The doubly-labelled-water (^{2}H$_2$^{18}O) method: principles and practice

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This is the third time in 3 years that nutritionists interested in energy expenditure in man have met in Europe to discuss what has become known as 'the doubly-labelled-water method'. During this time method application has at least run in parallel with method development: in fact the former may even have outstripped the latter because the developing literature provides ample testimony to the method's potential in human nutrition, but to the uninitiated may not give the impression that there is consensus about the fine detail of the methodology. This is an unfortunate situation which, it is to be hoped, this meeting does something to resolve. It will, I believe, become evident that there is substantial agreement as to what are the most important problems and only minor disagreement with respect to their solutions.

Problems arise because it is not possible to make observations on complex physiological systems in their entirety, but only on those parts of the system that are accessible to us. In this case, it is observed that when water labelled with ^{2}H is given to a subject, isotope disappearance, as measured in samples of urine for example, approximates to a single exponential. If water labelled with ^{18}O is used, disappearance is again exponential but the biological half-life of this isotope is shorter than that for ^{2}H. Single exponential curves do not prove a model (the model has to make physiological sense by itself) but Lifson & McClintock (1966) explained that these observations are compatible with a situation in which: (1) body-water is the single compartment which the isotopes label and from which the isotopes are lost; (2) ^{2}H is lost, only as water; (3) ^{18}O is lost, both as water and carbon dioxide, transference of O between water and carbon dioxide being the consequence of a rapid exchange promoted by carbonic anhydrase (*EC* 4.2.1.1); (4) fractional output rates of water and CO$_2$ output are constant; (5) water and CO$_2$ loss occurs at the same enrichment as that coexisting in body-water; (6) background isotope intake rates are constant.

With these assumptions a simple equation for CO$_2$ production rate (F_{CO_2}) emerges:

$$F_{CO_2} = (0.5V) (k_o - k_d). \qquad (1)$$

Quantities are expressed in mol, V is total body water, k is a rate-constant for isotope disappearance and subscripts d and o refer to ^{2}H and ^{18}O respectively.

To illustrate the nature of the method we can examine the practical consequences of this equation with reference to just two assumptions (1 and 5) using as basic information fifty observations we have made on eight pregnant women in which k_d averaged 0.76 (SD 0.0321) k_o.

The fact that total body water determined with ^{18}O is different from that measured with ^{2}H is well known. In the women in this example, V_d averaged 1.037 (SD 0.012) V_o; equations for F_{CO_2} can therefore be written in three different ways using either V_o as total body water:

$$F_{CO_2} = (0.5V_o) (k_o - 0.76k_o), \qquad (2)$$

or V_d as total body water:

$$F_{CO_2} = (0.5) (1.037V_o) (k_o - 0.76k_o), \qquad (3)$$

or the appropriate isotope distribution volume could be combined with its own rate-constant:

$$F_{CO_2} = 0.5[k_o V_o - (1.037)(0.76)k_o V_o].$$ (4)

If the result from equation 2 is given the value of 1, equation 3 is 1.0371 and equation 4 is 0.883. These differences are considerable and while they are the consequence of the ratio $V_d:V_o$, the size of the ratio $k_d:k_o$ is important in determining the magnitude of the difference between equation 4 and the others. If ^2H turnover is small relative to ^{18}O turnover the differences observed for equation 3 decrease; the reverse is also true. Clearly there are important choices to be made in respect of total body water assumptions.

When tracer leaves our model system it is assumed that enrichment in the outflow is the same as that co-existing in body water. This is not expected to be the case because the mass differences between tracer and tracee atoms lead to a phenomenon called fractionation, which is a measure of the relative ability of isotopically-labelled as compared with unlabelled molecules to partake in physical exchange processes such as (H_2O) vap $= (H_2O)$ liq. It is important therefore to consider both which components of loss are fractionated and which factors ought to be used to make appropriate allowances. Incorporating the appropriate factors into equation 1 gives:

$$F_{CO_2} = \frac{0.5V}{f_3}\left[k_o - k_d\frac{(f_2 x + 1 - x)}{(f_1 x + 1 - x)}\right],$$ (5)

where $f_1 = (^2H/^1H)$ vap$/(^2H/^1H)$ liq,
   $f_2 = (^{18}O/^{16}O)$ vap$/(^{18}O/^{16}O)$ liq,
   $f_3 = (^{18}O/^{16}O)$ CO$_2/(^{18}O/^{16}O)$ liq,
and x is the proportion of water loss that is fractionated.

