Validation of the doubly labeled water method in rats during isolation and simulated weightlessness

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Am J Physiol Regulatory Integrative Comp Physiol 279: R1964–R1979, 2000.—Total energy expenditure (TEE) of rats during simulated microgravity is unknown. The doubly labeled water method (DLW) reliably measures TEE, but the results depend on the methods of calculation. These methods were validated and appraised by indirect calorimetry in eight rats during isolation (7 days) and simulated microgravity (10 days). There were no effects on CO2 production in the method used to derive constant flux rates as in the regression models. rCO2 estimates were dependent on the assumed fractionation processes, the derivation of constant flux rate methods, and the selected pool models. Use of respiratory or food quotients did not influence TEE estimations, which were similar during isolation and simulation. During either isolation with growth or simulation with a stabilized mass, the one-pool model of Speakman (Speakman, JR. Doubly Labeled Water. Theory and Practice. London: Chapman and Hall, 1997) resulted in the more reliable validation (0.8 ± 2.2 and 2.2 ± 3.4% vs. calorimetry, respectively). However, during simulation, agreement was also observed with the single pool model of Lifson (Lifson N, Gordon GB, and McClintock R. J Appl Physiol 7: 704–710, 1955) (−2.5 ± 2.5%), and two two-pool models (Schoeller and McClintock R. J Appl Physiol 118: 1278–1289, 1988) (0.5 ± 3.1%) and Speakman (Speakman, JR. Doubly Labeled Water. Theory and Practice. London: Chapman and Hall, 1997) (−1.9 ± 2.7%). This latter finding seems linked to the stable body mass and to fractionation consideration close to the single-pool model of Speakman. During isolation or simulated microgravity, the other equations underestimated TEE by 10–20%.

deoxygen; energy expenditure; isolation; indirect calorimetry; microgravity

THE HINDLIMB TAIL-SUSPENDED rat was developed as a ground-based model for simulations of physiological responses to weightlessness (25). This model reproduces, through the hypokinesia, hypodynamia, and headward shift of body fluids the cardiovascular, muscular, and bone adaptations to space. As far as we are aware, no studies have investigated the energy metabolism adaptations to simulated or actual microgravity in rats. Such determination is essential, because weightlessness has been shown recently to induce fuel perturbations that are implicated in body mass and composition changes (45). Moreover, accurate estimates of energy requirements are a prerequisite for any long-term spaceflights, as they are foreseen in the International Space Station (19).

The doubly labeled water method (DLW) is currently the most relevant method for measuring free living energy expenditure (18). This method is based on the exponential disappearance from the body of the stable isotopes 2H and 18O after a bolus dose of water labeled with both isotopes. The 2H is lost as water, whereas the 18O is lost as both water and CO2. Thus the excess disappearance rate of 18O relative to 2H, after correction for isotopic fractionation, is a measure of the CO2 production rate (rCO2). This result can be converted to an estimate of energy expenditure using a known or estimated respiratory quotient (RQ) and the classical principle of indirect calorimetry. However, several assumptions should be considered with the technique: 1) the rates of carbon dioxide production and water loss/gains are constant, 2) the isotopic species leave the animal body at equal abundance, 3) the body water pool size is constant throughout the measurement period, 4) the isotopes turnover in the same pool equal to the body water pool, and 5) all substances entering the animal are labeled at the background level and there is no entry of unlabeled carbon dioxide and water via the skin. All these assumptions are invalid to a certain extent, because a number of complicated processes in mammals impinge on the accuracy of the calculated metabolic rate (36).

The favored method for assessing the accuracy of the DLW method has been to compare isotopically derived rCO2 with indirect calorimetry measurements. How-
ever, the assumptions may be violated and constants are only known within certain limits. Not all researchers apply the same assumptions, constants, or methods of calculation: the determination of the isotope pool spaces, the calculation of the constants elimination rate, the fractionation factors, and the mode of $r_{CO_2}$ conversion to energy expenditure all vary in approach (Fig. 1). Indeed, a number of variants of one calculation are used and the authors decide that the one that gives the best agreement between the indirect calorimetry and the DLW method is the appropriate choice. Thus, even if the validation is ascertained, it is tacitly assumed that the equation and constants used and the balance of errors that may cancel in the respiration chamber will do so in all physiological states and environmental conditions in which the method will be applied.

Taking into account the above considerations, selection of one typical way of calculation is not clear-cut, especially when environmental conditions and physiological adaptations are atypical, such as during simulated microgravity. This is why a longitudinal study was carried out to validate and appraise the published theoretical methods of DLW calculations during both a control period and hindlimb tail suspension in rats.

**METHODS**

**Animals**

A group of 50 male Wistar rats weighing 290 ± 27 g (mean ± SD) (Iffa Credo, les Oncins, France) were housed in controlled conditions of 21 ± 1°C at humidity of 60 ± 10% with a 12:12-h light-dark cycle. They were fed chow comprised of 23.5% protein, 5% lipid, 49.8% carbohydrate, 12% moisture, 4% fibers, 5.7% minerals with an energy equivalent of 13.4 MJ/kg (UAR, Epinay sur Orge, France). Food and tap water were given ad libitum. All procedures were conducted in accordance with the guiding principles of the American Physiological Society and the Veterinary Board of the French Space Agency.

**Experimental Protocol**

The 50 rats were divided into three groups. The first group ($n = 8$) was used to determine the $^2$H plateau of equilibration, This is a different approach than the classical validation studies that generally investigate the specific errors introduced in the DLW-derived $r_{CO_2}$ when one assumption is violated.

The objective was to determine the most accurate method to apply during simulated microgravity that could potentially be used during spaceflight.

**DLW**

**Levels of calculations**

\[
\text{Energy expenditure} = \text{[Average isotope dilution space - (18-Oxygen flux - Deuterium flux)] \times Energy equivalent}
\]

**ISOTOPE DILUTION SPACES**

<table>
<thead>
<tr>
<th>Initial</th>
<th>Final</th>
<th>Average</th>
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<tbody>
<tr>
<td>1- Plateau</td>
<td>1- Second isotope dose</td>
<td>Assuming changes:</td>
</tr>
<tr>
<td>2- Intercept</td>
<td>2- Scaled relationship</td>
<td>1- Linear</td>
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<tr>
<td></td>
<td>3- Inferred final mass</td>
<td>2- Exponential</td>
</tr>
</tbody>
</table>

**FRACTIONATION**

1- Proportion of water loss which is fractionated
2- Fractionation processes
   1. Kinetic
   2. Temperature

**ENERGY CONVERSION**

1- Respiratory quotient
2- Food quotient
3- Food quotient corrected for body composition changes
4- Background abundance of isotopes
5- Water influx to CO2 production ratio

**ISOTOPE CONSTANT FLUX RATES**

<table>
<thead>
<tr>
<th></th>
<th>Two-point method</th>
<th>Multi-point method</th>
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<tbody>
<tr>
<td>1- Slope resulting from two samples</td>
<td>1- Linear regression:</td>
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<tr>
<td></td>
<td></td>
<td>. Least square</td>
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<td>. Reduced major axis</td>
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<td>2- Non-linear regression</td>
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<td>. Exponential fit</td>
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<td>. Poisson fit</td>
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Fig. 1. Summary of the different methods for assessing the doubly labeled water (DLW) raw variables. The italic sentences refer to the methods that are not tackled in this study.
and the second \((n = 34)\) was required to fit a scaling relationship between body water pool size and body mass. The last group \((n = 8)\) was devoted to the validation study. The experimental procedures relative to each group will be chronologically detailed in the relevant parts of METHODS.

The experimental schedule of the validation study ran for 29 days and was broken down in four successive periods: 5 days of surgery recovery, 7 days of isolation, 7 days of attachment, and 10 days of simulated microgravity. The DLW method was tested twice against indirect calorimetry in each rat during isolation and during simulated microgravity. Weightlessness was simulated using the tail-suspension model of Morey et al. (24) modified in the laboratory. The attachment period allowed us to minimize the stress during the first days of suspension and wash out isotopes; the rats were kept in a horizontal position with their tails attached to the suspension device.

