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THE DOUBLY-LABELLED WATER METHOD FOR MEASURING ENERGY EXPENDITURE

Technical recommendations for use in humans

**A consensus report
by the
IDECG Working Group**

Editor: AM Prentice

I/D/E/C/G International Dietary Energy Consultancy Group



INTERNATIONAL ATOMIC ENERGY AGENCY

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Editor: AM Prentice

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NATHAN LIFSON

1911 - 1989

This book is dedicated to the memory of Nathan Lifson,
inventor of the doubly-labelled water method,
who sadly died on 31st December 1989.

Foreword

The doubly-labelled water method using stable isotopes of hydrogen and oxygen, is rapidly becoming established as an important new tool for investigating energy metabolism. It is the first genuinely non-invasive method for measuring energy expenditure in free-living people, providing estimates of habitual expenditure over a time period of 10-20 days. The accuracy and precision of these estimates should be superior to those obtained by traditional factorial methods.

The doubly-labelled water method, developed and applied to humans in eight research centres, has already been used in premature babies, neonates, infants, children, pregnant and lactating women, normal and obese adults, athletes, hospitalised patients and in the elderly. In spite of this, several aspects of the method have not been standardised.

This report is the outcome of a workshop convened to standardise the doubly-labelled water method. It was sponsored by the International Dietary Energy Consultancy Group (IDECG), financed by the Nestlé Foundation, organised by Dr Andrew Prentice, and took place in Cambridge, U.K., from September 26-29, 1988.

All the eight research centres using the method in humans and two who had done pioneering work in animals were represented at the workshop. Data sets and position papers on various aspects of the method were exchanged among centres prior to the meeting. The main issues discussed at the workshop were isotopic pool sizes and flux rates, estimates of error, fractionation effects, isotope exchange, effects of changes in isotopic background, the energy equivalent of CO_2 , problems of mass spectrometry and problems arising when the method is used in special groups of humans like premature babies and certain categories of

hospitalised patients. The discussion was very open and consensus could be reached on all important issues.

The Section of Nutritional and Health-Related Environmental Studies of the International Atomic Energy Agency (IAEA) supported the publication of this document within the framework of its Co-ordinated Research Programme on Applications of Stable Isotope Tracers in Human Nutrition Research. The methods described here are also expected to be applicable in some future IAEA projects dealing with other, more specific, aspects of energy metabolism in third world populations.

The organizations and scientists involved in this venture hope that this report will serve future users of the doubly-labelled water method by providing them with a sound and agreed-upon methodological basis.

Robert M. Parr
IAEA, Vienna

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PREFACE

Modern scientists are very quick to identify, assimilate and apply new techniques. This trait often leads to astonishing rates of progress in which new methods can become routinely used throughout the world in just a few years. Monoclonal antibodies are a good example of one such success story. However, in other instances the over-enthusiastic adoption of a new method, together with the pressure to publish new results as soon as possible, has led to the premature application of techniques which have not undergone the usual rigours of a full methodological work-up. This document, and the work leading up to it, are an attempt to ensure that the Doubly-Labelled Water (DLW) method does not fall into the latter category.

The method has undergone a period of arrested development with respect to its applications in man. It was over 30 years between Professor Nathan Lifson's initial idea and Dr Dale Schoeller's realisation in the early 1980's that improvements in the precision of isotope ratio mass spectrometers had reduced the cost of applying the technique to such an extent that it had become financially viable for studies in humans. There followed a period in which several laboratories built on the experience gained from applying DLW in small animals, and concentrated on refining the technique for use in man. The extrapolation from one to the other was by no means straight-forward. As indicated by Kleiber's Law, humans have a relatively low energy expenditure per unit body mass compared to small animals. Together with their relatively profligate use of water, and hence high water turnovers, this tends to increase the potential errors in the method. On the other hand humans have some distinct advantages over other animals. They are easier to dose and can supply regular serial samples for analysis thus removing the need to use

the traditional capture-release-recapture procedure.

The early papers on human applications of DLW all concentrated on theoretical and technical aspects, and on the results of new cross-validation studies. Many of the leading groups in the field participated in a methodological workshop during the XIII International Congress of Nutrition at Brighton in 1985. The first biological results were published in the same year, and it was announced that Nathan Lifson was to be awarded the Rank Prize in Nutrition for his pioneering work in developing DLW. In 1986 several groups from laboratories throughout the world participated in a symposium in Cambridge on 'Stable Isotopic Methods for Measuring Energy Expenditure'. This was an exciting time during which the results from many new studies were presented and when there was a general acceptance by most nutritionists that the method was probably working well. However, there remained a healthy scepticism which was encapsulated by Dr Elsie Widdowson in her now famous description of the method as "doubly-indirect calorimetry".

In addition to this residual scepticism the main proponents of the method had two other concerns. The first was that it might be difficult to make inter-laboratory comparisons of results if each laboratory used a slightly different variant of the technique. This problem has been particularly acute in another field where stable isotopes are used in nutritional studies, namely protein turnover. The diversity of tracers, end-products, dosing protocols and kinetic models employed, together with the sparsity of cross-validation studies, make it difficult to compare protein turnover results from different laboratories. The second concern was that new workers in the field may underestimate the complexities of DLW and publish invalid data which could potentially discredit the method.

In order to circumvent these problems it was decided to convene a workshop in which to seek a consensus view on the

various technical aspects of applying the method. The International Dietary Energy Consultancy Group (IDECG) endorsed this proposal and it was financed by the Nestlé Foundation. The International Atomic Energy Agency (IAEA) supported the publication of this document as part of their Co-ordinated Research Programme on Applications of Stable Isotope Tracers in Human Nutrition Research.

The workshop was held in Clare College, Cambridge in September 1988. It was preceeded by the exchange of 32 DLW data sets from 6 of the participating laboratories. These were re-calculated by a number of the participants using a total of 17 variants of the initial Lifson equation, and using different fractionation assumptions in order to quantify the maximum possible methodological discrepancies, and to identify the causes of such variance. The central participants prepared position documents which formed the basis of the subsequent discussions held over 4 days.

There was a remarkable concurrence of views concerning the causes, consequences and solutions to all of the major problems associated with DLW. Following the meeting these views were summarised by a number of the participants whose chapters were re-circulated for comments in order to ensure that the consensus had been fairly represented. The resultant recommendations contained in this report do not stipulate exact rules as to how the method should be applied, but instead provide a framework of guidelines which will ensure that published data is of a high quality. All current and potential users of the doubly-labelled water method are therefore stongly encouraged to make full use of these guidelines.

Readers who are completely unfamiliar with the doubly-labelled water method may find certain sections of this report rather impenetrable. They may find it easier to read Chapter 1 followed by Chapter 11, which contains some worked examples, before returning to Chapters 2 to 10 which examine the detailed arguments in support of the final recommendations.

Andrew Prentice
April 1990

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CHAPTER 1

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INTRODUCTION

1.1 Significance

The rate at which a human uses metabolic energy can reveal a great deal about their health and welfare. Energy is required to fuel the basic life processes, for work and activity, to meet challenges to survival such as disease, drought and famine, and to resist environmental stresses such as cold. Although metabolic rates can be measured routinely in laboratory settings either directly by assessing heat loss using whole-body calorimeters, or indirectly by measuring oxygen consumption and/or carbon dioxide production, measurements of energy metabolism by unrestrained humans in their normal surroundings had to await discovery of the doubly-labelled water (DLW) method.

1.2 Principle of the Doubly-Labelled Water method

The technique involves enriching the body water of a subject with an isotope of hydrogen (^3H) and an isotope of oxygen (^{18}O), and then determining the washout kinetics of both isotopes

as their concentrations decline exponentially toward natural abundance levels (Figures 1.1 & 1.2).

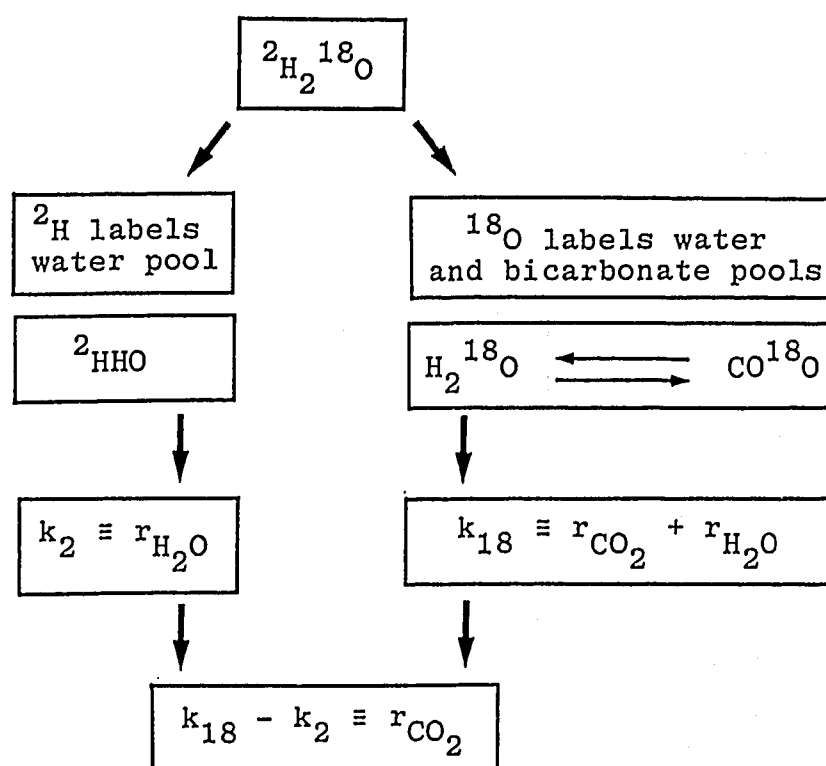
The concentration of the hydrogen isotope, nearly all of which remains associated with water molecules, decreases as a result of dilution of body water by new, unlabelled water (consumed as food and drink and produced during oxidation of foodstuffs), coupled with the simultaneous loss of labelled water via evaporation from lungs and skin, and via excretions and secretions. The rate constant for ^3H is derived as the slope of \log_e ^3H enrichment against time (Figure 1.2) and is a measure of the rate of water movement through the subject.

Most of the ^{18}O in a labelled subject is lost as water, but some is also lost as carbon dioxide because CO_2 in body fluids is in isotopic equilibrium with body water due to the action of carbonic anhydrase present in red blood cells and elsewhere. Thus the slope of the washout line representing ^{18}O is steeper than the line for ^3H (Figure 1.2), and the difference between slopes represents CO_2 production. This indirect measure of metabolic rate may then be converted to units of heat production by incorporating knowledge, or estimates, of the chemical composition of the foodstuffs being oxidised since this influences the energy equivalence of each litre of CO_2 produced.

Determination of the two rate constants requires a minimum of two post-dose samples of body fluid, over a time period of several days to several weeks, depending on the subject's age and rate of water consumption. The isotopes of choice in human studies are deuterium (^2H) and oxygen-18 (^{18}O) since these avoid the need to use any radioactivity and can be safely used in any subjects.

Figure 1.1

Principle of the doubly-labelled water method



k = experimentally-determined rate constant (see Fig 1.2)

r = production rate

Note

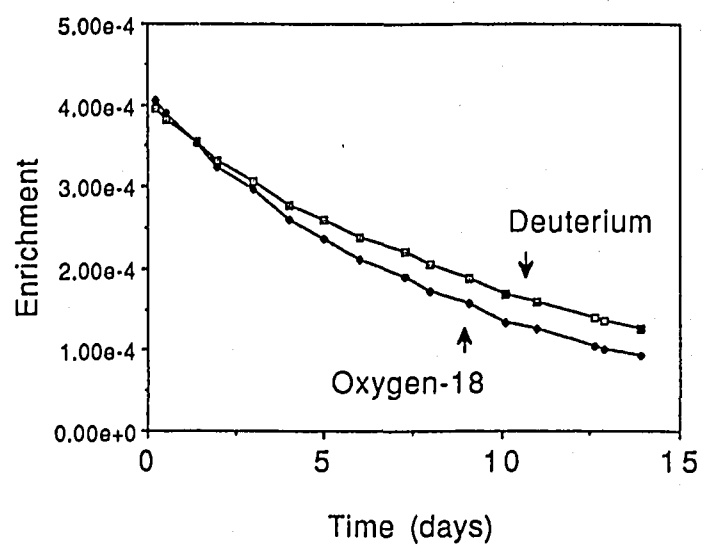
The carbonic anhydrase reaction in red blood cells and in the lung catalyses the following equilibria:



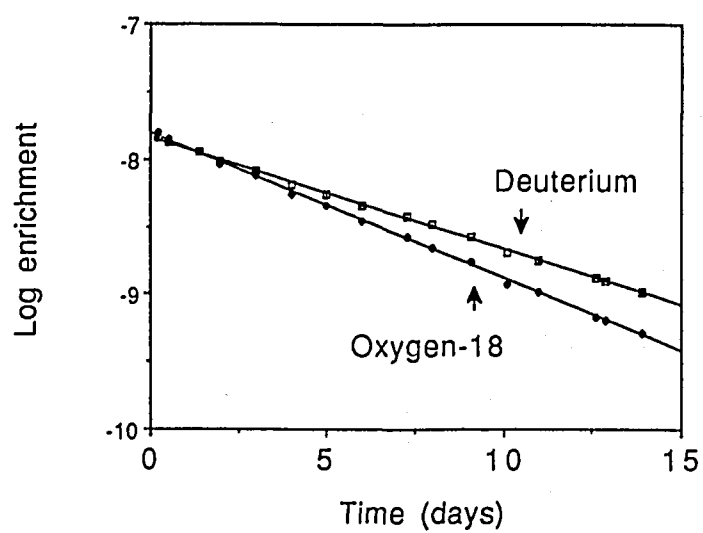
Figure 1.2

Examples of isotope disappearance curves

a) Untransformed data



b) Log transformed data



The initial values have been normalised on the y axis for clarity.

1.3 Origin of the method

Professor Nathan Lifson and his colleagues at the University of Minnesota invented the doubly-labelled water method in the late 1940's and early 50's ¹. Their discovery that the oxygen in respiratory carbon dioxide is in isotopic equilibrium with the oxygen in body water ² provided the key information upon which the DLW method is based. The simultaneous development of sensitive and accurate mass spectrometers by A.O. Nier, also at the University of Minnesota, was an essential ingredient in the invention process.

1.4 Development

Lifson and his colleagues performed subsequent studies on laboratory rats and mice to validate and evaluate the potential errors in the DLW method ^{1,3-7}. In 1966 they published a review paper which listed the assumptions inherent in the model which forms the basis of the technique, and considered the likely effects on accuracy of any deviations from this model ⁸. The paper concluded that the method was relatively robust to the sorts of deviations which might be encountered in reality, and emphasised the potential value of DLW in studies of free-ranging animals.

Aside from a study of the energetic cost of flight in pigeons by a student at the University of Minnesota ⁹, many years passed before researchers began using the DLW method. The reasons for this include the very high cost of the isotopes needed to enrich the body water of subjects (about US\$ 1500 per kilogram of body mass at prevailing prices and recommended doses in early 1960), and the technical difficulty and relative unavailability of isotope measurements.

1.5 Small animal studies

The substitution of tritium, which is much more easily measured than deuterium, and the development of the proton activation method for measuring oxygen-18 in the early 1970's^{10,11} facilitated implementation of DLW studies on small animals. Large doses of ¹⁸O were still required for the proton activation analysis to yield accurate results, so isotope costs were still very high, despite reductions in prices (down to about US\$ 200 per kilogram of subject). Thus, studies on large animals and humans remained impractical. However, research on small animals mushroomed, with more than 12 species of reptiles, 23 species of eutherian mammals, 13 species of marsupial mammals and 25 species of birds having been studied in their natural habitats up until the mid-1980's^{12,13}. Validation studies have compared metabolic rates determined using DLW with those determined using direct CO₂ or O₂ analysis or with other independent measures (Table 1.1). These yielded average errors of within $\pm 6\%$ in 10 studies on small mammals and 7 studies on small birds, but indicated that errors may be larger in reptiles ($\pm 8\%$) and especially in arthropods ($\pm 37\%$) due to their specialised water-conservation mechanisms.

1.6 Human studies

Research on humans with the DLW method had to await a reduction of isotope purchase costs. This was made possible in the late 1970's, not by further reduction in isotope prices, but by the increased sensitivity and accuracy of ¹⁸O measurements which permitted use of much lower doses. Lifson et al published an analysis showing that with ¹⁸O analyses performed on an isotope ratio mass spectrometer, which provides much more accurate measurements than other types of mass spectrometers, results having errors of less than 10% could be obtained from adult human subjects with doses of ¹⁸O costing only US\$ 75-250

Table 1.1

Cross-validation studies in animals

	<u>% error in DLW method</u>	
	<u>Mean</u>	<u>Range</u>
<u>Mammals</u>		
Mouse (<i>Mus</i>)	- 3	(+20, -21)
Mouse (<i>Mus</i>)	- 4	(+8, -12)
Mouse (<i>Perognathus</i>)	+ 0.9	(+6, -9)
Squirrel (<i>Ammospermophilus</i>)	+ 0.8	(+17, -12)
Chipmunk (<i>Tamias</i>)	+ 4.5	(+8, +1)
Chipmunk (<i>Tamias</i>)	+ 3.3	(+18, -19)
Rat (<i>Rattus</i>)	+ 2	(+10, -2)
Rat (<i>Rattus</i>)	+ 2	(+6, -9)
Rat (<i>Rattus</i>)	- 1	(+12, -13)
Gopher (<i>Thomomys</i>)	+ 3.7	(+15, -9)
<u>Birds</u>		
Pigeon (<i>Columba</i>)	+ 3.6	(+17, -12)
Martin (<i>Delichon</i>)	+ 3.6	
Sparrow (<i>Passerculus</i>)	+ 6.5	(+11, -0.2)
Starling (<i>Sturnus</i>)	+ 2.5	(+16, -15)
Sparrow (<i>Zonotrichia</i>)	+ 6.1	(+13, -4)
Parakeet (<i>Melopsittacus</i>)	- 0.04	(+6, -5)
Quail (<i>Callipepla</i>)	- 4.9	(+8, -17)
<u>Reptiles</u>		
Lizard (<i>Sceloporus</i>)	+ 3.2	(+18, -6)
Lizard (<i>Uta</i>)	- 7.3	(+12, -22)
Tortoise (<i>Gopherus</i>)	+ 2.2	(+25, -26)
<u>Arthropods</u>		
Locust (<i>Locusta</i>)	+ 7.2	(+60, -24)
Scorpion (<i>Hadrurus</i>)	+ 36.5*	(+71, +11)
Beetle (<i>Eleodes</i>)	+ 33.8*	
Beetle (<i>Cryptoglossa</i>)	+ 28.7	

See reference 31 for original citations.

* significantly different from zero.

Table 1.2

Cross-validation studies in humans

<u>Subjects</u>	<u>% error (SD)</u>	<u>Ref.</u>	<u>Calculation</u>	<u>Citation</u>
		<u>method</u>		
Adults, n=4	-0.4 (5.6)	I/B	S, 2 point	15
Adult, n=1	-4.6	RGE	L, multipoint	17
Adults, n=5	+1.5 (7.6)	RGE	S, 2 point	18
Adults, n=4	+1.9 (2.0)	RGE	C, multipoint	19
Exercising adults, n=2	-2.5 (4.9)	RGE	L, 2 point	20
Premature infants, n=4	-0.3 (2.6)	RGE	C, multipoint	21
Adults on TPN, n=5	+3.3 (5.9)	I/B	S, 2 point	22
Adults, n=9	+1.4 (7.7)	RGE	S, 2 point	23
Post-surgical infants, n=9	-0.9 (6.2)	RGE	S, 2 point	24
Infants, changing diet, n=8	-8.7 (12.9)	RGE	S, 2 point	25
Adults, n=5	+1.4 (3.9)	RGE	S, 2 point	26
Exercising adults, n=4x2	-1.0 (7.0)	RGE	S, 2 point	26
Soldiers in the field, n=16	+5.3	I/B	S, 2 point	27

I/B = Intake corrected for change in body stores.

RGE = Respiratory gas exchange.

S = Method of Schoeller ²⁸.

L = Method of Lifson ⁶.

C = Method of Coward ²⁹.

per subject ¹⁴. Shortly thereafter, gas isotope ratio mass spectrometers became available commercially. In 1982, Dale Schoeller and Edzard van Santen published the first study to show that DLW can yield an economical and accurate measurement of energy expenditure in humans ¹⁵. The first field applications of DLW in human subjects were reported in 1985 ¹⁶ and there has been a rapid expansion of the literature since then.

1.7 Human validation studies

The doubly-labelled water method has been validated in 13 separate studies by 4 independent research groups with excellent results (Table 1.2). Three mathematical models have been employed in these validations as detailed in later chapters. In general, the Lifson model tends to underestimate carbon dioxide production and hence energy expenditure by several percent in adults and up to 13% in infants or other subjects with high water turnover rates ²⁸. Because of this the Lifson model is not recommended for human studies. The remaining models of Coward ²⁹ and Schoeller ²⁸ have been found to be valid. Accuracy is generally in the order of 1 to 3% and precision 2 to 8%, with the Coward model using multi-point regression analysis of isotope elimination rates seeming to offer 2 to 3% better precision than the two-point method. The two major exceptions have both been studies in which the subjects changed their source of water during the isotope elimination period. These studies involved infants who were being weaned from total parenteral nutrition to oral nutrition ²⁵, and soldiers who were transported from their barracks to a field exercise ²⁷. The issues of changing water sources are discussed in Chapter 8, and are expected to lead to small losses of accuracy and precision. It should be noted, however, that the use of the intake/balance method based on self-monitored intake by the soldiers may have been more prone to error than the DLW method itself and is likely to be the cause of the large difference in this validation.

Perhaps the most important issue in the validations is that they have encompassed a range of circumstances. Studies in healthy adults have predominated, but they have included a wide range of subjects including: sedentary people; subjects exercising to exhaustion; subjects who were in energy balance; subjects who were underfed by 300 to 1500 kcal/day; and patients who were receiving total parenteral nutrition in excess of energy requirements. The other validations were performed in premature rapidly-growing infants, and in post-surgical infants. Thus the DLW method has been validated under a wide range of human conditions.

1.8 Purpose of the Cambridge workshop

The workshop was arranged to discuss different DLW techniques and procedures used by various laboratories, and to recommend standard procedures for use in further studies. This should render results comparable between laboratories, and benefit our goal of understanding human energetics.

The DLW method involves several assumptions about the behaviour of the isotopes, the body water pool and the exchange rates within that pool in the labelled animal ^{8,30}. These assumptions are:

- (1) The volume of the body water pool remains constant throughout the measurement period.
- (2) The rates of water influx, and water and CO₂ efflux are constant throughout the measurement period.
- (3) The isotopes label only the H₂O and CO₂ in the body.
- (4) The isotopes leave the body only in the form of H₂O and CO₂.

- (5) The concentrations of the isotopes in H_2O and CO_2 leaving the body are the same as those in body water at that time (i.e. there is no isotopic fractionation).
- (6) No H_2O or CO_2 that has left the body re-enters the body.
- (7) The natural abundance, or "background" levels of the isotopes remain constant during the measurement interval.

All of these assumptions are invalid to some degree in any DLW study, but a variety of corrections can be applied to completely or partially account for the resulting errors. New users of DLW are faced with a confusing array of technical decisions that must be made as part of this technique. Fortunately, the DLW method is sufficiently robust that making an inappropriate decision will, in most cases, cause less than a 10% error in the calculated rate of energy metabolism, provided that isotope concentration measurements (the largest potential source of error) are accurate.

The workshop participants pooled their knowledge, experience and different perspectives on the problems to generate the recommendations presented in this document. The recommendations are based on a variety of criteria including; (a) which procedure among several is theoretically correct in a given application; (b) which procedure is simplest and least prone to methodological errors; and (c) which procedure yields the lowest error in validation studies. We hope that this synthesis will clarify the many complex issues involved and hence encourage more researchers to use this exciting method to explore new areas of human biology and medicine.

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CHAPTER 2

Contributors: All participants

RECOMMENDED ABBREVIATIONS

2.1 Background

Early users of the doubly-labelled water method have tended to adopt their own variants and extensions of Lifson's initial notation. This causes unnecessary difficulty when the reader has to cross-refer to the author's particular usage, and in certain cases can lead to ambiguity. The working group therefore agreed to adopt the following notation for use in this and subsequent publications relating to human applications of doubly-labelled water. Others entering the field for the first time are strongly urged to employ the same symbols which will be referred to as 'IDECG Notation'.

2.1 IDECEG notation

<u>Variable</u>	<u>Notation</u>
<u>Isotopes</u>	
Unspecified isotopes of H or O	*H or *O
Oxygen	O or ^{16}O and ^{18}O
Hydrogen (protium)	H or ^1H
Deuterium	D or ^2H
Tritium	T or ^3H
<u>Dosing variables</u>	
Dose administered to subject	A (A_p and A_o)
Dose diluted for analysis	a (a_p and a_o)
Amount of water used for dilution	W
<u>Pool sizes and rate constants</u>	
Pool size	N (N_p and N_o)
Rate constant	k (k_p and k_o)
Production rate	r
Uncorrected production rate	r'

Fractionation factors

$^2\text{H}_2\text{O}$ vapour/liquid	f_1
H_2^{18}O vapour/liquid	f_2
$\text{C}^{18}\text{O}_2/\text{H}_2^{18}\text{O}$	f_3

Mass spectrometric variables

Isotopic enrichment (relative to a standard)	δ
Fractional abundance (concentration)	C
Atom percent excess	APE
Parts per million	ppm
Working standard	ws
Vienna-Standard Mean Ocean Water	V-SMOW or SMOW
Standard Light Antarctic Precipitation	SLAP

CHAPTER 3

Contributors: William Wong
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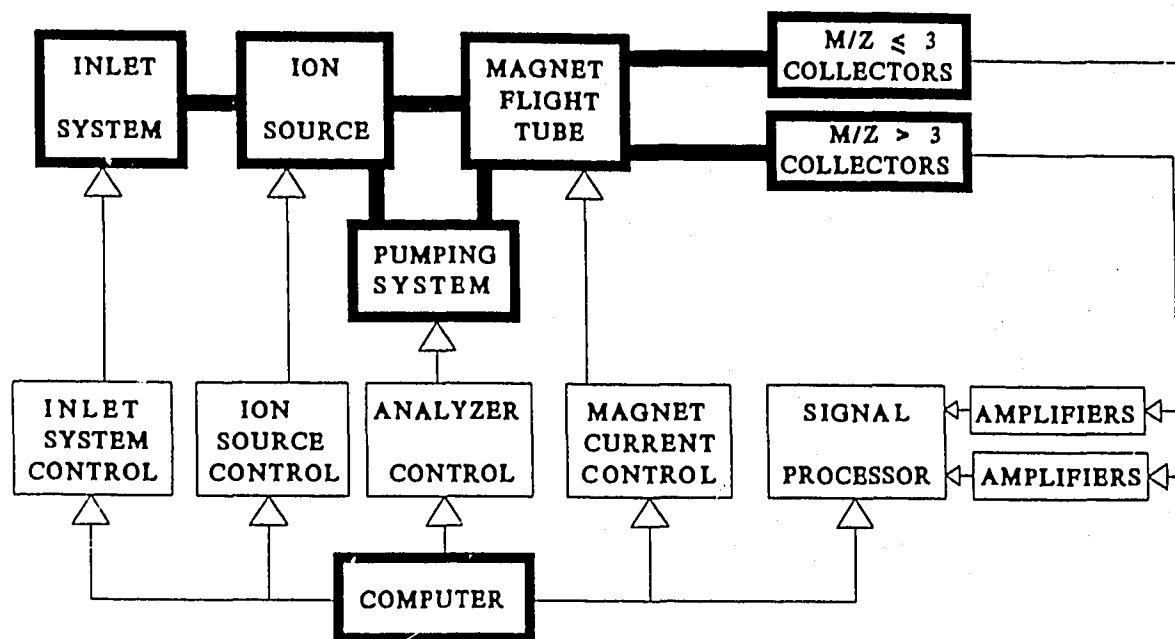
MASS SPECTROMETRIC ANALYSIS

3.1.1 History

Gas-isotope-ratio mass spectrometry (GIRMS) is considered to be the best analytical technique for the accurate and precise measurement of ^2H and ^{18}O content in physiological samples. The first GIRMS was described by Neir in 1940 ¹. The mass spectrometer consisted of an ion source, a permanent magnet, and a single collector. The vacuum of the mass spectrometer was maintained with a two-stage mercury diffusion pump. Gas sample was admitted into the ion source through a capillary leak. The gas molecules were ionised with electrons in the ion source and the positively charged molecules were propelled by an accelerating potential into the magnetic field where they were resolved into separate ion beams according to their masses. Because a single collector was used, the isotope ratio of the sample was measured by alternate focusing of each ion beam onto the collector after the adjustment of the ion accelerating voltage. Subsequently, a dual-collector for simultaneous measurements of the ion currents of two isotopic masses ^{2,3} and a dual inlet system ⁴ for alternate introduction of a sample and

Figure 3.1

Schematic diagram showing the general layout of a gas-isotope-ratio mass spectrometer



a standard gas into the mass spectrometer were incorporated into the Nier-type mass spectrometer.

3.1.2 Modern gas-isotope-ratio mass spectrometers

The basic design of the Nier-McKinney-type mass spectrometer persists in gas-isotope-ratio mass spectrometers today. With advances in electronic, vacuum and computer technologies, however, the present day instruments are fully automated and considerably more sensitive. A schematic diagram showing the general arrangement of the gas-isotope-ratio mass spectrometers used today is shown in Figure 3.1. After the sample and standard gases have been admitted into the inlet system, the entire process of valve sequencing, pressure matching between the sample and standard gases, ion source tuning and focusing, vacuum monitoring, and data collection and reduction is controlled completely by computer. Multiple collectors can be fitted to the mass spectrometer to measure the ion beam currents of different masses (eg 44,45 and 46 for $^{12}\text{C}^{16}\text{O}_2$, $^{13}\text{C}^{16}\text{O}_2$ or $^{12}\text{C}^{16}\text{O}^{17}\text{O}$ and $^{12}\text{C}^{18}\text{O}^{16}\text{O}$) simultaneously. For hydrogen isotope ratio measurement, a split-flight tube with a separate dual collector system can be fitted onto the same instrument. Therefore a single instrument with a single ion source can be used for both hydrogen and oxygen isotope ratio measurements.

3.2 Sample requirements

3.2.1 Sample-form requirements

Samples must be in a gaseous state before they are introduced to the ion source of the mass spectrometer. For hydrogen isotope ratio measurement, physiological fluids must be converted to hydrogen gas. For oxygen isotope ratio measurement

carbon dioxide was initially the preferred sample gas, but with the introduction of the twin mass spectrometer system, the oxygen-18 content of fluid samples can be measured directly from water vapour. This allows a constant sample flow, reduces problems with memory effects and minimises isotopic interference.

3.2.2 Sample-size requirements

The amount of sample required for measurement is related to the ease with which the sample can be transferred into the inlet system of the mass spectrometer. Hydrogen gas is difficult to transfer cryogenically. Therefore, for hydrogen measurements, approximately 20 μmol of H_2 is required. Carbon dioxide can be transferred easily with liquid nitrogen. Therefore, for accurate and precise oxygen isotope ratio measurements, as little as 0.5 μmol is sufficient. With the twin mass spectrometer system, as little as 55 μmol of H_2O is required for simultaneous hydrogen and oxygen isotope ratio measurements.

3.3 Isotope ratio measurements

3.3.1 Units of measurement

The sensitivity of gas-isotope-ratio mass spectrometer measurements is achieved by comparing the isotopic abundances of the sample to that of a standard under identical measurement conditions. Results are expressed in relative delta (δ) per mil (‰) units which are defined as follows:

$$\delta^2\text{H}_{\text{vs}} (\text{‰}) = \left[\frac{(^2\text{H}/^1\text{H})_{\text{sample}}}{(^2\text{H}/^1\text{H})_{\text{vs}}} - 1 \right] \times 1000$$

$$\delta^{18}\text{O}_{\text{ws}} (\text{‰}) = \left[\frac{(^{18}\text{O}/^{16}\text{O})_{\text{sample}}}{(^{18}\text{O}/^{16}\text{O})_{\text{ws}}} - 1 \right] \times 1000$$

where $\delta^2\text{H}_{\text{ws}}$ and $\delta^{18}\text{O}_{\text{ws}}$ are the delta values of the sample measured relative to the laboratory working standard (ws) and $(^2\text{H}/^1\text{H})_{\text{ws}}$ and $(^{18}\text{O}/^{16}\text{O})_{\text{ws}}$ represent the hydrogen and oxygen isotope ratios of the laboratory working standard respectively.

The natural abundances of $^2\text{H}/^1\text{H}$ and $^{18}\text{O}/^{16}\text{O}$ can be measured with instrument precisions (2 SDs, $n = 10$) of 0.1‰ and 0.03‰ respectively.

3.3.2 Normalisation

For ease of comparison, these $^2\text{H}_{\text{ws}}$ and $^{18}\text{O}_{\text{ws}}$ values are normalised against two international water standards: Vienna Standard Mean Ocean Water (V-SMOW) and Standard Light Antarctic Precipitation (SLAP) as follows ⁵:

$$\delta_{\text{sample/V-SMOW/SLAP}} = \frac{\delta_{\text{sample/ws}} - \delta_{\text{V-SMOW/ws}}}{\delta_{\text{SLAP/ws}} - \delta_{\text{V-SMOW/ws}}} \times \delta_{\text{SLAP}}^{\circ}$$

where $\delta_{\text{sample/ws}}$, $\delta_{\text{V-SMOW/ws}}$, and $\delta_{\text{SLAP/ws}}$ are the $\delta^2\text{H}$ or $\delta^{18}\text{O}$ values of the sample, V-SMOW, and SLAP measured relative to the working standard respectively. For $^2\text{H}/^1\text{H}$ and $^{18}\text{O}/^{16}\text{O}$ isotope measurements $\delta_{\text{V-SMOW}}$ is defined as zero and $\delta_{\text{SLAP}}^{\circ}$ has values of -428 and -55.5 ‰ for ^2H and ^{18}O respectively.

3.3.3 Atom percent and part per million units

The relative delta per mil values are convenient for expressing very small differences in ^2H and ^{18}O content. However, with high enrichment levels of these isotopes in tracer studies,

the results may be expressed more conveniently in terms of atom percent (atom %) or parts per million (ppm). By definition:

$$\text{atom \%} = \text{fractional abundance} \times 100$$

$$\text{ppm} = \text{fractional abundance} \times 10^6$$

Fractional abundance (C) for deuterium is calculated from the delta per mil value as follows:

$$C = R/(1 + R)$$

$$R = (\delta/1000 + 1) \times R_{V-SMOW}$$

where R is the $^2\text{H}/^1\text{H}$ ratio of the sample, δ is the normalised $\delta^2\text{H}$ value of the sample, and R_{V-SMOW} is the $^2\text{H}/^1\text{H}$ ratio of V-SMOW which has a value of 0.00015595 ⁶.

Fractional abundance of ^{18}O is calculated from the delta per mil value as follows:

$$C = {}^{18}\text{R}'/(1 + {}^{17}\text{R} + {}^{18}\text{R}')$$

$${}^{17}\text{R} = ({}^{18}\text{R}'/R_{V-SMOW})^{1/2} \times {}^{17}\text{R}_{V-SMOW}$$

$${}^{18}\text{R}' = (\delta/1000 + 1) \times {}^{18}\text{R}_{V-SMOW}$$

Where ${}^{17}\text{R}$ and ${}^{18}\text{R}'$ are the $^{17}\text{O}/^{16}\text{O}$ and $^{18}\text{O}/^{16}\text{O}$ ratios of the sample, δ is the normalised $\delta^{18}\text{O}$ value of the sample, and ${}^{17}\text{R}_{V-SMOW}$ and ${}^{18}\text{R}_{V-SMOW}$ are the $^{17}\text{O}/^{16}\text{O}$ and $^{18}\text{O}/^{16}\text{O}$ ratios of V-SMOW which have values of 0.000373 ⁷ and 0.002005 ⁸ respectively.

3.4 Water standards

The international water standards, V-SMOW and SLAP, can be

purchased from the International Atomic Energy Agency, Section of Isotope Hydrology, Wagramerstrasse 5, P.O. Box 100, A-1400 Vienna, Austria. Other water standards: Greenland Ice Sheet Precipitation (GISP), IAEA-302 ($\delta^2\text{H}$: 500 and 1000 ‰ vs V-SMOW), and IAEA-304 ($\delta^{18}\text{O}$: 250 and 500 ‰ vs V-SMOW), can also be obtained from the same agency.