This relationship allows the prediction of the consequences of real variations in the values of x, f_1, f_2 and f_3 in comparison with assumptions that might be made about them. For the purposes of illustration values assumed are those suggested by Lifson & McClintock (1966) (i.e. 0.5, 0.93, 0.99 and 1.04, respectively) and $k_d:k_o$ is given the value of 0.76, as before. Calculation from equation 5 shows that for an alteration in x of 20% (i.e. ± 0.1 when the assumed value is 0.5) energy expenditure measurements are changed by just over 2%. If the true proportion of water loss fractionated is 0.4 rather than 0.5 our assumed value will underestimate CO_2 production. It is also worth noting that the same directional changes in f_1 and f_2 have opposite effects and that (from equation 5) only changes in f_3 have an effect on the magnitude of the error that is independent of the relationship between k_d and k_o. As the ratio $k_d:k_o$ gets closer to 1.0, predicted errors increase and, as it falls, they decrease. For example, if k_d is 90% k_o an 8% error will be generated for a 20% error in x. In much the same way the significance of approximations in f_1 and f_2 diminishes if lower values for x can be realistically applied, but increases if x increases.

In the foregoing discussion, by no means comprehensive of the whole methodology, it will have become evident that there are rather too many choices to be made with respect to calculating results. Such a multiplicity of choices might undermine validation studies because a series of incorrect assumptions could quite conceivably produce the right answers. A validation only begins to prove a model if, in ways external to the system, it can be shown that assumptions that are made are reasonable.

Validation studies from Coward *et al.* (1985), Roberts *et al.* (1986) and Schoeller *et al.* (1986*b*) would lead us to suppose that for energy-expenditure determinations in human adults and very small infants, isotope distribution spaces should be matched with their own rate-constants for isotope disappearance:

$$F_{CO_2} = 0.5 \, (k_o V_o - k_d V_d).$$

It would, however, be comforting to find an alternative basis for this assertion. In dealing with a somewhat ill-defined system (we know our model is not perfect since $V_d \neq V_o$), it is preferable to make the minimum number of assumptions compatible with the solutions we require, that is estimates of the output rates of water (F_d) derived from the ^2H values, and those of water plus CO_2 (F_o) derived for the ^{18}O values. Provided that all input is to the primarily-labelled pool (in this instance 'body-water'), output rate is given as:

$$F = D/_o \!\int^\infty C(t)dt,$$

where D is the amount of dose given and $C(t)$ is isotope concentration at time t. This means that in order to calculate output we need to obtain the best estimate possible of the total area underneath the isotope-disappearance curve from the start of the experiment to infinite time. Because an extrapolation beyond the data points is required some assumptions about the system have to be made. If the system is defined as a single compartment both the ^2H and ^{18}O curves will be single exponentials with one slope (g_1) and one intercept (I_1), initial distribution volume will be given by the intercept divided into the dose (D/I_1) and the rate constant for output (k_d or k_o) will have the same value as that of the slope (g_1). The area under the curve will be given by the intercept divided by the slope (I_1/g_1). If, on the other hand, it is assumed that a more complex system exists (two or more interchanging compartments), the disappearance curve will be the sum of two or more exponentials with intercepts $I_1, I_2 \ldots I_n$ and slopes $g_1, g_2 \ldots g_n$.
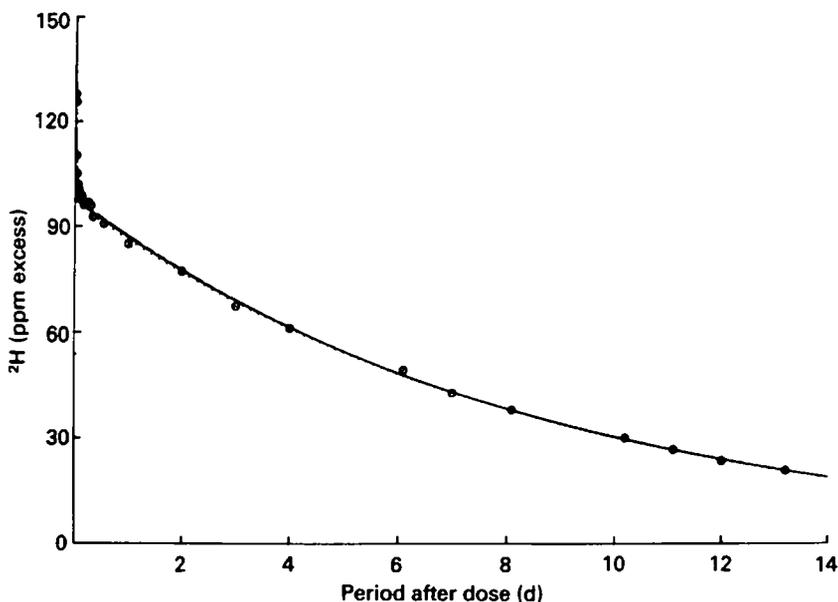