**Surgical Procedures**

Five days before the beginning of the experiments, the 50 rats were anesthetized with halothane (2% with oxygen) and were chronically cannulated using an aseptic technique. A polyethylene arterial catheter (ID 0.28 mm; OD 0.61 mm) was inserted into the abdominal aorta via the left femoral artery. After insertion, all cannulas were routed subcutaneously to the top of the head and exteriorized. The catheters were filled with a solution of polyvinylpyrrolidone in heparinized saline. During 5 days of recovery, the rats were housed in individual cages and were accustomed to human presence and handling.

**DLW Procedures**

**Determination of the deuterium equilibration time.** After a 200-μl blood sample (for background enrichment), 0.05-g/kg \(^2\text{H}_2\text{O} 99.9\%\) (Isotec, St. Quentin en Yvelines, France) was intraperitoneally injected into eight rats. The plateau of equilibration was determined in 30-min blood samples taken during the first 3 h. Other daily samples were taken over the subsequent 5 days. The 18-oxygen plateau is considered the one that was equilibrated. The 18-oxygen plateau is considered the one that was equilibrated.

**Validation study.** The first day of isolation, a 1-ml blood sample provided baseline \(^2\text{H}_2\text{O}\) and \(^{18}\text{O}_2\) enrichments. The rats were then intraperitoneally injected with \(^2\text{H}_2\text{O} 10\%\) (Isotec) mixed with \(^18\text{O}_2 99.9\%). Each rat received 0.05 g/kg \(^2\text{H}_2\text{O}\) and 1.5 g/kg \(^18\text{O}_2\). A second 1-ml blood sample was collected at the plateau of equilibration. Plasma was separated and stored as mentioned above. Daily urine samples were collected, centrifuged 5 min at 5,000 rpm, and stored at \(-20^\circ\text{C}\) until analysis.

**Multisamples methodology.** The \(K_2\) and \(K_d\) were calculated from the curves fitted to data collected along the isotope elimination track. This fitting was done with the least-squares (LS) the relative magnitudes of the error in time are not taken into account. The reduced major axis (RMA) is calculated as the LS gradient divided by the correlation coefficient. Residuals were plotted to the fitted relationships for both isotopes. This was performed only with the LS approach, because residuals, equivalent to the vertical distances from data to the fitted curve, have no meaning when the curve has been derived minimizing a different variable.

**Estimation of the Isotope Dilution Spaces**

**Initial isotope spaces.** The dilution spaces for \(^2\text{H}_2\text{O}\) and \(^18\text{O}_2\) (mole) were calculated using the following equation (9, 34)

\[
N (\text{mol}) = \frac{WA}{18.02/a} \left( \frac{\delta_w - \delta_i}{\delta_{w} - \delta_i} \right) (3)
\]

where \(N\) is the pool space; \(W\) is the amount of water used to dilute the dose injected; \(a\) is the amount of dose administered; \(\delta\) is the dose diluted for analysis, and \(\delta\) is enrichment of dose (a), dilution water (t), equilibrium enrichment of the isotopes in the body (s), and background levels of isotopes (p). Two procedures well described in the literature (9) were compared to determine \(\delta\).

**PLATEAU.** This approach takes \(\delta_{w}\) as the initial isotope estimated after equilibration of isotopes.

**INTERCEPT.** Given the gradient of the isotope elimination curves \(K_o\) and \(K_d\), the \(N_o\) and \(N_d\) were reevaluated by calculating the expected enrichment at the point of injection. The residual sum of squares was estimated. The plateau of the isotope elimination was then calculated along the same gradient and added to the log-converted initial excess enrichments to obtain the intercept value for \(\delta_{w}\).

**Final pool sizes.** Two potential methods for establishing the size of the \(^18\text{O}_2\) and \(^2\text{H}_2\text{O}\) dilution spaces at the end of the experiment were evaluated.

**Percentage mass.** The values for the initial pool sizes as percentages of the initial body mass are applied to the final body mass to estimate the final pool size.
Scaled relationship (36). The 34 remaining rats were intraperitoneally injected with $^2$H$_2$-18O following the same procedures as detailed above, and urine samples were taken on 2 consecutive days. The pool sizes were derived from estimates resulting from either the plateau or intercept approaches. A scaling relationship between the initial isotope spaces for each isotope and the initial body mass was derived using the LS regression. Using the observed final body mass and interpolating on these equations, we predicted the sizes of the final body water pools.

Average pool sizes throughout the experiment. The average N of the experiment can be calculated from $N_{\text{initial}}$ and $N_{\text{final}}$ by assuming a linear or exponential increase (18). In fact, the consequences of selecting one particular increase are very slight and the errors in doing so are negligible because an unrealistic 50% increase in N during the experiment will not introduce a difference >1.5% between the two estimates. The average pool size was assumed to be $N_{\text{average}} = (N_{\text{initial}} + N_{\text{final}})/2$. Using the intercept or plateau approach for the initial pool spaces and the percent mass approach or the scaled relationship for the final ones, we calculated four initial pool spaces and the percent mass approach or the intercept or plateau approach for the initial isotope spaces were compared.

Equation results in unrealistic 50% increase in N during the experiment will not introduce a difference >1.5% between the two estimates. The average pool size was assumed to be $N_{\text{average}} = (N_{\text{initial}} + N_{\text{final}})/2$. Using the intercept or plateau approach for the initial pool spaces and the percent mass approach or the scaled relationship for the final ones, we calculated four estimates of the average pool sizes for both oxygen and hydrogen. The pool size ratio (R) was calculated as $N_d/N_o$.

Correction Factors for Fractionation

Three fractionation factors (f) are defined depending on the temperature. These are first and second, the fractionation of hydrogen and oxygen in gaseous water relative to liquid water, respectively, and third, the fractionation factor of oxygen in carbon dioxide relative to oxygen in liquid water. The initial equation derived by Lifson et al. (18) for estimation of CO$_2$ output ignoring fractionation is

$$ r_{\text{CO}_2} \text{ (mol/day)} = \frac{N}{2} (K_o - K_d) $$

(4)

Completely derived, taking into account $f_1$, $f_2$, and $f_3$, the equation results in

$$ r_{\text{CO}_2} = \frac{N}{2f_3} (K_o - K_d) - r_{\text{H}_2\text{O}^*} \cdot \frac{f_1 - f_2}{2f_3} $$

(5)

where $r_{\text{H}_2\text{O}^*}$ is the rate of water leaving the system that is fractionated.

Estimation of CO$_2$ Production Rates

Ten different published techniques for estimating the CO$_2$ production by combination of the above-calculated parameters were compared.

Single-pool model using $^{18}$O isotope space. 

Equation of Lifson et al. (18). The oxygen dilution space is used as an estimate of the body water pool (N). Only the equilibrium fractionation factors, derived at 24°C, are taken into account: $f_1 = 0.93$, $f_2 = 0.99$, $f_3 = 1.04$. The fractionated water loss ($r_{\text{H}_2\text{O}^*}$) is assumed to be half the total water loss. The final equation simplified from Eq. 5 is

$$ r_{\text{CO}_2} = \frac{N}{2.08} (K_o - K_d) - 0.015 K_d \cdot N $$

(6)

Equation of Speakman 1997 (36). The in vivo kinetic and equilibrium fractionation factors at 37°C are combined and assumed to contribute in a ratio of 3:1 to the fractionated losses. It is assumed that 25% of the water loss is fractionated. In these conditions, the ratios of Eq. 5 are ($f_1 - f_3$)/2$f_3$ = 0.025 and $f_3 = 1.039$ (15, and reviewed in Ref. 36). This is a single pool approach, only the 18-oxygen dilution space is considered as the water pool size.

Two-pool models using individual isotope spaces or a population ratio. 