3.5 Sample preparation

3.5.1 Hydrogen isotope ratio measurements

3.5.1.i Uranium reduction

For hydrogen isotope ratio measurements, water in physiological fluids must be converted to hydrogen gas prior to mass spectrometric analysis. Conversion of water to hydrogen gas by passage over uranium at approximately 600°C follows the reaction:

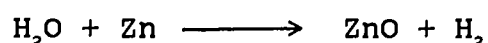


The reduction is usually performed within a vacuum line. Uranium metal, is obtained as turnings and is prepared for use by cutting the turnings into 1 cm lengths, degreasing with propanol and immersing them in concentrated nitric acid to remove surface oxidants. Because hydrogen gas is non-condensable at liquid nitrogen temperature it produced is compressed into a sample bulb/tube with a mercury Toepler pump. Complete reduction of the water and collection of the hydrogen gas is crucial to avoid isotope fractionation. Since samples of different ^2H enrichments are passed through the same uranium furnace a memory effect occurs. This is best minimised by flushing the line once or twice with the next sample prior to collection of the hydrogen

gas for analysis. Precision of the preparation ranges between 0.5 and 2 ‰.

3.5.1.ii Zinc reduction

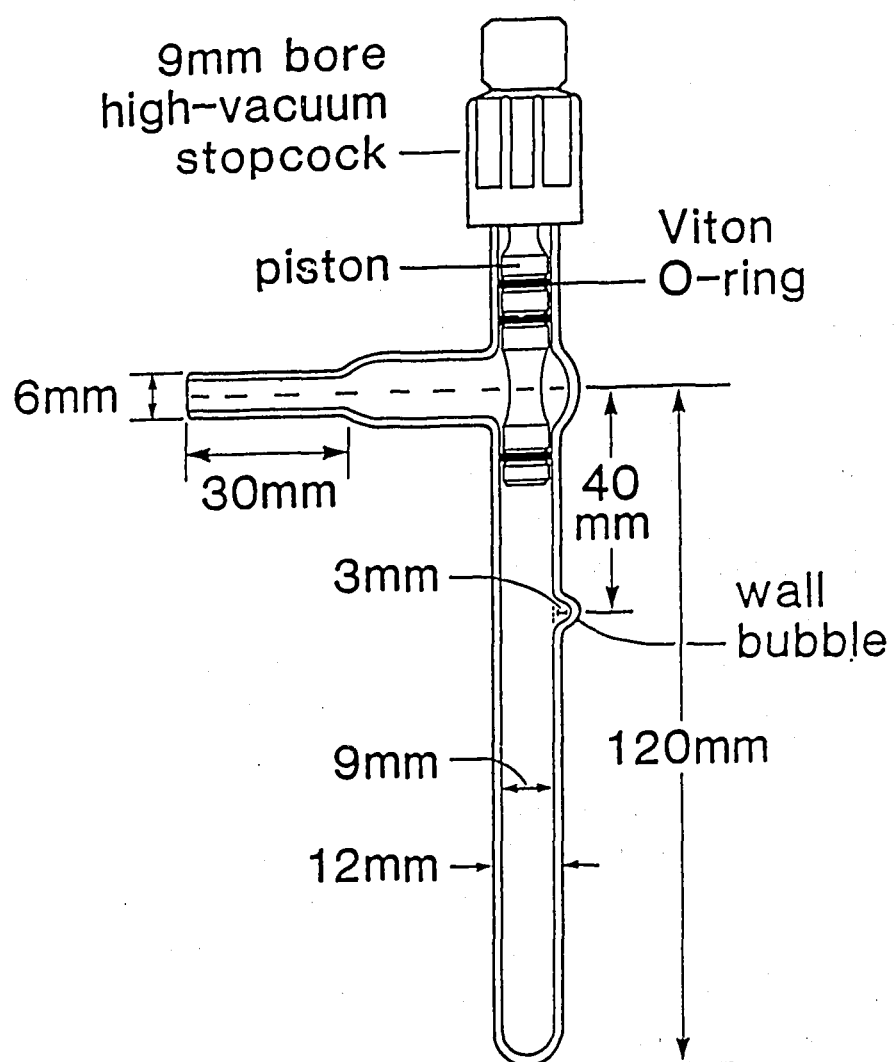
Water can alternatively be reduced to hydrogen with zinc at 450°C according to the following reaction:



Approximately 250 mg of <1 mm cleaned AnalaR zinc shot is transferred to a quartz reaction vessel (Figure 3.2). The vessel is evacuated to $<10^{-4}$ mbar and then filled with dry nitrogen. With the nitrogen flowing at approximately 50 ml/min, the stopcock of the vessel is removed and 10 mg of physiological fluid is placed at the wall bubble of the vessel using a micropipette. Immediately, the stopcock is replaced and the sample is frozen with liquid nitrogen. The vessel is again evacuated to 10^{-4} mbar. The water in the sample is reduced to hydrogen by heating the zinc shot to 450°C for 30 min. After cooling to room temperature the hydrogen is ready for isotope ratio measurement without further purification. Memory effect is minimised using this procedure because a fresh aliquot of zinc shot and an individual reaction vessel is used for each reduction. At natural abundances the deuterium values are accurate to -0.2 ± 1.2 ‰ (SD, $n = 68$) and reproducible within 1.2% (SD). At 600 ‰ the values measured from plasma, urine, saliva and human milk samples are accurate to -4.3 ± 4.8 ‰ ($n = 200$) and reproducible within 3.2 ‰ (SD).

Figure 3.2

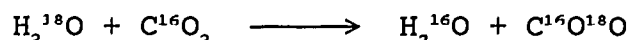
Quartz reaction vessel for the reduction of water to hydrogen gas
using zinc shot for hydrogen isotope ratio measurements



3.5.2 Oxygen isotope ratio measurements

3.5.2.i H₂O-CO₂ equilibration

The ¹⁸O content of aqueous fluids can be measured by equilibrating the sample with CO₂ of known ¹⁸O content according to the following reaction ¹⁰:



At the end of the equilibration, the CO₂ is isolated and purified from the equilibration vessel for isotope ratio measurement. The ¹⁸O content of the water is calculated according to the following equation:

$$\delta^{18}\text{O} (\text{‰}) = \delta^{18}\text{O}_t + a/k \times (\delta^{18}\text{O}_t - \delta^{18}\text{O}_0)$$

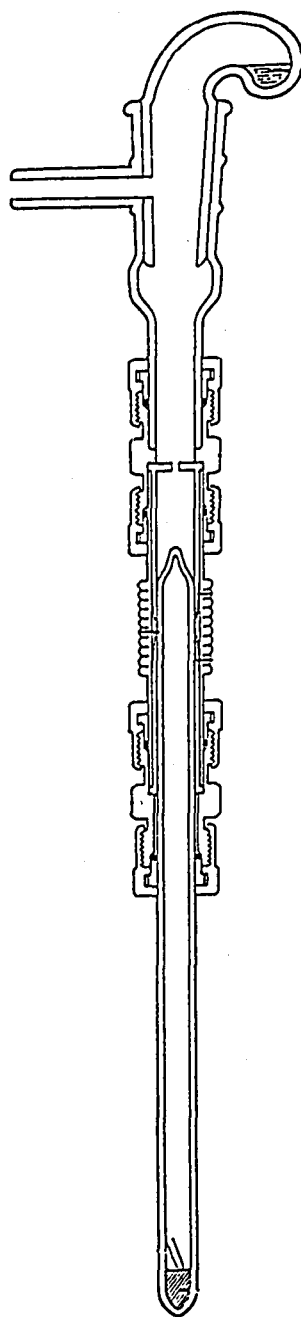
where $\delta^{18}\text{O}$ is the ¹⁸O content of the sample; $\delta^{18}\text{O}_0$ and $\delta^{18}\text{O}_t$ are the ¹⁸O content of the CO₂ before and after the equilibration respectively; a is the isotope fractionation factor between CO₂ and H₂O and has a value of 1.0412 at 25°C ¹¹; and k is the ratio of oxygen atoms between the water sample and the CO₂. When k is >800, correction for isotope fractionation becomes negligible. A sample size of approximately 0.5 g is sufficient for the equilibration procedure. The precision of this equilibration method ranges from 0.01 to 0.6 ‰ (SD).

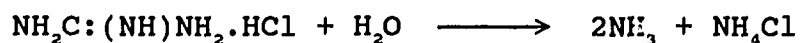
3.5.2.ii Guanidine hydrochloride conversion

When sample size is limiting, the guanidine hydrochloride method is appropriate for the conversion of 1-10 mg of H₂O to CO₂ for oxygen isotope ratio measurement ¹². Water is quantitatively converted to CO₂ with guanidine hydrochloride at 260°C for 16 h according to the following reaction:

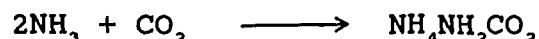
Figure 3.3

Reaction assembly for the conversion of H_2O to CO_2 with guanidine hydrochloride for oxygen isotope ratio measurements

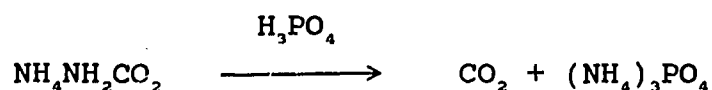




A diagram showing the reaction assembly is shown in Figure 3.3. Upon cooling, ammonium carbamate is formed as follows:



The CO_2 is released from the ammonium carbamate by heating the reaction assembly at 80°C for 1 h in the presence of 100% H_3PO_4 . The NH_3 released from the ammonium carbamate at 80°C is removed by H_3PO_4 as follows:



At the end of the incubation, the CO_2 is transferred to a sample bulb for isotope ratio measurement.

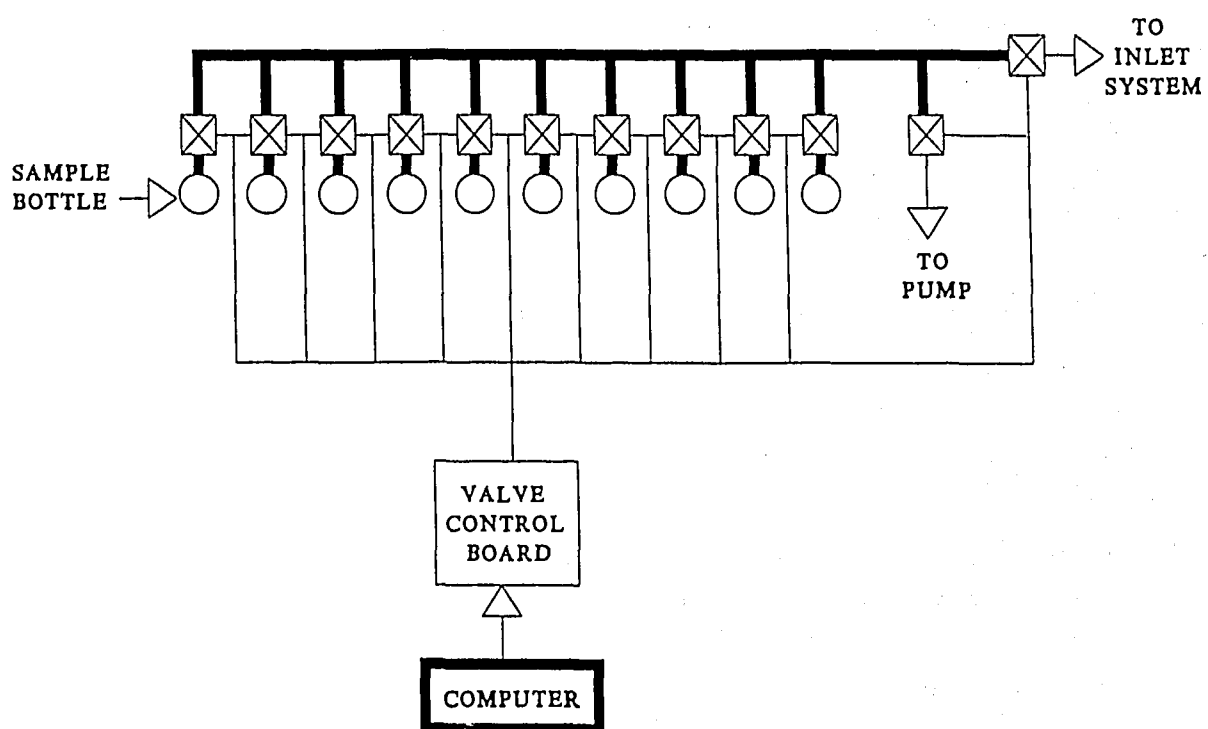
Using this method a precision of 0.08 ‰ was obtained for ^{18}O values measured from water samples with natural abundances of ^{18}O . At natural abundances, the $\delta^{18}\text{O}$ values of biological fluids (saliva, urine, plasma, human milk) were reproducible to within 0.16 ‰ and accurate to within 0.11 ± 0.73 ‰ (mean \pm SD) of the $\text{H}_2\text{O}-\text{CO}_2$ equilibration value. At a 250 ‰ enrichment level of ^{18}O the $\delta^{18}\text{O}$ values of these biological fluids were reproducible to within 0.95 ‰ and accurate to -1.27 ± 2.25 ‰.

3.6 Mass spectrometer accessories

Nutritional and biomedical applications of stable isotopes often require the ability to process many samples in a day. In order to increase sample throughput and optimise instrument usage, a number of accessories have been designed to operate in conjunction with the gas-isotope-ratio mass spectrometer without

Figure 3.4

Multiple sample inlet system for automatic sequential analysis of samples



[X] represents solenoid valve

operator intervention.

3.6.1 Multiple sample inlet system

A multi-sample inlet system (Figure 3.4) permits unattended sequential analysis of up to 50 samples. Sample bottles containing the gas samples (H_2 or CO_2) are attached to the solenoid valves with vacuum connectors. The manifold and the connections between the solenoid valves and the stopcocks of the sample bottles are evacuated to a preset vacuum level before sequential expansion of each gas sample into the inlet system of the mass spectrometer for analysis. Precision of ≤ 0.1 ‰ (SD, $n = 10$) can be obtained using the multiple sample inlet for CO_2 analysis. For $^2H/^1H$ isotope ratio measurements precision of approximately 0.5 ‰ can be expected.

3.6.2 Automatic cold finger

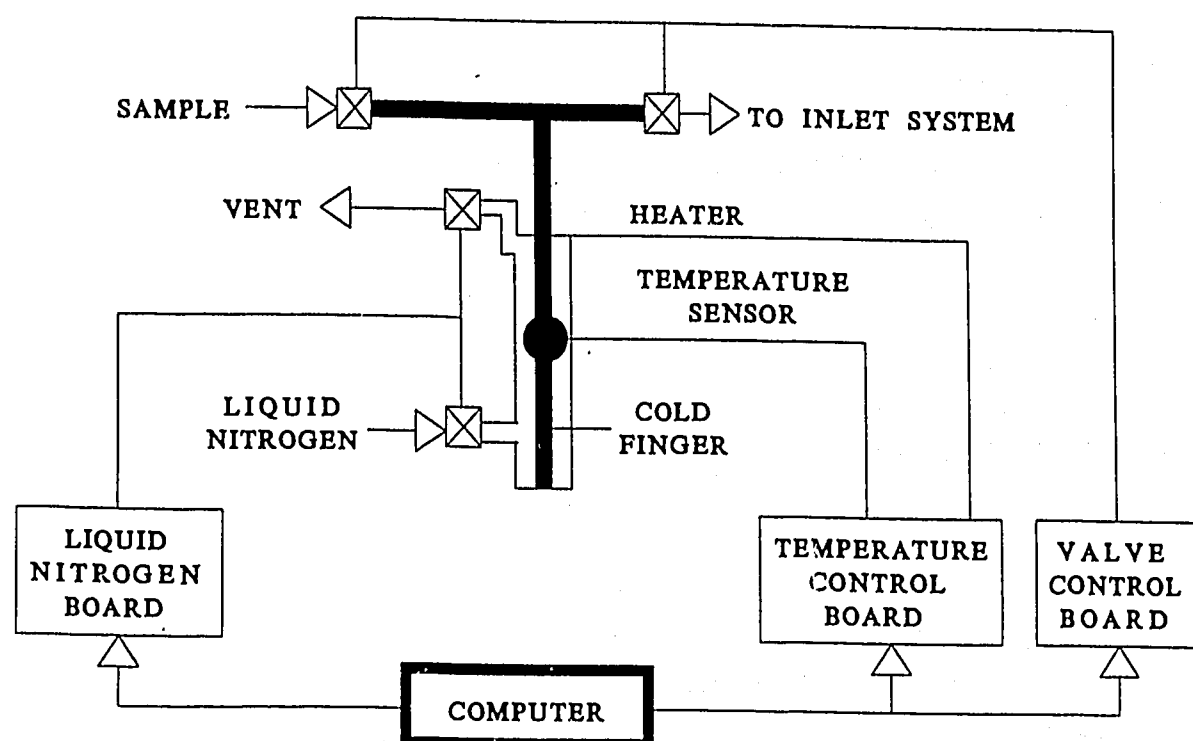
In working with small samples of CO_2 , the automatic cold finger (Figure 3.5) allows cryogenic transfer of the gas sample from an inlet system into the cold finger with liquid nitrogen. A gas sample as small as 0.05 μmol can be transferred and analysed with high precision and accuracy. The entire process of cooling, heating and valve sequencing is controlled by the computer. Precision of 0.01 ‰ and 0.03 ‰ can be obtained for ^{13}C and ^{18}O isotope ratio measurements respectively.

3.6.3 Breath CO_2 -purification system

A breath CO_2 -purification system is shown in Figure 3.6. This system can be used for purification of CO_2 used in the H_2O - CO_2 exchange reaction for the measurements of oxygen isotope ratios in physiological fluids. The system consists of a sample

Figure 3.5

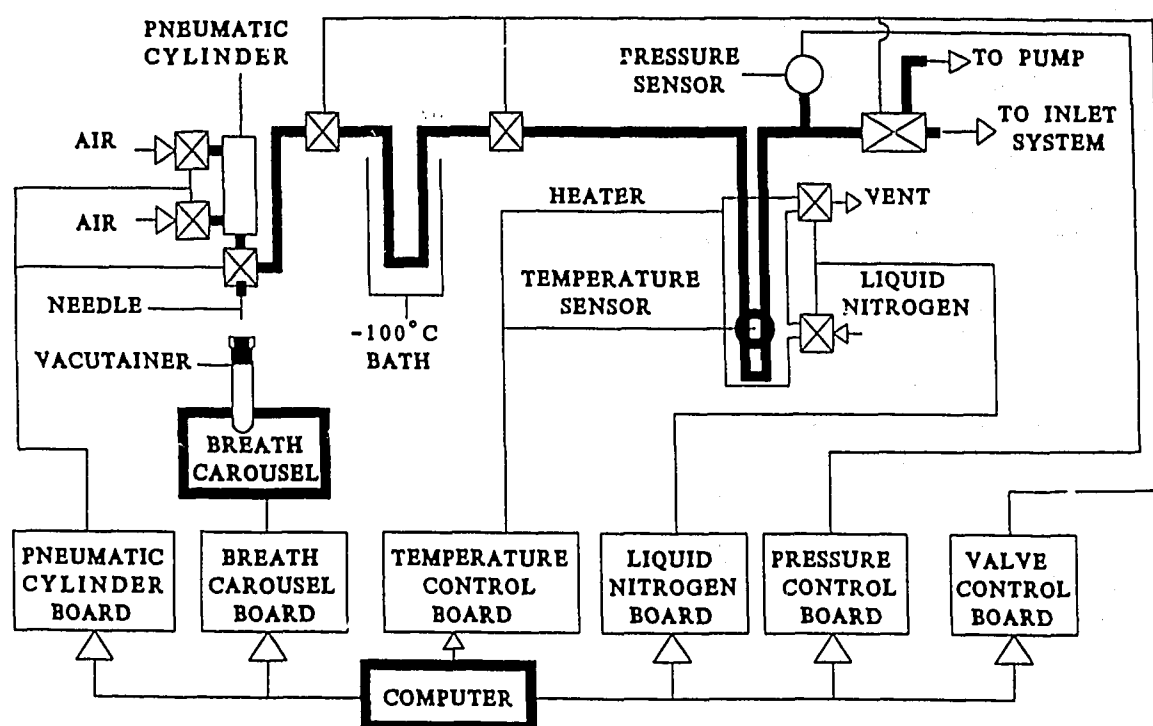
Automatic cold finger for cryogenic transfer of microliter quantity of CO₂ for oxygen isotope ratio measurements



[X] represents solenoid valve.

Figure 3.6

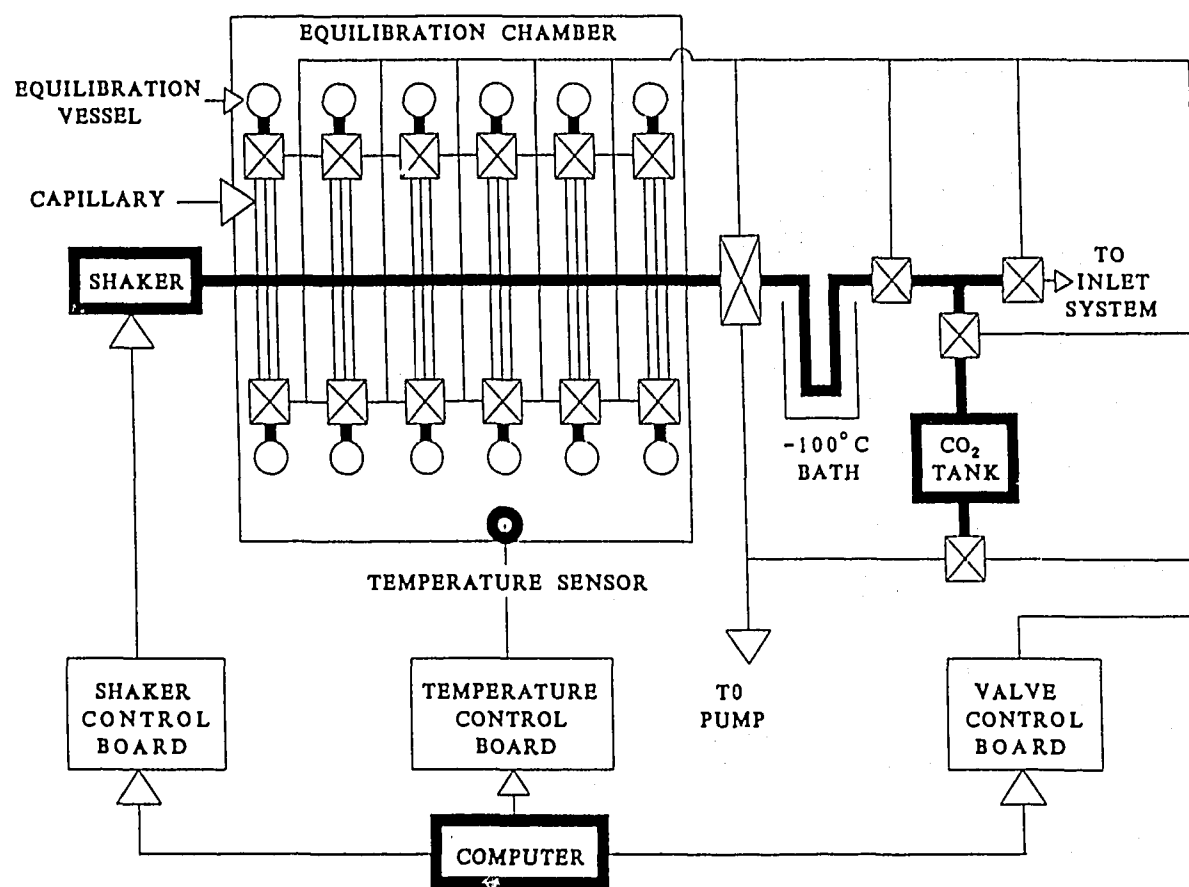
Breath CO₂-system for automated cryogenic purification of CO₂ for oxygen isotope ratio measurements



[X] represents solenoid valve.

Figure 3.7

Water-CO₂ equilibration system for oxygen isotope ratio measurements



[X] represents solenoid valve.

carousel, a pneumatic-cylinder system for puncture of the septum of a vacutainer, and a cryogenic purification system. Water vapour is removed by the -100°C trap, CO_2 is condensed in the liquid nitrogen trap and non-condensable gases are pumped away. Up to 50 samples can be processed sequentially with this system with a precision of 0.2 ‰ .

3.6.4 Water/carbon dioxide equilibration system

The $\text{H}_2\text{O}-\text{CO}_2$ equilibration system (Figure 3.7) is used to measure $^{18}\text{O}/^{16}\text{O}$ ratios in aqueous samples. The system utilises the difference in pumping speeds between gas molecules passing through a capillary to minimise the loss of water sample. Therefore, there is no need to freeze the water sample before the equilibration vessel is evacuated and CO_2 is admitted, or before the CO_2 is extracted after equilibration for isotope ratio measurement. Up to 48 samples can be accommodated. As little as 0.1 g of biological fluid is sufficient for accurate and precise oxygen isotope ratio measurements $^{\circ}$. The entire sequence of evacuation, CO_2 addition, shaking, temperature control, CO_2 extraction and isotope ratio measurement is controlled by computer.

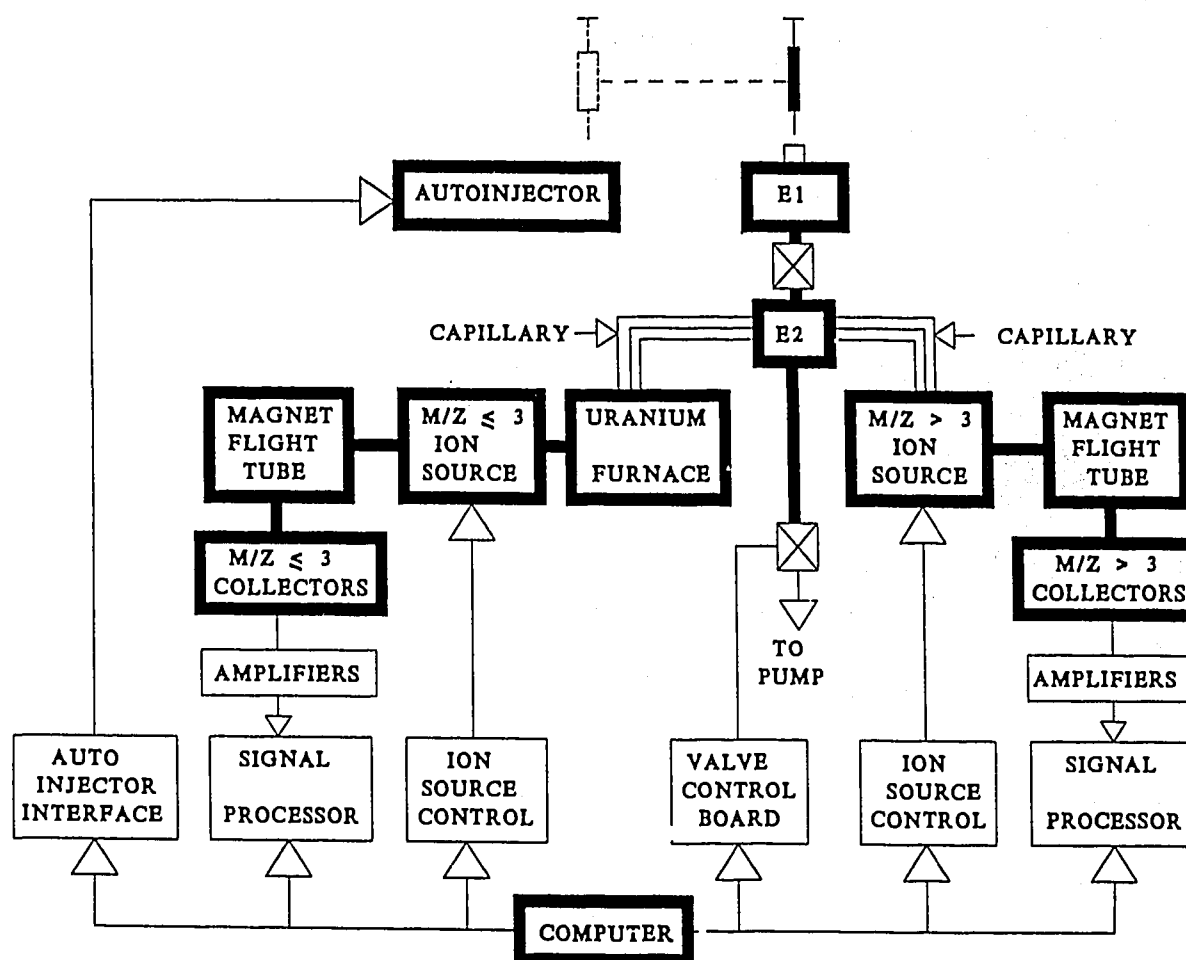
With water samples at natural abundances the $^{18}\text{O}/^{16}\text{O}$ ratios were accurate to $-0.05 \pm 0.50\text{ ‰}$ (mean \pm SD, $n = 52$) and reproducible within 0.21 ‰ . With biological samples at 250 ‰ an accuracy of $-0.32 \pm 0.87\text{ ‰}$ (mean \pm SD, $n = 200$) and a precision of 0.97 ‰ was obtained.

3.6.5 Dual isotope injection system

A twin mass spectrometer system 13 for simultaneous measurements of $^2\text{H}/^1\text{H}$ and $^{18}\text{O}/^{16}\text{O}$ ratios in aqueous samples is shown in Figure 3.8. Approximately $1\text{--}5\text{ mg}$ of sample is injected

Figure 3.8

Twin mass spectrometer system for simultaneous measurements of hydrogen and oxygen isotope ratios in aqueous samples



[X] represents solenoid valve.

into the expansion chamber, E1. After vaporisation, the water vapour is allowed to expand into expansion chamber, E2. A portion of the water vapour travels through a capillary into a uranium furnace at 620°C and is reduced to H₂. The H₂ enters the m/z ≤3 ion source and is ionised to ¹H₂⁺ and ¹H²H⁺ ions for hydrogen isotope ratio measurements. At the same time, another portion of the water vapour travels through the other capillary and enters the m/z ≥3 ion source, forming ¹H₂¹⁶O⁺ and ¹H₂¹⁸O⁺ ions for oxygen isotope ratio measurements. To maintain the water in vapour phase, the expansion chambers, the capillaries and the m/z ≤3 ion source are maintained at 150°C. Since the same paths are used by samples of different isotopic enrichments, multiple injection of the same sample is required in order to eliminate or to correct for memory effect. Correction for underestimation of deuterium enrichment by the twin mass spectrometer system is also necessary. At natural abundances of ²H and ¹⁸O, the ²H/¹H and ¹⁸O/¹⁶O ratios can be measured with a precision of 1.1 ‰ and 0.4 ‰ respectively.

3.7 Required precision of isotopic analyses

Without a doubt, the most difficult problem facing new users of the doubly-labelled water method has been obtaining isotopic analyses that are sufficiently accurate and precise. Small errors in the isotopic determinations lead to large errors in energy expenditure because the method depends on calculating the difference between the kinetics of ¹⁸O and ²H.

Accurate isotopic analyses are desirable if baseline isotopic abundances are to be interpretable against the extensive literature on isotopic hydrology (Chapter 8) and because day-to-day intra-laboratory and inter-laboratory comparisons are improved. Accuracy, however, is not an absolute requirement if all samples and the standard dilution of the dose are measured within the same day and if it can be assumed that the mass

spectrometer calibration does not change. Changes in calibration occur due to linear offsets in the calibration which move the scale up or down a fixed amount regardless of the abundance, or proportional errors which introduce a constant percentage error in the abundance relative to the working standard. Linear offset errors are cancelled when the enrichment of any sample is calculated relative to the pre-dose background abundance. Proportional errors are removed from elimination rates because this calculation involves a natural logarithmic transformation. However, proportional errors will produce errors in the apparent isotope dilution space. This is overcome if an aliquot of the loading dose given to the subject is gravimetrically diluted in a similar proportion to its *in vivo* dilution, and if this is analysed on the same day to determine the dose administered. Under these circumstances any proportional errors in the dilution space will cancel.

Despite the decreased importance of absolute accuracy, investigators should strive to achieve accuracy for the reasons stated above. This can be done by obtaining international isotopic standards and using these to check the performance of preparation procedures and mass spectrometry. (Suitable standards are available from: Dr Robert Parr, Section of Nutritional and Health Related Studies, IAEA, Wagramerstrasse 5, P.O. Box 100, A-1400 Vienna, Austria). Laboratory working standards (i.e. gravimetric dilutions) can then be used to ensure accuracy on a day-to-day basis.

Precision of isotopic analyses on the other hand is an obligatory requirement. The degree of precision needed depends on the isotope dose, the isotope elimination rate, the metabolic period, and the number of points used in the calculation of the elimination rate and dilution space. For the two-point method, it is recommended that precision of the isotopic analysis be better than one six-hundredth of the initial isotopic enrichment¹⁴. If adult doses are to be kept economically feasible (<US\$300) then

this translates into a required precision of 0.16 ‰ for ^{18}O and 1.1 ‰ for ^2H (where precision is defined as the standard error of multiple analyses obtained during the workup of samples for DLW studies). This will reduce the analytical error to less than 5% for metabolic periods of between 1 and 3 biological half-lives of ^{18}O ¹⁴. Requirements can be relaxed in proportion to the enrichment above baseline for highly enriched samples. The multi-point method can tolerate a reduction in precision in proportion to the square root of the number of points, except in the case of the baseline sample which must meet the standards set out above.

The most practical method for assessing adequacy of precision of isotopic analyses is to perform multiple analyses of a single set of samples from a subject. These should be done on separate days and the carbon dioxide production rate should be calculated independently for each set of analyses. For the two-point method, the coefficient of variation for carbon dioxide production should be about 4%. It should be 2 to 3.5% for the multi-point method. This standard should be relatively easily met in subjects whose ^2H elimination rate is less than 75% of their ^{18}O elimination rate, but difficult to meet for those in whom it is greater than 85%.

3.8 Sources of ^2H - and ^{18}O -labelled water

The ^2H - and ^{18}O -labelled water can be purchased from numerous stable isotope suppliers. Some of these suppliers are shown below:

Isotec Inc.
3858 Benner Road,
Miamisburg, Ohio 45342
USA.

CEA-ORIS,
Bureau de Stables Isotopes,
BP 21-91190,
Gof-sur-Yvette,
France.

Cambridge Isotope Laboratories
20 Commerce Way
Woburn, Massachusetts 01801
USA.

MSD Isotope
PO Box 899
Pointe Elaire-Dorval
Quebec
Canada H9R 4P7.

Isotope Department,
Weismann Institute of Science,
Rehovot,
Israel.

Delta Isotopes,
Wistaston Park,
Wistaston,
Crewe,
Cheshire, CW2 8JT,
UK.

Deuterium oxide is widely available from many sources with ^2H enrichment of 99.8 atom percent and above. Oxygen-18 labelled water is available in either low (10 atom % ^{18}O) or high enrichment (>95 atom % ^{18}O). The oxygen-18 labelled water is usually normalised with respect to hydrogen. Therefore deuterium oxide and the normalised ^{18}O labelled water can be purchased separately and then combined in the laboratory prior to the

study. However, oxygen-18 labelled water (>95 atom % ^{18}O) can be obtained without normalisation with respect to hydrogen. This labelled water usually has ^2H enrichment of approximately 60 atom percent. An alternative is to purchase the 'un-normalised' ^{18}O labelled water which has high enrichment of ^2H .

Water with low enrichment of ^{18}O (10 atom %) is recommended for use with older children, adolescents, and adults because it is less expensive and these subjects can tolerate larger volumes of the tracer water in a doubly-labelled water study. With small infants, water with high enrichment of ^{18}O (>95 atom %) is preferable because infants are less tolerant to large volumes of the tracer water.

3.9 Preparation of water tracer for human consumption

The deuterium oxide and the ^{18}O -labelled water are not made for human consumption. The amount of deuterium oxide and ^{18}O -labelled water used in a doubly-labelled water study will alter the natural abundances of ^2H and ^{18}O in the body fluid by approximately 0.03 and 0.06 atom %, respectively. Deuterium enrichment at this level is well below the toxicity level (10 atom %) reported for deuterium oxide. Studies for mice and primates indicated that replacement of the oxygen atoms in the body fluids and tissues with up to 60 atom % of ^{18}O has no physiological or pathological effects on these animals. However, in human studies involving infants, children, and pregnant and lactating women, it is important to make sure that the water tracer is bacteria and pyrogen free. Deuterium oxide is available bacteria and pyrogen free from MSD Isotopes. Bacterial contamination can be removed by filtration through sterile 0.2 μm filters. Pyrogens in the tracer water can be removed by ultrafiltration.

3.10 Deuterium and ^{18}O enrichments of the dose

To confirm the enrichments of deuterium and ^{18}O in the dose, a known amount of the dose must be diluted gravimetrically with water of known ^2H and ^{18}O content in a proportion similar to the dosage used in a doubly-labelled water study. To minimise instrumental effects on the accuracy of the isotope ratio measurements, it is recommended that the determination of the ^2H and ^{18}O enrichments of the dose and the actual isotope ratio measurements of the samples be done using the same instruments within the same time frame.