Fig. 1. ^2H disappearance from body-water in an adult male subject orally dosed with ^2H_2O. Values are ppm excess above predose values. For details of the fitted curves, see Table 1. (- - -), curve A; (. . . .), curve B; (———), curve C.

Table 1. *Effect of changing the model or sampling times, or both, on estimates of rate-constants* (k), *initial distribution volumes* (V), *or outflow rates* (F = kV) *in an adult male subject orally dosed with* $^2H_2^{18}O$

	Model	Values	k (% B)	V (% B)	F (% B)	
A	Two-pool	All	156·38	63·72	99·61	
B	One-pool	6, 7, 8 h; 1–12 d	100·00	100·00	100·00	^{18}O values
C	One-pool	1–12 d	99·10	101·16	100·24	
A			148·52	67·21	99·82	
B			100·00	100·00	100·00	^2H values
C			99·13	100·96	100·07	

These intercepts and slopes do not directly correspond to compartmental volumes or rate-constants for isotope disappearance, although the latter can be calculated from them, but of course the sum of the areas under each curve $I_1/g_1 + I_2/g_2 \ldots I_n/g_n$ corresponds to the total area. It is necessary to know therefore, if single-pool kinetics are to be applied to more complex systems, the relationship between areas under curves determined for each case. In addition, we also need to know about the consequences of making area estimates with restricted numbers of data points.

Fig. 1 shows three curves fitted to isotopic values from saliva sampling following an oral dose of ^2H_2O. Despite the fact that different assumptions are made and different sampling times used the curves are virtually indistinguishable. However, if a combination of a rate-constant for ^2H disappearance and a distribution volume for ^{18}O were to be used, for example, this would be the same as moving the point at which our ^2H curve intercepts the *y*-axis upwards, with obvious consequences for the area under the curve. Table 1 indicates the consequences for outflow rates of these differing assumptions for models or choice of data points. Particularly striking is the observation that the values for the rate-constant for output and initial distribution volumes, calculated from the *t*=0 intercept, are less stable than those for outflow. This is a consequence of the high degree of correlation that exists between errors on estimates of rate-constants and intercepts, or alternatively that the specific value of rate-constants and intercepts is a consequence of the assumptions made in fitting a particular model to a set of data points for isotope disappearance. There is nothing particularly sacrosanct about rate-constants or volumes in this context.

For these reasons it seems unsatisfactory to recommend the use of separate estimates of isotope-distribution volumes and slopes as suggested by Schoeller *et al.* (1986*b*) in a two-point method. Estimates of output rate obtained in this way are certain to be less precise. Nevertheless, on average, it might be expected that the two-point method and slope–intercept method should produce the same results, on average. Table 2 shows this comparison. For the two-point method, slopes were determined from data points 1 and 14 d after dosing and V_o and V_d determined from a single urine sample taken 6–8 h after the dose, these values were then divided by 1·01 and 1·04 respectively, and the result averaged and multiplied by 1·01 and 1·04 to obtain a weighted average value for each volume (see Schoeller *et al.* 1986*b*). For the slope–intercept method three urine samples were taken in the period 5–10 h after dosing, and usually one sample every subsequent day.