Equation of Coward and Prentice 1985 (10). The observed isotope pool sizes $N_d$ and $N_o$ are used in the estimate of CO$_2$ production. This is the simplest two-pool model. The fractionation factors are the same as those assumed in Eq. 6

$$ r_{\text{CO}_2} = \frac{N}{2.08} (N_o \cdot K_d - N_d \cdot K_o) - 0.015 N \cdot K_d $$

(8)

Equation of Schoeller et al. 1986 (32), modified 1988 (30). The author uses a fixed average pool size for oxygen and hydrogen pools relative to the body water pool. The oxygen pool is assumed to be 1.01 times and the deuterium pool 1.04 times the actual water space. Therefore, the isotope ratio is assumed to be 1.03. The fractionation factors derived at 37°C with fractionated water loss (31) equal 2.3 $r_{\text{CO}_2}$.

$$ r_{\text{CO}_2} = \frac{N}{2.078} \cdot (1.01 K_o - 1.04 K_d) $$

- $0.0246 \cdot N \cdot 1.05(1.01 K_o - 1.04 K_d)$

(9)

where

$$ N = \left( \frac{N_o}{1.01} + \frac{N_d}{1.04} \right) / 2 $$

(10)

Two-pool models using isotope spaces of the group studied. 

Equation of Speakman 1993 (38). This approach is related to the fact that there is no a priori reason to consider the fixed ratio derived from studies in humans to be valid in animal studies. Whatever the group, the mean dilution space ratio ($R_{\text{group}}$), should be substituted in the equation. The fractionation factors are the same as in Eq. 9

$$ r_{\text{CO}_2} = \frac{N}{2.078} (K_o - R_{\text{group}} \cdot K_d) $$

- $0.0246 \cdot N \cdot 1.05(K_o - R_{\text{group}} \cdot K_d)$

(11)

where

$$ N = \left( \frac{N_o}{1.01} + \frac{N_d}{R_{\text{group}}} \right) / 2 $$

(12)

Equation of Speakman 1997 (36). This is the two-pool equivalent of Eq. 7 but taking into account the data of the group dilution spaces. Thus

$$ r_{\text{CO}_2} = \frac{N}{2.078} (K_o - R_{\text{group}} \cdot K_d) - 0.006 \cdot N \cdot R_{\text{group}} \cdot K_d $$

(13)

where $N$ is calculated from Eq. 12.

Two-pool models using isotope spaces of the population and group studied. 

Equation of Speakman et al. 1993 (40). This is the same approach as Schoeller et al. (32), but using an alternative estimate of the constants derived from 211 measurements of the dilution space ratio in humans. This average ratio was 1.0427 for the pool size ratio and thus 1.01 and 1.0532 for the equation constants. A group mean dilution space ratio should be evaluated for the group of animals under investigation, and the statistical significance of the observed ratio should be tested against the observed popula-
tion level values for the constants. Thus

\[ r_{CO_2} = \frac{N}{2.078} (1.01K_s - 1.0532K_d) - 0.0246 \cdot N (1.01K_s - 1.0532K_d) \]  

(14)

where

\[ N = \left( \frac{N_s}{1.01} + \frac{N_d}{1.0532} \right) \]

(15)

EQUATIONS OF RACETTE ET AL. 1994 (28) AND SCHOELLER ET AL. 1995 (33). Another population dilution space ratio (1.034) is derived from the population, and further ways of combining the observed group and population dilution spaces are proposed.

AVERAGE APPROACH. The average dilution space of the group and the population is adopted

\[ R' = \frac{R_{group} + 1.034}{2} \]

(16)

leading to

\[ r_{CO_2} = \frac{N}{2.078} (1.01K_s - 1.01R' \cdot K_d) - 0.0245 \cdot N (1.01K_s - 1.01R' \cdot K_d) \]

(17)

where

\[ N = \left( \frac{N_s}{1.01} + \frac{N_d}{1.01 \cdot R'} \right) \]

(18)

WEIGHTED APPROACH. An average weighting of the group and population space ratio is used. Given a sample size of \( n_s \) for the \( R_{group} \) and a sample of 99 for the derived average dilution space for the population

\[ R' = \frac{(R_{group} \cdot n_s) + (1.034 \cdot 99)}{n_s + 99} \]

(19)

This estimate for \( R' \) can be then substituted in Eqs. 17 and 18 to obtain the \( r_{CO_2} \) estimation.

EQUATION OF COWARD ET AL. 1994 (11). This approach is a weighted calculation considering the relative variances of the sample and population means as the weights. The mean population estimate is suggested to be 1.034 [as Racette et al. (28)], with a standard deviation of 0.003. The estimated weighted dilution space ratio \( R_w \) is calculated as

\[ R_w = \left[ \left( \frac{R_g}{\text{var}_s} \right) + \left( \frac{R_p}{\text{var}_p} \right) \right] \left[ \left( \frac{1}{\text{var}_s} \right) + \left( \frac{1}{\text{var}_p} \right) \right]^{-1} \]

(20)

where \( R_g \) and \( R_p \) and \( \text{var}_s \) and \( \text{var}_p \) represent the sample and population mean dilution space ratios and variances. The \( R_w \) is then substituted in Eqs. 17 and 18 to yield \( r_{CO_2} \) and \( N \).

Estimation of the \( r_{CO_2} \) Precision

The \( CO_2 \) production using average values of the isotope enrichment at the start and end points of the experiments was derived with a precision error. These individual precision errors are combined to yield an overall precision error for the final estimate of \( CO_2 \) production. We use the empirical method described by Speakman (37) called the Jackknife calculation. The precision errors are made using the means of enrichment estimates. However, each time the calculation is done, one of the measurements is omitted. In each case, the means should be used to generate the final \( CO_2 \) production values. The Jackknife calculation proceeds using the means so that the distribution estimate is a distribution of means. Thus the standard deviation of the distribution is the standard error, and the 99% confidence limits are calculated as \( \pm 2.034 \) multiplied by the standard deviation of the resultant distribution.

Conversion of \( CO_2 \) Production Into Energy Demands

Two methods were compared. The first one uses \( RQ \) values obtained from indirect calorimetry, which are converted into an energy equivalent of \( CO_2 (E_{eqCO_2}) \) calculated by the Wier equation (47)

\[ E_{eqCO_2} (kJ/L) = \frac{15.457}{RQ} + 5.573 \]

(21)

Then TEE (kJ/day) is calculated as

\[ TEE = r_{CO_2} \cdot E_{eqCO_2} = 22.4 \]

(22)

where 22.4 is the conversion factor for moles of \( CO_2 \).

The second method consists of the estimation of the \( RQ \) from the food composition, so-called food quotient (FQ), calculated as follows

\[ FQ = \frac{(710.71P + (1377.06F) + (746C)}{(879.06P + (1948.34F) + (746C)} \]

(23)

where \( P, F, C \) are protein, fat, and carbohydrate intakes, respectively, expressed as grams per day (3).

Indirect Calorimetry

The rates of \( O_2 \) consumption and \( CO_2 \) production were assessed by an indirect calorimeter consisting of an open-flow gas analysis system using gas analyzers. Oxygen and carbon dioxide concentrations in downstream exhaust gases were successively measured in five different cages. To avoid errors resulting from sequential changes from one cage to another, common parts of the system were rinsed for 90 s, after which gas exchanges were measured for 40 s. The final value is a mean of 10 values obtained every 4 s. A computer-controlled system of three-way valves allowed for the sequential analysis of the five cages every 11 min. One cage was left vacant and served as reference for measuring ambient \( O_2 \) and \( CO_2 \). Air samples were pumped at a constant flow rate, controlled within strict limit by a mass flowmeter (precision <1%; Tylan, FM 380), and directed to a paramagnetic oxygen analyzer (range 0–100%, time delay <3 s; Klogor, Lannion, France) and an infrared carbon dioxide analyzer (range 0–1%, time delay <3 s; Gascard I, Edinburgh Sensors) after being dried through a Permapure system and calcium chloride, which were changed twice daily. The system was calibrated daily with pure nitrogen to set up the zero of the analyzers and with a standard gas mixture (CFPO) containing 20.5% \( O_2 \) (accuracy 20.44–20.56%), 0.5% \( CO_2 \) (accuracy 0.495–0.505%), and 79% nitrogen to set up the sensitivity. The measuring system was found to be accurate to within ±1% by bleeding known rates of \( O_2 \) and \( N_2 \) as known rates by the air flow through the cages, yielded the respiratory gas.
exchanges of animals. The energy expenditure is calculated from the Wier formula (47).