3.11 Concluding remarks

Gas-isotope-ratio mass spectrometers are very accurate and precise instruments. With proper training in the operation of the instrument and accessories, errors in isotope ratio measurements usually come from improper sample collection and/or sample preparation. When working with small quantities of physiological fluids, contamination of sample by moisture will dilute the enrichments of ^2H and ^{18}O particularly in the post-dose samples. Evaporation during storage or transit will also alter the isotopic enrichments of ^2H and ^{18}O in the samples. The effect is most critical with ^2H because of the large isotope fractionation effect during evaporation and condensation of $^2\text{H}_2\text{O}$. Isotope fractionation can also occur during sample preparation when the water sample is not converted quantitatively to H_2 (uranium/zinc reduction) or to CO_2 (guanidine hydrochloride). Each laboratory or institute must evaluate each sample preparation procedure which is to be adapted for preparation of physiological fluids for hydrogen and oxygen isotope ratio measurements. Prior to actual sample analysis, daily calibration of each instrument for optimal sensitivity and performance with laboratory working standards is recommended. Prior to the purchase of an instrument, it is advisable for the laboratory to consult current users to confirm instrument specifications and reliability. Accessibility

of service engineers and availability of replacement parts are important factors in the final selection of instruments.

3.12 References

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CHAPTER 4

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CALCULATION OF POOL SIZES AND FLUX RATES

4.1 Introduction

This chapter will discuss the basic theory underlying the isotope kinetic models employed in DLW studies, and will summarise the various methods available for calculating pool spaces, disappearance rates and hence CO_2 production rates. It is written in the expectation that the reader will be familiar with Lifson and McClintock's early work ¹, and the many publications derivative of it (see Chapter 1). Anyone new to the general field of kinetic studies with isotopes is also advised to have close to hand a text-book that explains the theory and techniques of compartmental analysis ². A brief introduction is also given in Appendix 1.

The public conception may be that there is considerable controversy about the appropriate methods of calculation and treatment of DLW data. Fortunately, close scrutiny of the subject indicates that this really is not the case and in fact there is a reasonable amount of common ground between the protagonists of two-point and multi-point methodologies. Identifying the common

ground enables us to highlight the significance of areas of disagreement.

The first point to make is that with non-invasive tracer techniques we can only deal with what we observe and although such observations may lead us to the conclusion that a certain model provides an appropriate basis for the treatment of the data the observations do not prove that the model is a valid one. The model has to make sense from the physiological point of view and if the model is inappropriate the answers will be incorrect, although apparently precise.

A simple example will illustrate this point. Suppose you are asked to calculate the rate of water-output from the only apparent exit in a water tank. Common sense would suggest that the best way in which to do this would be to directly measure the rate (r_{out}) at which water flows through that exit and this will give the correct result. If on the other hand this measurement cannot be made, an alternative method is to add a known amount of tracer to the tank, measure the tank volume (N) from the instantaneous dilution of tracer, and the rate constant for tracer exit (k) and calculate the outflow as Nk . If the tank has only one exit r_{out} will equal Nk , but if there is an exit in the form of a leak, Nk will be greater than r_{out} and if the presence of a leak is unsuspected r_{out} will be incorrectly estimated because the model was wrong although it fitted the data! The analogy should be evident. Direct measurement of r_{out} and Nk are both available when respirometry is combined with a doubly-labelled water study (as, for instance, during cross-validation studies), but in field applications of DLW the equivalence of these two values can never be checked. Our models and treatment of data must therefore be secure for all circumstances or, as it seems likely, there are points of insecurity these should be identified and their consequences understood.

4.2 The basic model

With these reservations we can now return to the assumptions originally made in the doubly-labelled water method ¹ and summarised in Section 1.8.

With these assumptions we can write:

$$r'_{\text{CO}_2} = N(k_o - k_d)/2 \quad \text{.....1}$$

but there is the immediate difficulty that many observations show us that the size of N is estimated differently by ^2H and ^{18}O , with N_d being about $1.03 \times N_o$, and N_o being closer to true body-water than N_d .

Thus an equation for CO_2 production could be written as:

$$r'_{\text{CO}_2} = N_d(k_o - k_d)/2 \quad \text{.....2}$$

$$\text{or } r'_{\text{CO}_2} = N_o(k_o - k_d)/2 \quad \text{.....3}$$

$$\begin{aligned} \text{or } r'_{\text{CO}_2} &= (N_o k_o - N_d k_d)/2 \\ &= N_o(k_o - 1.03k_d)/2 \quad \text{.....4} \end{aligned}$$

Equations 2 and 3 produce results that are different by 3% but for typical values of k_o (e.g. 0.130) and k_d (e.g. 0.105) the result from Equation 4 will be 13% less than that from Equation 3.

The solution to the dilemma of which equation to chose for studies in man emerges from both practical and theoretical work. Firstly, validations using equations respecting differences

between N_p and N_0 appear to work better than those that do not ³, and secondly theoretical treatment of the system suggests that this is the correct approach ⁴. We can assume that ^2H rapidly equilibrates with both body water and other exchangeable hydrogen ⁵, but this secondary pool cannot be a pool into which ^2H migrates never to return to body water because if this was the case initial dilutions of ^2H and ^{18}O in body water (the first diluting compartment) would be identical because body water would not be able to 'see' the secondary pool. Apparently different body-water volumes can only be explained by a rapidly exchanging secondary pool. In these circumstances it is not strictly speaking correct to use the product $N_p k_p$ to calculate water outflow from body-water. A correct solution in compartmental analysis is to be calculated from the slopes and intercepts of the exponentials that add together to produce a ^2H disappearance curve. By using the product $N_p k_p$ to calculate output, the two pools are being lumped together as one. When a secondary pool exchanges slowly with total body water large errors will be produced by treating two pools as if they were one, but if we imagine that rates of exchange increase to the extent that double exponential curves cannot or can rarely be observed the simple treatment of data becomes more acceptable. The reader is referred to the comments of Roberts et al ⁴ for a fuller treatment of these concepts.

Unfortunately there is very little experimental data that can be drawn on to test the view that total body water and a small subsidiary pool can be combined. Such data could be obtained by repeatedly sampling total body water in the first few hours after dose administration (preferably, dose administration by an intravenous route) and examination of these early parts of the curve for an exponential slope that is different from the terminal exponential. Coward has investigated this problem by oral administration of isotope and the collection of a large number of samples on the first day ⁶. Outflow by one- and two-compartmental procedures (see Table 4.1) was then calculated. In

these circumstances differences in outflow calculated by these methods were trivial even when no early data was used for the one compartment solution.

4.3 Calculation of pool sizes

Two different techniques are currently used for the determination of volume. In the slope/intercept multi-point procedure time zero distribution space is calculated from the same data as that used to measure the slope. In the two-point methodology volume is calculated from observed isotopic dilution at a plateau shortly after dose administration. In order to appreciate the differences between these two procedures it is instructive to consider the only circumstance in which they will produce identical results. That is when the rate constants for output are zero. In this case the intercept of values for all samples collected will be the same as the average of all values because a permanent plateau of isotope concentration exists. In all other conceivable circumstances the methods will not produce the same answers, and each individual method will be correct, assuming no analytical errors, in certain achievable circumstances.

The slope/intercept method will be correct, ignoring analytical errors, if mixing is instantaneous and if the rate constants for output do not change over time. Unfortunately output is never absolutely constant, but varies both within and between days, and an absolutely correct value cannot therefore be obtained. If the variations are random, however, the error can usually be reduced to 1% or less.

The plateau method will be correct, within the limits of analytical error, irrespective of the rate of isotope mixing if all isotope losses occurring during the equilibration period can be accounted for and subtracted from the dose administered. Unfortunately, only a fraction of all losses can be accounted for

Table 4.1

Effect of changing the model or sampling times or both on estimates of rate constants (k), initial distribution volumes (N), or outflow rates ($r' = kN$) in an adult male subject orally dosed with $^2\text{H}_2^{18}\text{O}$

	<u>Model</u>	<u>Samples</u>	<u>k(%B)</u>	<u>N(%B)</u>	<u>r'(%B)</u>
<u>Deuterium</u>					
<u>A</u>	Two-pool	All	156.38	63.72	99.61
<u>B</u>	One-pool	6,7,8hr 1-12d	100.00	100.00	100.00
<u>C</u>	One-pool	1-12d	99.10	101.16	100.24
<u>Oxygen-18</u>					
<u>A</u>	Two-pool	All	148.52	67.21	99.82
<u>B</u>	One-pool	6,7,8hr 1-12d	100.00	100.00	100.00
<u>C</u>	One-pool	1-12d	99.13	100.96	100.07

by urine collection for example, thus this procedure cannot ever produce an absolutely correct value. With collection of urine and estimation of other losses this error can also be reduced to 1% or less.

The question is: Do these differences from correct values and potential differences between the two methods matter? From first principles it would seem reasonable to suppose that the plateau method may slightly over-estimate volume because the dose remaining in the body may be over-estimated. Conversely, because of the time taken for mixing, an intercept procedure that ignores mixing will over-estimate initial isotope concentration and under-estimate volume.

For the plateau method Schoeller *et al*³ recommend that a fixed N_p/N_o ratio of 1.03 is assumed to exist and that if only one space measurement is made the other should be calculated from it. Alternatively, when both N_p and N_o are measured the observed values can be appropriately weighted. If N_p/N_o is 1.03 plus or minus some small SD for all conceivable subjects this procedure can be justified, but it ought to be preferable to use the observed N_p and N_o values if we are dealing with a genuine physiological variation in N_p/N_o .

It is important to determine the likely physiological range of N_p/N_o . The small SDs for N_p/N_o observed by both Coward⁶ (see Table 4.2) and Schoeller *et al*³ suggest a combination of small analytical errors and a relatively minor degree of between-subject variation. However, values obtained in the data sets exchanged prior to this meeting ranged from 0.939 to 1.329 (mean 1.045 ± 0.081 SD). In view of the theoretical considerations outlined in the next section it appears likely that a number of the outlying values were incorrect as a result of analytical errors.

Table 4.2

Ratios of isotope distribution spaces (N_p/N_o) in a variety of studies performed by the Dunn Nutrition Unit

<u>Subjects</u>	<u>Ratio (SD)</u>	<u>n</u>
Pregnant women (Cambridge)	1.036 (0.012)	8 subjects 42 measurements
Lactating women (Cambridge)	1.034 (0.012)	12 subjects 36 measurements
NPNL women (Cambridge)	1.037 (0.011)	17 subjects
Obese women (Cambridge)	1.040 (0.013)	8 subjects
Infants < 6 months (Cambridge)	1.035 (0.020)	136 subjects
Men (Northern Ireland)	1.034 (0.011)	16 subjects
Women (Northern Ireland)	1.036 (0.010)	16 subjects
Elderly women patients	1.028 (0.009)	14 subjects
Men (Gambia)	1.030 (0.024)	16 subjects 29 measurements

NPNL = non-pregnant, non-lactating.

Data from Coward.

Table 4.3

Exchangeable H in a reference man

	<u>kg</u>	<u>%</u> <u>exchangeable</u> <u>H</u>	<u>Total</u> <u>exchangeable</u> <u>H</u>	<u>%Total</u> <u>exchangeable</u> <u>H</u>
Water	41	11.11	4.555	94.758
Fat	14	0.35	0.049	1.019
Protein	13	1.49	0.194	4.036
CHO	0.5	1.85	0.009	0.187
Minerals	1.5	0	0	0
Total	70		4.807	

Predicted $N_D/N_o = 1.055$

Table 4.4

Exchangeable H in a reference man with modified body composition

	<u>kg</u>	<u>Total</u> <u>exchangeable</u> <u>H</u>	<u>%Total</u> <u>exchangeable</u> <u>H</u>
<u>Example 1</u>			
Water	41	4.555	95.734
Fat	0	0	0
Protein	13	0.194	4.077
CHO	0.5	0.009	0.189
Minerals	1.5	0	
Total	56	4.758	

Predicted $N_D/N_O = 1.045$

Example 2

Water	41	4.555	93.801
Fat	28	0.098	2.018
Protein	13	0.194	3.995
CHO	0.5	0.009	0.185
Minerals	1.5	0	
Total	84	4.856	

Predicted $N_D/N_O = 1.067$

Table 4.4 continued

	<u>kg</u>	<u>Total</u> <u>exchangeable</u> <u>H</u>	<u>%Total</u> <u>exchangeable</u> <u>H</u>
<u>Example 3</u>			
Water	41	4.555	96.709
Fat	14	0.049	1.040
Protein	6.5	0.097	2.059
CHO	0.5	0.009	0.191
Minerals	1.5	0	
Total	63.5	4.710	

Predicted $N_p/N_o = 1.034$

Example 4

Water	41	4.555	92.883
Fat	14	0.049	0.999
Protein	19.5	0.291	5.934
CHO	0.5	0.009	0.184
Minerals	1.5	0	
Total		4.904	

Predicted $N_p/N_o = 1.077$

4.3.1 Biological basis for differences between N_p and N_o

Culebras and Moore ⁵ provided a theoretical basis for the differences between N_p and N_o . Assuming that N_o correctly predicts body water, then N_p/N_o ratios of 1.055 are predicted by exchange of ^2H with ^1H in fat, protein and carbohydrate in a reference man (see Table 4.3). Clearly variations in body composition will alter the extent of ^2H exchange. However, these effects are relatively small and changing assumptions about body composition within very wide limits (see Table 4.4) does not produce the range of values that were found in the present data sets. One is driven to the view that the observed range was physiologically unreasonable, and this is the basis for the assumption that many of the outliers must have been wrong.

In later chapters it is proposed that the N_p/N_o value should be used as an initial screen in the detection of analytical problems. If extreme values for N_p/N_o ratios are obtained, the origin of these differences should be investigated and only if there is a good physiological explanation should they be used. (See Chapter 9 for further discussion of this point, and Chapter 11 for recommendations concerning the screening of data sets according to the between-measurement variance in N_p/N_o .)

4.3.2 Equation for calculating pool size from isotope dilution

The general equation recommended for use in human studies is:

$$N(\text{moles}) = \frac{WA}{18.02a} \times \frac{(\delta_a - \delta_t)f}{(\delta_s - \delta_p)}$$

where N is the pool space; W is the amount of water used to dilute the dose; A is the amount of dose administered; a is the dose diluted for analysis; and δ is enrichment of dose (a), tap water (t), post-dose sample (s) and pre-dose baseline (p). Since it possible to use this equation without fully understanding its

origin the full derivation has been put in Appendix 2.

4.4 Calculation of flux rates

Two procedures are commonly used for determining flux rates. These have become known as the two-point and multi-point methods. It may often be supposed that these procedures are basically the same, the two-point procedure being an abbreviated multi-point method. This is not, however, the case for although the underlying assumptions outlined in Section 4.2 apply in each case, the two-point procedure is more tolerant of deviations from the model than the multi-point method, essentially because the two-point method measures total flux between two points several days apart whereas the multi-point method attempts to provide a mean value for daily flux rates over the same time interval.

4.4.1 Two-point method

If N remains constant during an experimental period total outflow r' is correctly calculated for a period between t_1 and t_t as:

$$r' = \log \frac{(\delta_1 - \delta_p)}{(\delta_t - \delta_p)} \cdot N \quad \dots\dots\dots 5$$

where the subscripts refer to times 1 and t and pre-dose (p).

This is true even if the rate of outflow per unit time is not constant and varies in a random fashion or systematically. Imagine, for example, that r' measured over successive similar time intervals varies. In this case total flow for the period 1 to t is given by:

$$r'_{1t} = r'_{1,2} + r'_{2,3} + r'_{3,4} \dots\dots\dots r'_{(t-1),t}$$

or

$$r'_{1t} = \log \frac{(\delta_1 - \delta_p)}{(\delta_2 - \delta_p)} .N + \log \frac{(\delta_2 - \delta_p)}{(\delta_3 - \delta_p)} .N + \log \frac{(\delta_3 - \delta_p)}{(\delta_4 - \delta_p)} .N$$

$$\dots \log \frac{(\delta_{t-1} - \delta_p)}{(\delta_t - \delta_p)} .N \dots \dots \dots 6$$

Cross-cancellation gives:

$$r'_{1t} = \log \frac{(\delta_1 - \delta_p)}{(\delta_t - \delta_p)} .N \dots \dots \dots 7$$

The two-point methodology is also resilient when N changes with time as a consequence of r_{in} not equalling r_{out} . Coward *et al* ⁷ have shown that if r_{in} does not equal r_{out} , but both are constant so that N changes linearly, or if $r_{in}(t)/N(t)$ and $r_{out}(t)/N(t)$ are unequal but constant and N thus changes exponentially, total outflow is given by:

$$r'_{1t} = \frac{N_1 - N_t}{\log N_1/N_t} . \log \frac{(\delta_1 - \delta_p)}{(\delta_t - \delta_p)} + (N_1 - N_t) \dots \dots \dots 8$$

(A full derivation of this Equation can be found in Coward *et al* ⁷ and an alternative version is presented in Appendix 3.)

Because daily variation in the slope of the line between the two points does not matter in the two-point method obtaining the correct answer for the overall slope is entirely a matter of analytical accuracy and precision.

4.4.2 Multi-point (slope-intercept) method

The multi-point method is fundamentally different from the

two-point procedure because the basic intention is to obtain a mean value for flux rate with a standard deviation indicating the extent of random physiological variation.

Pool spaces (N) are measured from the zero-time intercept of a plot of isotopic enrichment versus time. The most important advantage of this manoeuvre is that it reduces the error on calculation of the products $k_o N_o$ and $k_p N_p$ because of the covariance of slopes and intercepts. For example, an error tending to make a slope steeper will increase the value of the intercept and consequently reduce N . Allowance can also be made for covariance between errors in the 2H and ^{18}O data in the determination of the SE of the estimate. These aspects are covered more fully in Chapter 5. Additionally random analytical error becomes less important than it is in the two-point method. This aspect is also covered fully in Chapter 5.

There are many possible ways of handling multi-point data. Three of these were explored in detail during the workshop and in the data analysis exercise leading up to it. They are summarised below. A fourth variant (the Product-Ratio Method) was developed by Cole and Coward and introduced at the workshop. It gives the same answer as the simple log fit described below, but provides a better estimate of error and the most informative way of inspecting the raw data. It is described in full in Chapter 5.

4.4.2.i Curve fitting to multi-point data

In order to obtain values for rate constants and intercepts (or volumes) using multi-point data it is necessary to fit an appropriate curve to the data which describes the mean decay over time. Different fitting procedures attach more or less importance to individual points and the choice of the most appropriate treatment should ideally be based on knowledge of the system's error structure. At extreme ends of a range of possibilities are:

a) that errors are proportional to the level of enrichment; or b) that the errors have a constant absolute value and are not dependent on enrichment. The first case might be represented by physiological variations such as random changes in water turnover. The second case could arise from constant analytical errors. In practice however it could be suggested that an ideal fitting procedure would be one that was appropriate to an error structure lying somewhere between the two extremes. A detailed discussion of this problem is given in Appendix 4, and further coverage appears in Chapter 5. However a few general statements can be made at this juncture and the following sections give a simple summary of 3 different data-fitting techniques and guidance as to which one is appropriate under different error conditions.

4.4.2.ii Log transformation

This procedure is the simplest that can be used since any computer package or calculator programme will fit the best straight line through log transformed data. The method assumes that errors are proportional; that is to say that residuals (differences between observed points and the fitted enrichment curve) become smaller as enrichment decreases or, in other words, are constant in size relative to enrichment. This fitting procedure is thus appropriate for errors described in case (a) above and has been used by most groups ^{8,12-15}. The curve fitting procedure described by Feldman ¹⁶ is used by the Cambridge group.

4.4.2.iii Exponential fit

Haggarty et al ¹⁷ at the Rowett have estimated volumes and rate constants by fitting exponentials directly to untransformed data. This approach was developed after inspecting residual plots which indicated a constant error structure throughout the

labelling period in the particular group of subjects studied ¹⁸. It was this process of scrutinising residual plots which alerted these authors to the need for alternative fitting methods under certain circumstances. In contrast to log transformation the exponential fit allows early points (higher enrichment) to have a greater effect on the outcome of the fitting than later points (lower enrichment). The curve fitting is not as readily available, but it is found in a number of more sophisticated statistical packages for computers. These workers use "Maximum Likelihood Program" (Numerical Algorithms Group, Oxford, UK) to fit models to data.

4.4.2.iv Poisson fit

Franklin, also from the Rowett, suggested a third "Poisson" model at the workshop which assumes that the error structure lies somewhere between the constant CV and constant SD situations covered above. The simplest ways to achieve this are to use either a weighted linear regression method or a 'generalised linear model' ¹⁹ with a logarithmic link function and a Poisson error distribution coupled with a heterogeneity factor. Essentially this allows the fitting of the log or exponential models with the standard deviation at each point being proportional to the square root of the enrichment. "Maximum Likelihood Program" also provides the facilities to carry out this type of analysis.

4.4.2.v Choosing the appropriate model

Firstly, it should be re-emphasised that with well-behaved data-sets it is virtually irrelevant as to which method of data reduction is employed since they will yield very similar answers. The workshop therefore recommended that users should adapt their software to calculate all data-sets in each of the 3 possible

ways. They can then predefine an acceptable level of agreement (say 3%). If the 3 results agree to within this tolerance then the particular version favoured by that laboratory can safely be used. If the results fail to satisfy this criteria then the data must be carefully scrutinised.

4.8 References

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CHAPTER 5

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ESTIMATES OF ERROR

5.1 Types of error

The calculations required to obtain the pool sizes and flux rates of the two isotopes have been described in Chapter 4. These quantities together with the relevant fractionation rates, lead to an estimate of the CO₂ production rate in individual subjects.

To be of most value, each estimate of CO₂ production rate requires an associated standard error, to show how closely the data satisfy the assumptions made by the method, and to provide reassurance that the error is small. The calculation of the error assumes that the data provide an adequate fit to the model of constant flux rate and constant pool size, so that the error is a measurement of *precision*. Quite separately there is the possibility of bias in the estimate, due to imperfections in the model, which defines the *accuracy* of the estimate.

5.2 Variance and covariance

The fundamental equation for CO₂ production rate ignoring fractionation is:

$$r'_{\text{CO}_2} = 0.5 [k_o N_o - k_h N_h] \quad \dots\dots\dots 1$$

where k_o and k_h are the flux rates of oxygen and hydrogen respectively, and N_o and N_h are the body water pool sizes as estimated by ¹⁸O and ²H. (Note that the use of a prime in r'_{CO_2} signifies production rates uncorrected for fractionation - see Chapter 2). The four quantities are assumed to be constant, and are estimated from the two regression lines of log isotope enrichment versus time. So long as there are at least 3 data points the estimates have standard errors, based on the departures from linearity of the observed log enrichments. It is assumed that the timing of each sample point is known to full accuracy, although in practice this may not be strictly true.

The departures from linearity are partly physiological, representing genuine changes in flux from day to day, and partly instrument measurement error. In the multi-point method, the two sources of error are combined in the residual variation about the regression line, whereas in the two-point method there is no residual variation.

5.2.1 Slope-intercept covariance

The regression of log isotope enrichment on time can take several forms, depending on whether the errors are assumed constant in enrichment units, or constant in coefficient of variation terms, or somewhere in between (see Section 5.9 and Chapter 4). In the simplest case a constant coefficient of variation is assumed, and k_o and k_h are estimated as the unweighted linear regression coefficients of log enrichment versus time. Each

regression coefficient has a standard error which is obtained from the regression analysis.

Similarly, estimates of I_o and I_p are obtained from the exponentials (or antilog) of the intercepts from these regressions, and again they have their corresponding standard errors. It is convenient to express the enrichments of ^{18}O and 2H as the fraction of dose per mole of body water. Then the intercepts I_o and I_p are by definition the reciprocals of the body water pools N_o and N_p , ie $I_o = 1/N_o$ and $I_p = 1/N_p$.

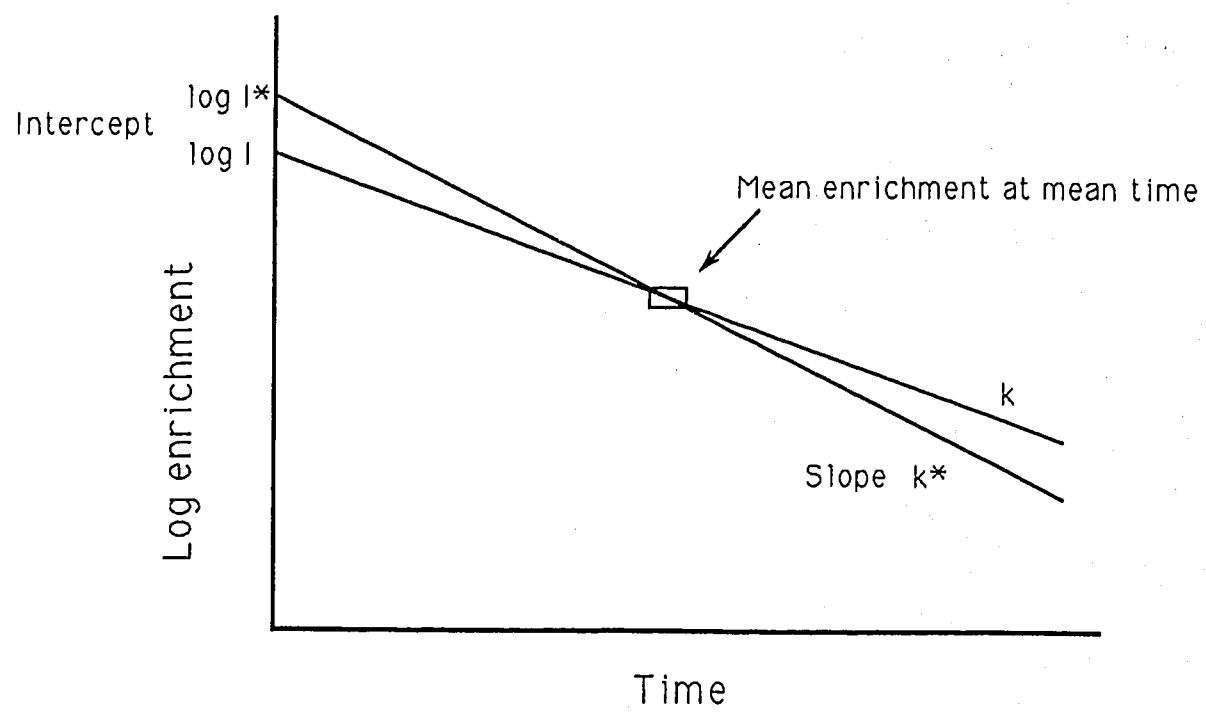
Given the standard errors for the flux rates and pool sizes, it might be thought straightforward to combine the errors, using Equation 1 to obtain a standard error for the CO_2 production. However there are complications, because of the way that the four parameters interact with each other.

Firstly the estimates of the slope k and intercept $\log I$ from each regression line are highly correlated (Fig 5.1). The fitted regression line passes, by definition, through the mean value of the data points, so that the line can pivot about its mean quite independently of the latter's location. Thus the mean \log enrichment and the regression slope are uncorrelated with each other. However when the slope changes the intercept changes in the same direction - the slope k and intercept $\log I$ are correlated. (It should be emphasised that k is treated here as being positive even though it is actually negative - the negative sign is included in the equations). Thus the standard error of the ratio k/I (or equivalently the product kN) needs to reflect this covariance relationship.

From Fieller's theorem ¹ the variance of k/I is given by:

Figure 5.1

Schematic illustration of slope-intercept correlation



$$\text{Var}(k/I) = (1/I^2)\text{Var}(k) + k^2\text{Var}(\log I) - 2r^*(k/I)\sqrt{\text{Var}(k)\text{Var}(\log I)} \dots\dots\dots 2$$

where $\text{Var}(k)$ and $\text{Var}(\log I)$ are the variances of k and $\log I$, and r^* is the correlation between k and $\log I$. This correlation is given by:

$$r^* = \bar{\epsilon}/\bar{\epsilon} \dots\dots\dots 3$$

where $\bar{\epsilon}$ is the arithmetic mean of the sampling times and $\bar{\epsilon}$ is the root mean square of the sampling times (ie square the sampling times, add them together, divide by the number and take the square root). This correlation lies between zero and +1, and is a function only of the chosen sampling times, not of the measured enrichments. This takes account of the covariance between k and I for each isotope, but there is the further problem that after fitting the two regression lines, the two residuals at a particular time tend to be correlated.

5.2.2 Correlated residuals

If the observed ^{18}O enrichment is greater than the value predicted by the regression line, there is a high probability that the ^2H enrichment will also be elevated. This correlation arises in two ways. Firstly, ^2H and ^{18}O are both influenced by water turnover, but only ^{18}O is affected by CO_2 production. So if the water turnover from one sampling time to the next is anomalously high say, then the latter hydrogen enrichment is depressed. The oxygen enrichment is also depressed, but less than for the hydrogen because only a fraction of the ^{18}O is lost as water. The converse is true for low water turnovers. So, depending on the fraction of oxygen lost as water, the deviations of ^2H and ^{18}O from their fitted lines will be correlated to a greater or lesser extent.

The second source of the correlation is physiological, in that if energy expenditure (ie CO₂ production) increases sufficiently, there is likely to be a corresponding increase in water turnover through sweating. Thus both the CO₂ and H₂O components of ¹⁸O may rise together, depending on the precise nature of the increase in expenditure. In practice though, this source is likely to be far smaller than that due to the slope-intercept interaction.

The estimate of error for CO₂ production given by Coward et al ² fails to take either of these sources of correlation between the residuals into account. The correlation cannot be predicted on a theoretical basis, and the best method of incorporating it in the estimate of the variance of CO₂ production is by no means clear.

5.3 Ratio plots and product plots

CO₂ production rate can be thought of as ¹⁸O turnover adjusted for ²H turnover. Thus much of the information about CO₂ production is contained in the difference between the isotope flux rates ($k_o - k_p$). This can be estimated directly from the log regression of the enrichment ratio δ_o / δ_p plotted against time. The appeal of this 'ratio plot' regression is that the errors in ²H and ¹⁸O caused by water turnover fluctuations nearly cancel each other out, so that the fit to the line is much closer. In fact the linearity of the plot is simply a measure of the constancy of CO₂ production.

To make use of the ratio plot, Equation 1 defining CO₂ production has to be recast in terms of the ratio plot slope and intercept, k_r and I_r say. (Note that although $k_r = k_o - k_p$, the relationship between I_r , I_o and I_p is not a simple ratio). Results from the analogous 'product plot', ie $\delta_o \times \delta_p$ plotted on a log scale against time, are also required - here the slope and

intercept are k_p and I_p .

Although the correlation between residuals is much reduced by analysing the ratio and product plots, it is not removed entirely. There may still be the correlation between water turnover and CO_2 production discussed earlier, albeit on a much smaller scale.

5.4 The variance of CO_2 production

The assumption of exponential disappearance implies that:

$$\delta^* = I^* e^{-k^*t} \quad \dots\dots\dots 4$$

where the suffix (*) is either D or O. The corresponding equations for the ratio plot and product plot are, respectively:

$$\delta_o / \delta_D = I_r e^{-k_r t} \quad \dots\dots\dots 5$$

and

$$\delta_o \times \delta_D = I_p e^{-k_p t} \quad \dots\dots\dots 6$$

Equation 1 can then be expressed in terms of I_r , k_r , I_p and k_p as follows:

$$\begin{aligned} 2r'_{\text{CO}_2} &= k_o N_o - k_D N_D \\ &= (k_o - k_D)(N_o + N_D) + (k_o + k_D)(N_o - N_D) \\ &= k_r A_r + k_p A_p \quad \dots\dots\dots 7 \end{aligned}$$

where:

$$A_r = (N_o + N_D)/2 = (1/\sqrt{I_r I_p} + \sqrt{I_r/I_p})/2 \quad \dots\dots\dots 8$$

and:

$$A_p = (N_o - N_D)/2 = (1/\sqrt{I_r I_p} - \sqrt{I_r/I_p})/2 \quad \dots\dots\dots 9$$

Using Equation 2 the variance of Equation 7 is given by:

$$\begin{aligned} \text{Var}(r'_{\text{co}_2}) = & A_r^2 \text{Var}(k_r) + B_r^2 \text{Var}(\log I_r) \\ & - 2A_r B_r r^* \sqrt{\text{Var}(k_r) \text{Var}(\log I_r)} + A_p^2 \text{Var}(k_p) \\ & + B_p^2 \text{Var}(\log I_p) - 2A_p B_p r^* \sqrt{\text{Var}(k_p) \text{Var}(\log I_p)} \end{aligned} \quad \dots\dots\dots 10$$

where:

$$B_r = (k_o N_o + k_D N_D)/2 = (k_r A_p + k_p A_r)/2 \quad \dots\dots\dots 11$$

$$B_p = (k_o N_o - k_D N_D)/2 = (k_r A_r + k_p A_p)/2 \quad \dots\dots\dots 12$$

and r^* is the slope-intercept correlation given in Equation 3.

Similarly the $\text{Var}(*)$ indicate the variances (i.e. the squares of the standard errors) for each of the parameters k_r , k_p , $\log I_r$ and $\log I_p$.

Equation 10 is rather intimidating. Fortunately it can be reformulated in terms of the residual variances of the two regression lines s_r^2 and s_p^2 , and the chosen sampling times t_i , where $i = 1 \dots n$:

$$\text{Var}(r'_{\text{CO}_2}) = (B_r S_r)^2 \left[\frac{1}{n} + \frac{(\bar{t} - A_r/B_r)^2}{\Sigma t^2} \right] \\ + (B_p S_p)^2 \left[\frac{1}{n} + \frac{(\bar{t} - A_p/B_p)^2}{\Sigma t^2} \right]$$

.....13

Here \bar{t} is the mean of the sampling times t_i and Σt^2 is the corrected sum of squares of the t_i , where:

$$\Sigma t^2 = \sum_{i=1}^n t_i^2 - n\bar{t}^2$$

The equation consists of terms which depend on n , \bar{t} and Σt^2 . It is clear that if both n and Σt^2 are large, then the variance of CO_2 production will be small.

5.5 Optimal design to minimise the variance

Equation 13 gives the required variance, but in addition it also defines the optimal choice of sampling times to minimise the variance. For example if the mean of sampling times, \bar{t} , is arranged to exactly equal A_r/B_r , then the first term in Equation 13 becomes zero; alternatively if $t = A_p/B_p$ the second term vanishes.

The ratio A_r/B_r is given by $(N_o + N_d)/(k_o N_o + k_d N_d)$, which is the reciprocal of a weighted sum of the flux rates k_o and k_d . Similarly A_p/B_p , which is the same as $(N_o - N_d)/(k_o N_o - k_d N_d)$, is a reciprocal of a weighted difference of the two flux rates.

The importance of Equation 13 cannot be overstated - it indicates how to design doubly-labelled water experiments optimally, providing the smallest possible variance for the CO_2

production. Typical values for A_x , B_x , s_x and A_p , B_p , s_p are first required for the subjects under study, and then by suitable choice of the sampling times, n and \bar{t} and Σt^2 can be set to minimise Equation 13.

Based on the 30 sets of data provided for the workshop, the ratio A_x/B_x averages about 10 days while A_p/B_p is about -1 days. This poses a problem, for in order to make both the second and third terms in Equation 13 vanish, \bar{t} needs to be equal to 10 and -1 simultaneously.

The best choice of \bar{t} is a compromise between these two values, the exact choice depending on the relative sizes of $(B_x s_x)^2$ and $(B_p s_p)^2$. If they are similar in size, then \bar{t} should be set to the average, ie 4 or 5 days. The terms s_x^2 and s_p^2 represent the noise about the fitted ratio and product lines, and s_x^2 is typically a tenth of s_p^2 (although it varies widely about this figure). However B_x is very much greater than B_p , so that in practice the ratio $(B_x s_x)^2 / (B_p s_p)^2$ for the workshop datasets is close to 6. This makes the optimal choice for t equal to 7.7 days, as compared with the actual mean of 7.5 days, indicating that the experiments were close to optimally designed on the whole.

This discussion has assumed that the flux rate k is known in advance of the experiment, which of course it is not. The optimal value for \bar{t} can only be chosen approximately. To ensure that the second and third terms in Equation 13 are kept small, the value of Σt^2 should be as large as possible, which requires the sampling times to be widely spaced. The possible range of times is bounded in both directions of course, at time zero at one end and the time when the signal gets lost in background at the other, so that the optimal design has one group of samples near the start and another group near the end, arranged symmetrically around \bar{t} .

The first term of Equation 13 is inversely proportional to the reciprocal of the number n of sampling times in the experiment. In the workshop datasets, where n averages 14, almost exactly 50% of the total variance was contributed by this term. If n were to be reduced to 5 (while keeping the other variables unchanged) then the first term's contribution would be increased 3 times and the total variance doubled. Put the other way, this indicates that using 14 sampling points, as opposed to 5, halves the variance emphasising the improvement in precision that multi-point sampling provides.