Table 2 is interesting for several reasons. Essentially it shows that there were no significant differences in any of the rate-constants or volumes, but the *t* of 1·8 for the 2% on average lower estimate for F_{CO_2} using the slope–intercept method may be an indication of something real. The two-point procedure forces a 1·03:1 relationship

Table 2. *Comparisons between rate-constants for ^{18}O and ^{2}H disappearance (k_o and k_d), isotope distribution volumes (V_o and V_d) and carbon dioxide production rate F_{CO_2}, calculated using equation (4) (p. 210) estimated using the slope–intercept and two-point methods (n 50)*

		Slope/intercept (A)	Two-point (B)	A–B Mean	A–B SD	Statistical significance of difference: t
k_o	Mean	0·1138	0·1132	0·0006	0·0026	1·01
	SD	0·0144	0·0144			
V_o	Mean	34624	34671	−48	929	−1·00
	SD	3889	4102			
k_d	Mean	0·0868	0·0864	0·0004	0·0027	1·11
	SD	0·0134	0·0137			
V_d	Mean	35893	35706	188	887	1·01
	SD	4009	4222			

F_{CO_2}, mean difference (% A) = −1·9 (SD 7·4), paired t 1·82.

between V_d and V_o, whereas the average value for this data-set was 1·037. Simple calculation shows that for a mean $k_d:k_o$ of 0·76 this difference in values for volumes will produce a 2% lower value for CO_2 production. The SD of the mean differences for F_{CO_2} of 7% of the slope–intercept value may seem rather large; it is, however, compatible with average errors for F_{CO_2} in the slope–intercept method of about 3% and errors of roughly double this for the two-point method.

Elsewhere at this meeting Speakman & Racey (1988) have suggested that models such as these, with H and O disappearance from total body-water only to the outside, will be confounded by incorporation of either element in tissue as a sink. This is indeed the case and unfortunately isotope-disappearance curves cannot be used to say whether or not it is happening, nor can any mathematical treatment of isotope values resolve the problem. Of particular concern is that high rates of *de novo* fat synthesis would increase the apparent water loss and therefore decrease calculated CO_2 production rates. The suspicion is that this will always be of greatest concern to zoologists interested in energy-balance studies in small animals because in this case there may be high rates of fat accretion from non-fat dietary sources. On the other hand, if for a pregnant woman laying down 4 kg fat in 9 months the view is taken that this is entirely *de novo* synthesis, and 50% of all the H in it are derived from water (Jungas, 1968), the underestimate of CO_2 production rate would be of the order of 1%. Losses of ^{2}H or ^{18}O to the outside, occurring not as water, but as H and O fixed in urinary or faecal solids, present a similar problem. However, Schoeller *et al.* (1986a) have estimated their magnitude in adult man at 0·47% of ^{2}H turnover and 0·34% of ^{18}O turnover and concluded that they are quantitatively unimportant.

The fourth assumption is that output rates of water and CO_2 are constant in relation to pool size (for a discussion of the appropriate mathematical treatment of volume changes during the experimental period, see Coward *et al.* 1982). Both the multipoint and especially the two-point methodologies will be sensitive to unusual fluctuations occurring at the beginning or end of a measurement period. However, only in the former case will information be available (from standard errors of intercepts, slopes and areas under curves) that will allow inferences with respect to this type of variation to be made. In the data base we have considered here, standard errors for k_d, I_d, k_o, and I_o averaged

1·58, 1·08, 1·29 and 1·12%, respectively, and allowing for the correlation of errors on slopes and intercepts these values translate into errors on F_d as F_o of 1·00 and 0·81%, respectively. Since F_d and F_o are also bound to be correlated (F_d is a large proportion of F_o) we should also include a term for the correlation of errors on F_d and F_o. I can see no specific way of calculating the value of this correlation for an individual set of values but estimates made of the relationship between F_d and F_o in successive days within individuals indicates that a value of 0·8 is a modest one. This would lead to an average error for F_{CO_2} measurements in this set of values of 2·43%. By the use of computer simulations it can be shown that this level of error is equivalent to a variation in output rates with a standard deviation of about 3·5%/d or a standard deviation for each data point of 2·5%.