**Software**

The $r_{\text{CO}_2}$ estimations from the different approaches were calculated using Microsoft Excel 98. The precision of the DLW-derived $r_{\text{CO}_2}$ was assessed using the software DLW version 1.0 developed by Professor Speakman and Dr. Lemen (Aberdeen, UK).

**Statistical Analysis**

Because of the small number of rats and the inherent loss in statistical power, a Student’s paired $t$-test was used to compare 1) the raw variables obtained from the different methods, 2) the DLW-derived $r_{\text{CO}_2}$ against the indirect calorimetry results, and 3) the isolation against the simulated microgravity periods. The impacts of the different methods of deriving $r_{\text{CO}_2}$ at the different levels of DLW calculations (pool sizes, constant elimination rates, fractionation processes, and equations) during the two environmental conditions (isolation and suspension) were evaluated by a multiway factorial ANOVA (F-ANOVA). When a significant difference ($P < 0.05$) was noted, post hoc tests were performed using Fisher’s protected least-significant difference (PLSD) test. A Bland and Altman test (6) examined the agreement of the DLW method with indirect calorimetry. The differences between the indirect calorimetry and the DLW method were plotted against the average of the two methods. Bias and precision were defined as the mean difference ± 2 SD. The biases were compared through a Student’s paired $t$-test. To simplify the results, the Bland and Altman test was used on five of the ten equations described above selected for representing a typical way of calculation. All analyses were performed with STATVIEW 4.5 (Abacus Concepts, Berkeley, CA, 1992), and values are expressed as means ± SD, with $P < 0.05$ considered to be statistically significant.

**RESULTS**

**Energy Intake and Body Mass**

Energy intake (Fig. 2) has to be considered carefully, because we estimate an accuracy of ±10% in the measurements due to losses. Therefore, the results are more qualitative than quantitative. During isolation, rats grew normally, with an average increase of $9.1 ± 1.0\%$ at the end of the period, associated with a constant daily energy intake varying between 323 ± 14 (isolation day 3) to 363 ± 19 kJ/day (isolation day 6). During suspension, body mass was stabilized (from 15.4 ± 1.3% on suspension day 1 to 15.7 ± 2.4% on day 10). The energy intake throughout this period varied between 256 ± 34 and 295 ± 16 kJ/day on suspension days 5 and 10.

**Kinetic of Deuterium Equilibration**

The deuterium equilibration in the rat body fluids was determined from the plateau observed on the curve of isotope enrichment as a function of time elapsed from dose administration (Fig. 3). It was observed at 120 min ($284.12 ± 9.91$ ppm).

**Scaled Relationship Between Isotope Dilution Spaces and Body Masses**

One of thirty-four rats was excluded from the study because of a sealed catheter. From the plots, four equations were derived and used predictively (Fig. 4). The population isotope space ratio resulting from this
analysis was $1.04391 \pm 0.03212$ from plateau and $1.04396 \pm 0.03118$ from intercept.

Indirect Calorimetry

The produced CO$_2$, consumed O$_2$, and RQ are presented in Table 1; there were no differences between the periods of isolation and suspension.

Isotope Backgrounds, 2-h Equilibration, and Total Sampling Times

The isotope enrichments of daily tap water measurements throughout the experiment were unchanged (H$_2^{18}$O: $1.980.34 \pm 0.54$; H$_2$O: $145.24 \pm 0.36$ ppm). The data for isotope backgrounds and equilibration are presented in Table 2. During the simulated microgravity period, two rats were withdrawn from the study at, respectively, 7 and 9 days due to tail injury from the suspension device. The rCO$_2$ was calculated until the rats were removed.

Initial Isotope Dilution Spaces

During isolation, the H$_2^{18}$O and H$_2$O dilution spaces estimated from the plateau were higher than from the intercept, whereas the dilution space ratios were similar (Table 3). During simulated microgravity, the H$_2^{18}$O and H$_2$O dilution spaces were also statistically distinct without differences between the ratios. The suspension did not modify the isotope spaces of $^{18}$O and $^2$H from either plateau or intercept. Conversely, higher ratios were observed during suspension for both the intercept estimates and the plateau.

Isotope Constant Elimination Rates

To simplify the results (Table 4), we focus on the $K_{o}$/$K_{d}$ differences, which pass directly into rCO$_2$. The differences calculated by RMA and LS were similar during both isolation and suspension. This lack of difference was also observed between the LS and two-point method during both experimental periods. The $K_{o}$/$K_{d}$ ratios were comparable to the LS during isolation but were lower during suspension. The residual standard deviations were within the range of published values and therefore acceptable. For the above variables, no difference was noted between isolation and simulated microgravity.

Appraisal of the Different Methods of Calculations

Constant flux rates. We did not observe any global effect of the constant flux rate calculations, i.e., two- or multipoint methods ($F = 1.664, P = 0.197$). This was also noted when the analysis was split by groups: during isolation ($F = 0.235, P = 0.628$) and during suspension ($F = 2.192, P = 0.139$). Therefore, we will focus the following statistical results of the F-ANOVA on the multipoint methodology.

Pool models. The choice of the pool model is an important determinant of the rCO$_2$ estimations ($F =$

Table 1. Indirect calorimetry results

<table>
<thead>
<tr>
<th></th>
<th>VO$_2$, mmol/day</th>
<th>VCO$_2$, mmol/day</th>
<th>RQ</th>
</tr>
</thead>
<tbody>
<tr>
<td>Isolation</td>
<td>568</td>
<td>514</td>
<td>0.915</td>
</tr>
<tr>
<td>SD</td>
<td>138</td>
<td>105</td>
<td>0.046</td>
</tr>
<tr>
<td>Suspension</td>
<td>585</td>
<td>509</td>
<td>0.871</td>
</tr>
<tr>
<td>SD</td>
<td>92</td>
<td>77</td>
<td>0.026</td>
</tr>
</tbody>
</table>

VO$_2$, daily consumption of oxygen; VCO$_2$, daily carbon dioxide production; RQ, respiratory quotient.

Table 2. Isotope backgrounds and total sampling times

<table>
<thead>
<tr>
<th></th>
<th>Oxygen-18, ppm</th>
<th>Deuterium, ppm</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Duration, days</td>
<td>Baseline</td>
</tr>
<tr>
<td>Isolation</td>
<td>7.217</td>
<td>1,994.8</td>
</tr>
<tr>
<td></td>
<td>0.075</td>
<td>6.8</td>
</tr>
<tr>
<td>Suspension</td>
<td>9.499</td>
<td>1,997.2</td>
</tr>
<tr>
<td></td>
<td>0.970</td>
<td>2.3</td>
</tr>
</tbody>
</table>

ppm, Particles/min.
among the two-pool models, \( R \) estimated from \( P \) period (9.797, \( \text{estimates} \) pool model and the two-pool models using individual \( N \) showed only significant differences between the one-population (\( 5 P < 0.0001 \)). Splitting the analysis by period, these results yielded a higher \( rCO_2 \) assuming a fractionated water loss of 25% the total water loss yielding a higher \( rCO_2 \) than \( R \) from the group (\( P < 0.0001 \)) or the group and population (\( P < 0.0001 \)). Splitting the analysis by period, these results were unchanged during the suspension period (\( F = 9.797, P < 0.0001 \)). Conversely, within the isolation period (\( F = 2.555, P = 0.0389 \)), the Fisher’s test showed only significant differences between the one-pool model and the two-pool models using individual \( N \) estimates (\( P = 0.006 \)) or \( R \) from the group and the population (\( P = 0.002 \)).

