Physiol*. **264**, E912-E917.

Speakman, J. R. and Thomson, S. C. (1997). Validation of the labeled bicarbonate technique for measurement of short-term energy expenditure in the mouse. *Z. Ernährungswiss*. **36**, 273–277.

Vache, C., Gachon, P., Ferry, M., Beaufrere, B. and Ritz, P. (1995). Low-cost measurement of body composition with ^{18}O-enriched water. *Diabete & Metabolisme* **21**, 281–284.

Webb, P. M., Crocker, D. E., Blackwell, S. B., Costa, D. P. and Le Boeuf, B. J. (1998). Effects of buoyancy on the diving behavior of northern elephant seals. *J. Exp. Biol.* **201**, 2349–2358.

Wells, J. C. K., Ritz, P., Davies, S. W. and Coward, W. A. (1998). Factors affecting the ^2H to ^{18}O dilution space ratio in infants. *Ped. Res*. **21**, 242–246.

Westerterp, K. R., Wouters, L. and van Marken Lichtenbelt, W. D. (1995). The Maastricht protocol for the measurement with labeled water. *Obes. Res*. **3**, 49–57.

Wong, W. W., Butte, N. F., Garza, C. and Klein, P. D. (1990). Comparison of energy expenditure estimated in healthy infants using the doubly labelled water and energy balance methods. *Eur. J. Clin. Nutr*. **44**, 175–184.

Wong, W. W., Cochran, W. J., Klish, W. J., Smith, E. O., Lee, L. S. and Klein, P. D. (1988). In vivo isotope-fractionation factors and the measurement of deuterium- and oxygen-18-dilution spaces from plasma, urine, saliva, respiratory water vapor, and carbon dioxide. *A. J. Clin. Nutr*. **47**, 1–6.

Zar, J. H. (1996). *Biostatistical Analysis*. New Jersey: Prentice-Hall, Inc.