We saw earlier how assumptions made about the value of four factors, x, f_1, f_2 and f_3, can affect the results obtained in the doubly-labelled-water method and provisionally ascribed values of 0·5, 0·93, 0·99 and 1·04 to them. It is clear that the use of average values for the proportion of total water losses fractionated (x) reduces the usefulness of the method as applied to an individual subject. As far as respiratory water loss is concerned it has been suggested that this unknown can be eliminated from equations by expressing it in terms of CO_2 production rate assuming that expired air contains 35 ml CO_2/l and is saturated with water vapour at 37° (Schoeller et al. 1986b). This gives a value of 1·42 ml water/l CO_2. In contrast to this theoretical value we have obtained an average value of 0·83 g/l CO_2 in young adults both exercising and at rest. For our population of pregnant women these amounts represent 726 or 424 ml/d respectively (23 and 14% total water turnover).

If we assume that sweat is not fractionated, the remaining component of fractionated water loss is insensible perspiration. For this Schoeller et al. (1986b) have suggested that a value of 30% of estimated breath losses, to be substituted in the calculations. For these subjects this would represent 218 ml to provide a total of 944 ml (30·3% total water turnover). In contrast we have obtained values of about 500 g/d for young adults lightly clothed at 22°. Fortuitously this value, combined with our value for respiratory water losses, gives total fractionated losses of 924 ml (29·7% total water turnover). It seems likely that more independent experimental values should clarify the situation with respect to respiratory water loss and its equivalence to CO_2 production but we cannot be as confident for skin water losses. In our experiments there is no convincing correspondence to weight or surface area and it is well known that the ability of the stratum corneum to retain water depends both on temperature and the relative humidity of the surrounding air (for a review, see Kuno, 1956).

The situation is clearer with respect to the value of fractionation factors in vivo although again more direct, independent measurements are certainly required. A value of 1·038 for the equilibrium $(^{18}O/^{16}O)CO_2/(^{18}O/^{16}O)$liq has been found both in vivo and in vitro at 37° (Friedman & O'Neil, 1977; Pflug et al. 1979). For respiratory water loss, geochemical values for equilibrium conditions suggest values in the range 0·9371–0·9408 for $(^2H/^1H)$vap/$(^2H/^1H)$liq (Friedman & O'Neil, 1977) and Schoeller et al. (1986a) calculated values of 0·946 (SE 0·002) from the values of Halliday & Miller (1977). In contrast, for seven subjects breathing dry air at 22° we have obtained 0·9346 (SE 0·0007). Corresponding values for $(^{18}O/^{16}O)$vap/$(^{18}O/^{16}O)$liq are: geochemical values 0·9916–0·9923 (Friedman & O'Neil, 1977), in vivo 0·991 (SE 0·001) (Schoeller et al. 1986a) or our estimates of 0·9927 (SE 0·001). If we assume that insensible water loss is fractionated under non-equilibrium conditions, geochemical values would suggest 0·917–0·930 for $^2H/^1H$ and 0·976 for $^{18}O/^{16}O$ (Dansgaard, 1964), whereas Schoeller et al. (1986a) have obtained values of 0·935 (SE 0·008) and 0·981 (SE 0·001) and we have measured 0·9333 (SE
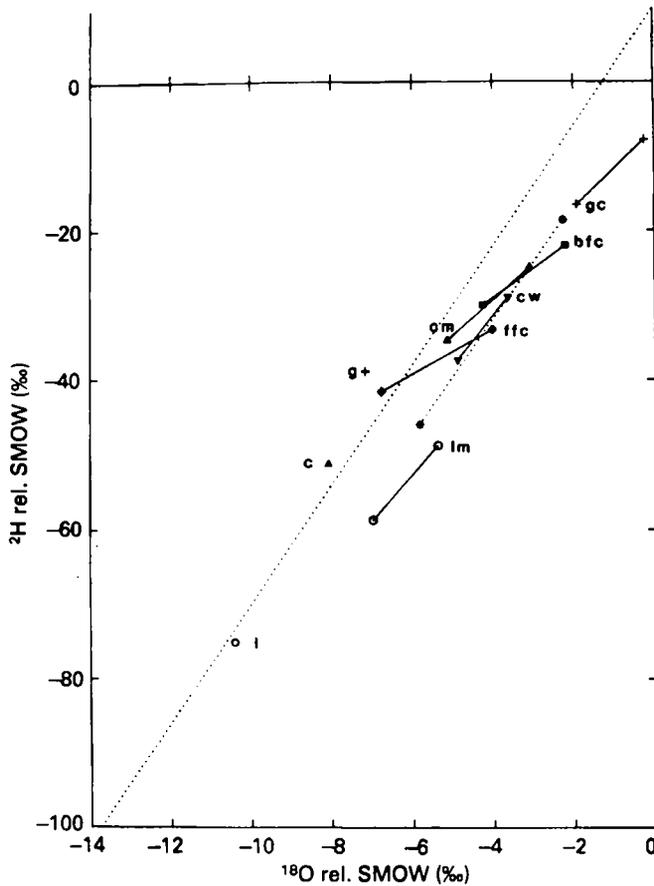
Fig. 2. ^2H and ^{18}O enrichment in the body water of a variety of subjects, and in their local water supplies. Values are ‰ rel. SMOW. (....) Meteoric water line (^2H‰ = 8 ^{18}O‰ + 10) for the northern hemisphere (Dansgaard, 1964). (lm), Lausanne men; (ffc), formula-fed Cambridge children; (bfc), breast-fed Cambridge children; (cm), Cambridge men; (cw), Cambridge women; (gc), Gambian children. Values are means and 1 SD. (∗....∗), Mean and standard deviation for all the body-water values. Points, 1, c, g are Lausanne, Cambridge and Gambian water supplies.