Proportion of fractionated water loss and fractionation processes. Regardless of the calculation used, assuming a fractionated water loss of 25% the total water loss yielded a higher \( rCO_2 \) than an assumption of 50% (\( F = 9.883, P = 0.002 \)). An overall effect of the fractionation processes used has been demonstrated, i.e., kinetic and/or equilibrium processes at 24 or 37°C (\( F = 26.991, P < 0.0001 \)). Assuming kinetic and equilibrium fractionation processes at 37°C resulted in a higher estimate of the DLW-derived \( rCO_2 \) than both equilibrium processes at 37°C (\( P < 0.0001 \)) or at 24°C (\( P < 0.0001 \)). These results are similar during either isolation (\( F = 4.629, P = 0.010 \)) or suspension (\( F = 11.902, P < 0.0001 \)).

20.625, \( P < 0.0001 \)). Overall, from the PLSD Fisher’s test it appears that the results of \( rCO_2 \) were higher when a single pool (\( N_p \)) was used than when two-pool models using individual \( N \) (\( P < 0.0001 \)) or two-pool models using either an \( R \) estimate from the population (\( P < 0.0001 \)) from the group (\( P = 0.001 \)), or from the group and the population studied (\( P < 0.0001 \)) was used. Among the two-pool models, \( R \) estimated from the population gave higher \( rCO_2 \) results than \( R \) from the group (\( P < 0.0001 \)) or the group and population (\( P < 0.0001 \)). Splitting the analysis by period, these results were unchanged during the suspension period (\( F = 9.797, P < 0.0001 \)). Conversely, within the isolation period (\( F = 2.555, P = 0.0389 \)), the Fisher’s test showed only significant differences between the one-pool model and the two-pool models using individual \( N \) estimates (\( P = 0.006 \)) or \( R \) from the group and the population (\( P = 0.002 \)).

Proportion of fractionated water loss and fractionation processes. Regardless of the calculation used, assuming a fractionated water loss of 25% the total water loss yielded a higher \( rCO_2 \) than an assumption of 50% (\( F = 9.883, P = 0.002 \)). An overall effect of the fractionation processes used has been demonstrated, i.e., kinetic and/or equilibrium processes at 24 or 37°C (\( F = 26.991, P < 0.0001 \)). Assuming kinetic and equilibrium fractionation processes at 37°C resulted in a higher estimate of the DLW-derived \( rCO_2 \) than both equilibrium processes at 37°C (\( P < 0.0001 \)) or at 24°C (\( P < 0.0001 \)). These results are similar during either isolation (\( F = 4.629, P = 0.010 \)) or suspension (\( F = 11.902, P < 0.0001 \)).

Average \( N \) throughout the study. The \( N_{\text{average}} \) estimation method, either intercept or plateau assuming a scaled relationship or an inferred final mass evaluation, did not significantly influence the \( rCO_2 \) estimates (\( F = 1.714, P = 0.162 \)). This lack of significant effect was also noted during isolation (\( F = 0.820, P = 0.484 \)) and simulated microgravity (\( F = 1.587, P = 0.192 \)).

Estimation of \( rCO_2 \): Global Effects of the Different Equations. The association of the above-derived variables into the published equations had an overall significant impact on the \( rCO_2 \) (\( F = 13.088, P < 0.0001 \)) (Table 5, Fig. 5). More precisely, during isolation, this effect was maintained (\( F = 1.910, P = 0.049 \)). The PLSD Fisher’s test showed that Eq. 8, using the individual \( N \), gave the lower \( rCO_2 \) estimation. These estimates were significantly different from the one-pool models in Eqs. 6 and 7. We noted also that the different ways of combining \( R \) obtained from group or population or both (Eqs. 11, 14, 16, 17) result in significantly lower \( rCO_2 \) production than the one-pool model (Eq. 7), although not in the two-pool model (Eq. 13). During the suspension period, the impact of the equation was also significant (\( F = 5.681, P < 0.0001 \)). Between the equations, we observed the same differences as noted during isolation, but other patterns can be dissociated from the results. Effectively, the \( rCO_2 \) derived from Eq. 8 (the basic 2-pool model) was significantly lower than all the other equations. On the other hand, Eq. 9, which is also a two-pool model but using an \( R \) fixed at 1.03, yields significantly higher results than the other two-pool models using \( R \) from group and/or population,
### Table 5. Algebraic percentage deviation of the DLW measurement from indirect calorimetry

<table>
<thead>
<tr>
<th>Equation &amp; Reference</th>
<th>K Derivation</th>
<th>Plateau, %</th>
<th>Intercept, %</th>
<th>Plateau, s</th>
<th>Intercept, s</th>
</tr>
</thead>
<tbody>
<tr>
<td>Eq. 6 (18)</td>
<td>TP</td>
<td>$-4.3 \pm 4.2$</td>
<td>$-6.6 \pm 4.0$</td>
<td>$-6.7 \pm 5.3$</td>
<td>$-9.3 \pm 5.1$</td>
</tr>
<tr>
<td>Eq. 7 (36)</td>
<td>MP(LS)</td>
<td>$0.1 \pm 4.4$</td>
<td>$-1.9 \pm 4.1$</td>
<td>$-2.3 \pm 5.5$</td>
<td>$-5.1 \pm 5.3$</td>
</tr>
<tr>
<td>Eq. 8 (30)</td>
<td>MP(LS)</td>
<td>$0.8 \pm 2.2$</td>
<td>$-1.3 \pm 2.3$</td>
<td>$-2.0 \pm 3.1$</td>
<td>$-4.6 \pm 3.3$</td>
</tr>
<tr>
<td>Eq. 9 (30)</td>
<td>TP</td>
<td>$-8.1 \pm 4.8$</td>
<td>$-5.5 \pm 4.9$</td>
<td>$-7.0 \pm 5.6$</td>
<td>$-8.7 \pm 5.6$</td>
</tr>
<tr>
<td>Eq. 11 (35)</td>
<td>MP(LS)</td>
<td>$10.6 \pm 4.7$</td>
<td>$-12.4 \pm 4.5$</td>
<td>$-13.7 \pm 5.2$</td>
<td>$-15.4 \pm 5.1$</td>
</tr>
<tr>
<td>Eq. 13 (36)</td>
<td>MP(LS)</td>
<td>$1.9 \pm 2.7$</td>
<td>$-3.9 \pm 2.8$</td>
<td>$-5.6 \pm 3.3$</td>
<td>$-7.1 \pm 3.4$</td>
</tr>
<tr>
<td>Eq. 14 (40)</td>
<td>TP</td>
<td>$-11.6 \pm 4.6$</td>
<td>$-13.4 \pm 4.5$</td>
<td>$-14.7 \pm 5.2$</td>
<td>$-16.3 \pm 5.1$</td>
</tr>
<tr>
<td>Eq. 16 &amp; 17 (28)</td>
<td>TP</td>
<td>$-10.2 \pm 4.7$</td>
<td>$-11.9 \pm 4.6$</td>
<td>$-14.4 \pm 2.7$</td>
<td>$-15.8 \pm 2.8$</td>
</tr>
<tr>
<td>Eq. 17 &amp; 19 (35)</td>
<td>MP(LS)</td>
<td>$-9.5 \pm 2.3$</td>
<td>$-11.4 \pm 2.3$</td>
<td>$-12.9 \pm 2.8$</td>
<td>$-14.4 \pm 2.9$</td>
</tr>
<tr>
<td>Eq. 18 &amp; 20 (11)</td>
<td>TP</td>
<td>$-10.1 \pm 4.7$</td>
<td>$-11.9 \pm 4.6$</td>
<td>$-13.3 \pm 5.3$</td>
<td>$-14.9 \pm 5.2$</td>
</tr>
<tr>
<td>MP(LS)</td>
<td>$-9.5 \pm 2.3$</td>
<td>$-11.4 \pm 2.3$</td>
<td>$-12.9 \pm 2.8$</td>
<td>$-14.4 \pm 2.8$</td>
<td></td>
</tr>
</tbody>
</table>

Results are means ± SD. $K$, constant isotopic elimination rates calculated by 2 points (TP) or multipoints with least-square regression [MP (LS)]. The body water pool sizes were evaluated by either plateau or intercept methods and using either assumption of a fixed percentage (%) or a scaled relationship (s). *$ P < 0.05$ and †$P < 0.01$ vs. indirect calorimetry results. Results in bold refer to nonsignificant differences with indirect calorimetry.

although not in Eq 13. There was no difference between these latter two. Interestingly, these equations used fractionation processes similar to those of Eq. 7.