Another consideration is the timing of the first sample. It is argued above that the first sample should be near to zero time, but in fact this is not a strong requirement from the precision point of view. The term Σt^2 is only of secondary importance compared to n and \bar{t} , and there are strong practical reasons for avoiding a sample immediately after dosing. According to Equation 13, a first sample as late as 1 or even 2 days after dosing would not have much impact on the variance of CO_2 production so long as \bar{t} is chosen appropriately. Such a delay means that the pool size is being determined through extrapolation and hence increases the importance of selecting the correct model.

5.6 Variance and the two-point method

The use of Equations 10 or 13 to calculate the variance of the CO_2 production rate requires knowledge of the residual variances about the ratio and product regression lines. This precludes its application to two-point data. However it is quite valid to substitute the known instrument measurement errors into Equation 13, where the errors on ^2H and ^{18}O , δ_{D} and δ_{O} are independent so they combine vectorially. Thus:

$$s_r^2 = s_p^2 = \delta_{\text{D}}^2 + \delta_{\text{O}}^2 \quad \text{.....14}$$

This variance is only a fraction of the total variance, and takes no account of day-to-day variation in water and CO_2 production. Thus the variance estimated from Equation 13 will be a substantial under-estimate of the true variance, and for this reason it cannot be used for experimental design purposes.

5.7 Bias in the estimation of pool size

The calculation of CO_2 production rate (Equation 1) assumes that the flux rates of ^2H and ^{18}O are constant, leading to linear plots of log enrichment against time. It is also valid if the plots are non-linear so long as the body water pool size N remains constant during the experiment. Water volume is likely to be constant with adults in non-clinical situations unless they are dieting or there is serious dehydration, but in small children who are growing rapidly or in pregnant women there is a chance that the water volume will increase during the study and introduce a bias. This bias shows itself as a curved plot of log enrichment versus time.

The pool sizes, obtained from zero time enrichments, are estimated from the intercepts of the fitted regression lines. In practice the plots may not be linear, but may oscillate systematically above and below the fitted lines. This phenomenon is called *serial correlation* or *auto-correlation*. When this happens, the intercepts are biased estimates of the zero time enrichments, leading to a corresponding bias in the estimate of CO_2 production.

Systematic non-linearity in the data, and the magnitude of the bias, can be seen most easily from residual plots of the data (see figures in Chapter 11). The plots for ^{18}O and ^2H show the biases for the two water spaces separately, but the biases can also be obtained from the 'product' and 'ratio' plots. The advantage of using the product and ratio plots is that the bias

is partitioned into an average pool size effect (bias in I_p) and an effect due to the N_D/N_O ratio (bias in I_r). When the biases in the two water spaces are covariant, as they often are, the resulting bias in CO_2 production rate is contained largely in the product plot, while the ratio plot bias is far smaller.

To see how the biases in I_p and I_r relate to the bias in r'_{CO_2} Equation 7 can be rearranged to give:

$$r'_{CO_2} = 1/\sqrt{I_p}(k_o/\sqrt{I_r} - k_D/\sqrt{I_r}) \quad \dots\dots\dots 15$$

Differentiating Equation 15 and rearranging leads to:

$$\delta r'_{CO_2} = -\frac{1}{2} \left[\delta I_p + \delta I_r \frac{k_o N_o + k_D N_D}{k_o N_o - k_D N_D} \right] \quad \dots\dots\dots 16$$

where $\delta r'_{CO_2}$, δI_p and δI_r are the fractional or percentage biases in r'_{CO_2} , I_p and I_r respectively. If δI_r is zero, Equation 16 shows that δI_p needs to be halved and its sign changed to give the bias $\delta r'_{CO_2}$ in CO_2 production rate. From the analyses performed prior to the workshop we recommend that the value for δI_p is given by the residual from the product plot about 5 to 8 hours post-dose.

Similarly, the bias in I_r is shown by a series of residuals from the ratio plot between 5 and 8 hours post-dose which are consistently non-zero. As there is a lot of noise on the ratio plot, a single large residual during this time should not be treated as bias, it may equally be due to measurement error. However if bias is present (i.e. δI_r is non-zero), its contribution to Equation 16 needs to be taken into account. The value of the multiplier $(k_o N_o + k_D N_D)/(k_o N_o - k_D N_D)$ is typically 9 or a little more, so that δI_r should be multiplied by 9/2 to give the bias in r'_{CO_2} .

Thus a bias of say +2% in I_p leads to a -1% bias in r'_{CO_2} , whereas a +2% bias in I_r biases r'_{CO_2} by -9%. In practice it is

difficult to detect bias in the intercept of the ratio plot. Nevertheless Equation 16 makes clear that if the N_p/N_o ratio is at all aberrant, its percentage effect on r'_{CO_2} is magnified up some 4 or 5 times.

Unlike the multi-point method, the two-point method explicitly uses an early enrichment to derive the pool size. As a result it avoids the possibility of bias in the estimation of pool size. However this gain in accuracy relative to the multi-point method is offset by a corresponding loss in precision, owing to the smaller number of samples.

Chapter 9 gives further coverage of the potential errors introduced by systematic and random variations in water and CO_2 flow rates, and by errors in estimating N_p/N_o .

5.8 Precision and accuracy in the multi-point and two-point methods

In this section the ten Cambridge workshop datasets are used as examples to illustrate the precision and accuracy of estimated CO_2 production.

The standard error of r'_{CO_2} calculated from Equation 13 ranges from 0.7% to 4.0% over the ten datasets, with a median of 1.6%. The bias for each dataset is obtained from the residual for the first sample after 5 hours on the 'product' line, halving it and changing its sign. The bias from the 'ratio' plot is assumed here to be zero, since all the N_p/N_o ratios are reasonable in value. The 'product' bias over the ten datasets has a mean of -0.1% and a standard deviation of 2.1%. Thus the standard deviation for precision is 1.6% and for accuracy it is 2.1%. These two errors are independent, so they can be combined as their root mean square. This gives a combined error of 2.6%. The overall mean bias of 0.1% is trivially small. It should be noted that the

Cambridge datasets were selected from sequential analyses from a large database, and were not selected as being particularly well-behaved data.

It is instructive to repeat this exercise for the two-point method. Here there is no bias, so the error is all in the precision. Normally it is not possible to calculate the precision as there are no degrees of freedom for error. However an estimate of the precision can be obtained by assuming that the errors around the 'product' and 'ratio' lines in the multi-point method, s_p and s_r in Equation 13 are the same as in the two-point method. On this basis the terms involving s , A and B in Equation 13 are unchanged, but n is reduced to 2, and Σt^2 is calculated from just the first and last points of each dataset. This then provides an estimate of the variance of r'_{co_2} which can be compared with the multi-point method.

Doing this sum for the Cambridge datasets shows that the precision variance is increased by a median factor of 5.0 (range 4.2 to 5.9), giving a median error of $\sqrt{5.0} \times 1.6\% = 3.6\%$, where 1.6% is the multi-point precision. Thus the multi-point method has a combined precision and accuracy of 2.6%, whereas for the two-point method the figure is 3.6%.

5.9 Constancy of errors

The log regression analysis assumes that the error about the regression line does not change systematically with the level of enrichment (ie the absolute error is proportional to the enrichment). In theory (Section 9.3) physiological error should satisfy this assumption quite well, whereas analytical error tends to be relatively smaller at high enrichments. So, depending on the relative magnitude of the two sources of error, enrichment error should lie somewhere between constancy and proportionality. Although the analytical error varies from laboratory to laboratory,

it *should* be much smaller than the physiological error unless enrichments are small, so that the assumption of proportional errors is usually reasonable on average over a large number of subjects. Some individuals may exhibit error structures which deviate from this model (see for example James et al ²) largely because of temporal variability in water intake.

When there is evidence that the errors are nearer to constant than proportional, an absolute form of analysis can be used which fits an exponential curve to the absolute enrichments. This approach may suit the analytical error structure, but it is usually inappropriate for the physiological error, which being relatively constant gets smaller in absolute terms as the enrichment falls. As a result the exponential fit concentrates on the large errors at the start of the experiment and pays little attention to the later data (see also Section 9.2.2). Figure 5.2 illustrates residuals from 4 data-sets which show equally large residuals at the end of the experiment as at the beginning, and for which use of an exponential fit may therefore be preferable.

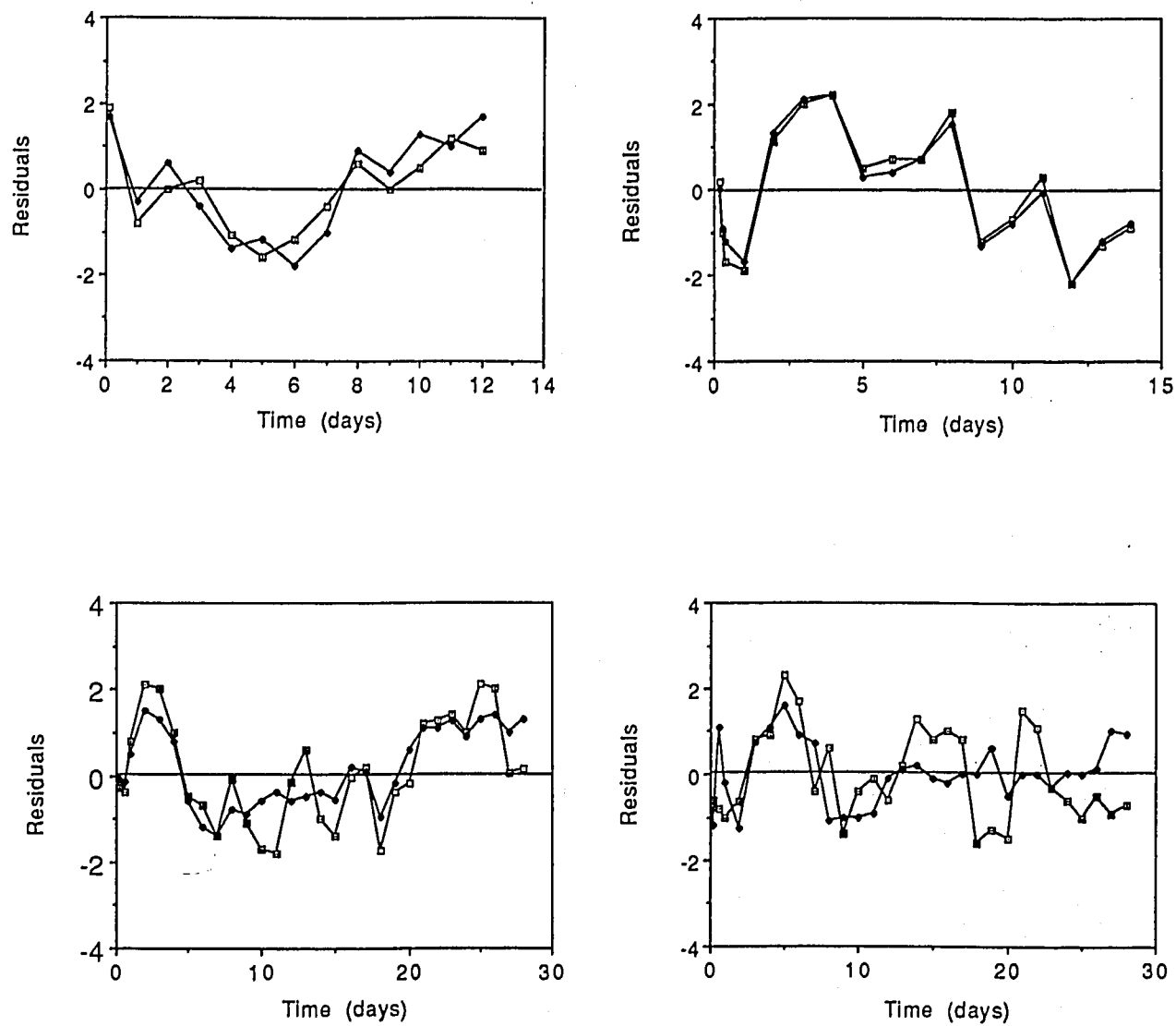
There is an intermediate form of analysis which assumes Poisson type errors, where the errors increase as the square root of the enrichment. This can be viewed as a compromise analysis, to be used when the error structure is neither constant nor proportional. It is possible to compare the effects of the three approaches in an approximate way by using weighted log regression, where the individual points are given weightings as follows:

- a) for errors proportional to enrichment, use weighting = 1
- b) for constant errors, use weighting = enrichment²
- c) for in-between errors, use weighting = enrichment.

If the results change substantially using the different weighting systems, this suggests a pathological pattern of residuals which inspection of the residual plots (see Section

Figure 5.2

Examples of data-sets showing rather constant error structures



Note The units (ppm normalised to 100 at zero time) differ from those used in Chapter 11.

5.10) should clarify. (Note that the weighted regressions (b) and (c) require, in theory, an iterative procedure for the true weightings are unknown, but in practice use of the observed enrichments usually suffices.)

It is probable that the unweighted log regression (equivalent to (a) above) is the most useful analysis, as it is easy to do even on a hand calculator, and it fits in with the ratio and product plot procedures described earlier.

5.10 Data and residual plots

The production of diagnostic plots is an important stage in the fitting process. There are two useful forms of plot, data plots and residual plots. In general the values for ^{18}O and ^2H can be plotted on the same graph, while the product and ratio data need to be treated separately.

Each data plot indicates the observed enrichments, on a log scale, plotted against time, with the fitted linear regression line superimposed. The residual plot shows the residuals from each enrichment (ie the observed values less the value predicted from the fitted line) plotted against time. In general, the residual plots show whether or not the assumption of a constant proportional error variance is valid. If the magnitude of the residuals appears to increase systematically with time then this is evidence against, and one of the alternative analyses discussed in Section 5.9 may be considered more suitable.

The natural log scale on the y axis of the residual plot can be rendered more comprehensible by multiplying it by 100, and calling it a percentage scale. This is highly accurate for log values up to ± 0.1 , ie $\pm 10\%$, but becomes progressively less accurate for higher values. Thus a residual of -0.06 indicates that the observed enrichment is 6% less than the enrichment

predicted by the regression line at that time.

There are other features to look for in each type of plot, which are dealt with separately.

5.10.1 ^{18}O and ^2H plots

It is important that the results for the two isotopes should be plotted on the same graph - they then show the degree of covariance between ^{18}O and ^2H . Both data and residual plots also give a measure of the size of the errors, evidence for any systematic departures from linearity, and evidence for errors increasing or decreasing through the experiment.

5.10.2 Product plot

The most important part of the product data plot is the intercept of its regression line, as this estimates the zero time enrichment and hence the body water pool size. The product plot often shows signs of serial correlation (also known as autocorrelation), where the residuals at particular time points are similar to the residuals near them in time. Put another way, it means that the data follow a curve which differs systematically from a straight line. The product plot is better than the ^{18}O and ^2H plots for seeing signs of this non-linearity, as the two trends are combined.

If there is evidence of serial correlation then the intercept is likely to be a biased estimate of the zero time enrichment (see Section 5.7), and the magnitude of this bias can be read off the residual product plot. However it is important to remember to halve the value so obtained, as it is the sum of the biases for the two isotopes. The CO_2 production rate is then biased to this extent *in the opposite direction*, as the intercept

is the inverse of the pool size.

It is possible to test formally for the presence of curvature in the product plot, but in practice it is more important to read off the likely bias of the intercept from the residual plot.

5.10.3 Ratio plot

The ratio plot is a very sensitive way of presenting information about the CO_2 production rate, and as such is useful for assessing the linearity of the plot and constancy of production. In addition, the intercept of the ratio data plot is the log of the N_p/N_0 ratio, and should normally be about +0.03, corresponding to a ratio of 1.03. Since the plot declines with time, the fitted line normally crosses the time axis during Day 2. If there are several residuals during the first day, and if they are all systematically non-zero, then this may indicate bias in the N_p/N_0 ratio. Section 5.7 shows how this bias translates to a five-fold bias in the CO_2 production rate.

Worked examples illustrating many of the points raised here are presented in Chapter 11.

5.11 References

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2. James WPT, Haggarty P & McGaw BA (1988) Recent progress in studies on energy expenditure: are the new methods providing answers to old questions? *Proc Nutr Soc*; 47: 195-208.

CHAPTER 6

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ISOTOPE FRACTIONATION CORRECTIONS

6.1 Isotope Fractionation

6.1.1 General Terminology

Isotope fractionation is the physical phenomenon which causes changes in the relative abundance of isotopes due to their differences in mass. There are two categories of isotope effects: equilibrium and kinetic.

An equilibrium isotope effect will cause one isotope to concentrate in one component of a reversible system that is in equilibrium. If it is the heavier isotope that concentrates in the component of interest, then that component is commonly referred to as enriched or heavy. If it is the light isotope that concentrates then the component is referred to as depleted or light. In most circumstances the heavy isotope concentrates in the component in which the element is bound more strongly and thus equilibrium isotope effects usually reflect relative differences in the bond strengths of the isotopes in the various compo-

nents of the system.

A kinetic isotope effect occurs when one isotope reacts more rapidly than the other in an irreversible system or a system in which the products are swept away from the reactants before they have an opportunity to come to equilibrium. Normally, the lighter isotope will react more rapidly than the heavy isotope and thus the product will be lighter than the reactant.

It should be noted that isotope fractionation will only occur in systems in which there is both an isotope effect and a reaction that does not proceed to completion. Thus, even in the presence of an isotope effect, there will be no isotope fractionation if all the reactant goes to a single product because all the atoms have reacted and thus the ratio of the heavy to light isotope must be the same in the product as it was in the reactant.

The magnitude of an isotope effect is expressed as a fractionation factor. This is defined as the ratio of the heavy to light isotope in the product divided by the ratio of the heavy to light isotope in the reactant. Among users of doubly-labelled water the fractionation factor has generally been represented by the symbol "f". Other common symbols that have been used by other communities of users include α and ϕ .

Stated mathematically:

$$f = \frac{(\text{heavy/light})_{\text{product}}}{(\text{heavy/light})_{\text{reactant}}}$$

When f is greater than 1, the product is heavy or enriched. When it is less than 1, the product is light or depleted. Most fractionation factors lie between 0.9 and 1.1, but deuterium isotope effects can result in much smaller or larger fractionation factors. A fractionation factor of 1.050 is often referred to as a 5% isotope effect.

For further basic information on isotope fractionation, the reader is referred elsewhere ^{1,2}.

6.1.2 Influence of isotope fractionation in vivo

When stable isotopes are used as tracers for *in vivo* metabolic studies, an investigator is typically measuring the tracer flux and making inferences about the flux of the tracee (i.e. the material being traced). When there is no isotope fractionation, the flux of the heavy tracer is equal to the flux of the lighter major isotope and hence the tracee flux. The rates derived from the isotope kinetic analysis are thus exactly equal to the material flux. When there is isotope fractionation, however, the flux of the heavy tracer and the tracee are not equal and thus flux derived from the kinetic analysis of the tracer is not equal to that of the tracee. For example, ^{18}O is fractionated between water and carbon dioxide. The fractionation factor is 1.037 at 37°C. Thus, the rate of removal of ^{18}O by CO_2 is 3.7% greater than the rate of ^{16}O removal. Because ^{16}O comprises 99.8% of the oxygen pool, the rate of carbon dioxide production is essentially equal to the rate of ^{16}O removal by CO_2 . Therefore, the true rate of CO_2 production will not be equal to that measured from ^{18}O , but will be 3.7% less. In other words, isotope fractionation leads to an error in the calculated tracee flux, unless the tracer rate is corrected for the fractionation.

6.1.3 Isotope fractionations influencing doubly-labelled water

One of the basic assumptions of the doubly-labelled water method is that isotope only exits the body water pool as water and carbon dioxide. The processes of interest are therefore the exchange of ^{18}O between water and carbon dioxide and the elimination of water as vapour or liquid.

The isotopic abundances of plasma water, carbon dioxide,

water vapour, urine and other physiologic samples for a subject living in the Chicago metropolitan area are shown in Figure 6.1. As can be seen, there is little or no isotope fractionation with respect to deuterium or ^{18}O for urine or sweat, which are excreted as liquids. Water vapour, however, is isotopically fractionated with respect to both isotopes and carbon dioxide is fractionated with respect to ^{18}O . As a matter of convention factors for these three isotope fractionations have been termed f_1 , f_2 , and f_3 , where:

$$f_1 = \frac{(^2\text{H}^1\text{HO}/^1\text{H}_2\text{O})_{\text{vapour}}}{(^2\text{H}^1\text{HO}/^1\text{H}_2\text{O})_{\text{liquid}}}$$

$$f_2 = \frac{(\text{H}_2^{18}\text{O}/\text{H}_2^{16}\text{O})_{\text{vapour}}}{(\text{H}_2^{18}\text{O}/\text{H}_2^{16}\text{O})_{\text{liquid}}}$$

$$f_3 = \frac{(\text{C}^{18}\text{O}^{16}\text{O}/\text{C}^{16}\text{O}_2)}{(\text{H}_2^{18}\text{O}/\text{H}_2^{16}\text{O})}$$

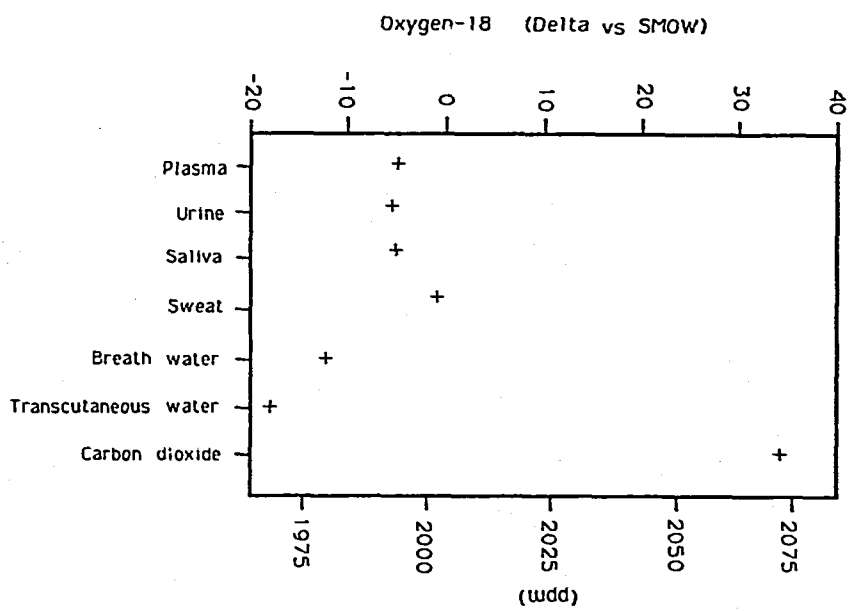
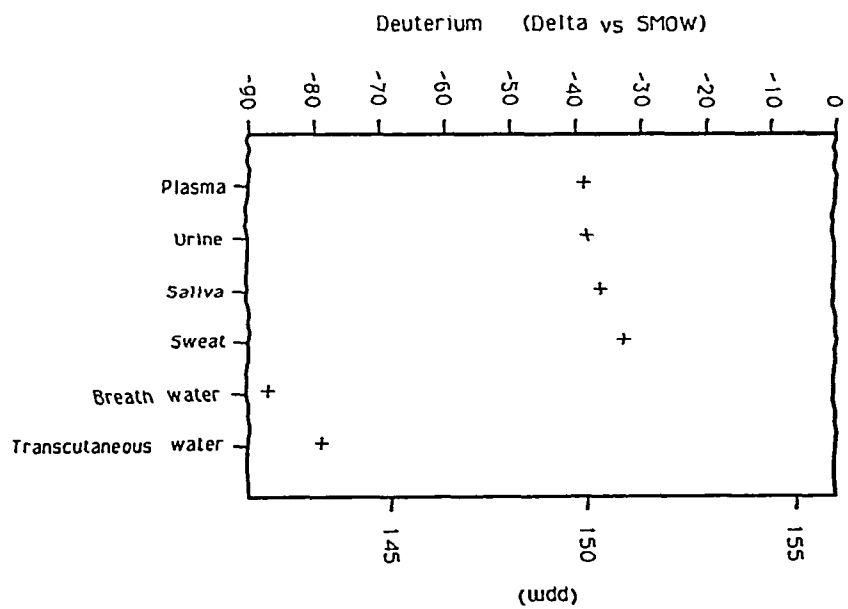
6.2 Experimental determinations of fractionation factors *in vivo*

6.2.1 Fractionation factors measured *in vivo*

In the original work by Lifson and coworkers ³, the values of the fractionation factors were taken from *in vitro* experiments performed at 25°C. In what was probably the first attempt to measure the values *in vivo*, Nagy ⁴ and Nagy and Costa ⁵ measured the change in isotopic abundance of tritium and ^{18}O in a toad during progressive dehydration. The results did not support Lifson's contention of *in vivo* isotope fractionation. However, neither they did they constitute a disproof because the isotope measurements were of only moderate precision. More recently, investigators have taken advantage of the greater precision of

Figure 6.1

Isotope abundances in different body fluids



gas isotope ratio mass spectrometry to measure f_1 , f_2 , and f_3 in humans under steady-state conditions ⁶⁻⁹. In each of these four studies, samples of excreted water vapour, carbon dioxide and/or sweat were quantitatively collected and isotopically compared with either plasma water or urine. In each study samples were expressed in per mil relative to a standard or plasma water. It can be shown from the definition of per mil and the definition of fractionation factor, that the latter can be calculated as:

$$f = (\text{product} + 1000)/(\text{reactant} + 1000)$$

The observed values of f_1 , f_2 , and f_3 are summarised in Table 6.1. Where there are values from more than one study, they are quite similar. In the case of f_3 , a small effect was noted with increasing exercise intensity ⁷. Because this was consistent with the predicted effect for a small increase in temperature, the authors suggested that it simply reflected the increase in core temperature with increasing exercise intensity.

The observed values for the fractionation factors are quite similar to those that have been measured *in vitro* and adjusted to body temperature (Table 6.1). This is particularly true for breath carbon dioxide and breath water, which are nearly identical to the *in vitro* values for an equilibrium effect. Water collected from the arm, however, is suggestive of a kinetic isotope effect rather than an equilibrium isotope effect. The value, however, from the total water collected from the arm indicates that even at rest, water loss is a mixture of unfractionated sweat losses and fractionated transcutaneous water loss. When the fractionation factor (as listed in Table 6.1) is recalculated for only the estimated transcutaneous water loss, the value is quite similar to the value determined *in vitro*. It should be noted that these *in vitro* values for kinetic isotope effects are not known as precisely as the equilibrium fractionation factors and thus a range is included in Table 6.1.

Table 6.1

Isotope fractionation factors

<u>f₁</u>		<u>f₂</u>		<u>f₃</u>	<u>Ref</u>
<u>Equilibrium</u>	<u>Kinetic</u>	<u>Equilibrium</u>	<u>Kinetic</u>		
<u>in vivo</u>					
-	-	-	-	1.038	7
0.946	-	-	-	-	6
-	0.940	0.991	0.981	-	8
0.944	-	0.989	-	1.039	9
<u>in vitro</u>					
0.941	0.923 ^a	0.992	0.976	1.038	7,20

^a Range 0.917 - 0.93

Recent studies at the Rowett laboratory ¹⁰ have indicated that water lost from the skin during exercise may not behave as a combination of unfractionated sweat loss plus fractionated transcutaneous water loss (Table 6.2). They observed that during a 20 minute period of exercise at 75% VO_{2max} the entire water vapour lost from the arm (sweat plus transcutaneous) was kinetically fractionated.

Because f_1 and f_2 differ for equilibrium and kinetic fractionation effects, there has been some concern about which value is most appropriate for application in doubly-labelled water studies. Schoeller et al ⁸ have suggested the use of a weighted average, but in truth the use of one or other has little influence on final doubly-labelled water results. This is because the fractionation correction for water vapour loss depends on the difference between f_1 and f_2 rather than on the absolute values. This difference is almost identical for an equilibrium isotope effect ($0.992 - 0.941 = 0.051$) as for a kinetic isotope effect ($0.976 - 0.923 = 0.053$).

Because values for the fractionation factors measured *in vivo* do not differ from literature values, it is recommended that the literature be used because they are based on more extensive and precise measurements.

6.3 Incorporation into calculations

As stated above, isotopic fractionation introduces inequality into the relationship between the kinetics of the isotopic tracer and the tracee. As such, the calculation of CO_2 production rate from the isotopic data must include a correction term for fractionation.

Three more or less equivalent equations have been suggested. Originally it was assumed that a constant proportion of true

Table 6.2

Isotope fractionation factors at rest and while working at 75%

VO_{2max}

	<u>f₁</u>	<u>f₂</u>	<u>f₃</u>
<u>Transcutaneous</u>			
<u>water</u>			
At rest	0.966 (0.005)	0.992 (0.001)	-
Exercising	0.911** (0.008)	0.983** (0.002)	-

Breath CO₂

At rest	-	-	1.035 (0.001)
Exercising	-	-	1.036 (0.002)

** p<0.01, SE in parentheses.

Data supplied by the Rowett group.

water output (x) could be regarded as being fractionated ³. In which case:

$$\begin{aligned} k_D N_D &= x f_1 r_{H_2O} + (1 - x) r_{H_2O} \\ &= r_{H_2O} (x f_1 + 1 - x) \end{aligned} \quad \text{.....1}$$

$$\begin{aligned} \text{and } k_O N_O &= 2 f_3 r_{CO_2} + x f_2 r_{H_2O} + (1 - x) r_{H_2O} \\ &= 2 f_3 r_{CO_2} + r_{H_2O} (x f_2 + 1 - x) \end{aligned} \quad \text{.....2}$$

From Equation 1:

$$r_{H_2O} = \frac{k_D N_D}{(x f_1 + 1 - x)}$$

and substitution in Equation 2 gives:

$$k_O N_O = 2 f_3 r_{CO_2} + k_D N_D \frac{(x f_2 + 1 - x)}{(x f_1 + 1 - x)}$$

$$\text{and } r_{CO_2} = \frac{k_O N_O}{2 f_3} - \frac{k_D N_D (x f_2 + 1 - x)}{2 f_3 (x f_1 + 1 - x)} \quad \text{.....3}$$

which can be approximated by:

$$r_{CO_2} = k_O N_O - k_D N_D (1 + (f_2 - f_1) x) \quad \text{.....3a}$$

(As $f_2 > f_1$ the effect of fractionation is equivalent to increasing k_D or N_D).

Schoeller ¹⁵ recommends another approach which involves different terminology, and removes the need to supply a value for x in Equation 3. The routes of water loss are separated into non-

fractionated ($r_{H_2O_1}$) and fractionated routes ($r_{H_2O_f}$):

$$k_D N_D = r_{H_2O_1} + f_1 f_{H_2O_f} \dots\dots\dots 4$$

$$\text{and } k_O N_O = r_{H_2O_1} + f_2 r_{H_2O_f} + 2f_3 r_{CO_2} \dots\dots\dots 5$$

Substitution for $r_{H_2O_1}$ in the second equation and rearrangement to solve for r_{CO_2} yields:

$$r_{CO_2} = \frac{(k_O N_O - k_D N_D)}{2f_3} - \frac{(f_2 - f_1)}{2f_3} r_{H_2O_f} \dots\dots\dots 6$$

Using the literature values for the fractionation factors for 37°C for human studies in which the subject has a normal body temperature yields:

$$r_{CO_2} = \frac{(k_O N_O - k_D N_D)}{2.078} - 0.0246 r_{H_2O_f} \dots\dots\dots 7$$

A third elaboration proposed by Coward ¹⁶ further divided fractionated water loss into that occurring as respiratory loss (r_r) and those skin losses (r_s) that are fractionated. Arithmetic analogous to Equations 4 and 5 generates:

$$k_O N_O - k_D N_D = 2f_3 r_{CO_2} + r_r (f_2 - f_1) + r_s (f_2 - f_1) \dots\dots\dots 8$$

It is then assumed that there is a constant relationship between respiratory water loss and CO₂ loss ($r_r = q r_{CO_2}$) so rearranging Equation 8 gives:

$$r_{\text{co}_2} = \frac{k_o N_o - k_b N_b - r_a (f_2 - f_1)}{2f_3 + q(f_2 - f_1)} \quad \dots\dots\dots 9$$

Values suggested for q and r_a are 1.1 mole water/mole CO_2 and about 27 mole/day for normal adults in a temperate climate. Thus:

$$r_{\text{co}_2} = \frac{k_o N_o - k_b N_b - 27(f_2 - f_1)}{2f_3 + 1.1(f_2 - f_1)} \quad \dots\dots\dots 10$$

Substitution of typical values for a human adult ($k_o N_o - k_b N_b = 36$ mole/day, $f_3 = 1.04$, $f_2 - f_1 = 0.052$) yields values of 16.4 from Equation 7 and 16.2 mole/day from Equation 10. The close agreement between the equations in terms of the result is somewhat coincidental because rather different relationships between respiratory and skin water losses are assumed to exist in the establishment of the constants.

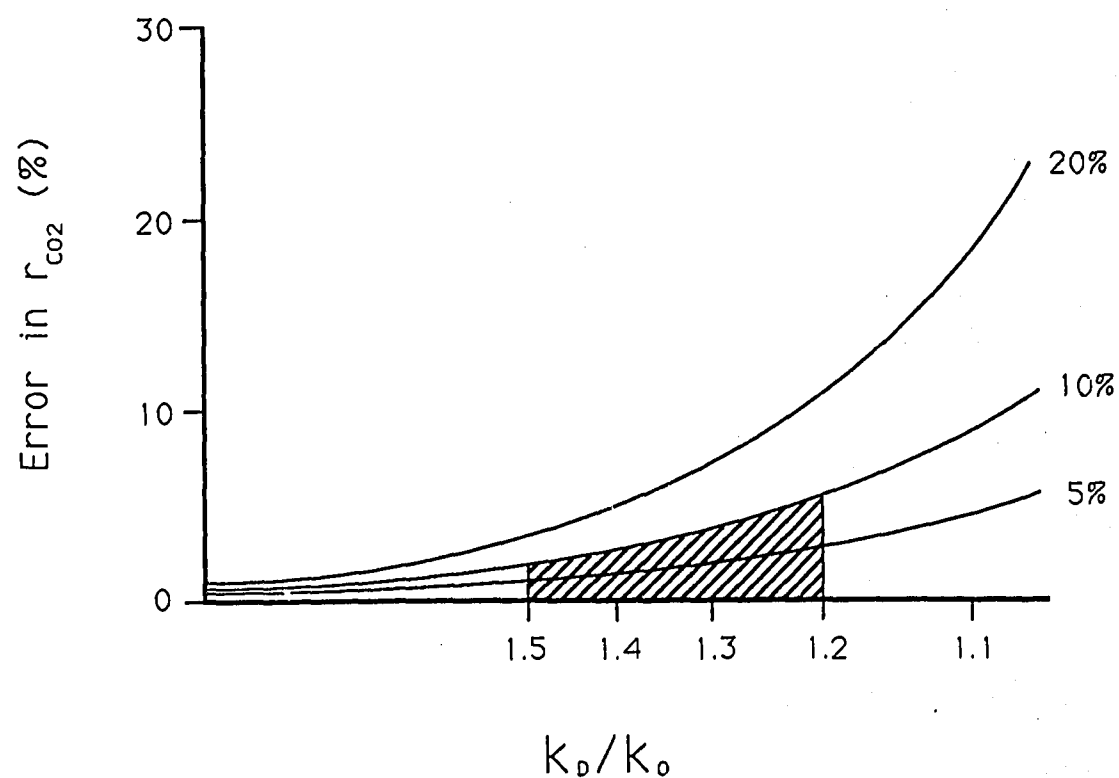
6.4 Potential errors in r_{co_2}

Inspection of the above equation indicates a practical problem, which is that some estimate of the rate of fractionated water loss is required for the calculation of CO_2 production. In the vast majority of applications of doubly-labelled water, it is impossible to know the exact rate of fractionated water loss. The error made in estimating the rate of fractionated water loss will therefore introduce some error or uncertainty into the calculated rate of carbon dioxide production.

The potential error is presented graphically in Figure 6.2 for virtually any potential case in human studies. For healthy adults, the potential range of uncertainty is rather small. Indeed, for a typical adult living in a moderate climate ($N_o k_o / N_b k_b = 0.095/0.07 = 1.35$), the relative rate of fractionated water loss only needs to be estimated to the nearest 15% of

Figure 6.2

Potential errors caused by incorrect fractionation assumptions



Curves represent different levels of error (5, 10 and 20%) in the proportion of water turnover assumed to be fractionated.

Hatching shows the range within which most measurements will fall.

total water output to reduce the uncertainty in the calculated carbon dioxide production rate to less than 2%. Potential for error in neonates is larger, but the largest potential error probably occurs in burn patients who may have a very large water turnover and hence a large deuterium elimination relative to the rate of oxygen elimination ($N_o k_o / N_b k_b = 0.33/0.30 = 1.10$). In burn patients, the relative rate of fractionated water loss must be known to the nearest 5% to reduce the uncertainty in the calculated carbon dioxide output rate to less than 2% (see Chapter 13 for further consideration of this point).