0·002) and 0·9855 (SE 0·0005). For water losses Schoeller *et al.* (1986a) suggested the use of a 3:1 weighted average of the fractionation factors for respiratory and transcutaneous water losses. In contrast we would suggest a value close to 1:1 which would lead, using our values, to 0·934 for $^2H/^1H$ and 0·989 for ^{18}O. Differences between this latter value and values suggested by Lifson & McClintock (1966) are trivial and changing our $^2H/^1H$ fractionation value to 0·934 from 0·93 would increase CO_2 estimates by less than 1%.

For workers used to tracer experiments with radioactive isotopes the need to be concerned with variations in isotope intake from sources outside the experimental manipulations will be unfamiliar. Stable isotopes, however, occur naturally and are in everything we eat, drink or breathe. Individuals thus take on a pattern of isotope enrichment that reflects, but is not the same as, their isotopic intake. As far as ^2H and ^{18}O are concerned the ultimate source of variance is that brought about by evaporation and condensation processes where isotopic fractionation can occur. Similarly, for the

microcosm of the individual human subject, his characteristic pattern of isotope fractionation will determine isotopic enrichment in his body water. Schoeller *et al.* (1986*a*) have made very substantial investigations in this area, but a few general points are pertinent. World-wide background isotopic enrichment in man follows the same pattern as that seen for his water supplies and ^{2}H and ^{18}O enrichments are co-variant (Fig. 2). The consequences of this fact are that it should be advantageous in the methodology to choose doses of ^{2}H and ^{18}O that in the enriched subject will have the same relationship to one another as in the background because if this is done, the effects of variation in backgound will tend to cancel (Schoeller, 1983).

Apart from the obvious circumstances of a subject moving from one place to another and changing his backgound intake there are others that are worth mentioning. For example, Cambridge breast-fed babies are more enriched than bottle-fed ones (see Fig. 2), and presumably at weaning breast-fed babies gradually become less enriched, and 57% of the variation in ^{18}O background in Gambian infants is accounted for by consideration of the time of year. The value for ^{2}H was 54% (Eccles and Coward, unpublished observations). The most rapid rate of change (an increase of 8·8‰ rel. SMOW for ^{2}H and 1·3‰ rel. SMOW for ^{18}O) occurs between September and October and for children given doses of 0·1 g ^{2}H$_2$O and 0·28 g H$_2$^{18}O/kg body-weight, and if these changes took place entirely in the study period, would produce an underestimate of CO$_2$ production rate of 1·6%. There is, as yet, insufficient information to indicate whether or not this magnitude of variation is the exception or the rule for field consumptions.