**Comparison with indirect calorimetry.** A paired t-test comparison of the DLW-derived $r_{CO_2}$ with indirect calorimetry raised evidence that most equations are statistically different (Table 5). The test was performed with the $r_{CO_2}$ estimates from two Naverage calculations: plateau (P) or intercept (I), assuming a scaled (s) or percentage mass (%) relationship. Within isolation, either with the multipoint or two-point methods, no difference from calorimetry was observed for Eq. 7 (P%, I%, and Ps) and Eq. 13 (P%). During suspension using the multipoint, we noticed nonstatistical differences for Eq.
7 (P%, I%, and Is), Eq. 13 (P%, I%, Ps, and Is), Eq. 6 (P%, Ps, and Is), and Eq. 9 (Ps and Is). With some exceptions, the results were similar with the two-point method.

**Precision Errors from Eq. 7 [SPEAKMAN (36)].** The precision estimates were compared with the deviation of the DLW estimates (Fig. 6). These comparisons were realized with either the two- or multipoint methods. To simplify the results, the scaled relationships are not represented. Except for a few animals, we observed that the two-sample method used during isolation or simulated microgravity lay to the right line of identity. Thus the deviations between the DLW and indirect calorimetry measurements were less than the observed precision of the DLW, pointing to precision as a key problem. For the multipoint method, results fit near the line of identity and were below 5% for both deviation and precision (1 rat did not fit within 5%). Therefore problems arising with the DLW technique are approximately equal to the precision and are highly acceptable.

**Bland and Altman Test.** The agreement between the two methods tested by the Bland and Altman test (4) is represented in Fig. 7. The results show that for the five equations tested, the individual data are in the range of agreement (mean difference ± 2SD), except for one rat. However, we can note that apart from Eq. 7, the mean differences are not close to the zero (the statistical significance of this deviation is equivalent to the test represented in Fig. 6 and Table 5) and the higher biases are observed by using individual N estimates (Eq. 8). Compared with our single pool reference model (Eq. 7), the paired t-test during either isolation or suspension, for both multi- or two-point methods, indicated significant higher biases by using individual N estimates (Eq. 8), a fixed ratio (Eq. 9), and the initial model Eq. 6. No effect on the biases using either a single pool (Eq. 7) or two pools with a group ratio (Eq. 13) was noted. However, the biases were significantly increased from a two-pool model using a group ratio (Eq. 13) to a two-pool using a fixed ratio (Eq. 9) and from this latter to the individual N estimates (Eq. 8), whatever the period or the flux calculations. On the other hand, biases were higher using the two-point method either during isolation or suspension. An exception is observed with Eq. 7 where the biases of the two- versus multipoint method were similar during isolation.

**rCO₂ Conversion to Energy Expenditure**

The selection of an RQ or an assumed FQ from the diet did not significantly modify the energy expenditure estimations that were similar during isolation and suspension (Table 6).

**DISCUSSION**

Numerous methods have been published to determine the energy expenditure from the DLW method, but the validation studies supporting these methods used constants and assumptions that may be valid only within the environmental conditions of the experiment. Therefore, the selection of one method is far from trivial. This appears to be particularly important when considering the spaceflight environment, where the experimental conditions are poorly controlled and unusual. The energy requirements of spaceflight have been poorly investigated in humans (19, 45) and are unknown in rats. Therefore, this study was undertaken 1) to summarize and validate the different methods of DLW-derived rCO₂ production using the hindlimb tail suspended rat model and 2) to appraise the best method to use during simulated microgravity that could be extrapolated to spaceflight.

**General Considerations of the Study**

A validation study relies on the measurements of the reference method. The indirect calorimetry system
used has proven reliability in different environmental and physiological situations (1, 8, 23).

The hindlimb tail-suspended rat model is used to mimic the physiological consequences of spaceflight, and there has been wide acceptance of its usefulness. Suspension experiments using the Morey et al. model (24) generally result in a constant growth, as observed in flight (5). During our study, the body mass was unexpectedly stabilized and this may be due to the duration of the study and the use of mature rats. However, we cannot exclude a stress response due to both confinement in the small calorimetric cages for 29 days and suspension. This is supported by the decrease in energy intake observed during suspension, keeping in mind our limitation in energy intake records. In the same way, the stress response may explain the lack of changes observed in TEE and the nonsignificant RQ decrease. Studies conducted in humans were unable to demonstrate changes in TEE and the nonsignificant RQ decrease. Studies conducted in humans were unable to demonstrate changes in TEE, and this was attributed either to the cost of physical activity in space or to stress (20). Overall, the unexpected lack of growth during suspension allows us to observe unexpected results concerning the application of the DLW method in rats during simulated microgravity, leading to the conclusion that during growth or simulated microgravity without growth, Eq. 7 (Speakman (36)) results in the more reliable estimate of TEE. In the following discussion, the influence of each calculation step of on the final rCO2 production is appraised.

### Table 6. Energy expenditure

<table>
<thead>
<tr>
<th></th>
<th>Indirect Calorimetry, kJ/day</th>
<th>Multisamples</th>
<th>Two-Samples</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>RQ, %</td>
<td>FQ, %</td>
<td>RQ, %</td>
</tr>
<tr>
<td>Isolation</td>
<td>260.77</td>
<td>-0.78</td>
<td>-1.90</td>
</tr>
<tr>
<td>Means</td>
<td>69.89</td>
<td>2.15</td>
<td>4.42</td>
</tr>
<tr>
<td>Suspension</td>
<td>266.16</td>
<td>2.19</td>
<td>-0.35</td>
</tr>
<tr>
<td>Means</td>
<td>41.21</td>
<td>3.43</td>
<td>4.28</td>
</tr>
</tbody>
</table>

FQ, food quotient. The expenditures are calculated from Eq. 7 (36). With N average from plateau and % of final mass. The DLW-derived expenditures are expressed in percentage of calorimetry results.

### Estimation of the Isotope Pool Space

The calculations of rCO2 from the isotope data assumed that the initial dilution space estimate of the body water pool is accurate and precise. The technique of choice for measuring the dilution space in animal studies is the plateau technique. We observed that the...
sequence is an increase in the exchange pool of $^2$H with pally driven by fat deposition (35) and the direct con-
unrealistic situation during growth. This question is of
exponent of body pool size on body mass to be 1.0. It
misuse of ratios (27), because it assumes a scaling
ual. Nevertheless, this latter approach represents a
approach (36, 41, 44) is to calculate the initial body pool
2r

an (44) studied this effect on long-eared bats and observed
and hence in body water as well. Consequently, the
excess of the body water pool and, if they are appropriately timed, can result in an accurate estimate. We did not observe an effect of the way of deriving $N_{\text{initial}}$ but this may be due to the lack of statistical power linked to the small number of ani-
imals. Overall, it appears from our results that when
one equation is validated, the best agreement with calorimetry is observed using the plateau estimate whether the rats are isolated or suspended.