6.5 Estimation of fractionated water loss

6.5.1 Assumed constant percentage of water output

In work with amphibians and other small animals, Nagy proposed that no fractionation correction be made. Evidence to date indicates that this assumption cannot be made for human applications of the doubly-labelled water method as breath and transcutaneous water vapour have been definitively shown to be isotopically fractionated.

In the original equation developed by Lifson ³, it was proposed that the rate of fractionated water loss be assumed to be 50% of water output. Human studies, however, have demonstrated that this is an overestimate for most populations. In a single adult, Klein *et al* ¹¹ estimated maximal fractionated water loss to be 25% of water turnover, which would have introduced a 6% error in the calculated carbon dioxide production rate if the assumption of 50% had been made. Roberts *et al* ¹² estimated the fractionated water loss to be 16% in premature neonates, which would have introduced a 15% error in the calculated carbon dioxide production if the assumption of 50% had been made. Thus, the 50% assumption is not universally applicable in human

studies. Indeed, any assumed constant fraction of water loss can easily lead to modest ($\pm 5\%$) systematic errors in CO_2 production when applied to all populations.

6.5.2 Individual estimates of fractionated water loss

From a physiological viewpoint, it is unlikely that any single constant percentage of water output can be assumed to be subject to isotopic fractionation. This is because fractionated water output does not change as a function of water turnover, but rather varies as a function of other physiologic variables. Specifically, only transcutaneous water vapour and breath water are subject to isotope fractionation. Classic studies of transcutaneous water loss indicate that non-sweat water loss is relatively constant in adults for most skin surfaces ¹³. The apparent exceptions are the palms of the hands and the forehead, but the larger water loss rates reflect sweating from glands that are constantly active ¹³. As such these excess water losses are probably not isotopically fractionated. Transcutaneous water loss per unit of skin surface is nearly the same for infants and adults ¹⁴, except for premature infants during the first few weeks of postnatal life in whom transcutaneous water loss is 2 to 8 fold greater due to the immaturity of the skin ¹⁴. Breath water loss is also not dependent on water turnover, but is probably related to the ventilation volume as significant quantities of water vapour exit during the expiratory phase of breathing.

Within this framework, two approaches to estimating fractionated water loss have been used as indicated in Section 6.3. Schoeller and co-workers ¹⁵ have estimated water loss from body surface area and ventilatory volume and expressed these both as functions of CO_2 production. Coward ¹⁶ has also estimated breath water loss as a function of CO_2 production, but suggests a constant value for transcutaneous water loss.

In the approach used by Schoeller *et al* ¹⁵, it is assumed that expired CO₂ averages 3.5% of the 24 hour ventilatory volume and that expired air is 95% saturated with water vapour at 36°C ¹⁷. The latter two figures are taken from direct measurements in infants (and are slightly smaller than previously published assumptions of Schoeller *et al* ¹⁵). Assuming atmospheric pressure, the rate of respiratory water loss is therefore: $r_{\text{CO}_2}(1/0.035) \times (44.5/760) \times 0.95 = 1.59r_{\text{CO}_2}$. Transcutaneous water vapour loss is estimated from body surface area (m²) using the mean rate of non-sweating vapour loss of 0.14 g/min per m² ¹³, which is also slightly smaller than previous estimates used by Schoeller *et al* ¹⁵. Finally it is assumed that clothing reduces the rate of loss to 0.07 g/min per m² for the areas covered by clothing, which is estimated as 85% of the body in adults and 25% of the body surface area in infants in Western cultures. The reduction is assumed because of studies that have shown a reduction in non-sweat water losses when barriers are placed between the skin and the source of air flow ¹⁸.

In an adult, the calculated daily transcutaneous loss is therefore $[0.85(0.7) + 0.15(0.14)] \times 1440 = 116 \text{ g/d per m}^2$, or 6.4 mole/d per m². For a typical adult this averages 30% of the respiratory loss, so the two terms are combined for convenience to yield a total fractionated water loss of $r_{\text{H}_2\text{O f}} = 2.1r_{\text{CO}_2}$.

For an infant the transcutaneous water loss is closer to 65% of respiratory losses because of the larger ratio of surface area to CO₂ production, so the two terms are combined to yield a total fractionated water loss of $r_{\text{H}_2\text{O f}} = 2.6r_{\text{CO}_2}$. For comparisons with other values, these correspond to about 30% and 13% of total water turnover for typical adults and infants living in temperate climates.

Coward ¹⁶ does not combine the terms for respiratory water loss and transcutaneous water loss, but does make individual fractionation corrections. He measured the two components of water

vapour loss in adults and observed that breath water vapour was only 55% of the saturation value and thus suggest a value of $1.1r_{\text{CO}_2}$ (moles) for breath water loss. Measurements of transcutaneous water loss showed a high level of variability and did not detect any significant relationship to body surface area in adults. Therefore a constant value of 500 g/day was used.

6.5.3 Measured water vapour loss

As an alternative to estimating fractionated water vapour loss several investigations have either measured insensible water loss or calculated it by difference from water balance studies. Before applying these methods, however, it should be remembered that insensible water loss does not necessarily equal fractionated water loss. Specifically, sweat losses are not subject to isotopic fractionation and will be erroneously totalled with fractionated water loss if insensible water loss is measured by weight change or water balance under conditions where sweating occurs. Note, that even under cool conditions, sweat is produced on the palms and forehead ¹³. Conversely, measured insensible water loss may underestimate fractionated water loss under humid conditions. Atmospheric water vapour does enter the body both through the lungs and the skin ¹⁹. Thus, there will be a unidirectional influx of unlabelled water that will reduce the net insensible water loss, while not altering the unidirectional loss of isotopically fractionated water loss. For example, if a person inhales 1 litre of air saturated with water vapour at 20°C (19 mg/l) this water will be absorbed and replaced with labelled body water, which will be exhaled at 36°C and 95% saturation ¹⁷ (45 mg/l). The net insensible water loss will be $45 - 19 = 26$ mg, but the unidirectional water loss will be 45 mg. Water loss estimated from weight loss is thus a 40% underestimate of the true value, but even this would only introduce an error in carbon dioxide production of about 2% (Figure 6.2). If sweating occurs, then the error will be reversed because sweat losses increase the

weight loss and hence the estimation of insensible water loss, but sweat is not subject to isotopic fractionation ⁸.

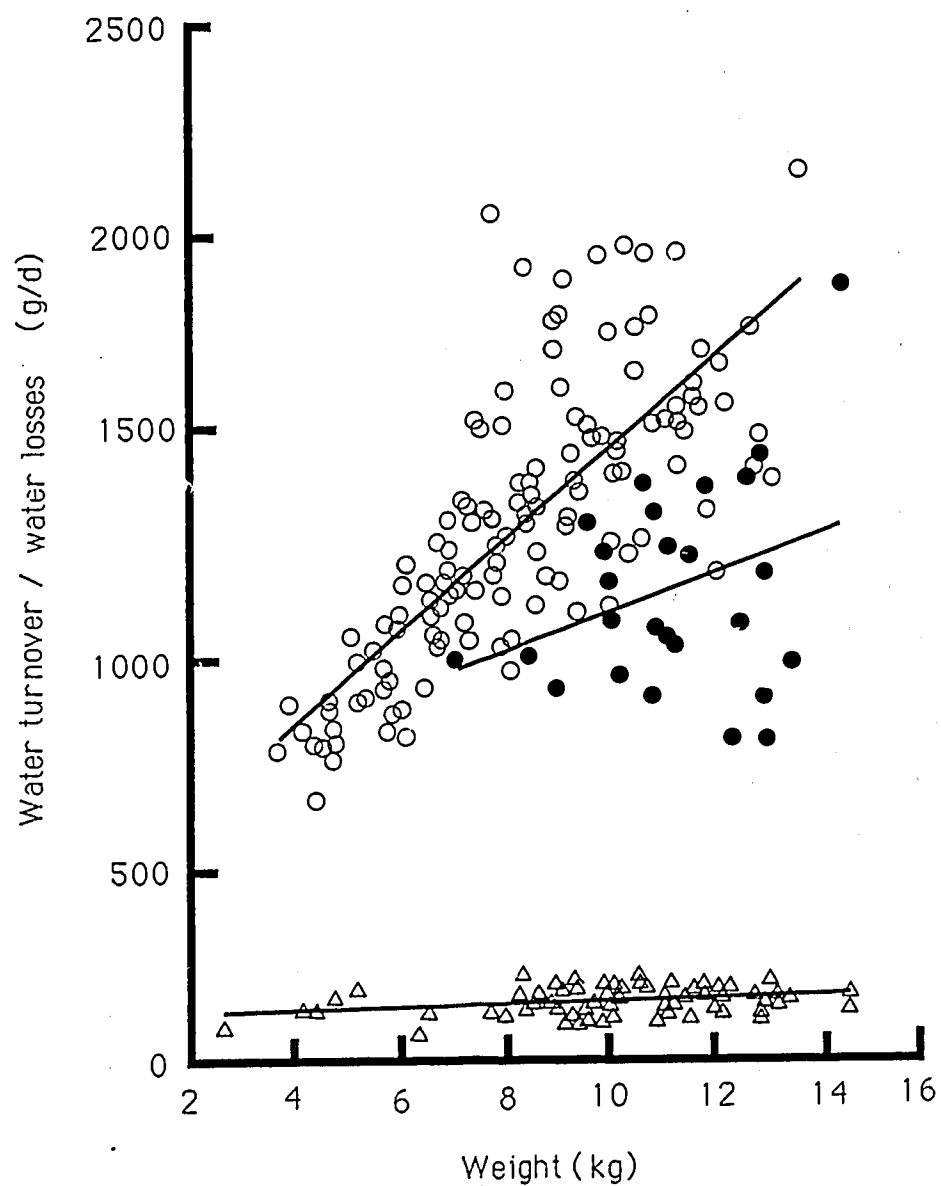
Recently, Vasquez-Velasquez has measured water vapour loss in infants living in The Gambia, West Africa and Cambridge, UK (PhD Thesis, University of Cambridge). Total water turnover was measured by deuterium elimination and insensible water loss was measured by the increase in water content of air passing over the infant while it was sleeping in an open circuit indirect calorimeter. Results are shown in Figure 6.3. Using a regression model, it was estimated that insensible water vapour loss was: $4.8wt(kg) + 96 (g/d)$. For the infants living in the UK this was always 13% of total water turnover. For the Gambian infants however, insensible water loss was about 17% of water turnover at 2 kg body weight and 8% at 16 kg body weight due to the different water turnover rates. It should be noted, however, that these are net insensible water losses. The true insensible water loss will be higher as it is partially obscured by insensible water input from the ambient humidity. Assuming 40% relative humidity at 25°C, the underestimate would be about 35% at 2 kg body weight and 25% at 16 kg body weight.

6.5.4 Triply-labelled water

An objective method for measuring the rate of fractionated water loss has been proposed by Haggarty et al ¹⁰. This is the triply-labelled water (TLW) technique. With this method 3H_2O , 2H_2O and $H_2^{18}O$ are co-administered. The tritiated water is added to the usual loading dose because the fractionation factors between water and water vapour differ for deuterium and tritium. Thus, a small but measurable difference in the deuterium and tritium elimination curves will be introduced as the percentage of water lost via fractionated routes increases.

Figure 6.3

Estimates of routes of water loss in Gambian children measured
at a cool temperature while asleep or resting



Data from Vasquez-Velasquez.

Equations 1 and 2 are not specific to deuterium and oxygen-18 but apply equally well to other isotopic species in water such as tritium or oxygen-17. Therefore after substitution of the appropriate tritium and oxygen-17 factors in these equations water flux and CO₂ production could be derived from tritium and oxygen-17 flux rates. Considering only the case of heavy hydrogen; if the true evaporative loss (x) and fractionation factors are known the corrected deuterium flux must equal the corrected tritium flux. Conversely, if the fractionation factors are known and the flux rates are determined experimentally, x can be solved using the following relationship:

$$x = \frac{F_D - F_T}{F_D(1 - f_{1T}) - F_T(1 - f_{1D})}$$

The obvious advantage of this method is that it provides an individual estimate of fractionated water loss and thus could minimise the error in calculated CO₂ production. The difference between the deuterium and tritium fractionation factors must be known with a relative accuracy of 25% if the triply-labelled water method is to offer much advantage over the other techniques. However, as discussed in Section 6.2.1 the relative contribution of kinetic and equilibrium isotope effects has little effect on the DLW method if the total amount of fractionated water loss is known. The same is true of the TLW method and the maximum error on the calculation of CO₂ production resulting from uncertainty about the predominant type of fractionation is only $\pm 0.3\%$ ¹⁰. There are values for tritium and deuterium fractionation factors in the literature, but further work needs to be done to improve the confidence in TLW. Schoeller et al¹⁵ have pointed out the difficulties of measuring fractionation factors *in vivo* but for the purposes of TLW it is only necessary to determine fractionation in a mixture of tritiated and deuterated water *in vitro*; this is currently the subject of experimental investigation.

The TLW method has the obvious disadvantage of using a radio-active isotope and will therefore be impossible to use in many circumstances. However, even if it does not provide much improvement in individual estimates of fractionated water loss, it will provide some valuable group estimates that should all but eliminate the already small potential errors in the fractionation correction.

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CHAPTER 7

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THE EFFECT OF ISOTOPE SEQUESTRATION AND EXCHANGE

7.1 Isotope incorporation into tissue and its effect on the DLW technique

This chapter sets out to describe the process of isotope sequestration and the nature of its effect on the DLW method; to present appropriate constants and numerical methods whereby the effect of sequestration may be calculated; to outline the evidence that sequestration occurs in human subjects; and to assess the likely limits of error in normal human subjects and in special groups where the problem of sequestration is exacerbated.

7.1.1 Labile and non-labile hydrogen

Label movement into and out of products other than CO_2 and water may be reduced to two distinct processes:

- a) exchange of water hydrogen and oxygen with labile hydrogen and oxygen;

- b) incorporation of water hydrogen and oxygen into, and release from, non-labile positions.

Labile hydrogens are found on carboxyl, hydroxyl, amino and sulphhydryl groups ¹, and non-labile hydrogens are those bonded to carbon ². Exchange is a rapid process but sequestration (ie incorporation of water hydrogen into, and release from, non-labile positions) occurs over longer periods. It is important to distinguish between these processes because they are dealt with in different ways in the DLW technique.

7.1.2 Exchange

The most obvious manifestation of the exchange process is found in the difference between body water and deuterium and oxygen-18 distribution spaces. This difference occurs because the $^2\text{H}_2\text{O}$ and H_2^{18}O distribute throughout the body water, but deuterium can also exchange with the labile hydrogens in body solids resulting in a dilution space greater than the body water pool.

Exchange of ^{18}O with bicarbonate also occurs, indeed the DLW technique is based on this fact, therefore the ^{18}O distribution space will also be greater than the body water space. Taking values derived by Irving et al ³ for the bicarbonate pool-size in man (11300 $\mu\text{mol/kg}$ body weight), together with the assumption that ^{18}O is in equilibrium with this pool and that 1 mole of bicarbonate can exchange oxygens with 3 moles of water it follows that the bicarbonate pool is equivalent to 0.6105 g water/kg body weight. Thus in a 50 kg subject with a body water pool size of 28 kg the ^{18}O distribution space will over-estimate body water by 30.525 g or 0.1%. In reality it has been observed that H_2^{18}O dilution over-estimates body water by approximately 1% suggesting that there are other exchange reactions involving ^{18}O .

Whatever factors are involved in the exchange processes the

only measurable effect on the DLW technique is to increase the isotope distribution spaces. The use of independent pool sizes ^{4,5} which are not necessarily equivalent to body water, is sufficient to deal with this phenomenon, at least with respect to exchange within the body. Exchange with material which is subsequently exported from the body (eg urine, faeces or milk) will for all practical purposes appear as sequestration and will therefore not be adequately dealt with by using independent pool sizes.

7.1.3 Sequestration

Deuterium incorporation into body solids during reductive biosynthesis creates the greatest concern with respect to sequestration. The term 'reductive' is used to describe any process whereby oxygen is removed from a compound and/or hydrogen is added. In many cases the added hydrogen has its origin in water. This is not surprising since water is the universal solvent for chemical reactions in the body and the hydrogen of water is rapidly exchangeable with many of the intermediates involved in the biosynthetic process. That sequestration occurs is well documented, and H isotope incorporation from water into protein and fat has even been used to measure turnover rates *in vivo* ^{6,7}. Unlike exchange, where the simple use of independent pool sizes is sufficient to deal with its effect on the calculation of r_{CO_2} , the process of sequestration can only be dealt with by estimating the rate of hydrogen isotope incorporation and by correcting the derived parameters of $r_{\text{H}_2\text{O}}$ and r_{CO_2} by the appropriate amount.

Water hydrogen is known to be incorporated into stable bonds but the important questions are: Does it occur to such an extent that it will have a measurable effect on the technique? If so, what is the likely magnitude of error? In order to answer these questions we must first obtain information on the stoichiometry

of water hydrogen incorporation into the main constituents of the body and second, we must know the rate of synthesis of these constituents.

7.2 The stoichiometry of sequestration

7.2.1 Fat

Fat is used here to describe free fatty acids, mono-, di- and tri-glycerides. The fatty acids which form the largest part of the glycerides are synthesised from 2-carbon units by the enzymes acetyl CoA carboxylase and fatty acid synthetase. The reductant in this biosynthetic process is NADPH and the hydrogen in water is freely exchangeable with the NADPH hydrogen, thereby providing the route of entry of water H into the stable carbon-hydrogen bonds of the fatty acid molecule. The stoichiometry of incorporation can be deduced from the empirical observations of early workers in the field who maintained mice on a constant intake of deuterated water over a period of weeks and then measured the deuterium content of the body fat. Only the saturated fats were studied and it was observed that 43 - 46% of the hydrogen atoms of newly synthesised fatty acids were derived from body water ^{2,8-10}. However, it has subsequently been pointed out by Jungas ² that the close agreement between these studies was fortuitous since the time interval used (21-98 days) "did not allow for the *de novo* synthesis of the entire carcass pool of saturated fatty acid" but that approximately "53% of the hydrogens of newly synthesised fatty acids would be derived from water as assayed with deuterium". Jungas was careful to specify deuterium because mass effects will cause the factor to be different for tritium. In studies where both isotopes were used Jungas observed that 17% less ³H than ²H was incorporated into newly synthesised fat. For the purposes of the calculation of sequestration the value of 53% will be used for the stoichiometry

of water H isotope incorporation when assayed with ^2H and 44% when assayed with ^3H .

In order to calculate the extent of incorporation we must also know the composition of synthesised fat. From data on the composition of human adipose tissue the average fatty acid formula can be calculated as $\text{C}_{17.13}\text{H}_{31.65}\text{O}$ (molecular weight 254 a.m.u.) for normal adult humans ¹¹. This gross composition changes very little between males and females or in pathological conditions such as coronary heart disease, diabetes and obesity. The empirical formula for the triglyceride of this fatty acid is $\text{C}_{54.41}\text{H}_{99.97}\text{O}_6$ (molecular weight 850 a.m.u.). Assuming that 53% of the hydrogens of newly synthesised fatty acid will be derived from water labelled with ^2H , synthesis of 1 mole of fatty acid will result in the incorporation of 16.78 moles of ^2H and deposition in the form of triglyceride will result in the sequestration of 50.34 moles of ^2H per mole of triglyceride, or 59.19 mmoles per gram. Were incorporation to be assayed with tritiated water the values would be 13.93, 41.78, and 49.13 respectively. In terms of equivalent water flux this translates into 0.5333 g of water/g fat synthesised (using $^2\text{H}_2\text{O}$) and 0.4427 g of water/g fat synthesised (using $^3\text{H}_2\text{O}$).

7.2.2 Protein

Similar experiments to those described for fat have also been carried out, largely by the same workers, to study the incorporation of water H into the stable carbon-hydrogen bonds of the amino acids which go to make up the body proteins. Unlike fat, however, there are a large number of exchangeable hydrogens in protein ¹. Early workers therefore took great care to remove these by washing before analysing the amino acids for deuterium in stable carbon-hydrogen bonds. Foster, Rittenberg and Schoenheimer ¹² measured the incorporation of ^2H from water into non-labile positions in nine amino acids of the hydrolysed protein of

rats maintained on deuterated water for 10-19 days and found that, on average, 0.9 atoms of ^2H derived from water were incorporated into stable carbon-hydrogen bonds per amino acid. The hydrogen of water is thought to be incorporated into the α -carbon of the amino acid during transamination⁷. In studies on transamination *in vitro*, Hilton *et al*¹³ found that 1.7 deuterium atoms were incorporated per molecule of aspartate. More recently Commerford *et al*¹⁴ performed similar *in vivo* experiments where mice were labelled with tritiated water from conception and looking at 16 amino acids they found that, on average, 0.41 atoms of heavy hydrogen were incorporated per amino acid. Using the figure of 17% less incorporation for ^3H than ^2H (derived for fat synthesis), we can calculate that approximately 0.5 atoms of ^2H from water would be incorporated per amino acid. The discrepancy between the values of 0.9 and 0.5 may partly be explained by the smaller number of amino acids used by Foster *et al*, since if Commerford *et al*'s analysis is restricted to the same amino acids the average is closer to 0.6. For the purposes of the calculation of sequestration the more conservative figure of 0.5, based on a larger sample of amino acids, will be used for water labelled with ^2H and 0.4 for water labelled with ^3H .

In order to calculate the extent of incorporation we must also know the composition of synthesised protein. The main proteins in the human body are collagen, actin, tropomyosin and myosin which respectively have 0.8965, 0.7919, 0.8133 and 0.8227 mmols of amino acids per gram of protein¹. Since these values are very similar, and in the absence of any knowledge of the respective rates of synthesis of each in the body, an average of 0.8227 mmols of amino acids per gram of synthesised protein will be used. Using this and the values for water H incorporation we can calculate that 0.4114 mmols will be incorporated per gram of protein when assayed with ^2H and 0.3291 mmols when the isotope is ^3H . In terms of water flux this represents 0.003706 g water/g protein synthesised (using $^2\text{H}_2\text{O}$) and 0.002965 g water/g protein synthesised (using $^3\text{H}_2\text{O}$).

7.2.3 Carbohydrate

The main storage form of carbohydrate in the body is glycogen and the stoichiometry of water hydrogen incorporation into liver glycogen has again been studied in small animals using deuterated water. Stetten and Klein ¹⁵ observed that about 38% of the hydrogen in liver glycogen arose from the body water when assayed with deuterium and that this deuterium was uniformly distributed among the non-exchangeable positions. This value varies slightly depending on the precursors available but 38% is a good approximation. Carbohydrate, like protein, has a large number of exchangeable hydrogens therefore care has to be taken to remove these before estimating the carbon bound deuterium. Stetten and Boxer ¹⁶ found that 34% of the hydrogens of glycogen are freely exchangeable with water when the label is ²H. From the chemical formula of glycogen it can be calculated that 22.33 mmoles of ²H derived from water will be sequestered per gram of glycogen synthesised. This translates into 0.2012 g water/g glycogen synthesised (using ²H₂O) and (using the ³H/²H mass discrimination effect observed during glycogen synthesis of 8% ¹⁷) 0.1851 g water/g glycogen synthesised (using ³H₂O).

For ease of reference the values derived above are summarised in Table 7.1.

7.3 The effect of sequestration on the performance of DLW

In order to illustrate the method of calculation of the effects of isotope sequestration it is helpful to use a completely defined system where all the relevant parameters have been experimentally determined. This is only possible in animal models where the end products can be extracted and their isotopic composition determined. The necessary analyses have been performed on the obese Zucker rat for fat and protein. This animal will serve as a useful example since its high rate of

Table 7.1

Water equivalents incorporated into fat, protein and carbohydrate
(assayed with $^2\text{H}_2\text{O}$)

	<u>Water equivalents (g/g)</u>		
	<u>Fat</u> ^a	<u>Protein</u> ^b	<u>Carbohydrate</u> ^c
Sequestration	0.5333	0.003706	0.2012
Exchange	0	0.1332	0.1801

^a As triglyceride: chemical formula $\text{C}_{54.11}\text{H}_{99.97}\text{O}_6$.

^b Consisting of an equal mixture of collagen, actin, tropomyosin and myosin.

^c As glycogen.

lipogenesis relative to body size should exacerbate the effect of sequestration on the performance of the DLW technique.

7.3.1 Calculation for an animal model

In order to clarify the calculation the usual units for r_{H_2O} and r_{CO_2} and synthesis rates of grams and litres have been converted to moles. All the relevant raw data was obtained in rapidly growing immature obese Zucker rats (25 days old). The rate of 3H flux in such animals is around 1.906 moles/day, and since we are assuming no fractionated water loss the value of 1.906 moles/day will be taken as equal to r_{H_2O} . r_{CO_2} measured by indirect calorimetry is approximately 0.2245 moles/day¹⁸. Because each CO_2 molecule contains two oxygen atoms the $H_2^{18}O$ flux which occurs as a consequence of r_{CO_2} will be 0.4489 moles/day. Whole-body lipogenesis measured by incorporation of tritium from 3H_2O was 0.002731 moles/day⁶. If deuterium is the hydrogen label then this will result in 53% of the hydrogens in newly synthesised fat being derived from water. Following the logic of Section 7.2.1 we estimate that 0.04582 moles/day of 3H will be incorporated. Since each molecule of water contains two atoms of hydrogen the extra water flux resulting from sequestration will be 0.02291 moles/day.

Protein synthesis was measured in rats of the same age and weight using the flooding-dose phenylalanine technique¹⁹. The rate of whole-body protein synthesis was 1.914 g/day. Using the composition of the average human protein we calculate that the rate of amino acid incorporation into protein was 0.001575 moles/day. Using an average figure of 0.5 water hydrogen atoms sequestered per amino acid we can calculate the atoms of water hydrogen incorporated to be 0.000788 moles/day. Again, since each molecule of water contains two atoms then the extra water flux resulting from sequestration will be half this figure: 0.000394 moles/day.

When calculating the consequences of sequestration on the DLW technique it is very important to distinguish between the effect on r_{H_2O} and r_{CO_2} . This concept is readily illustrated in Figure 7.1 for the obese rat calculation where the components of flux are represented graphically. It is apparent from the figure that the absolute amount by which r_{H_2O} is over-estimated is the same as that by which r_{CO_2} is under-estimated. Therefore, because r_{CO_2} is less than r_{H_2O} the percentage error on r_{CO_2} is greater. It is in fact greater by the molar ratio of $r_{H_2O}/(2.r_{CO_2})$. We can use this to calculate the error on r_{CO_2} as:

$$\frac{r_{H_2O}}{(2.r_{CO_2})} \times \text{the proportional effect on } r_{H_2O}$$

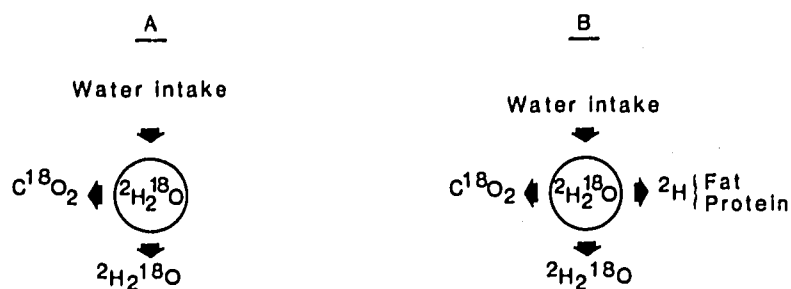
This ratio (or its analog which is often expressed in terms of the rate constants k_p/k_o) has already been shown to be important in many aspects of the heavy water technique (see Chapters 5 & 6). However, in this case the ratio is not of primary importance since the error on r_{CO_2} can be predicted simply from the rate of sequestration and the true r_{CO_2} . It serves only as an alternative method of calculation. Thus, fat synthesis in these animals will cause r_{H_2O} to be over-estimated by 0.019016 moles/day and the percentage effect on r_{H_2O} is +1.20%.

The effect of protein synthesis may be calculated in the same way and is +0.02%.

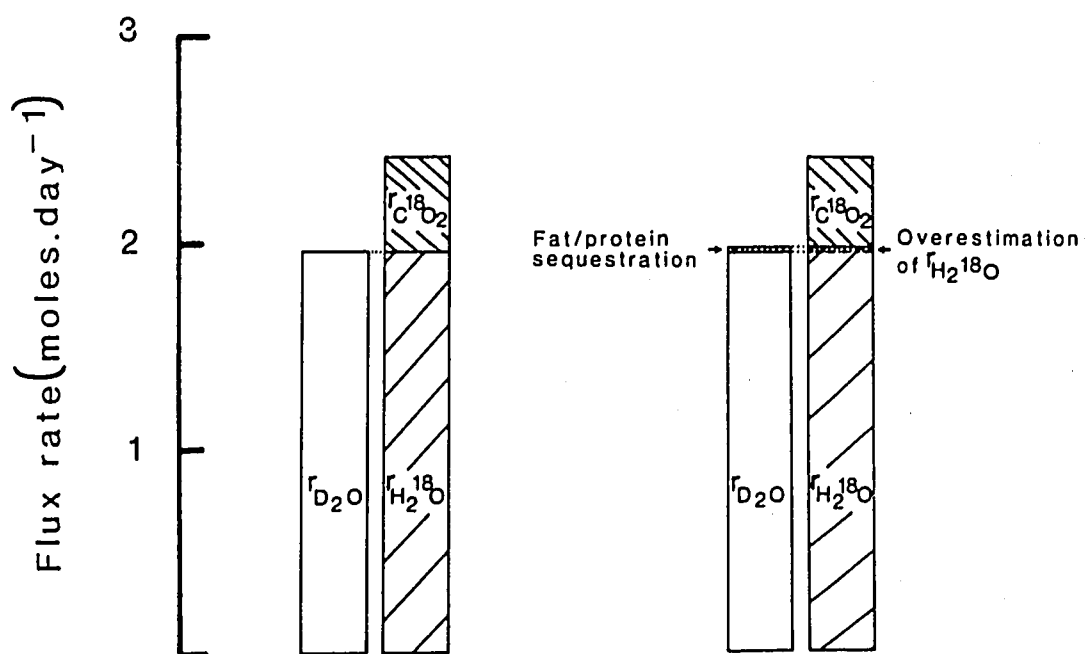
Using the molar ratio ($r_{H_2O}/2.r_{CO_2}$) of 4.25 and the above equation we can calculate the effect on r_{CO_2} of sequestration resulting from fat synthesis as -5.10% and that resulting from protein synthesis as -0.09%.

Figure 7.1

- a) Simple model describing $^2\text{H}_2^{18}\text{O}$ flux (A) and a model incorporating sequestration (B)



- b) The components of isotope flux in the genetically obese Zucker rat



7.3.2 Calculation of the effect in human subjects

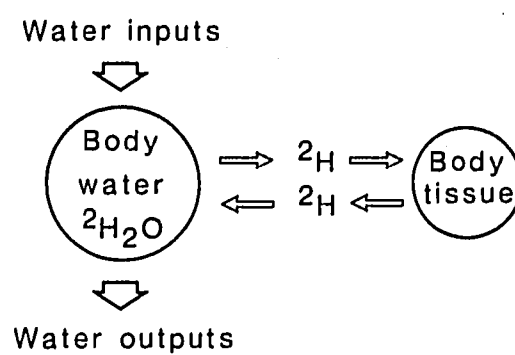
Unfortunately the process of sequestration in humans is very difficult to quantify because it is almost impossible to distinguish between ^2H lost from the water pool as water and that lost by incorporation into stable carbon-hydrogen bonds. In order to do this it would be necessary either to sample the fat and protein of the body in order to measure isotope incorporation, as in the obese rat, or to compare the true water flux, determined by meticulous water balance studies, with isotopic estimates of water flux; the difference being that component which had entered non-labile positions in the body. The former is not possible in human subjects and the latter would be futile since the errors on the measurements would greatly exceed the likely magnitude of sequestration. Since it is not feasible to directly measure the process of incorporation, indirect methods must be found.

7.3.2.i Experimental evidence for sequestration in humans

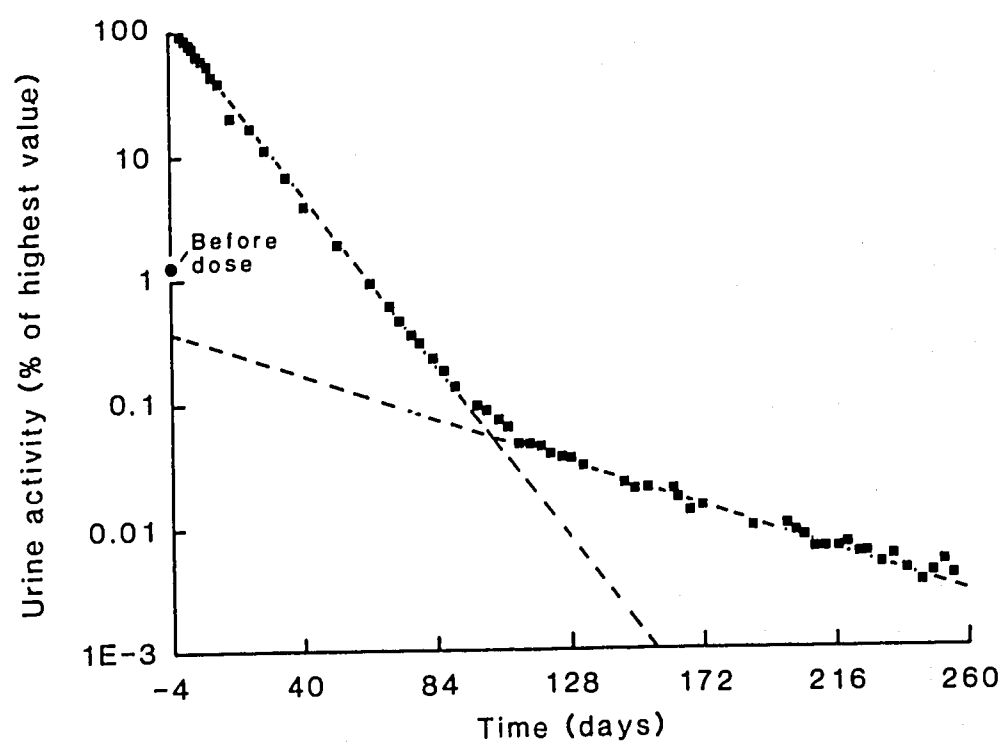
Isotopic hydrogen from water is incorporated into tissue but this is not simply a uni-directional process, it occurs as a result of the constant turnover of the constituents of the body, and we can therefore predict that ^2H will be re-released into the body water pool when labelled material is catabolised. This simple model of sequestration and release of water hydrogen is illustrated in Figure 7.2a. The body tissue is shown as a single pool and the single arrow in and out implies one rate of turnover. However, this is a simplification since we would expect the body tissue pool to be heterogeneous consisting, for example, of protein, fat and carbohydrate, and even of sub-pools turning over at different rates. Whatever the precise nature of this pool it should be possible to quantify the rate of incorporation from the amount re-released. However, in order to observe this recycling we have to sample the body water for much longer than the 14-21 days normally used in a human DLW study. Such extended

Figure 7.2

a. Model for isotope sequestration and release



b. Tritium concentration over 260 days in a subject exposed to $^3\text{H}_2\text{O}$ (from Snyder et al ²²)



monitoring of H isotopes in the body water has been carried out for workers in the nuclear industry who have been accidentally contaminated with high activity tritiated water. The subjects were monitored for periods of up to one year to assess the radiological risk associated with persisting isotope in the body. The data obtained from such subjects uncovers a number of points which we do not see during the relatively short time course of a DLW experiment, points which help to elucidate the process of sequestration. These studies show that isotope decay is more complex than our simple models suggest. They yield double exponentials ²⁰⁻²², triple exponentials ²³ and exponentials in combination with sines and damped sines ²⁴. The latter two models imply that the sequestered hydrogen resides in a heterogeneous pool with more than one rate constant. Whatever the number of pools, these secondary components to the curve only become apparent at around 100 days after dosing (see Figure 7.2b) but the effect of this slowly turning over compartment (half-life approximately 34 days), although masked, is present throughout the labelling period. Figure 7.2b was drawn from experimental data presented by Snyder *et al* ²² for an adult (41 years) weight-stable male after contamination by tritiated water. Apart from the appearance of the second exponential at 100 days there is some evidence of a sine wave at 200-260 days. These secondary components represent labelled hydrogen which has been incorporated from water into stable carbon-hydrogen bonds during reductive biosynthesis. Because the compounds are constantly turning over then this hydrogen is released back into the body water during catabolism thereby superimposing the kinetics of a secondary pool on the primary process of single exponential decay which we normally see in DLW studies.