The translation of values for CO$_2$ production rate into values for energy expenditure poses a final problem. Conventionally, CO$_2$ production rate by itself, is not regarded as a good indicator of energy expenditure because, in the short-term respiratory quotient (RQ) can vary considerably (for example 0·8 and 0·9 in the fasting and postprandial states, respectively). However, it is average estimates of total energy expenditure over relative long periods of time that are relevant here. In these circumstances RQ can be calculated from the composition of dietary intake with necessary adjustments for deposition or mobilization of fat taking place during the measurement period. Within individuals there seems to be little variation in dietary RQ (coefficient of variation (CV) 0·7% for Cambridge women) and even between individuals the variation is small (CV 1·5%) so that a population mean RQ and 2 SD is equivalent to a 2·3% error in an energy-expenditure measurement. Errors in ignoring fat mobilization or storage are similarly trivial. For example if 10% of total energy expenditure is derived from stored fat, even when dietary RQ represents a low-fat diet, errors will only be of the order of 2% (Black *et al.* 1986).

At the beginning of the present paper it was suggested that we ought to be able to see our way towards some agreement as to protocols for universal application of the doubly-labelled-water method. As far as dose levels are concerned there is no reason why the regimens suggested by Schoeller (1983) should not be used except if in particular circumstances analytical constraints demand that more or less isotope should be given. However, whatever dose levels are used, the aim ought to be to achieve precision on estimates of F_d and F_o of the order of 1%. Protocols with respect to dose administration are also worth consideration. It is customary to ask subjects to rest for a few hours while the dose 'equilibrates' within their body water. This is a tradition adopted from total body-water measurements and is not entirely logical when we are interested in turnover. In this case it might be better to avoid breaks in normal behaviour patterns. The length of time required for observation will depend on the biological half-lives of the isotopes within the subject and both Schoeller's (1983) work and our own suggests that two to three half-lives is a suitable length of time. It is always well worth considering computer

simulations to test the relative merits of different initial enrichment levels and sampling times on predicted precision before starting experiments. The system we use (Coward *et al.* 1988) enables us to investigate these variables for any level of constant analytical or proportional biological error. There remains the question of how many samples to take. The so-called two-point method does not really use two points. Schoeller *et al.* (1986*b*) now recommend the addition of a third point as a check for linearity, and use a fourth point for the determination of volume. On the other hand measuring samples collected every day is time-consuming and simulation shows that improvements in precision are by no means proportional to the number of data points added. I suspect that individual laboratories will come up with their own particular sampling regimens (there will always be a need to consider what is convenient for both the subject and the field worker) but whatever method is used it is not unreasonable to expect that published values give some indication of observed precision. This can be quite easily calculated from errors on slopes and intercept or volume determinations. As far as the values for fractionation factors are concerned more 'in vivo' work is required, especially with respect to the $(^2H/^1H)$vap/$(^2H/^1H)$liq equilibrium. However, the most pressing issue must be about the proportion of water loss fractionated. At best, we can say that some estimates look reasonable (i.e. about 30% for ordinary adults in a temperate climate). In most other circumstances the situation is much less clear and physiological investigations on insensible water losses should be done in parallel with any isotope study. The particular group of subjects used as examples here had water turnover rates that were not exceptionally high (3115 g/d) but we are familiar with circumstances in The Gambia where turnover rates can be double this. Unless the increment of turnover is unfractionated the decreased difference between k_o and k_d or F_o and F_d considerably increases the errors involved in making assumptions about the amounts fractionated.

It has been the intention to present a realistic description of the methodology from the point of view of both its potential and risk. The potential is that of the only viable method for the measurement of energy expenditure in free-living men and women. The risks are those associated with the indiscriminate application of the method in total ignorance of the subject's physiology; such pit-falls can and should be avoided.

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