In the above discussion, we assumed that $N_{\text{initial}}$ is constant throughout the measurement period. This may be realistic during suspension, but not during isolation, where growth results in a progressively changing pool size. Consequently, the initial dilution space does not adequately reflect the average pool size throughout the experimental period. Even if the variations in $\text{CO}_2$ production occur at random and even if the rate of $\text{CO}_2$ production is constant, this is a signif-
icant problem. The best methods to evaluate the final pool size are to kill and desiccate the animals or to perform a second isotope injection, but the blood sam-
pling and fasting necessary would result in a stress response. We have tested two other classical noninva-
sive approaches. The first was to establish empirically a relationship between body mass and the initial body pool size (36). An error in individual estimates, how-
ever, is introduced by using this method because ani-
mals of the same mass may also vary in composition and hence in body water as well. Consequently, the predictive equation will not be perfect. Speakman et al.
(44) studied this effect on long-eared bats and observed an $r^2 = 0.67$. The data from this study give a better agreement, with an $r^2$ ranging from 0.83 (for deute-
rium pool size estimate from plateau) to 0.91 (for 18-
oxgen from plateau). The second more widely used approach (36, 41, 44) is to calculate the initial body pool as a percentage of the body mass for the same individ-
ual. Nevertheless, this latter approach represents a misuse of ratios (27), because it assumes a scaling exponent of body pool size on body mass to be 1.0. It also
assumes that there are no changes in body com-
position associated with the mass change, which is an unrealistic situation during growth. This question is of
importance in our study, because rat growth is princip-
ally driven by fat deposition (35) and the direct con-
sequence is an increase in the exchange pool of $^2$H with labile hydrogen of lipids.

We were unable to demonstrate statistically any superiority of the scaled relationship or of the inferred percentage of final mass for estimating the final pool size on the $r_{\text{CO}}$ calculations. However, during isolation when a particular equation validates the DLW method, we can observe that the percentage of final mass ap-
proach results in the best agreement with the calorim-
etry. On the other hand, with the stabilization of body mass occurring during suspension, this difference is no
longer observed. This point emphasizes the above con-
considerations on the body mass and composition changes, as suggested in growing pigs (16), and suggests that the scaled relationship should be considered cautiously when body mass changes during an experiment. In addition, the results demonstrate that the hydroelec-
trolytic adaptations to simulated microgravity are less significant on the DLW procedures than growth.

**Effects of Pool Models**

In the original formulation of Lifson et al. (17, 18) both isotopes are assumed to turn over only in the body water pool. Hence, flow rates are calculated as $K_d\cdot N$ and $K_o\cdot N$. However, this is an obvious contradiction, because the fundamental basis of the technique is that the oxygen isotope is in exchange equilibrium with the oxygen in dissolved $\text{CO}_2$ and bicarbonate. Hence, oxygen must spread not only into the body water pool but also into the carbonate and dissolved $\text{CO}_2$ pools. In humans, Schoeller et al. (34) suggested that the body water from oxygen dilution is overestimated by $\sim 0.7%$. Because the dilution space technique has an analytic sensitivity ranging from 0.2 to 1.3% for oxygen (9, 34), the correspondence between the oxygen isotope dilu-
sion space and the body water estimated from desicca-
tion is good. For the deuterium dilution space, in 79 animal studies of 35 species (reviewed in Ref. 36), it exceeds the oxygen space and the body water by desicca-
tion by 4.57% ($\sim 5.6-20\%$), suggesting the existence of a rapid exchange pool for hydrogen, apart from the hydrogen in body water. It is generally assumed to represent a reversible exchange with labile hydrogen on amino groups in protein and lipids, as previously stated.

The first equation developed by Lifson et al. (18) ignores this difference in pool sizes and assumes the body water pool (N) to be equivalent to the oxygen dilution space. Later, Schoeller et al. (32) and Coward and Prentice (10) suggested that the different pool sizes should be taken into account in the calculations. However, the theoretical superiority of the two-pool models is not evident in all the conditions. Lifson et al. (18) first evaluated these effects and observed that the two-pool model yielded a difference of $\sim 2\%$ (SD 11%) against $\sim 3\%$ (SD 10%) for the single pool. In the same way Speakman (36) concluded that it would be better to use a single pool because of a large variation in the accuracy of the individual estimates (23%) that did not allow significant difference between the pool models. Nowadays, such comparisons are not easy because there are three different approaches that can be em-
ployed for the two-pool model. In the first approach, the individual measure of dilution spaces for a given ani-
mal are used in the Eq. 8 of Coward et al. (10). From
our results, there is clear evidence that this is not the best procedure to apply in rats. Regardless of period and body mass changes, this equation results in an underestimation of $\sim 20\%$ of $r_{\text{CO}}$. This suggests that in animals of such body mass or at least in rats, such equations are inadequate independent of growth.
Schoeller et al. (30, 32) introduced a second variation for the pool ratio based on large samples of pool size measurements. During isolation when the pool size increased, this equation results in an underestimation of rCO₂ between 5 and 10%, significantly different from indirect calorimetry data. Conversely, the same equation is validated during simulated microgravity. Thus when body weight is stable over the experimental period, a two-pool model with the use of a fixed R is valid. The third approach calculates an average pool size ratio for the group of animals under study and uses that ratio to derive a group-specific modification to the individual equation. In the latter, there are related methods that advocate using both population and group means: a combination method is to calculate an unweighted average of the group and population means (28, 33) and another method is to calculate an average weighted by sample size (28, 33) or variance (11). Whatever the period studied, all these equations yielded approximately the same underestimation of the rCO₂ (10–15%), except for Eq. 13 [Speakman (36)], which uses R estimated from the group studied. This suggests that regardless of the R combination method (from group and/or population), the impact on the energy requirement calculations is equivalent. The reason Eq. 13 of Speakman (36) differs from the other equations may be attributable to the fractionation processes that are discussed later. We should mention that validation studies covering a range of body sizes [barnache geese (26), tufted duck (2), and sea lions (7)] indicated that some form of the two-pool model is superior. Conversely, when Speakman recalculated rCO₂ from the original studies of McClintock and Lifson (21, 22) to compare the single- and two-pool model, he observed that the single pool is more reliable (36). Thus the selection of a pool model to apply to small animals is not clear-cut.

From our study it seems difficult to prove the superiority of the single- or the two-pool models. As with Speakman (36), we found that the single-pool model is more reliable in small animals. But undoubtedly some two-pool models using R from the group data give good agreement with calorimetry when the pool space is constant during the DLW measurement. Therefore, in absence of clear indication from the literature, we suggest that the one-pool model yields more reliable DLW results during both isolation and simulated microgravity in rats.

**Methods of Calculation of the Isotope Constant Elimination Rates**

**LS and RMA fitting approaches.** Given the impact of the different regression models on the derived gradients, the choice of which model to employ depends on the relative magnitudes of error in time and isotope abundance variables. Precision estimates for both isotope measurements by ratio mass spectrometry vary between 0.5 and 1.5%. The question that arises is the precision error for the sample timing. Primarily, this precision is very good especially for blood samples. The precision on timing is so great relative to the error in mass spectrometry that it will always be appropriate to use the LS fit procedure. In the case of urine sampling, the timing precision is reduced because of the difficulty of ascribing a time to samples that have been stored in the bladder over a variable period of time. In addition, these samples may include different proportions of sample from different times. Thus the decision to use LS or RMA to derive the elimination rate is not clear-cut. During our study, the urine samples were taken within the half-hour devoted to daily maintenance, so that special attention was paid to the 24-h interval. Significantly higher K₁ and K₂ from RMA related to LS were noted, but the K₁ and K₂ differences that pass directly into the CO₂ production were not modified and the effects on energy expenditure were <0.5% (data not shown). This suggests that respecting the 24-h interval overcomes the problem of urine storage in the bladder and that LS regression can be used without any transformation to RMA during either isolation or simulated microgravity in rats.