The theoretical premise that sequestration should occur in humans is therefore borne out by this complicated description of hydrogen isotope decay but the question remains as to the likely effect of such a process on the DLW technique? Snyder *et al* ²²

calculate that approximately 1.7% of the ^3H flux passed through the slowly turning over secondary pool, or pools, which represent the carbon-bound hydrogen in body tissue. This would cause a direct over-estimate of $r_{\text{H}_2\text{O}}$ of 1.7% and an even greater under-estimate of r_{CO_2} . The actual effect can be calculated from knowledge of the error on $r_{\text{H}_2\text{O}}$ and the molar ratio of $r_{\text{H}_2\text{O}}/(2.r_{\text{CO}_2})$ as described in Section 7.3.1. A typical ratio of $r_{\text{H}_2\text{O}}/(2.r_{\text{CO}_2})$ in human subjects is 3.64²⁵ which results in an error on r_{CO_2} of -5.59%. Also, this effect was estimated using ^3H as the hydrogen isotope and, because of mass effects, the error on r_{CO_2} would be increased to -6.74% with ^2H (see Section 7.2.1).

This calculation was based on the assumption that the total H isotope ingested by the subject was in the form of water at zero time. We can however see that 3 days before contamination, the subject already had significant levels of ^3H in the body water. This will have the effect of over-estimating the proportion of ^3H flux which passes through the secondary pool since some of the re-released isotope will have been incorporated at an earlier time and will not be derived from the estimated dose at the beginning of the decay curve. Therefore, studies on such subjects serve only to illustrate qualitatively that sequestration of water hydrogen does occur, that it can be experimentally detected and that we can obtain information on the half life of the sequestered hydrogen. Quantitative conclusions cannot be drawn from this example.

7.3.2.iii Calculation of sequestration from rates of deposition

Another method of estimating the effect of sequestration on the DLW technique would be to assume that any fat and protein deposited is synthesised *de novo*, and to calculate the effect of tissue deposition from knowledge of the stoichiometry of ^2H incorporation into fat and protein^{4,26}. The results of such hypothetical calculations, using data on changes in body composi-

Table 7.2

The effect of weight gain on the DLW technique (using $^2\text{H}_2^{18}\text{O}$)

Composition of gain ^a

Weight (g/day)	+143.6
Fat (g/day)	+87.9
Protein (g/day)	+9.5

The effect of protein deposition ^b

% error on $r_{\text{H}_2\text{O}}$	+0.02
% error on r_{CO_2}	-0.07

The effect of fat deposition ^b

% error on $r_{\text{H}_2\text{O}}$	+1.43
% error on r_{CO_2}	-5.19

^a From Norgan and Durnin ²⁷.

^b Calculated assuming values for an adult male of: body water = 44 l; $r_{\text{H}_2\text{O}}$ = 3.3 l/day; r_{CO_2} = 560 l/day; and molar ratio $r_{\text{H}_2\text{O}}/(2.r_{\text{CO}_2})$ = 3.64 (Schoeller et al ²⁵).

tion during extreme weight gain in adults ²⁷, are shown in Table 7.2. As in the obese rat by far the greatest effect arises from the synthesis of fat (5% underestimate of r_{co_2}). This is quantitatively more important than protein synthesis not only because more fat is deposited but also because fat incorporates 144 times more water H than an equal weight of protein.

In two respects the example given in Table 7.2 is likely to give an exaggerated assessment of error. Firstly it assumes a weight gain of 1 kg/week which is normally only achieved by deliberate over-feeding or during rehabilitation from illness. Secondly, it assumes that all of the fat gained was synthesised *de novo* which is known to be untrue on the high fat diets (35-40%) typical of developed countries. When energy requirements are exceeded such diets provide an excess of fat which may be deposited directly, therefore the assumption of total *de novo* synthesis will over-estimate the effect on r_{co_2} . However, in under-privileged rural communities in most areas of the developing world the average diet contains only 5-20% of energy from fat and 70-85% from carbohydrate. This level is comparable with the very low fat diets fed to small animals and which result in high levels of lipogenesis. Therefore when assessing the likely magnitude of sequestration in DLW studies it is important to distinguish between groups on high and low fat diets. Set against this is the fact that developing country diets are usually marginal and the people consuming them are unlikely to be depositing much fat. A third limitation of the calculation would result in it being over-optimistic. This relates to the fact that it uses a figure for net synthesis based on deposition and takes no account of fat and protein turnover which could result in fat synthesis and therefore sequestration without any change in the amount of body fat. Such a situation did occur in the weight-stable subject studied by Snyder et al ²² (see Section 7.3.2.i).

It would be preferable to avoid these ambiguities by using rates of synthesis measured directly with tracer techniques, and

such data have been obtained for protein synthesis in humans in many different physiological states. Using the calculations described above it can be shown that one of the highest rates of protein synthesis observed (286 g/day²⁸) accounts for only 1 g/day of water flux leading to a maximum error on r_{CO_2} of 0.11% in normal adults. In infants $r_{\text{H}_2\text{O}}$ would be over-estimated by around 0.03% and r_{CO_2} under-estimated by 0.21%. These estimates will be further reduced if a proportion of the synthesised protein is broken down on the same day. The magnitude of degradation rates suggest that this could occur but we have no direct evidence.

Unfortunately, whole-body fatty acid turnover has not been measured in human subjects largely because there are no equivalent techniques to the ¹⁵N glycine and carbon labelled amino acid procedures, therefore we cannot draw quantitative conclusions as to the likely effect of fatty acid turnover in the body. Glycogen turnover has also proved difficult to measure but in this case we know that at least in adult weight-stable subjects, the effect on r_{CO_2} is less than 1%; if all the estimated 500 g of glycogen in the body turned over within a 14 day DLW study this would result in the sequestration of 100 g of water equivalents or 7.1 g/day, and for a typical molar ratio of $r_{\text{H}_2\text{O}}/2.r_{\text{CO}_2}$ in an adult human of 3.64 this would translate into an error on r_{CO_2} of -0.8%.

7.3.2.iv Validation studies

Another way to estimate the effect of sequestration on the DLW technique would be to compare the values for r_{CO_2} derived isotopically with independent measurement of respiratory gas exchange for example. If a process such as sequestration were to have a measurable effect on the calculation of r_{CO_2} we might expect to find discrepancies between r_{CO_2} measured by independent methods and that measured isotopically. A number of these validations have been carried out in adults (see Chapter 1, Table

1.2). In all but one of these studies DLW over-estimated r_{CO_2} ; the average over all the studies was +2%. In two of the validation studies in infants DLW provided a 0-1% under-estimate of r_{CO_2} whilst the third study found that DLW under-estimated r_{CO_2} by 8.7%. Thus with the exception of this last study, DLW has been reported to yield values which, within the stated precision of the method, agree well with independent estimates of r_{CO_2} and if there is any bias it is toward over-estimation of r_{CO_2} . Since sequestration would lead to an under-estimate of r_{CO_2} we might come to the conclusion that no sequestration had occurred in these subjects or that the effect was negligible. Indeed these results would be very encouraging were it not for the fact that r_{CO_2} was calculated by each group in a different way. This highlights the problem of using such validation studies to assess the validity of individual assumptions such as 'there is no loss of isotope in products other than CO_2 and water' or that 'our estimates of fractionated water loss are correct'. In order to use this approach we have to be sure that, with the exception of the assumption to be tested, all the other factors which affect the calculation are known and that they are invariant. For example, Haggarty et al ²⁹ have attempted to estimate the effect of sequestration by comparing r_{CO_2} measured by DLW and by respiratory gas exchange in rapidly growing pigs. They found that r_{CO_2} measured by DLW was under-estimated by 4.8% during rapid growth. However, the calculation of r_{CO_2} from isotope flux rates is very sensitive to the estimate of fractionated water loss ^{4,30} and the 4.8% under-estimate of r_{CO_2} could be completely removed by reducing the assumed value for fractionated water loss by 0.25. Since we would expect sequestration in human subjects to be lower than that found in rapidly growing pigs on a low fat diet then the errors on r_{CO_2} due to sequestration could easily be obscured by small errors in our estimate of fractionated water loss.

7.3.2.v Special groups

The validation studies described in the previous section, although spanning a large age range, were performed in what might be termed 'normal' subjects in that they were not in any unusual physiological state with the possible exception of the validation of Westerterp et al ³¹ where subjects underwent strenuous activity. When considering sequestration our concern is with physiological states which involve increased reductive biosynthesis or accelerated turnover of the body constituents, states which often occur simultaneously. Apart from the growth of infants and children the main states in which reductive biosynthesis occurs are during pregnancy and lactation.

Pregnancy

Protein synthesis will not materially affect the calculation of r_{CO_2} during pregnancy. With respect to fat synthesis Coward ⁴ has calculated the effect of a pregnant woman depositing 4 kg of fat in nine months (approximately 15 g/day). He suggested that the error introduced into the DLW technique (making the exaggerated assumption that the deposited fat is entirely synthesised *de novo*) would be of the order of 1%. Using slightly different assumptions we have also found the error on $r_{\text{H}_2\text{O}}$ and r_{CO_2} to be small; +0.20% and -1.72% respectively. However, it has already been pointed out that this approach is flawed because the contribution of dietary fat to deposition is not known and because no account is taken of the continual turnover of the body fat (Section 7.3.2.iii).

Lactation

Protein can be ignored since it has been shown above that a protein synthesis rate 100 times that of the protein exported in

milk has little effect on r_{CO_2} . Assumptions about the maximum likely amount of *de novo* fat synthesis during milk production can be drawn from the fatty acid composition of milk. On this basis Prentice & Prentice ³² have estimated that women on high and low fat diets synthesise approximately 12% and 36% of their milk fat respectively giving absolute daily synthesis rates of about 3.6 and 10.8 g/day. (The remainder is transferred directly from the diet.) From these values it can be calculated that exported fat in milk could cause r_{CO_2} to be under-estimated by 0.32 - 1.00% (Table 7.3). The export of lactose may result in a further error of about 1.11% giving a combined effect for sequestered ²H of about 1.4 - 2.1%.

7.3.3 The effect of sequestration on the assumption of steady state

So far we have only considered the effect of sequestration on the calculation of $r_{\text{H}_2\text{O}}$ and r_{CO_2} over the full duration of a DLW study but there is another possible consequence which would arise because of discontinuous sequestration and release of H isotope. Such a process would introduce variability into the deuterium decay data and consequently reduce the precision with which $r_{\text{H}_2\text{O}}$ and r_{CO_2} are calculated without necessarily causing the over-estimation of $r_{\text{H}_2\text{O}}$ and under-estimation of r_{CO_2} described previously. The main concern would be fluctuations in glycogen, the levels of which change markedly in response to carbohydrate supply and demand. Liver glycogen turns over very rapidly being reduced by an overnight fast to less than half its pre-fasting value. Depletion and repletion of muscle glycogen can also be very rapid during and after exercise. Rapid sequestration associated with the replenishment of muscle glycogen after exercise will introduce variability into deuterium decay by causing transient sequestration of ²H which may be re-released during subsequent bouts of exercise. For instance, deposition of 300g of muscle glycogen over a 12 hour period would cause a transient 3.7% over-estimate in $r_{\text{H}_2\text{O}}$ and a consequent 13.3%

Table 7.3

The effect on the DLW method of sequestration into the components of human milk ^a

	<u>Well nourished</u>	<u>Poorly nourished</u>
<u>Fat</u>		
Water equivalents in fat (g/day) ^b	1.92	5.75
% error on r_{H_2O}	+0.06	+0.19
% error on r_{CO_2}	-0.32	-1.00
<u>Lactose</u>		
Water equivalents in lactose (g/day) ^c	8.73	8.73
% error on r_{H_2O}	+0.22	+0.22
% error on r_{CO_2}	-1.11	-1.11
<u>Combined effect of export of sequestered hydrogen</u>		
% error on r_{H_2O}	+0.28	+0.41
% error on r_{CO_2}	-1.43	-2.11

^a Calculated using values for a lactating woman of: body water = 30 l; r_{H_2O} = 3.0 l/day; r_{CO_2} = 370 l/day; and molar ratio $r_{H_2O}/(2 \cdot r_{CO_2})$ = 5.05 (Prentice et al, unpublished data).

^b Calculated from Table 7.1.

^c Calculated from Table 7.1 (assuming stoichiometry of sequestration is the same as for glycogen).

under-estimate in r_{CO_2} .

7.3.4 Sequestration of oxygen

The above discussion has concentrated exclusively on the problems of ^2H sequestration largely because the reduced state of the body constituents (H:O ratio for fat = 32, for protein = 5, and for carbohydrate = 2) means that ^2H sequestration will have a much greater impact on the DLW method than ^{18}O sequestration. However, it must be remembered that there are possible routes of ^{18}O sequestration. These have not been quantified and cannot therefore be discussed in detail, but their effect will always be to offset some of the error on r_{CO_2} incurred due to ^2H sequestration.

7.4 Export of exchangeable hydrogens

As already pointed out the exchange of water hydrogen and oxygen with material which is subsequently exported from the body will for all practical purposes appear as sequestration and should be dealt with as such.

7.4.1 Urine and faeces

Schoeller et al ³³ have estimated isotope losses in urine and faeces in adult man to be 0.47% of ^2H flux and 0.34% of ^{18}O flux. Note that these authors found that no more than 10% of isotope losses in urine and faeces were exchangeable suggesting that most are the result of true sequestration and not just due to export of exchangeable hydrogen. The resulting error on $r_{\text{H}_2\text{O}}$ in such subjects would be +0.47% which represents 15.46 g of water equivalents/day. This additional flux taken alone would cause r_{CO_2} to be under-estimated by 1.7%. However, because of the

simultaneous loss of 0.34% of the ^{18}O flux these additional losses will largely cancel out resulting in a small error on r_{CO_2} of -0.75%.

7.4.2 Milk

There are no exchangeable hydrogens in milk fat if we assume that it is all in the form of triglyceride.

The number of exchangeable hydrogens on protein can be calculated using the procedure described by Culebras & Moore ¹. The main proteins in milk are casein and lactalbumin and from their amino acid composition it can be calculated that they contain respectively, 1.665 and 1.789 moles exchangeable hydrogen per 100g protein. Taking an average value of 1.727 moles it can be estimated that 100 g of milk protein will exchange with 0.8634 moles of water equivalents (0.1556 g water equivalents/g protein). From this we can calculate the error on $r_{\text{H}_2\text{O}}$ and r_{CO_2} for an average lactating mother as shown in Table 7.4. As with true sequestration resulting from protein synthesis the effect on r_{CO_2} of export of exchangeable hydrogen in protein is very small at less than 0.15% (Table 7.4).

Using the same procedure it is possible to calculate the potential error on $r_{\text{H}_2\text{O}}$ and r_{CO_2} introduced by export of exchangeable hydrogen in lactose which has 8 hydroxyl groups and therefore 8 exchangeable hydrogens (Table 7.4). The number of exchangeable hydrogens per gram of protein and lactose is very similar but since lactose production greatly exceeds that of protein in milk the effect on r_{CO_2} will be larger (-0.9 to -1.2%).

The combined effect on r_{CO_2} of export of exchangeable hydrogen on protein and lactose is -1.0 to -1.3%. Since exchange is known to occur and since it can be estimated relatively accurate-

Table 7.4

The effect on the DLW method of export of exchangeable hydrogen in human milk ^a

	<u>Well nourished</u>	<u>Poorly nourished</u>
<u>Lactose</u>		
Water equivalents in exchangeable groups (g/day) ^b	9.13	6.74
Error on r_{H_2O}	+0.23	+0.17
Error on r_{CO_2}	-1.16	-0.85
<u>Protein</u>		
Water equivalents in exchangeable groups (g/day) ^c	1.14	0.87
Error on r_{H_2O}	+0.03	+0.02
Error on r_{CO_2}	-0.15	-0.10
<u>Combined effect of export of exchangeable hydrogen</u>		
Error on r_{H_2O}	+0.26	+0.19
Error on r_{CO_2}	-1.31	-0.95

^a See Footnote A in Table 7.3.

^b Molecular weight of lactose = 342 a.m.u.. Eight exchangeable hydrogens per molecule of lactose.

^c 1.727 moles exchangeable hydrogen/100 g protein.

ly from the chemical formulae of compounds this value is likely to be a true estimate of the effect on r_{CO_2} and is not subject to the same qualifications as the estimation of sequestration in fat.

7.5 The effect of sequestration/exchange on related techniques

There are two techniques related to the DLW method which would also be subject to errors introduced by H isotope incorporation into products other than water. These are the triply-labelled water (TLW) method of measuring fractionated water loss and r_{CO_2} ³⁰ and the milk transfer technique ³⁴.

TLW depends on the different fractionation factors for evaporative $^3\text{H}_2\text{O}$ and $^2\text{H}_2\text{O}$ loss but the mass effects which give rise to the differential fractionation also cause isotope discrimination during sequestration. Preferential incorporation of ^2H over ^3H into non-labile positions would appear as fractionated water loss and would therefore over-estimate the TLW-derived value for fractionated evaporative water loss.

In the milk transfer method one of the assumptions is that ^2H is transferred from mother to baby only in the form of water. However, ^2H can also be transferred from mother to baby in sequestered positions and on exchangeable groups in the components of milk. Taking a nominal milk water transfer of 600 ml/day we can predict from the values presented in Table 7.4 that export of exchangeable hydrogen to the baby will result in an over-estimate of milk transfer of 1.3 to 1.7%. Transfer of H in sequestered positions in lactose will further increase this over-estimate to 2.7 to 3.2%. When H sequestered into fat is included the final error on estimated milk transfer is 3.5 to 3.7%.

7.6 Summary and conclusions

Any loss of ^2H or ^{18}O as products other than CO_2 and water will introduce an error into the calculation of $r_{\text{H}_2\text{O}}$ and r_{CO_2} . In addition to ordinary water flux, hydrogen can be lost from the body water as sequestered hydrogen bound to carbon in fat, protein and carbohydrate or as hydrogen in the exchangeable positions of compounds which are subsequently exported from the body. To a limited extent ^{18}O may also be sequestered and exported as exchangeable ^{18}O and this will partially offset any error due to ^2H loss. The proportional effect of these processes on the isotopically-derived values for $r_{\text{H}_2\text{O}}$ depends on the rate of sequestration/exchange and the true $r_{\text{H}_2\text{O}}$. From the molar ratio of ^2H flux in water to ^{18}O flux in CO_2 in humans it can be predicted that r_{CO_2} is 3 to 5 times more sensitive to these processes than $r_{\text{H}_2\text{O}}$.

Experimental constraints make it very difficult to quantify the effect of sequestration in human subjects. There are however some things that we can state with some degree of certainty:

- 1) The effect of protein synthesis can be ignored when calculating the effects of sequestration.
- 2) Glycogen turnover is unlikely to introduce significant bias into the DLW technique although cycles of sequestration and release could introduce additional variability into ^2H decay.
- 3) The largest sequestration effect is likely to occur during fat synthesis simply because the fatty acids are the most reduced class of compound in the body.
- 4) Even in weight-stable subjects there is constant turnover of the body constituents (notably fat) which will result in sequestration. However, we cannot as yet assess the

74
magnitude of this effect.

- 5) Loss of isotope in urine and faeces will introduce an error into r_{co_2} corresponding to approximately -4 l/day in 'normal' adult male subjects therefore workers may wish to add a 'correction factor' of +4 l/day to their estimate of r_{co_2} .
- 6) During lactation the export of exchangeable hydrogen bound to solids in milk will result in an under-estimate of r_{co_2} of 1.0 - 1.3%. Isotope sequestration may further increase this value to 1.5 - 3.4%.
- 7) Under extreme anabolic conditions and using pessimistic assumptions regarding *de novo* fat synthesis the maximum error on r_{co_2} due to ^2H sequestration was estimated at -5%. Although there are a number of uncertainties inherent in this calculation it seems unlikely that the error would be as high as this under many circumstances.

The relatively small effects which can be quantified should not be dismissed too lightly since all the processes outlined here cause r_{co_2} to be under-estimated and will therefore be additive perhaps resulting in measurable consequences for the DLW technique. However, until more information is obtained on turnover of body constituents we must conclude from the circumstantial evidence of the validation studies that, within the limits of our knowledge of the other factors which affect the DLW technique (fractionated water loss for example), ^2H sequestration does not have a measurable effect on the calculation of r_{co_2} in 'normal' subjects.

7.7 References

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CHAPTER 8

Contributor: Dale Schoeller

CHANGES IN ISOTOPIC BACKGROUND

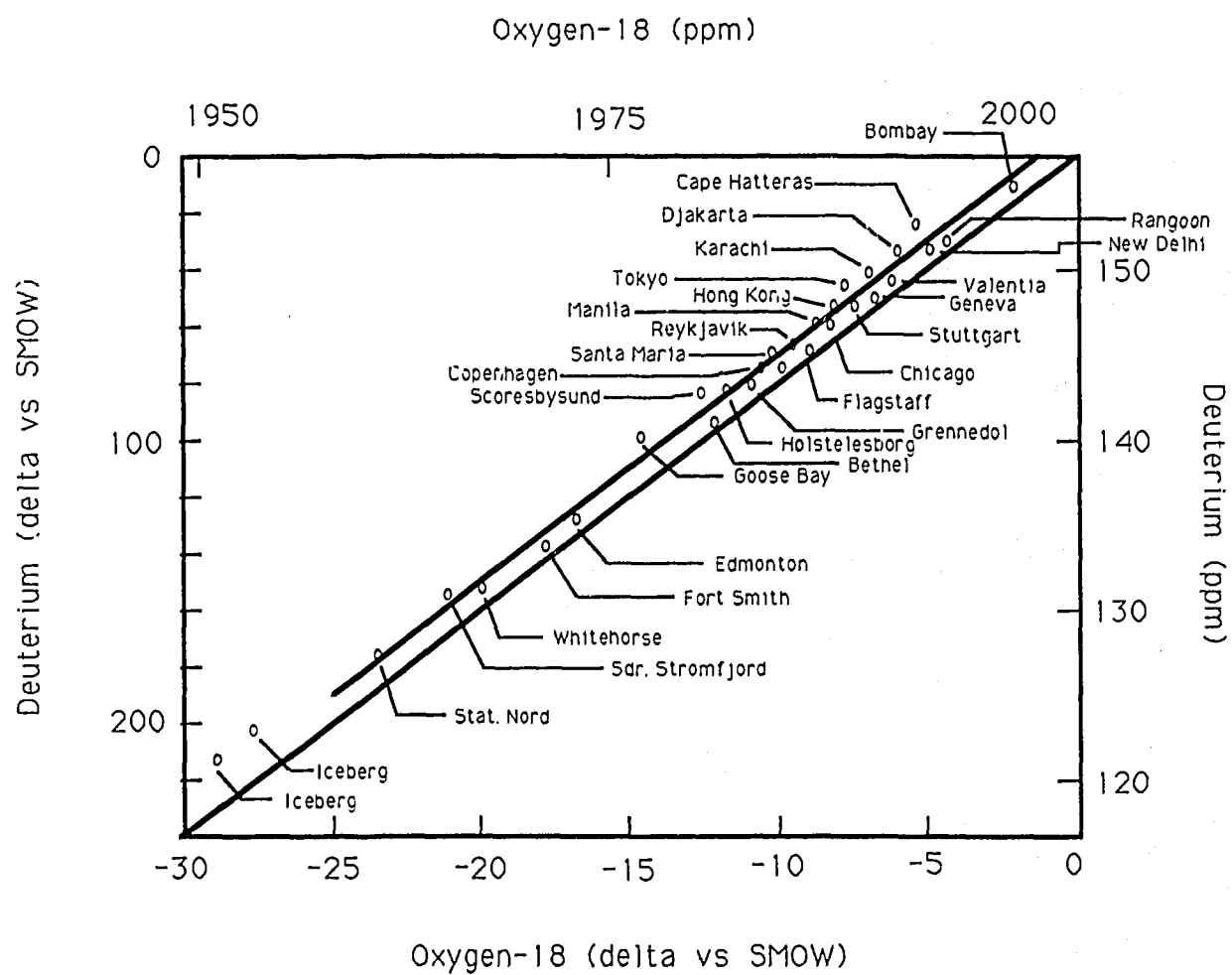
8.1 All water is not created equal

Both deuterium and ^{18}O are naturally occurring isotopes that are present in the body's organic compounds prior to the administration of doubly-labelled water. As such, tracer studies depend not on measurement of isotope concentration, but rather on concentration in excess of natural abundance or background isotope concentration. This would present little problem if the natural abundances were constant or if the isotopic tracers were dosed far in excess of natural abundance. Unfortunately, there are variations in isotopic natural abundances and these variations are not insignificant at economic doses of doubly-labelled water. The problem is most significant for ^{18}O because of its high cost.

The nominal natural abundances of ^2H and ^{18}O are 155 and 2000 ppm respectively, but range from 80 to 200 ppm for ^2H and 1900 to 2100 ppm for ^{18}O . This may appear to be a small variation, but typical doses of doubly-labelled water only produce excess isotope abundances of 100 and 200 ppm for ^2H and

Figure 8.1

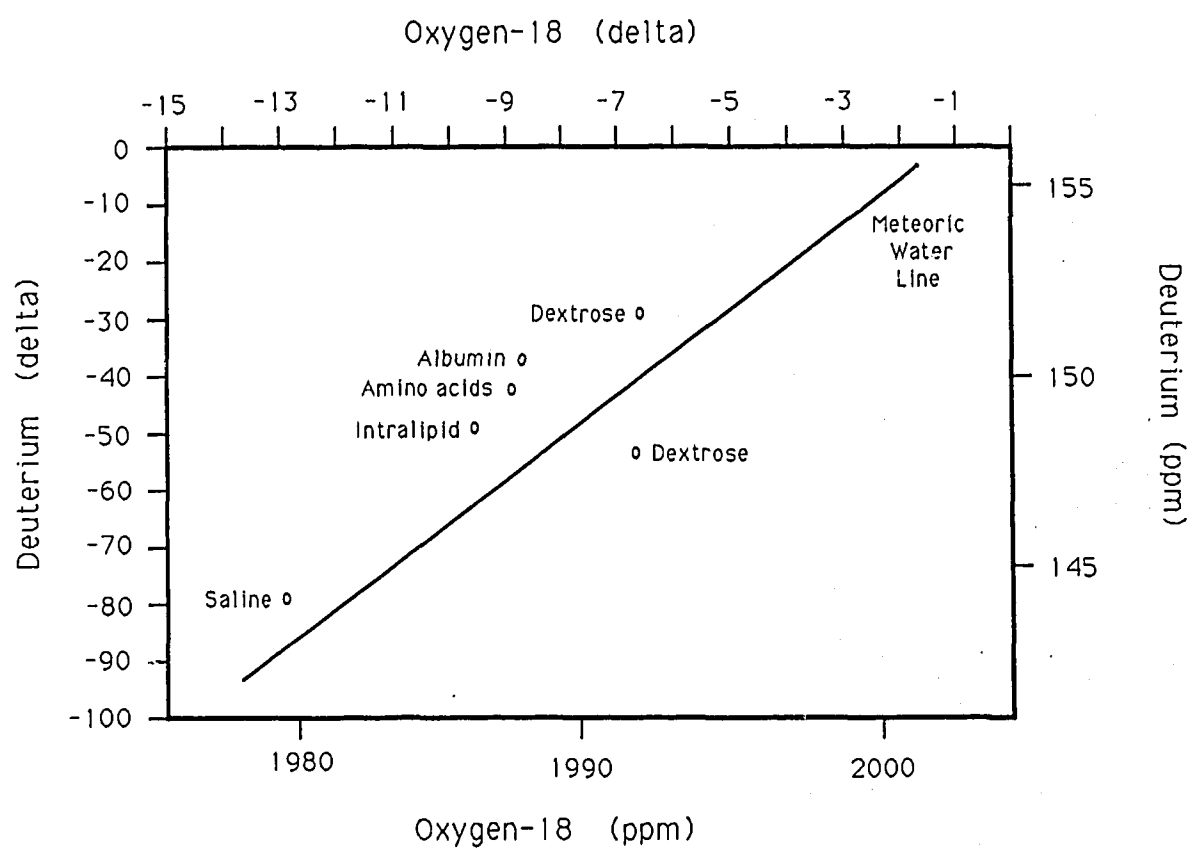
Global relationships between ^2H and ^{18}O abundances in precipitation



Adapted from Dansgaard ¹

Figure 8.2

Altered relationship between ^2H and ^{18}O in products manufactured from distilled water



¹⁸O respectively.

As discussed in Chapter 3, natural variations in isotopic abundance are easier to discuss in 'per mil' units relative to an international standard. Per mil units (‰) are defined as the difference in the ratio of the heavy to light isotope relative to the ratio in the standard, times 1000, or alternatively as 10 times the percent change:

$$\delta = \frac{R_u - R_{std}}{R_{std}} \times 1000, (\text{‰})$$

Where R is the ratio of the heavy to light isotope, u is the unknown or sample, and std is the international standard. The commonly used standard for ²H and ¹⁸O in water is Standard Mean Ocean Water (SMOW). A relative abundance of 0 ‰ is identical with SMOW, a negative relative abundance indicates less heavy isotope than SMOW, and a positive relative abundance indicates more heavy isotope than SMOW. Recast in per mil units, the natural range of deuterium abundances is -450 to +50 ‰; and that of ¹⁸O is -60 to +50 ‰ (Figure 8.1).

These natural variations result from the accumulation of isotope effect as these elements are cycled through the hydro and biospheres. Of these natural variations, those of water have the greatest influence on the doubly-labelled water method. This is because water is the major source of hydrogen and oxygen that flows into body water and it has the major influence on the isotopic abundances of ²H and ¹⁸O in body water. Water is ingested either as a beverage or as moisture in food and is largely from fresh water derived from precipitation. The processes controlling the isotopic abundances of ²H and ¹⁸O in fresh water have been extensively described in hydrology literature¹. In brief, most meteoric water is largely derived from the oceans by evaporation. This evaporative process is governed by a kinetic isotopic process that depletes both ²H and ¹⁸O relative to SMOW.

There is 3 ‰ change in the per mil enrichment of ^2H for each 1 ‰ change in ^{18}O enrichment under these conditions. This corresponds to a 0.25 ppm change for ^2H for each 1 ppm change for ^{18}O .

As the water vapour travels inland and away from the equator, the air cools, reaches the point of supersaturation and rain or snow falls. Again this process is subject to isotope fractionation. Under most conditions however, this fractionation is governed by an equilibrium process in which the precipitation is more enriched than the remaining water vapour. Thus, as this water vapour continues to travel inland, it becomes more and more depleted and the precipitation becomes more and more depleted. Thus, on the simplest level, rain near the equator has an isotopic abundance near that of SMOW, while that near the poles is highly depleted. Because this process is governed by equilibrium isotope fractionation, the ^2H abundance changes 8 ‰ for each 1 ‰ change in ^{18}O abundance. Meteoric water, when plotted in per mil units with ^2H on the y axis and ^{18}O on the x axis therefore falls on a line with a slope of 8 (Figure 8.1) ¹. This corresponds to a slope of 0.6 when the data is plotted in ppm.

As this water falls through the atmosphere and when it collects on the surface of the earth, it is again subject to evaporation. This evaporative process is again subject to a kinetic isotope effect which carries off the lighter isotopes leaving progressively heavier water behind. This process is most noticeable in areas subject to long dry periods, the extreme examples of which are semi-arid areas. Changes in ^{18}O of up to 20 ‰ have been noted ².

In addition to natural variations, human intervention can also introduce isotopic differences in source water. The most obvious example is heating to either distill or boil water. Under most circumstances, this will lead to a kinetic isotopic effect. If the water is partially distilled, then the recaptured water vapour will be lighter than the mother liquor. Solutions for

parenteral administration are good examples of this phenomenon (Figure 8.2). The change in deuterium enrichment will be about 3 ‰ for each 1 ‰ change in the ^{18}O enrichment, which will displace the solution to the right of the meteoric water line. Conversely, boiled water will be heavier than the starting water as the lighter isotopes are lost as water vapour. The degree of fractionation will be dependent on the percent of water lost during boiling.

8.2 Observed baseline changes

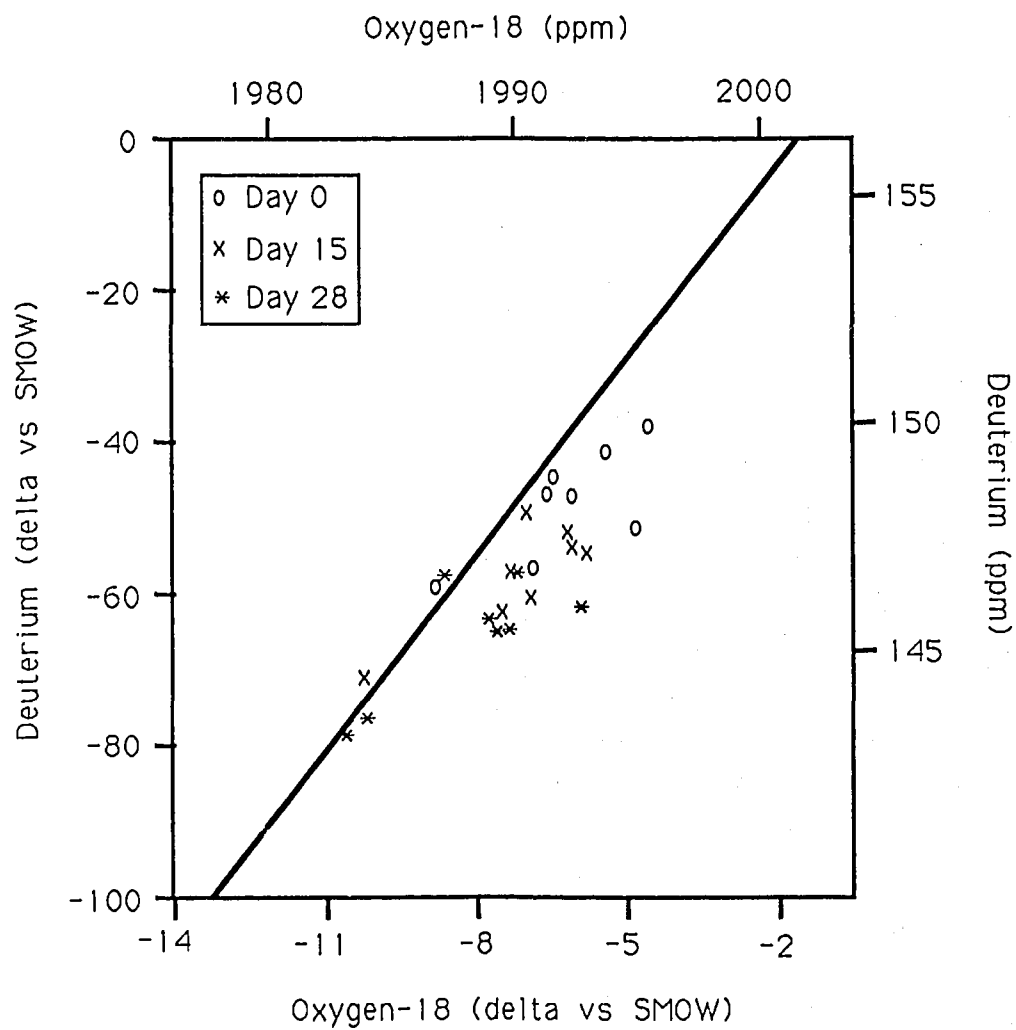
Several doubly-labelled water studies have been performed under conditions that result in changes in the baseline isotopic enrichment. DeLany et al ³ were investigating energy expenditure in soldiers during an exercise in which the soldiers moved from their base camp to a field camp in a low mountain range. Urines were collected periodically during the one month exercise and a significant decrease in the isotopic enrichment was noted (Figure 8.3). The changes in ^2H and ^{18}O isotopic abundances were 17 and 2 ‰ for ^2H and ^{18}O respectively. This is very close to the ratio of 8 to 1 expected for changes in drinking water in which both water sources lie on the meteoric water line.

Comparable changes in isotopic backgrounds would be observed in the case of the subject moving to another country or hemisphere during the DLW measurement, as calculated by Klein et al ⁴.

Seasonal variations have also been noted in two recent studies. Riumallo et al ⁵ performed a series of repeated doubly-labelled water studies in subjects living in Santiago, Chile. Baseline samples were collected at 12 week intervals when all measurable excess doubly-labelled water should have been washed out of the subjects. Deuterium abundances, however, increased from -81 to -59 ‰ (vs SMOW) and ^{18}O abundances increased from

Figure 8.3

Change in ^2H and ^{18}O abundances in soldiers during a one month field exercise in Vermont



-9.4 to -8.7 ‰. These changes do not illustrate the typical 8 to 1 ratio, and thus may not be due solely to a seasonal change in drinking water. Unfortunately, drinking water samples were not collected and thus isotopic analyses could not be performed. In a more complete baseline study, Coward et al (unpublished), analysed baseline samples collected throughout the year from subjects living in The Gambia, Africa. These samples showed a marked seasonal variation between the wet and dry seasons (Figure 8.4). The large variations are not atypical for locales which alternate between a very wet and very dry climate. The isotopic enrichment observed in the dry season is probably caused by the partial evaporation of surface water which leaves the water progressively heavier as the dry season progresses. Both of these observations contrast the nearly constant isotopic abundances observed in individuals living in Chicago ⁶. Drinking water in Chicago comes from either Lake Michigan or deep wells which are both large bodies of water that show little seasonal variation due to their large size relative to annual input from precipitation.

The third example of baseline change was reported by Schoeller et al ⁷ in patients placed on total parenteral nutrition. The water in the parenteral fluids was isotopically unusual because it was obtained by distillation. The ¹⁸O abundance was fortuitously similar to Chicago drinking water so there was little change in the ¹⁸O abundance, but there was a large shift in the ²H abundance (Figure 8.5). Because this was a simple step change in isotopic abundance, the isotopic abundance of body water demonstrated a classic mono-exponential change as the body equilibrated to the new isotopic input.

On the basis of a cross-sectional study, Roberts et al ¹⁰ reported significantly different isotopic backgrounds between breast-fed and formula-fed infants. In addition, as with the studies described above, the ratio of ²H to ¹⁸O abundance changes in the groups was substantially less than 8:1. The authors calculated the effects of an infant undergoing weaning

Figure 8.4

Seasonal variation in ^2H and ^{18}O abundances in subjects living in The Gambia

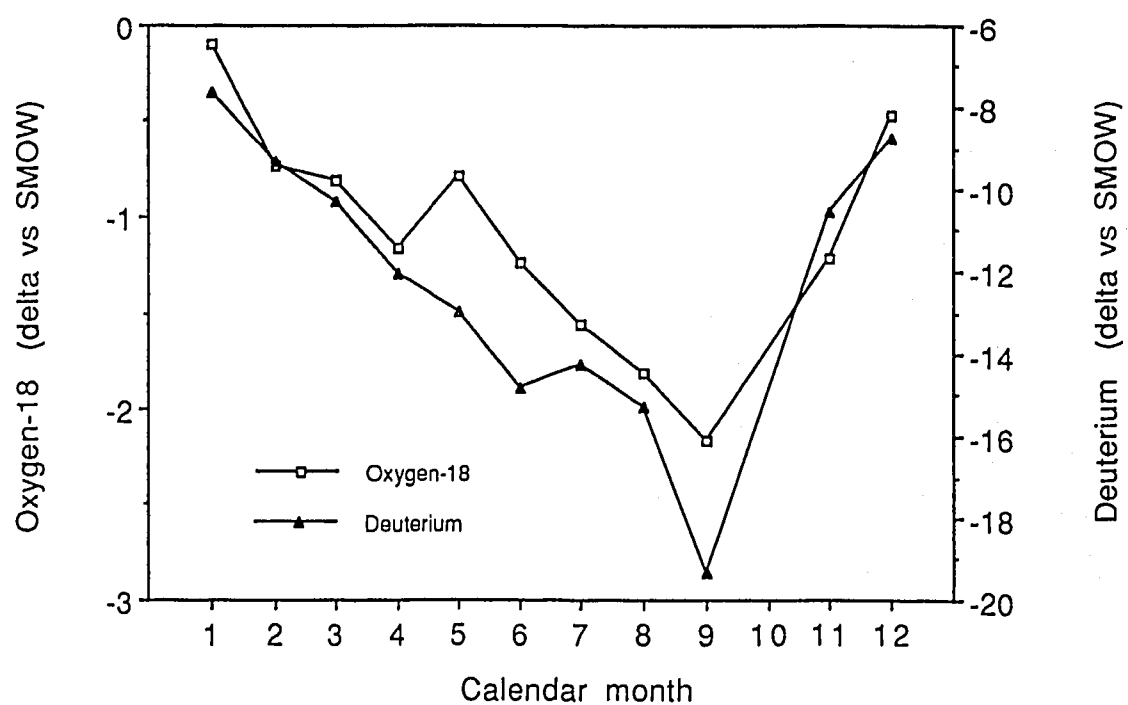
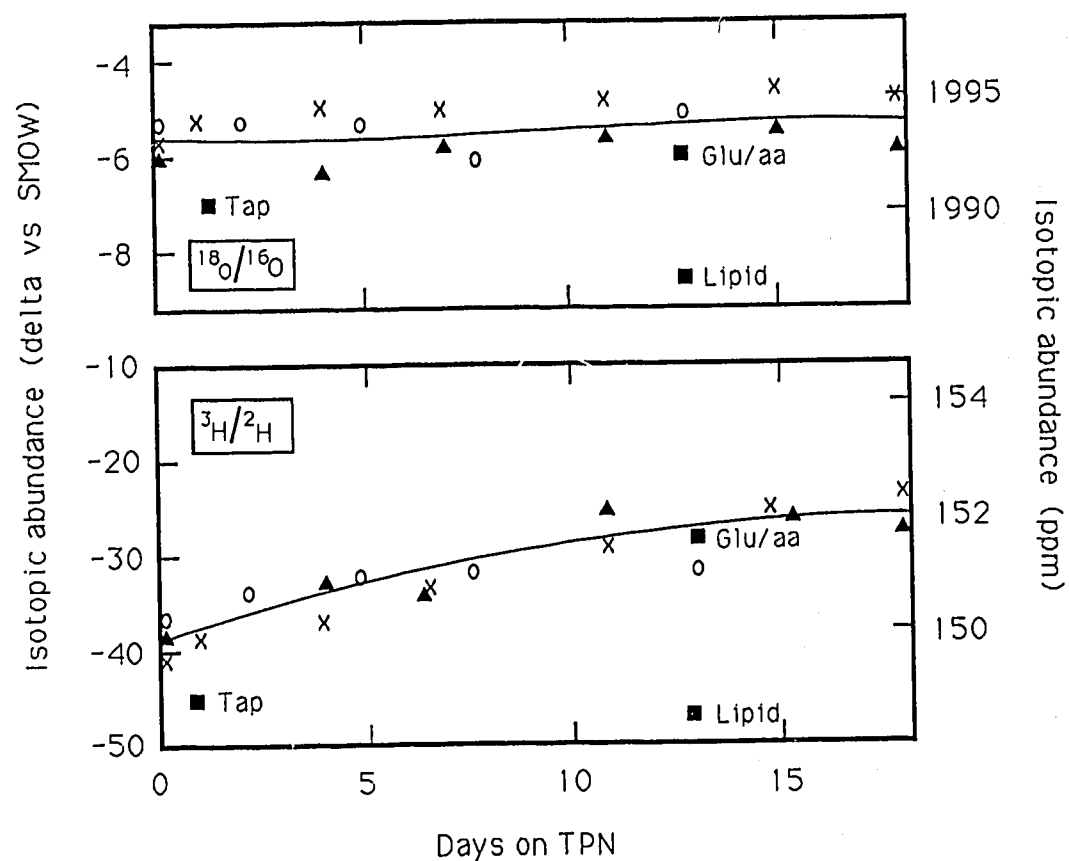


Figure 8.5

'Imbalanced' change in ^2H and ^{18}O abundances in patients started on total parenteral feeding



Adapted from Schoeller et al ⁷

during a DLW measurement from breast milk to formula. The magnitude of error varied greatly with study duration and isotope dose intake, ranging from 1-20%. It should be noted that this represents an extreme scenario in which it would be imprudent to use DLW.

8.3 Model of isotopic abundance of body water

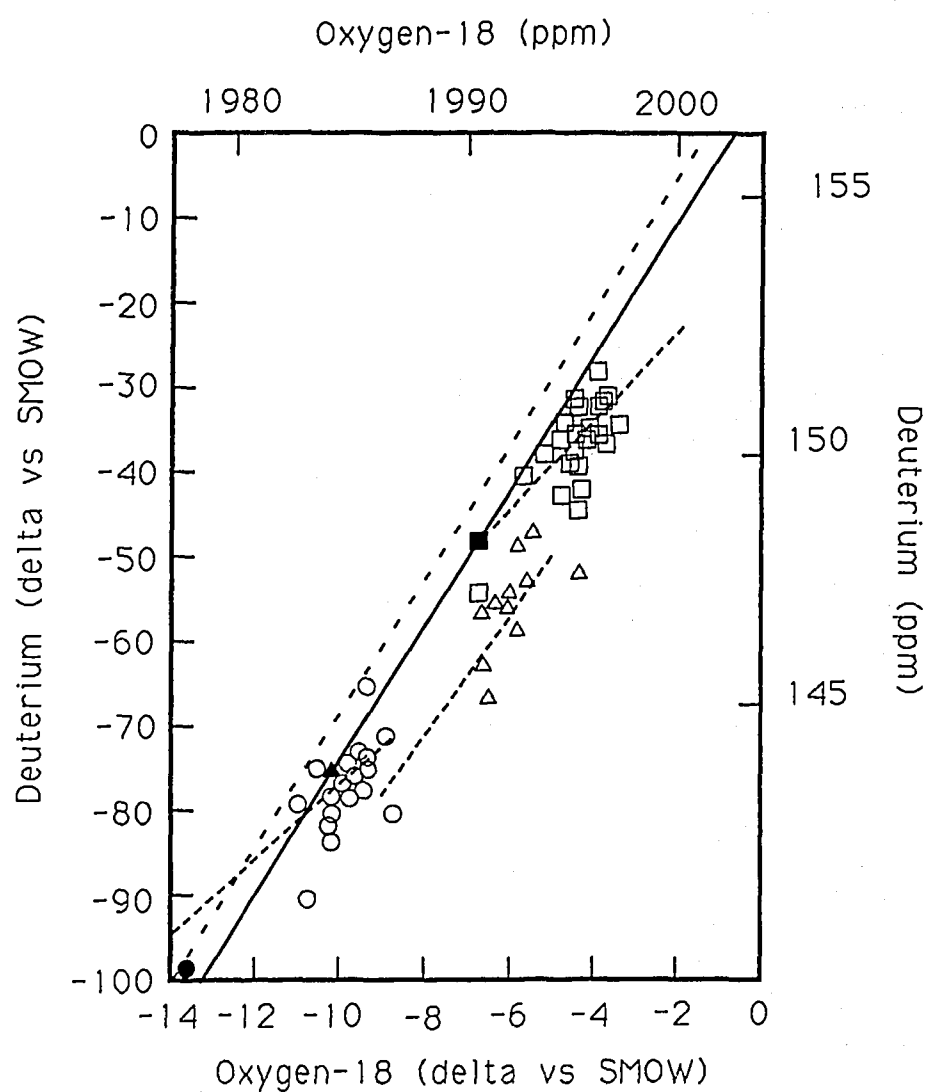
As indicated above, the isotopic abundance of body water and hence the baseline isotopic abundances for use with doubly-labelled water are highly influenced by the isotopic abundances of preformed water in food and beverages. The abundance, however, is not equal to that of preformed water, but rather tends to be enriched in both isotopes relative to preformed water (Figure 8.6). This reflects the effects of isotope fractionation during evaporative water loss caused by processing.

The isotopic abundance of body water is actually a balance between the isotopic abundances of all the hydrogen and oxygen entering the pool and the isotope effects that tend to carry the lighter isotopes out of the body. Under these conditions, the isotopes reach a steady state at enrichments greater than the input material at the time when average isotopic abundances of all material entering the water pool equal those of all material leaving the pool. The inputs to body water include preformed water, metabolic water, molecular oxygen, and traces of water vapour; while the outputs include liquid water, fractionated water vapour, small amounts of solid waste carbon dioxide (Figure 8.7). The steady state equations for the isotopic abundances are therefore ^{18}O :

$$\begin{aligned} & r_{\text{H}_2\text{O}} R_{\text{H}_2\text{O}} + r_{\text{FO}} R_{\text{FO}} + 2r_{\text{O}_2} f_4 R_{\text{O}_2} \\ & = r_{\text{H}_2\text{O}} R_{\text{BW}} + r_{\text{H}_2\text{O}} f_2 R_{\text{BW}} + 2r_{\text{CO}_2} f_3 R_{\text{BW}} \end{aligned}$$

Figure 8.6

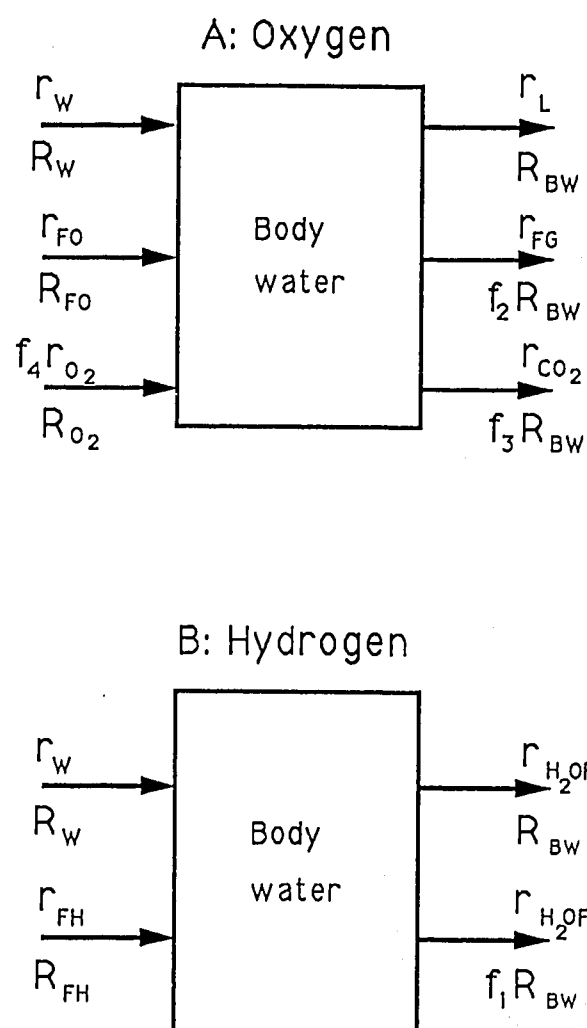
Deuterium and ^{18}O abundances in humans (open symbols) are enriched relative to drinking water (solid symbols)



Meteoric water lines for the Northern (---) and Southern (- - -) Hemispheres are drawn for reference. Regression lines and individual data points from top to bottom are for Chicago, USA (open boxes); Lausanne, Switzerland (open triangles) and Lima, Peru (open circles). Adapted from Schoeller et al ^a.

Figure 3.7

Models for oxygen and hydrogen isotopic balances



Isotopic abundances are expressed as the ratio (R) of heavy-to-light isotope; isotope fractionation relative to body water is symbolised by f_1 , for ^2H in water vapour, f_2 for ^{18}O in water vapour, f_3 for ^{18}O in CO_2 and f_4 for O_2 . Rates (r) are expressed in moles/d. Subscripts indicate the following: local water source (W), O_2 from food (FO), hydrogen from food (FH), molecular O_2 , CO_2 , body water (BW), liquid water loss (H_2OL) and fractionated water vapour loss (H_2OF). Adapted from Schoeller et al ⁸.

and ^2H :

$$r_{\text{H}_2\text{O}} R_{\text{H}_2\text{O}} + r_{\text{FH}} R_{\text{FH}}/2 = r_{\text{H}_2\text{O}} R_{\text{BW}} + r_{\text{H}_2\text{O}} f_1 R_{\text{BW}}$$

where the symbols are defined in Figure 8.7. This is similar to the model published previously by Schoeller et al ⁸ except that it includes a fractionation factor for the uptake of molecular oxygen.

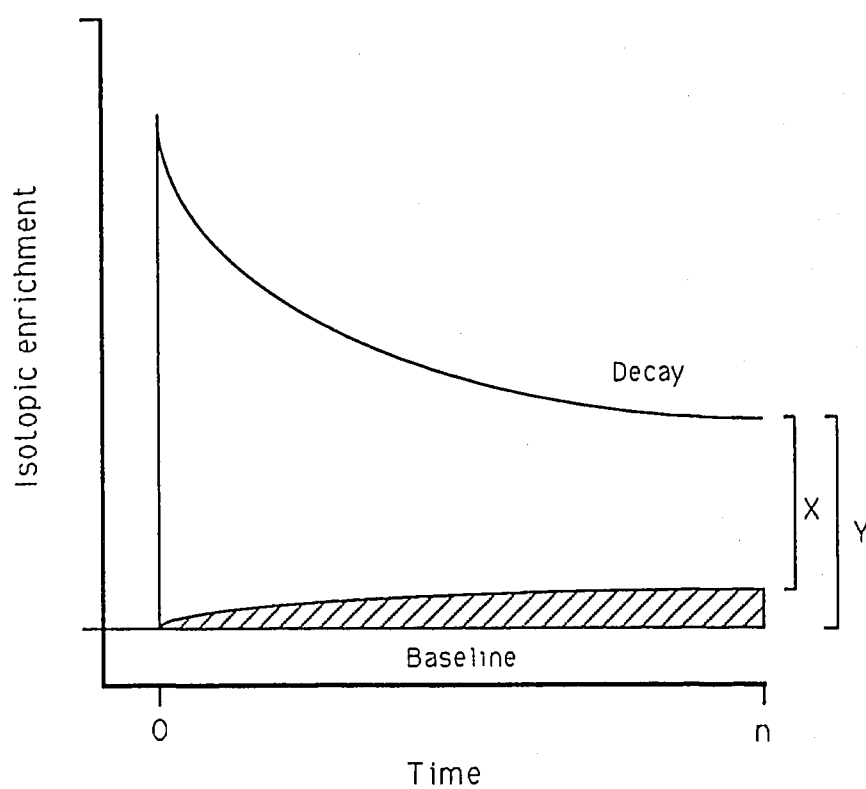
8.4 Use of doubly-labelled water under conditions of changing baseline

A change in the baseline isotopic abundance of body water can introduce a significant error into a doubly-labelled water study. Figure 8.8 illustrates the problem in which a change in the baseline results in an erroneously low calculated turnover rate resulting from an undetected increase in the isotopic baseline during the washout period. The central problem is that the change in the baseline cannot be detected in an individual after administration of the isotope unless the washout period is extended for about 10 elimination half-lives such that all the excess isotope is eliminated. This obviously requires a long time and is rather impractical.

Because a major cause of a baseline change is the change in the abundances of water coming into the body water pool, one of the simplest methods of reducing potential errors is to ensure that the subjects are fully equilibrated on the water source to be used in a study. This is not typically a problem when the subjects remain in the same locality and maintain a relatively constant diet ⁶. If there is a dietary change, however, such as with the implementation of total parenteral nutrition, then the investigator can sometimes afford to wait for a new baseline to be established before administering doubly-labelled water and in this way avoid any error due to the changing baseline. If the

Figure 8.8

Effect of change in the basal isotopic abundance on the apparent enrichment of samples collected during the elimination period



The true enrichment X will be erroneously estimated as Y . Adapted from Jones *et al* ⁹.

change in the isotopic abundances of the input is not too large - for example <2 ‰ or 3 ‰ for ^2H or ^{18}O , respectively, then this only requires 2 to 3 biological half-lives of water turnover. This approach was used by Schoeller et al ⁷ in a validation of doubly-labelled water in patients receiving total parenteral nutrition.

If the delay for re-equilibration is impractical, then other approaches can be used. The first is to include a placebo group as part of the doubly-labelled water study. These subjects are treated the same as the treatment group, except that they do not receive doubly-labelled water. Physiologic samples are collected from the placebo group in parallel to the treatment group. The baseline change is measured in the placebo group and used to correct the apparent enrichments in the treatment group. This method was used by DeLany et al ³ in soldiers during a field exercise.

The third approach is to model the anticipated baseline change from knowledge of the initial baseline and the change in isotopic abundances of the input water. This method is more complex than the placebo method, but is more flexible because it does not require the assumption that all subjects will behave identically to the change in the isotopic abundance of the input water. This approach has been used by Jones et al ⁹ in infants being weaned from total parenteral nutrition.

Although the placebo and calculated baseline change approaches have been used and seen to give valid results, the coefficient of variation of the doubly-labelled water method did increase ^{3,10}. The exact loss of precision is not known, but probably ranges between 2 and 10% depending on the magnitude of the baseline change and the dissimilarity of the subjects' responses.

The final, and possibly the most important method, for

reducing the error associated with changing isotopic backgrounds on the calculated CO_2 production rate is either to increase the isotope dose given, or to reduce the study duration within the recommended range of 1-3 half-lives for ^2H disappearance. This is because it is the magnitude of isotopic abundances of body water remaining at the end of the study that determine the importance of isotopic background changes, and these are in turn determined by the isotope dose intake and study duration. An example of the effect of these factors was given by Roberts et al ¹⁰ in the study of isotopic backgrounds in infancy. They determined that for an infant undergoing complete weaning during a doubly-labelled water measurement, the error associated with the determination of CO_2 production rate would be 6-18% for a study lasting 7 days (equivalent to 3 half-lives for ^2H disappearance) when a moderate isotope dose was used compared to only 3-8% where a 5 day study was employed.

Another important factor that influences the precision of the doubly-labelled water method in the face of changes in baseline is the ratio of ^2H to ^{18}O in the loading dose. Because the doubly-labelled water method depends on the difference in the elimination rates, error in elimination rates can be tolerated if it is identical for both isotopes. Thus, if the loading dose is adjusted such that the per mil enrichments of ^2H and ^{18}O above initial baseline are in the same ratio as the baseline changes in abundance, then no error will be introduced to the calculated CO_2 production rates ^{6,9}. Schoeller ⁶ has discussed the selection of optimal dosing ratios, and further consideration is given in Appendix 5 which presents a simple formula for predicting the required doses.

8.5 References

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CHAPTER 9

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PRACTICAL CONSEQUENCES OF DEVIATIONS FROM THE ISOTOPE ELIMINATION MODEL

9.1 Assumptions and the model

The model for the doubly-labelled water method described by Lifson ¹, is based on two key assumptions about the behaviour of body water. These are that the amount of body-water is constant and that it turns over at a constant rate. This means that disappearance curves for $^2\text{H}_2\text{O}$ and H_2^{18}O added to the system are predicted to be mono-exponential.

Strictly speaking neither of these assumptions is correct for any living organism. Water intake and excretion and carbon dioxide production rates change with time, and such changes will affect the linearity of isotope disappearance curves. It will be obvious that if such deviations from the average value are small and of short duration relative to the total measurement period they can be considered as random physiological noise and will have little effect on our calculations. However it is not entirely inconceivable that such changes could be large both in deviation from average values and in terms of duration. In this

case it is possible that our techniques for calculation may not be appropriate and may produce biased results. A further factor to consider is random measurement error in all the parameters that enter the model. Thus, the key question is how much error in carbon dioxide production is introduced by random and systematic deviations when current methods of data reduction are applied.

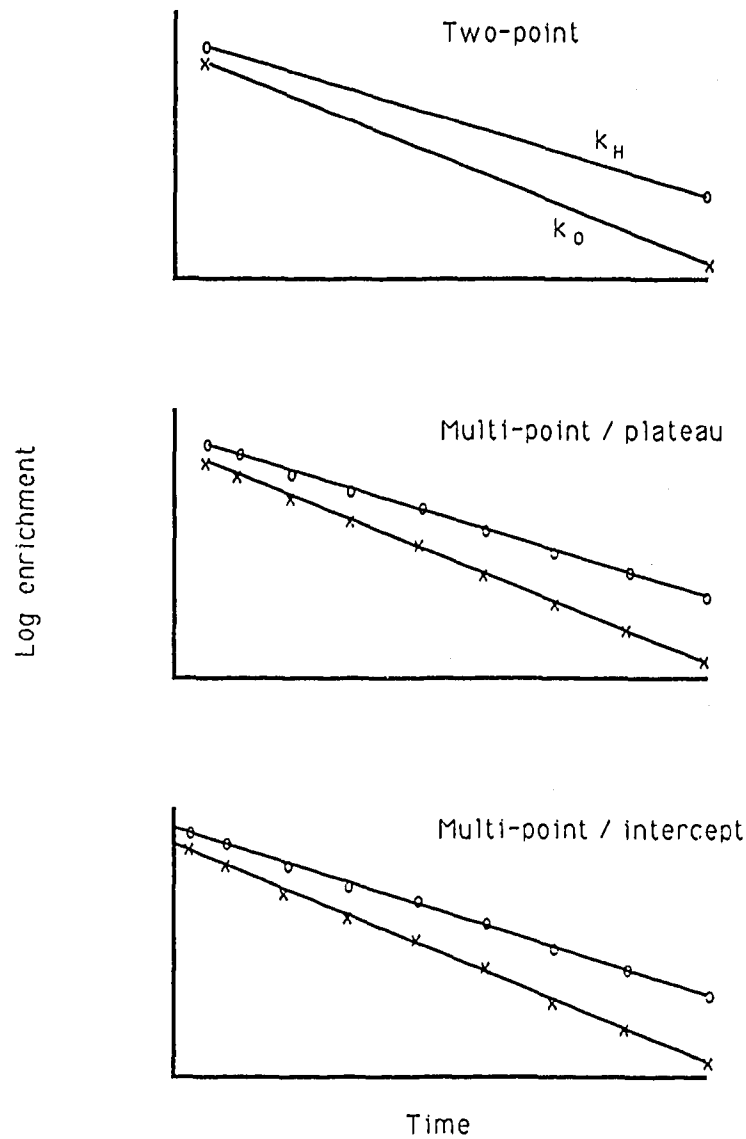
As has been discussed in Section 4.2 the basic formula for reducing the isotopic data for the calculation of CO_2 production is:

$$r_{\text{CO}_2} = 0.5 (k_o N_o - k_p N_p)$$

Investigators, however, have chosen three basic approaches to calculating the elimination rates. They have been calculated using the original two-point method ^{1,2} in which the isotopic enrichment is measured at the start and end of the metabolic period, a multi-point method in which the elimination rates are calculated by linear regression of natural log transformed isotopic enrichments ^{3,4}, or a multi-point method in which elimination rates are calculated from a non-linear curve fit of untransformed data to a mono-exponential equation ⁵ (Figure 9.1). At this meeting two further approaches were suggested, one in which an error structure intermediate between linear and non-linear regression (i.e. Poisson) is assumed, and a fifth in which the elimination parameters are calculated from a regression analysis of the isotope enrichment products ($C_o C_p$) and ratios (C_o/C_p) (see Chapter 5). This method, however, leads to the identical CO_2 production rate as the single isotope fits and only the estimate of precision is changed because covariance of ^2H and ^{18}O data is taken into account. In addition, each of these methods has two possible variants, one in which a single isotope dilution space is measured and the other space is assumed to be a fixed proportion of the first ² and the second variant in which both isotope dilution spaces are measured ^{3,4}. For the two-point method, the dilution spaces are typically measured by the plateau

Figure 9.1

Schematic representation of the 3 types of fitting procedure assumed in the simulations



Note In practice all current users of the multi-point version employ variable pool spaces. Comparisons between two-point and multi-point with fixed pools are therefore somewhat unnecessary.

method in which the isotopic enrichment is measured at an equilibrium which is assumed to occur two to six hours after the dose. For the multi-point method, the dilution spaces are usually calculated from the zero-time isotope enrichments predicted by the regression line.

9.2 Systematic changes in isotopic turnover

Two factors could systematically alter isotope turnover rates in doubly-labelled water studies. These are step changes in either carbon dioxide production or water flux. In order to determine how sensitive the doubly-labelled water method is to such systematic variations, a series of synthetic data sets were computer generated.

9.2.1 Methods

The model assumed a typical adult male receiving a loading dose of 0.119 g $^2\text{H}_2\text{O}$ and 0.247 g H_2^{18}O (initial isotopic enrichments of 660 and 110 ‰, respectively) and having isotope elimination rates of 0.1050 and 0.1300 d^{-1} for ^2H and ^{18}O respectively. N_D was 2217.8 moles (40l) and N_O was 2153.2 moles (38.3l). N_D/N_O was thus 1.03. Daily isotopic enrichments were calculated for these basal conditions and then for 30% increases in CO_2 production and water flux in which sustained increases occurred during Days 1-6, 7-12 or 13-18 of an 18 day measurement period. Isotope dilution spaces and elimination rates were then calculated from the synthetic data and the accuracy of the data reduction method was determined as the percent error relative to the true average CO_2 production rate.

It should be noted that the assumption of a 30% change in carbon dioxide production is rather extreme. For example, a subject with a daily energy expenditure of $1.5 \times \text{BMR}$ would have

Table 9.1

Effect of a 30% increase in CO₂ production during one third of a two half-life metabolic period for energy expenditure

<u>Method/Period of increase</u>	<u>Model</u>	
	<u>Fixed pool</u> ¹	<u>Variable pool</u> ²
	(% error)	(% error)
<u>Two-point</u> ³		
Beginning	0.0	-----
Middle	0.0	-----
End	0.0	-----
Every 3 days	0.0	-----
<u>Linear regression</u> ⁴		
Beginning	-1.6	4.8
Middle	3.7	0.8
End	-1.6	-5.2
Every 3 days	0.0	-1.2
<u>Non-linear regression</u> ⁵		
Beginning	4.5	7.5
Middle	2.1	0.0
End	-6.2	-7.1
Every 3 days	-0.8	-1.7

¹ Assumes $N_p/N_o = \text{constant (1.03)}$.

² Uses individually calculated values of N_o and N_p .

³ Calculated from initial and final enrichment.

⁴ Calculated from least squares regression of the transformed enrichment.

⁵ Calculated from least squares fit $c = c_o e^{-kt}$.

to increase this to $1.95 \times \text{BMR}$. Physical activity would have to increase by 150% in order to achieve this since BMR and obligatory thermogenesis account for a relatively constant $1.15 - 1.20 \times \text{BMR}$. The postulated increments in CO_2 production may therefore be regarded as somewhat unphysiological and clearly, if the frequency of the changes is increased, errors will be smaller. This is indicated by the values for 3-day rather than 6-day cycles in Table 9.1.

9.2.2 Systematic errors due to changes in CO_2 production

Systematic errors in CO_2 production for the three basic data reduction techniques are compared in Table 9.1 for a metabolic period of 18 days. In each case the two-point method shows no error because it provides an exact average when rates vary, if, as assumed here, N_o and N_p are correctly determined from plateau values (see Section 4.4.1). When a fixed pool model is used (ie it is assumed that N_o/N_p is constant) errors for the linear regression technique are only -2 to +4%. In contrast the non-linear regression method is in error by -6 to +5% with the largest errors occurring when the increase occurs early or late in the metabolic period. This is because the non-linear method heavily weights the early data points and thus the elimination rates reflect the values of the first half of this data rather than the average. It should however be noted that no group using multi-point data employ fixed pool size ratios.

When a variable pool model is used (ie both isotope dilution spaces are calculated) the errors in CO_2 production increase dramatically as both methods show errors in CO_2 production in excess of 5%. This is because the intercepts no longer give the correct values for N_o and N_p . Inspection of residuals of differences between fitted and observed values exposes this problem (see Fig 9.2).

9.2.3 Systematic errors due to changes in water flux

The errors in CO_2 production due to systematic variation in water flux are compared for the three basic methods of data reduction in Table 9.2. As was observed for increases in CO_2 production, the two-point method always gives the exact average and hence zero systematic error if N_0 and N_D are correctly estimated. Similarly if N_0 and N_D are correctly measured from an isotope plateau, the linear regression method was also very robust as errors are less than 1%. The non-linear regression method, however, is still subject to large systematic errors of up to 6%.

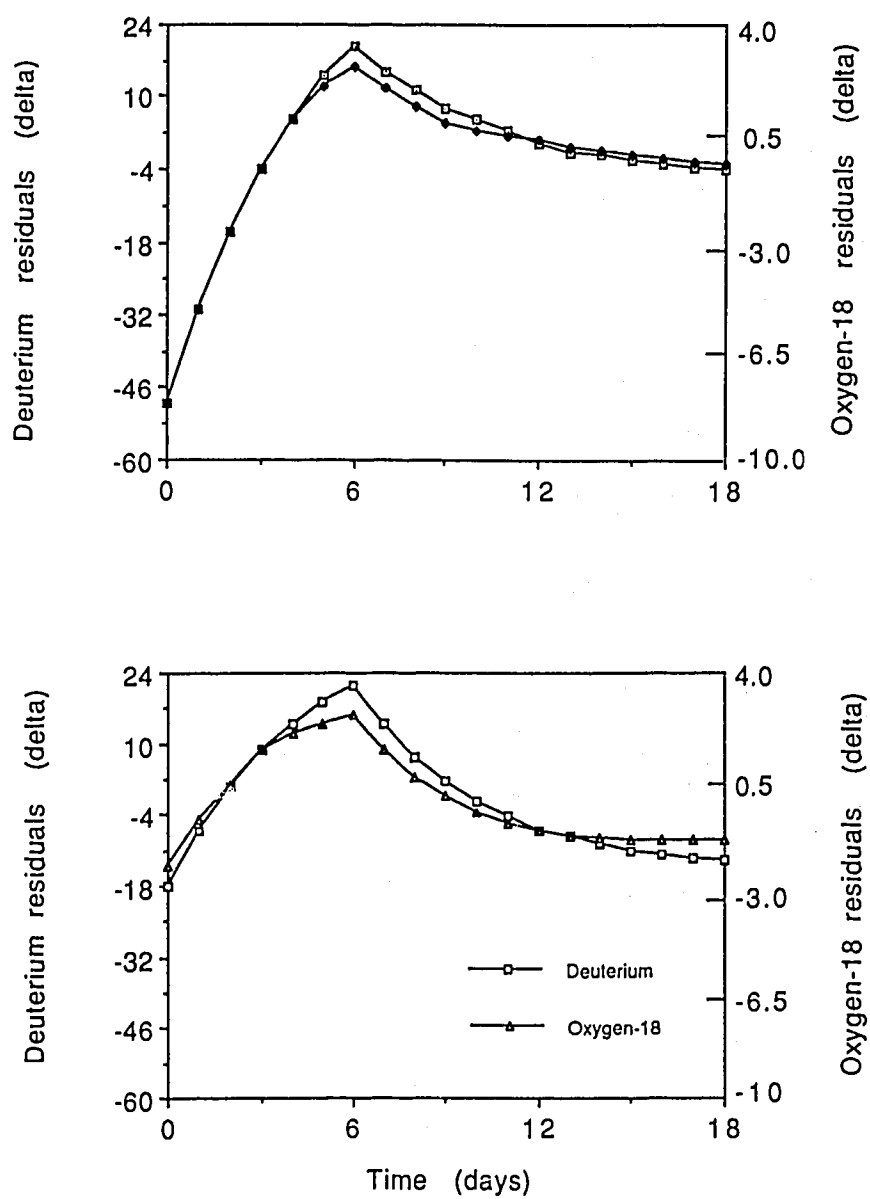
When the variable pool model is used and both isotopic dilution spaces are calculated by back extrapolation to zero time, the systematic errors again increase for the linear regression method. This is due to a systematic difference between the intercept calculated from the regression line and the true zero-time value that is evident from the early elimination data as evident from the residual plots (Figure 9.3). There is, however, little effect on the non-linear regression method, because it heavily weights the early data and thus leads to smaller residuals and hence error in the intercept.

In common with systematic increases in CO_2 production, there is very little error if the increased water flux occurs at regular intervals throughout the metabolic period.