**Multisampling versus two-sampling method.** By using the two-point approach, the intricacies of the patterns of decline are absolutely irrelevant. The two-point technique measures the average rate at which the isotopes decline throughout the measurement period. The error in the elimination rate using the two-sample approach depends on two things: the precision and accuracy of the initial and final isotope estimates and the timing of these samples. The two-sample techniques ignore the temporal variation in isotope abundance in the time between the samples and this led Coward and Prentice (10) to suggest that the true error is underestimated because there is also some contribution of the variation in isotope abundance during the measurement period. On the other hand, and we agree with this opinion, Speakman et al. (39) concluded that this is not possible because the calculation for the two-sample estimate cannot perceive these variations. So, the two-point method is robust to variations in the pattern of decline of isotope.

This robustness does not extend to the multisampling approach. Asymmetry in the pattern of decline leads to a shallower fitted curve compared with the actual average rate of decline. Temporal variation in CO₂ production, which can produce patterns of decline in oxygen abundance, can thus result in errors in the estimated gradient of isotope elimination. Speakman and Racey (43) showed that variations in the activity of small animals over 24 h would introduce systematic errors in the multipoint method of 4–30%, dependent on the exact variation pattern. However, the day-to-day variation in CO₂ production induced an error that was lower (at least 2%) than that anticipated from within-day variation of small mammals (42). Based on the residual standard deviations of our regression lines, we can conclude that the rCO₂ was constant during either isolation or simulated microgravity.

The bottom line from these considerations is that there may be substantial discrepancies between the multisample and the two-sample estimates of gradient...
for the same data set. Welle (48) suggested that as the two-sample approach accurately measures the average rate of elimination; these discrepancies indicate that the multisampling approach provides less reliable results. On the other hand, Goran et al. (14) observed differences of 2.76% (SD 0.16–5.02%); these results are in agreement with ours. We observed a nonsignificant difference of 0.9% between the two- and multipoint method during isolation. The difference was increased during suspension to 1.9% but still remains highly acceptable. During these two periods, the multipoint gave better agreement with the calorimetry than the two-point method, and the Bland and Altman (6) analysis confirms this.

Thus we can conclude that the use of the two-sample approach results in a decline in precision not accuracy. This is of special importance when considering the microgravity environment. A two-sample approach is the only way to proceed, because in-flight manipulation of animals is limited. On the other hand, the loss of precision linked to the two-sample methodology may be reduced using a Penington or Maastricht protocol (12, 50) that we have not addressed here. Lastly, the application of a two-sample calculation reduces the cost of the experiment, which is not negligible with stable isotope experiments.

Effects of Fractionation Processes

The estimates of CO₂ production and water flux can be strongly affected by fractionation. If the material leaving the body is assumed to be of the same composition that is left behind but is actually depleted in the heavy isotope, then we will underestimate the true loss. There are two different fractionation processes: the equilibrium and kinetic fractionations, both depend on temperature.

Initially, Lifson et al. (18) only used equilibrium fractionation without any information on the proportion of water that was being lost by the fractionated and unfrac- tionated routes. They assumed that half the water loss is fractionated (rH₂O = 0.5 rH₂O). This method of calculation (Eq. 6) has been widely used in animal studies (cited in Ref. 36), but it is evident that this equation is based on false assumptions and simplifications. First, endotherms regulate their body temperature at a level much higher than 25°C; second, the loss of fractionated water and CO₂ is likely to represent a mix of kinetic and equilibrium fractionation processes; and third, the actual proportion of fractionated water loss is unlikely to be 50% of total losses. More recently, Haggarty et al. (15) found that fractionation losses in pigs weighing ~38 kg amounted to ~25% of water loss. Webb et al. (46) partitioned the water losses by three species of small insectivorous bats (6–13 g) and found that evaporative water losses accounted for 20 and 40% of the total water losses. Similar proportions of fractionated loss across a wide range of body masses and species may indicate that losses <50% are generally applicable and, in the absence of direct measurements for any particular species, a figure of 25% may be more suitable.

A major issue of our study is the importance of these factors in the accuracy of the derived rCO₂. Effectively, Eq. 7 [Speakman (36)], taking the more recent considerations for fractionation processes, gives the more accurate rCO₂ results regardless of period. Interestingly, two two-pool model equations (Eq. 13: R group; Eq. 9: R = 1.031) also give good results when the pool space is not modified throughout the measurement period. The particular of these equations are fractionation factors close to those in Eq 7. Overall, during either isolation or suspension, we observed that the best agreement with indirect calorimetry is observed when, as suggested by Speakman (36), in vivo kinetic and equilibrium fractionation factors are combined in a ratio of 3:1 at 37°C, with the assumption of 25% fractionated water losses.

Methods of rCO₂ Conversion Into Energy Demand

The problem of converting estimated gas exchange (indirect calorimetry) to energy demands has been widely discussed in the past, and this cannot be related directly to the method per se. In general, when the RQ is known, the conversion of CO₂ production to energy demands is accurate and involves an error of <1%. Gessaman and Nagy (13) assessed the impact of assuming a fixed RQ of 0.8 and found that the errors in the derived caloric equivalence of consumed oxygen were relatively small (<5%). Therefore, no important errors are introduced by assuming an RQ of 0.80 in the estimate of daily expenditures. However, the more precise this value is, the less error will be introduced. Three possible approaches are available to estimate the RQ. Among these three methods, only one is applicable for a long duration of measurement: an RQ from the food composition is calculated (assuming no changes in body composition). During isolation we observed that using either the two- or multipoint method, the expenditure calculations with RQ or FQ did not result in different estimates. We noted that the standard deviations were higher with the FQ and may be due to the body composition changes. The results are similar during suspension.

In conclusion, the unexpected lack of growth during suspension provided us with unexpected results on the application of the DLW. This allowed us to study the physiological consequences of simulated microgravity without any concomitant changes in the isotope pool spaces. In these conditions, the errors introduced in the DLW calculations by suspension are fewer than those introduced by growth occurring from isolation.

We can conclude that Eq. 7 [Speakman (36)] provides the best estimation of the DLW-derived rCO₂ production with the animals in isolation or in simulated microgravity conditions. This is a one-pool model equation using revised fractionation factors (kinetic/equilibrium: 3/1 at 37°C) and a proportion of fractionated water loss of 25%. With the use of this equation, a better agreement with indirect calorimetry is observed.
when $N_{initial}$ is calculated at the plateau and $N_{final}$ is estimated from the inferred percentage of final mass. The isotopic constant elimination rates can be accurately calculated by a two-point method, which is of particular importance for application during spaceflight where multisampling is not realistic. Lastly, the use of an FQ results in accurate estimates of TEE.

From a more general point of view concerning the use of the DLW application on small animals, we can conclude that some two-pool models using an R estimated from the group studied [Eq. 13, Speakman (36)] or fixed to 1.031 [Eq. 9, Schoeller et al. (32)] can accurately estimate $r_{CO_2}$ when the isotope dilution space is constant throughout the experimental period. The fractionation processes of these equations are close to those of Speakman (Eq. 7). The other equations resulted in an underestimate of $\sim 10$–15% of $r_{CO_2}$, the higher discrepancy being observed with Eq. 8 [Coward et al. (10)] (20%). Lastly, we should mention that the results do not extend to human applications, because the majority of these equations are proven reliable.

**Perspectives**

As mentioned above, the energy requirements of spaceflight have been poorly investigated. Only two studies are published in humans (20, 45), and adaptation of rats is unknown. Such a determination is a strong prerequisite for scheduling any long-term spaceflight. Because the hindlimb tail suspension rat model is the more reliable animal model to simulate physiological adaptations of humans to space, the study of rat energy metabolism adaptations is essential to ensure the validity of the model. This study provides evidence that the DLW method can be used accurately in simulated microgravity. However, one limitation is the large constraint placed on the animals during a validation study, which may explain the lack of changes in energy expenditure expected from human studies (4, 19, 45). The clear next step of this study is to assess the energy expenditure of rats during a classical suspension protocol and during a space shuttle mission.

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**REFERENCES**