This analysis identifies potential pitfalls associated with multi-point methodology. The discrepancies do not arise from an error in the method, but instead are an example of the wrong answer being generated by using a procedure in circumstances for which it was not designed. It should be noted that either the propagation of error analysis suggested in Chapter 5 or a simple residual plot of the multi-point data would quickly identify a data-set suffering from such a discontinuity. It should then be

Figure 9.2

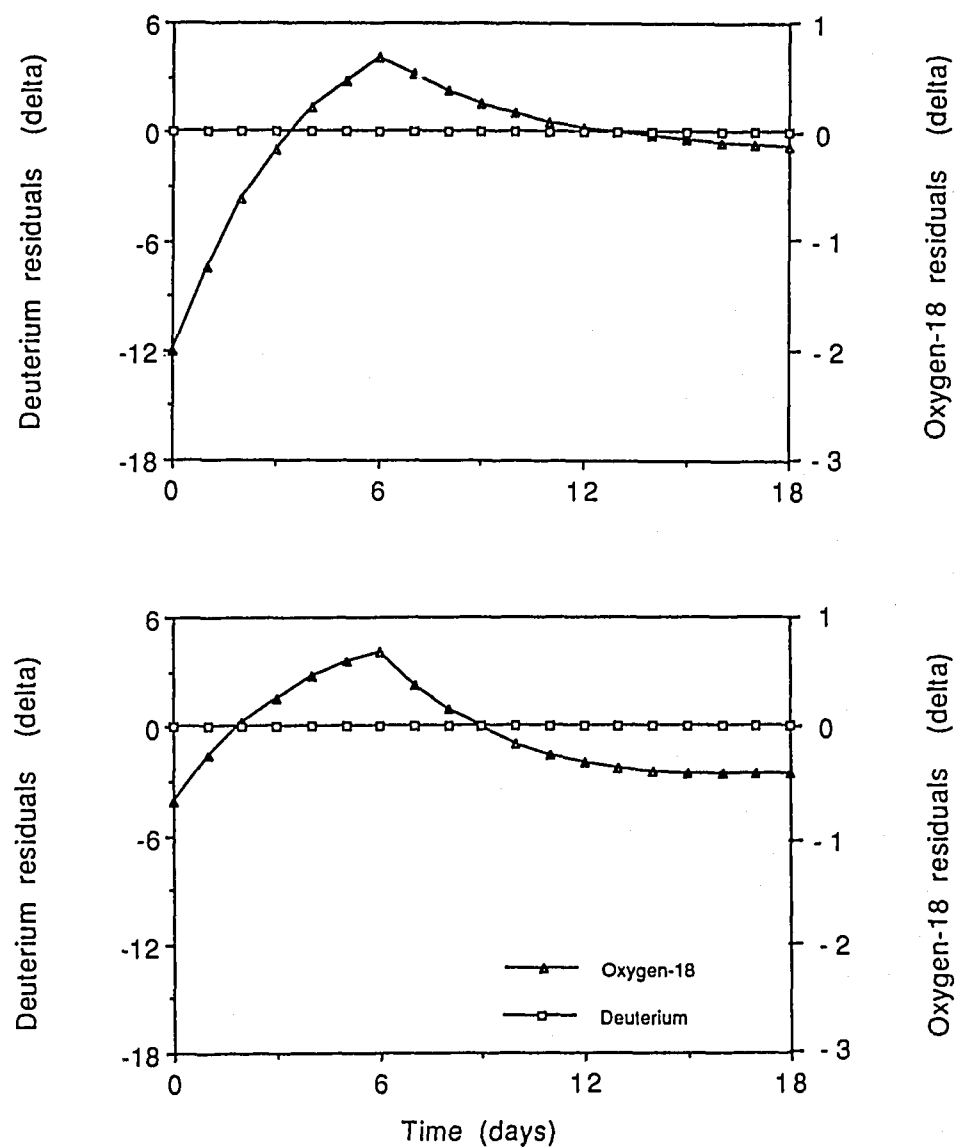
Plot of residual enrichments when CO₂ flux is increased by 30% in the first third of a metabolic period



Upper panel - Linear regression
Lower panel - Non-linear regression

Figure 9.3

Plot of residual enrichments when H₂O flux is increased by 30% in the first third of a metabolic period



Upper panel - Linear regression
Lower panel - Non-linear regression

Table 9.2

Effect of a 30% increase in water turnover during one third of a two half-life metabolic period for energy expenditure

<u>Method/Period of increase</u>	<u>Model</u>	
	<u>Fixed pool</u> ¹	<u>Variable pool</u> ²
	(% error)	(% error)
<u>Two-point</u> ³		
Beginning	0.0	-----
Middle	0.0	-----
End	0.0	-----
Every 3 days	0.0	-----
<u>Linear regression</u> ⁴		
Beginning	0.2	5.4
Middle	-0.5	-2.7
End	0.3	-2.4
Every 3 days	0.0	-0.9
<u>Non-linear regression</u> ⁵		
Beginning	6.0	5.8
Middle	-3.6	-3.9
End	-2.4	-1.9
Every 3 days	-0.8	-1.2

Superscripts as in Table 9.1.

rejected or recalculated by the two-point method if the sampling protocol included collection of 'plateau' post-dose samples.

9.3 Random error in the isotopic enrichment

Each of the data reduction techniques differs in robustness with regard to the effect of random error in isotopic enrichment which occurs during sample collection, treatment and isotopic analysis. This question of random analytical error is especially critical for the first time user of doubly-labelled water because high precision isotopic measurements are quite demanding. As indicated in other chapters, the isotopic enrichments are small with regard to the absolute amount of excess isotope and analytical errors that result from isotope fractionation during sample handling and analysis can be significant relative to the actual enrichment. This introduces an error in the calculated CO_2 production and reduces the precision of the technique.

9.3.1 Methods

Random error was added to the initial data set and the CO_2 production rate calculated using the same data reduction methods as in the above section on systematic error. Each data set included fifteen decay curves with random error which were then subjected to the three modes of data reduction. The precision of the doubly-labelled water method with regard to CO_2 production was calculated from the standard deviation about the mean. This was expressed as the percent of total CO_2 production. Random error was added to the isotopic enrichment at each time point using a computer program that assigns error randomly based on the normal Gaussian distribution. To do this, we assumed a random error level from literature ⁶ in which the ^{18}O analytical error was 0.18 ‰ at zero enrichment and increased linearly to 0.97 ‰ at an enrichment of 260 ‰. The analytical error for the

deuterium was taken to be 1.2 ‰ at zero enrichment and increased linearly to 3.2 ‰ at an enrichment of 590 ‰.

In addition, the effects of a high analytical error were investigated assuming random errors equal to 3 times the above errors. Two error analyses were performed. In the first analysis, it was assumed that there was no error in the baseline measurement and that all the error was contained in the enrichment data. In the second analysis it was assumed that error was present in the baseline and the sample. Thus, the enrichment data for each data-set contained a baseline error or offset and random error in each time point.

The effect of random errors in the two-point method of calculating CO_2 production were initially discussed by Lifson ⁷ and Nagy ⁸. This has been extended by Schoeller ⁹. In these discussions, it was generally observed that the best precision of the doubly-labelled water method occurs between one and three biological half-lives of the isotopes. For shorter metabolic periods, the isotopic change is small relative to analytical error and precisions of less than 5% are hard to obtain at economical isotopic doses. For longer metabolic periods, the final isotopic enrichment is small relative to the analytical error and precisions of less than 5 to 10% are hard to obtain. The sensitivity to analytical error was therefore calculated for metabolic periods of 2 biological half-lives.

9.3.2 Effect of random error on the precision of calculated CO_2 production

The effect of random error in the enrichment data is summarised in Table 9.3. In contrast to the previous systematic physiologic effects, the multi-point methods were more robust in the face of random error than the two-point method by a factor of between 1.5 and 2. At the lower levels of analytical error,

however, all three methods provided precisions of 5% or better. When the variable pool method was used and the two isotopic dilution spaces were calculated from the enrichment data, there was a 39% improvement in the precision of the multi-point methods. This improvement illustrates the effect of the negative covariance of the slope and intercept of a regression line in the presence of random error (see Chapter 5).

When the large analytical error was applied, the precision of the two-point method was very poor (Table 9.3 and Figure 9.4), and is not improved by using the two calculated dilution spaces. The precision of the linear regression technique was also poor, but was improved when intercept data rather than plateau data was used for volume determinations. The extent of the improvement (35%) was about the same as that observed in the case of moderate analytical error.

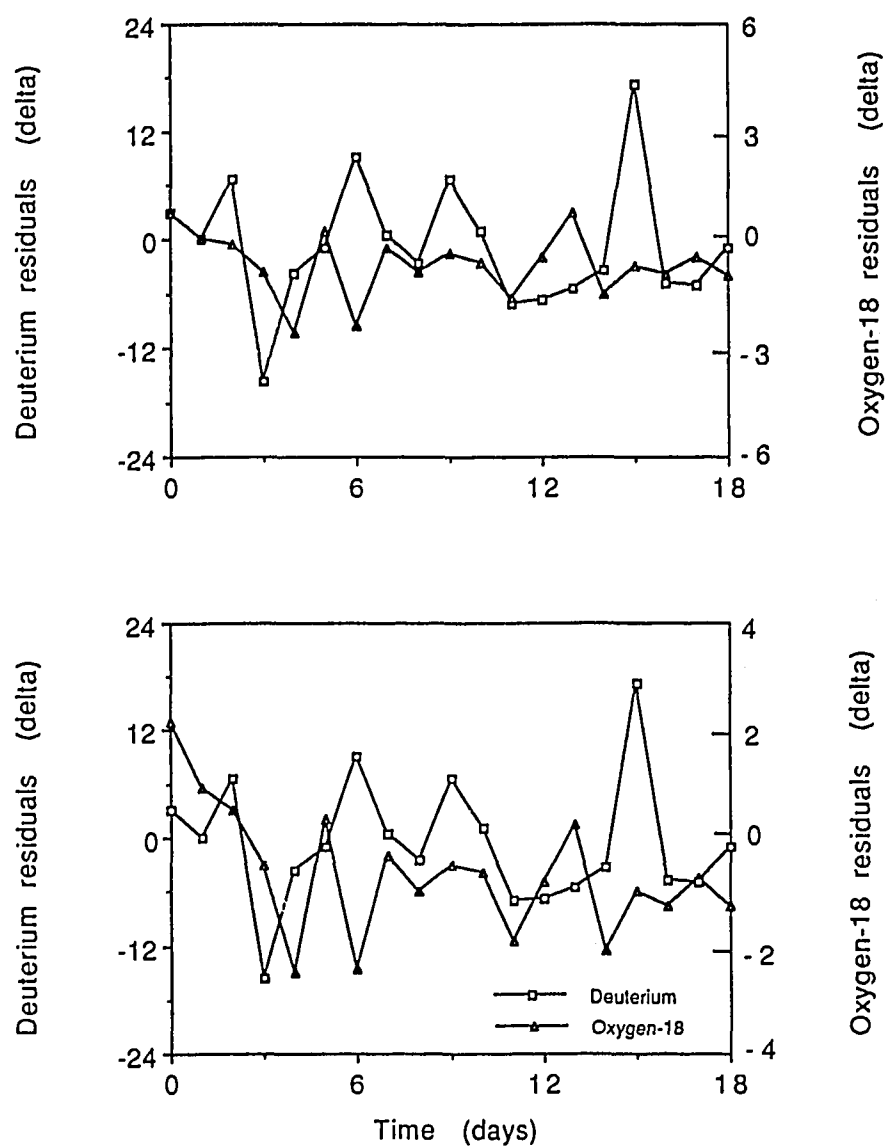
When the effect of baseline random error is added to the analysis, precision for all methods worsens (Table 9.4). With the large analytical error, the precision of the two-point method is quite poor and would be unacceptable for almost all applications. The multi-point method performs much better, but precisions are still worse than 8% and thus unacceptable for many applications of the method. In general, the non-linear regression technique provided better precision than the linear regression method because it weighted the average in favour of the earlier time points that had a smaller relative error.

9.4 Summary of theoretical comparison of methods

A general conclusion from the foregoing analyses is that the two-point method for calculating CO_2 production is the most robust in the presence of systematic changes in CO_2 and water production, provided that N_0 and N_p are correctly measured, and that the non-linear regression method is most sensitive to

Figure 9.4

Residuals arising from a simulation with large random errors in isotope enrichments



Upper panel - Linear regression
Lower panel - Non-linear regression

Table 9.3

Effect of moderate and large random errors in isotopic enrichment on the precision of the doubly-labelled water method for measuring energy expenditure during a 2 half-life metabolic period

<u>Method/Period of increase</u>	<u>Model</u>	
	<u>Fixed pool</u>	<u>Variable pool</u>
	Relative standard deviation (%)	
<u>Two-point</u>		
Moderate error ¹	5.1	4.4
Large error ²	20.2	22.3
<u>Linear regression</u>		
Moderate error	2.3	1.4
Large error	10.3	6.7
<u>Non-linear regression</u>		
Moderate error	2.1	1.9
Large error	4.9	4.4

¹ $\sigma\delta_o = 0.18 + (\delta_o/260) \times 0.79$, and $\sigma\delta_p = 1.2 + (\delta_p/600) \times 2$.

² Large random error = 3 x moderate random error.

Table 9.4

Combined effects of random analytical error in isotopic enrichment and baseline isotopic abundance on the precision of the doubly-labelled water method for measuring energy expenditure during a 2 half-life metabolic period

<u>Method/Period of increase</u>	<u>Model</u>	
	<u>Fixed pool</u>	<u>Variable pool</u>
Relative standard deviation (%)		
<u>Two-point</u>		
Moderate error ¹	5.9	6.0
Large error ²	21.8	24.5
<u>Linear regression</u>		
Moderate error	3.6	3.8
Large error	13.4	13.1
<u>Non-linear regression</u>		
Moderate error	3.0	3.7
Large error	8.9	11.5

Superscripts as in Figure 9.3.

systematic changes. Both multi-point methods perform better when there is a high degree of covariance in the ^2H and ^{18}O data. Evidence from most data sets (see Chapter 5) and physiological commonsense indicates that a degree of covariance will normally exist. However, in all cases where large systematic changes occur, initial isotope distribution spaces will be incorrectly estimated from the zero-time intercepts of fitted curves. Thus bias can occur with this procedure. On the other hand, at all levels of analytical error, combining slopes and intercepts substantially improves precision.

The non-linear regression method further improves precision in the face of analytical error but this improvement is small and does not compensate for the potential inaccuracies in the presence of the particular systematic changes in the isotope turnover rates specified in this simulation. However, as stated in Chapters 4 and 5 the fitting procedure should be chosen after inspecting the residual plot. Using this approach only some of the simulations would be identified as appropriate material for this type of fit: e.g. the variable r_{CO_2} (middle) and variable $r_{\text{H}_2\text{O}}$ (end) simulations in which the non-linear regression method performed marginally better than linear regression (see Tables 9.1 and 9.2).

The apparent advantages of the two-point method portrayed by the above analyses may however be somewhat misleading. As has been mentioned, it was assumed that one space or the other (N_o or N_p) was correctly measured from plateau determinations and the relationship between them was such that $N_p/N_o = 1.03$. There are therefore two questions that must be asked. Firstly, what level of precision and accuracy can be achieved by making measurements at plateaus of enrichment near to the start of an experiment? Secondly, are we justified in fixing a relationship at $N_p/N_o = 1.03$ for all conceivable subjects. As already indicated (Section 4.3.1) our knowledge in this area is relatively scanty. For American adults a combination of analytical accuracy and noise

Table 9.5

Energy expenditure values calculated on the assumption that
 $N_D/N_o = 1.03$ compared to true values obtained when $N_D/N_o \neq 1.03$

<u>True N_D/N_o</u>	Deviation from true energy expenditure if N_D/N_o assumed to be 1.03	
	_____ (%)	
1.01	-11.0	
1.02	- 5.8	
1.03	0	
1.04	+ 6.6	
1.05	+14.1	

about plateau values gives a precision of about 1.5% for volume determinations using saliva samples at 3-6 hr after a dose (recalculated from ref. 10) but we do not know if this time interval is appropriate in all physiological circumstances. This error need not worry us too much however since if N_p/N_o really does equal 1.03 estimates of CO_2 production will only be incorrect by the % inaccuracy in the plateau determination of body-water.

What is of greater concern is the appropriateness of $N_p/N_o = 1.03$. The evidence is that this is a reasonable average figure (see Section 4.3 and Table 9.6) but we really do not know its physiological range for all conceivable subjects. Table 9.5 indicates the problem for a case where k_p and k_o are 0.1050 and 0.1300 respectively. It can be seen that even small physiological variations away from $N_p/N_o = 1.03$ could cause a substantial inaccuracy in estimates of CO_2 production.

9.5 Practical comparisons of the two-point and slope-intercept methods

We have shown in Chapter 4 and earlier in this chapter that the two procedures are quite different from the point of view of what is intended to be measured. The two-point method will produce the correct value for total flux between two time points even in circumstances where systematic variations occur to such an extent that the calculation of an average flux rate per day could almost be said to be inappropriate. In contrast a multi-point method only produces a correct result when variations in rate constants are random. The multi-point method does, however, provide an estimate of the variation about the daily average production rate which combines effects of instrumental and physiological variation (see Chapter 5).

There is little data that allows comparisons to be made

between two-point and multi-point methodologies. In the comparison provided by Coward ¹¹ there was no significant bias between the two methods (see Table 9.6). The SDs of the differences between the two procedures are slightly higher than would be predicted from the theoretical uncertainties of about 3% for the slope/intercept approach and about 4.5% for the two-point method. In this comparison the small average difference between the two procedures almost certainly originated from the fact that the volume ratio N_p/N_o averaged 1.037 in the slope/intercept method but was normalised to 1.03 in the two-point method.

In contrast Schoeller & Taylor ¹² compared two-point methods in which either a plateau method was applied to measure N_p and N_o and the values normalised to $N_p/N_o = 1.03$, or individual N_p and N_o were calculated from the intercepts of disappearance curves generated between two points. In these circumstances the differences between the results obtained using each method can only be attributed to different estimates of N_p and N_o because slopes used were the same. For a period 0-7 days the intercept method produced an average value that was 15% lower than that obtained using the plateau procedure (see Table 9.7) and if values of k_p and k_o are taken to be 0.105 and 0.130 respectively this difference is equivalent to an average N_p/N_o ratio of about 1.06. In the worst case (Subject D) where the difference was 26%, the same assumptions produce an N_p/N_o ratio of 1.08. These differences from the value of 1.03 on which spaces were normalised are clearly important and contrast markedly with the data from Table 9.6 where mean N_p/N_o ratios were 1.037 ± 0.012 SD and with the more extensive data in Table 4.2. It is impossible to say for certain whether marked deviations from the value of 1.03 are genuine physiological differences, in which case the use of 1.03 as a normalising factor is unlikely to be inappropriate, or whether such large ratios have a non-physiological origin, in which case normalisation is an adequate procedure.

Table 9.6

Comparisons between rate-constants for ^{18}O and ^2H disappearance (k_o and k_p , d^{-1}) isotope distribution volumes (N_o and N_p , g) and carbon dioxide production rate F_{CO_2} , estimated using the slope-intercept and two-point methods ($n = 50$)

		<u>Slope/ intercept</u>	<u>Two-point</u>	<u>(A-B)</u>		Statistical significance of difference
		(A)	(B)	<u>Mean</u>	<u>SD</u>	(t)
k_o	Mean	0.1138	0.1132	0.0006	0.0026	1.01
	SD	0.0144	0.0144			
N_o	Mean	34624	34671	-48	929	-1.00
	SD	3889	4102			
k_p	Mean	0.0868	0.0864	0.0004	0.0027	1.11
	SD	0.0134	0.0137			
N_p	Mean	35893	35706	188	887	1.01
	SD	4009	4222			

Mean difference in F_{CO_2} (as % of A) = -1.9 (SD 7.4),
paired-t = 1.82.

Table 9.7

Energy expenditure (MJ/day) for 7 subjects calculated using the two-point method with N_p and N_o estimated from plateau or intercept

<u>Subject</u>	<u>Two-point/ plateau</u>	<u>Two-point/ intercept</u>
A	11.0	9.2
B	13.6	10.4
C	11.5	10.3
D	8.9	6.6
E	9.9	9.2
F	10.5	9.6
G	11.5	10.3
<u>Mean</u>	10.99	9.37
<u>SD</u>	1.48	1.33

Data from Schoeller & Taylor ¹².

9.6 Conclusions

It will now be evident that the main difficulty with the methodologies we have been discussing lies not with the measurement of slopes of isotope disappearance curves but with the estimates of volume.

The use of multi-point data with fitting procedures appropriate to the error structure will provide a good estimate of the average difference between rate constants when there is a high degree of covariance between ^2H and ^{18}O data and even in cases where only CO_2 production is increased and there is no such covariance the errors are fairly small even where 30% changes in CO_2 production occur that persist for one third of a total measurement period. If such a change happened at regular intervals, such as every third day as might occur with a recreational runner, errors are less than 1% and can be ignored. There is also the additional factor to consider that, in the case of any level of analytical error, multi-point methods will reduce errors to about half of those obtained with a two-point method assuming that great care is taken to minimise analytical error in the measurement of baseline abundance. Furthermore if no systematic physiological deviations from linearity occur negative covariance will further improve precision. However, in the presence of large systematic variations in either CO_2 production or water output, bias will occur in estimates of CO_2 production if isotope distribution spaces are measured from the zero-time intercepts of isotope disappearance curves. This error will not always be observed in an estimate of a regression coefficient as these are almost invariably better than 0.99 but will be observable in plots of residuals near time zero (see Figure 9.2). This illustrates the importance of drawing and inspecting a residual plot for all multi-point studies. When systematic deviations are detected, then it will be theoretically preferable to obtain intercept data from the analysis of a subset of data near to zero-time.

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In many ways the conclusions that should be drawn from the inadequacies of the two-point methodology are very similar to those outlined for the multi-point method. Provided there is the assurance of analytical accuracy in the determination of a slope the problem is likely to lie with the measurement of volume. The adequacy of this value will only be improved by making several measurements at different times on a plateau near to zero-time; in other words on a similar subset of data to that suggested for the multi-point method when systematic variation occurs.

There remains the problem of the relationship between N_p and N_o . The balance of current evidence suggests a value for N_p/N_o of about 1.035 and if this relationship cannot be established during an experiment it is not unreasonable to use this average value, on the understanding that if the true value for any subject is 1% different from this, the error produced in the measurement of CO_2 production will be about 5%. On the other hand if it is possible to measure both N_p and N_o with an accuracy of 0.5 - 1.5% in experiments it is theoretically preferable to use these values. However, experience from the literature and from the data exchange exercise prior to this meeting suggests that values of 1.03 (plus or minus some small SD) are not always found. Until consistent findings for particular populations indicate otherwise it is commonsense to treat N_p/N_o ratios differing markedly from these values with some suspicion. The IDECG Workshop recommended that 1.015 - 1.060 should be adopted as the acceptable range.

At this meeting Speakman suggested that a general rule might be applied when choosing methods for calculating energy expenditure measurements and his view fairly encapsulates all the arguments propounded in this chapter. That is, that with low isotopic enrichments and relatively small temporal variation in water turnover or carbon dioxide production it will be preferable to use a multi-point slope-intercept method. This is because relatively low enrichments put a premium on the analyses and, in the absence of much temporal variation, fitting single

exponentials to data is a satisfactory procedure. When temporal variation is large, as it may often be in wild animals, a two-point method will certainly be preferable from both the theoretical and practical point of view ¹³. The theoretical reasons are evident, the practical reason is that wild animals are difficult to catch more than a few times in any measurement period. There is the further advantage that relatively high enrichments are often used in experiments such as these and this is an advantage when analytical precision is considered, especially in the two-point method.

9.7 References

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CHAPTER 10

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CONVERTING CARBON DIOXIDE PRODUCTION TO ENERGY EXPENDITURE

10.1 Introduction

The fact that the doubly-labelled water method estimates carbon dioxide production rather than oxygen consumption is something of a drawback since the energy equivalent of carbon dioxide ($E_{eq_{CO_2}}$) varies to a much greater extent than the energy equivalent of oxygen. The variation is governed by the substrate mixture being oxidised and hence the respiratory quotient (RQ). This is illustrated in Tables 10.1 and 10.2 which show, for example, that the $E_{eq_{CO_2}}$ for carbohydrate oxidation is 30% lower than for fat, and 10% lower than for alcohol. The $E_{eq_{CO_2}}$ for protein oxidation is intermediate between that of fat and carbohydrate.

As a result of this variation it is necessary to have quite an accurate estimate of the mean RQ (or better still the mean $E_{eq_{CO_2}}$ over the whole of a DLW measurement period in order to convert CO_2 production to energy expenditure. The earliest human studies assumed an average value for the RQ of 0.85 for all subjects, but acknowledged that this was an approximation. In 1986

Black et al ¹ suggested a more sophisticated approach based on a knowledge of the macronutrient content of each subject's diet. This can be summarised in the form of a food quotient (FQ = calculated CO₂ produced/O₂ consumed during oxidation of foods). Under conditions of perfect nutrient balance the FQ must equal the RQ. Black et al provided methods for converting FQs into RQs under conditions of imbalance, and demonstrated that under most circumstances it was possible to reduce the potential error from this source to below 2%.

This chapter provides a more detailed analysis of the issues involved.

10.2 Estimating Eeq_{co2} for different oxidation mixtures

The Eeq_{co2} for an oxidation mixture depends both on the proportion of energy derived from individual fuels and on the end products of metabolism ^{2,3}. The variability of these two factors in different circumstances therefore requires some discussion. However it is first necessary to briefly consider the methods available for estimating the energy equivalent of CO₂ for a subject (Eeq_{co2}-body) and for a mixed diet (Eeq_{co2}-diet).

10.2.1 Estimation of the Eeq_{co2}-body

The Eeq_{co2} for an oxidation mixture in a subject is simply given by:

$$\text{Eeq}_{\text{co2}}\text{-body} = \frac{\text{Total energy expenditure}}{\text{Total CO}_2 \text{ produced}} \quad \dots\dots\dots 1$$

The total CO₂ released is equal to the quantity of CO₂ produced from the oxidation of individual fuels:

$$\begin{aligned}
\text{Total CO}_2 &= \text{CO}_2 \text{ from protein oxidation (CO}_2\text{-prot)} \\
&+ \text{CO}_2 \text{ from fat oxidation (CO}_2\text{-fat)} \\
&+ \text{CO}_2 \text{ from carbohydrate oxidation (CO}_2\text{-carb)} \\
&+ \text{CO}_2 \text{ from alcohol oxidation (CO}_2\text{-alc)} \\
&\dots\dots\dots 2
\end{aligned}$$

Substituting Equation 2 into Equation 1 gives:

$$\text{Eeq}_{\text{CO}_2}\text{-body} = \frac{\text{Total energy expenditure}}{\text{CO}_2\text{-prot} + \text{CO}_2\text{-fat} + \text{CO}_2\text{-carb} + \text{CO}_2\text{-alc}} \dots\dots\dots 3$$

The volume of CO₂ released during the oxidation of an individual fuel is given by:

$$\text{Volume of CO}_2 \text{ (l)} = \frac{\text{Energy released from oxidation of fuel (kJ)}}{\text{Eeq}_{\text{CO}_2} \text{ of fuel (kJ/l)}} \dots\dots\dots 4$$

Therefore the Eeq_{CO₂}-body for a fuel mixture can also be expressed as:

$$\text{Eeq}_{\text{CO}_2}\text{-body (kJ/l)} = \frac{100}{\frac{p}{23.33} + \frac{f}{27.46} + \frac{c}{21.12} + \frac{a}{30.49}} \dots\dots\dots 5$$

where p, f, c and a represent the percentage of energy derived from the oxidation of protein, fat, carbohydrate and alcohol respectively, and 23.33, 27.46, 21.12 and 30.49 are the associated values of Eeq_{CO₂} for these fuels in kJ/l (Table 10.1).

In practice it is not often that information is available about the exact proportion of energy derived from the oxidation of individual fuels especially in human studies carried out over

Table 10.1

The energy equivalent of O₂ and CO₂ and the respiratory quotient of fat, protein, carbohydrate and alcohol

	<u>RO</u>	<u>Energy equivalent of gas (kJ/l)</u>	
		<u>O₂</u>	<u>CO₂</u>
Fat	0.710	19.50	27.46
* Protein	0.835	19.48	23.33
** Carbohydrate	1.000	21.12	21.12
Alcohol	0.667	20.33	30.49

* End products assumed to be urea, ammonia and creatinine in the nitrogenous ratio 90:5:5.

** Glucose polysaccharide.

From Livesey & Elia ⁵.

extended periods of time. Therefore to calculate $E_{eq_{CO_2}}$ -body it is necessary to estimate either the percentage of energy derived from individual fuels, or the overall RQ of the oxidation mixture which is given by:

$$RQ = \frac{0.835 \times O_{2prot} + 0.71 \times O_{2fat} + 1.00 \times O_{2carb} + 0.667 \times O_{2alc}}{O_{2prot} + O_{2fat} + O_{2carb} + O_{2alc}} \dots\dots\dots 6$$

where O_{2prot} , O_{2fat} , O_{2carb} and O_{2alc} represent the O_2 utilised during the oxidation of protein, fat, carbohydrate and alcohol respectively, and 0.835, 0.71, 1.00 and 0.667 are the respective respiratory quotients of these fuels ⁴.

The volume of O_2 consumed during the oxidation of individual fuels is given by Equation 7 which can be substituted into Equation 6:

$$\begin{aligned} &\text{Vol } O_2 \text{ consumed (l)} \\ &= \frac{\text{Energy expended during oxidation of fuel (kJ)}}{E_{eq_{O_2}} \text{ for that fuel}} \dots\dots\dots 7 \end{aligned}$$

where $E_{eq_{O_2}}$ is the energy equivalent of O_2 (Table 10.1).

The $E_{eq_{CO_2}}$ of a carbohydrate/fat oxidation mixture is related to the RQ according to Equation 8 (see Elia and Livesey ⁴ for derivation):

$$E_{eq_{CO_2}} = \frac{15.457}{RQ} + 5.573 \text{ (kJ/l)} \dots\dots\dots 8$$

The RQ of the fat/carbohydrate oxidation mixture is almost linearly related to the proportion of energy derived from carbohydrate oxidation (c) (Equation 9) and inversely related to the

proportion derived from fat oxidation:

$$RQ = \frac{0.2133 c + 0.71}{1 - 0.0767 c} \quad \dots\dots\dots 9$$

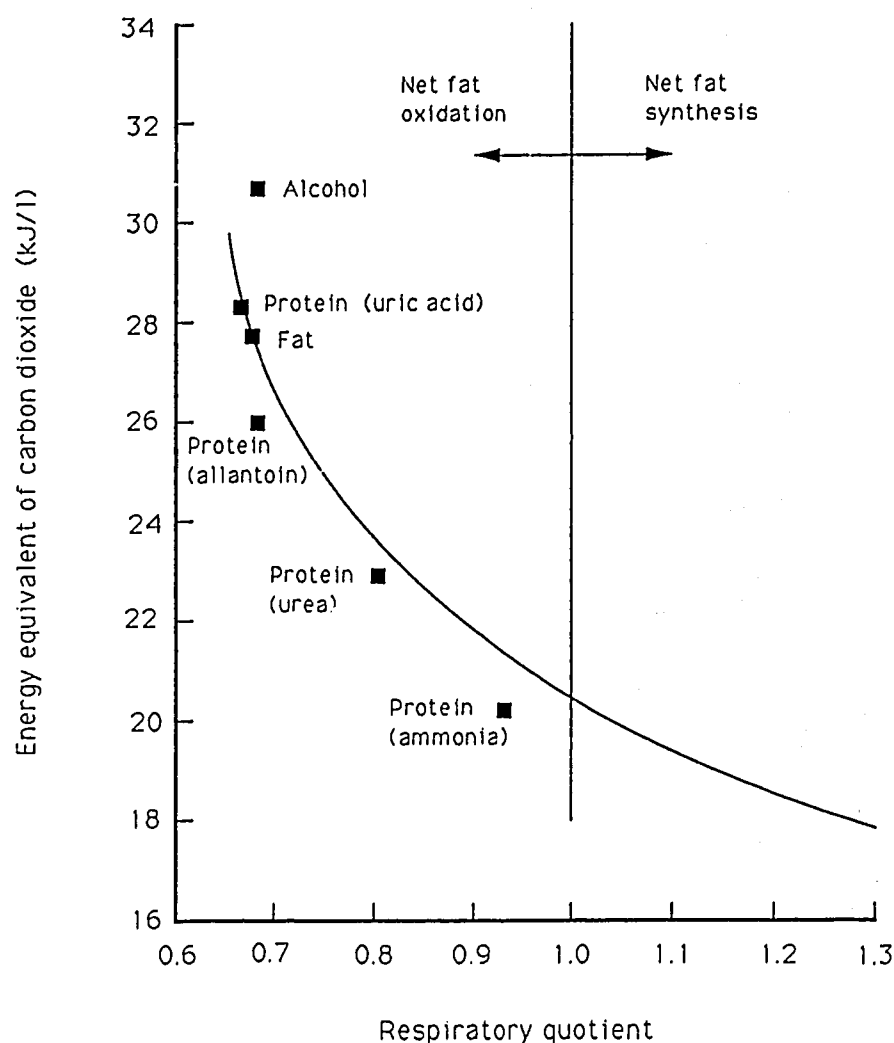
The variation in $E_{eq_{CO_2}}$ with the RQ of a carbohydrate/fat oxidation mixture is shown in Figure 10.1. Values of RQ below 1.0 refer to the net oxidation of fat and carbohydrate, and those above 1.0 to the oxidation and conversion of carbohydrate to lipid ⁴.

In practice fat and carbohydrate are never oxidised in isolation and although they are the primary determinants of RQ it is necessary to consider the effects of protein and alcohol. The oxidation of protein to different nitrogenous end products is associated with various values of $E_{eq_{CO_2}}-prot$ (Table 10.2), which would cause a slight deviation below the curve generated by a pure fat/carbohydrate oxidation mixture (Fig 10.1). The more reduced the nitrogenous end product, the greater the deviation of the value from the fat/carbohydrate curve. In contrast alcohol oxidation would cause a slight deviation above the curve. However, all these values are so close to the fat/carbohydrate curve in Fig 10.1 (within 5-6%) that if the overall respiratory quotient of a fuel mixture were known, the associated $E_{eq_{CO_2}}$ of that mixture can be closely predicted ¹. A general formula for calculating the $E_{eq_{CO_2}}$ of a carbohydrate/fat/protein oxidation mixture from the RQ of the oxidation mixture is given by Equation 10 (see also Equation 5), which assumes that about 12% of energy expenditure is derived from protein oxidation and that urinary N is distributed in urea, creatinine and ammonia in the ratio 90:5:5 ^{4,5}.

$$E_{eq_{CO_2}}-body = \frac{15.48}{RQ} + 5.55 \text{ (kJ/l)} \quad \dots\dots\dots 10$$

This relationship is also illustrated in Table 10.3.

Figure 10.1



The relationship between the energy equivalent of CO₂ and the respiratory quotient of a carbohydrate fuel mixture (see curve). The energy equivalent of CO₂ and the respiratory quotient associated with the oxidation of individual fuels are also indicated: carbohydrate (Carb); fat (Fat); alcohol (Alc) and protein (Protein). Four values are indicated for protein to take into account its conversion to urea, ammonia, allantoin and uric acid. See text for details.

Table 10.2

The respiratory quotient and energy equivalent of O₂ and CO₂ associated with the oxidation of Kleiber's standard protein to different nitrogenous end products.

			<u>Energy equivalent of gas (kJ/l)</u>	
			<u>O₂</u>	<u>CO₂</u>
		<u>RQ</u>		
Protein	> urea	0.826	19.48	23.58
Protein	> ammonia	0.950	19.68	20.72
Protein	> uric acid	0.707	19.57	23.58
Protein	> allantoin	0.749	19.53	25.08

Kleiber's standard protein = C₁₀₀H₁₅₉N₂₆O₃₂S_{0.7}

From Elia ².

Table 10.3

Relationship between the respiratory quotient and the energy equivalent of CO₂ for a carbohydrate/fat/protein oxidation mixture

<u>Respiratory quotient</u>	<u>Energy equivalent of CO₂</u> (kJ/l)
0.75	26.19
0.80	24.90
0.85	23.76
0.90	22.75
1.00	21.03
1.05	20.29
1.10	19.62

Derived from Equation 10.

As indicated above, there is an increasing error in the overall $E_{eq_{CO_2}}$ as the proportion of energy derived from protein oxidation increases (the maximum error is 3.2% when all the energy is derived from protein). A small error will also occur when the equation is used to calculate the overall $E_{eq_{CO_2}}$ for an oxidation mixture that includes alcohol (maximum error 5.8% when all the energy is derived from alcohol). Therefore the direct approach suggested by Equations 3 and 5, is theoretically more sound than the indirect approach which involves calculation of the RQ.

10.2.2 Estimation of the FQ and $E_{eq_{CO_2}}$ -diet

The $E_{eq_{CO_2}}$ can be calculated by equations analogous to Equations 3 and 5 using the metabolisable energy of fuels ^{1,2}. In this respect it should be noted that the energy derived from the oxidation of given quantities of endogenous nutrients such as fat or protein, is greater than the metabolisable energy derived from the same quantity of dietary nutrients. This is because digestibility does not have to be considered when endogenous fuels are mobilised for oxidation (eg the heat of combustion of fat is about 39.3 kJ/g, whereas the metabolisable energy is about 37.7 kJ/g). For subjects in nutrient balance the metabolisable energy intake of individual fuels is expected to equal the energy derived from the oxidation of these fuels by the body. Under these circumstances the $E_{eq_{CO_2}}$ -diet should equal the $E_{eq_{CO_2}}$ -body. Similarly, (under these circumstances of nutrient balance), the food quotient will equal the respiratory quotient. However, differences between $E_{eq_{CO_2}}$ -body and $E_{eq_{CO_2}}$ -diet (or FQ and RQ) will occur when there is energy or nutrient imbalance (see below). Therefore the value of $E_{eq_{CO_2}}$ -body, which is of primary importance in the estimation of energy expenditure by the doubly-labelled water technique, depends both on the extent of nutrient imbalances as well as on the composition of the diet.

10.2.2.1 Variation in $E_{eq_{CO_2}}$ -diet and FQ

The $E_{eq_{CO_2}}$ -diet for groups of individuals in Britain is found to be remarkably constant (at about 23.85 kJ/l) irrespective of whether it is calculated from data of food intake obtained from dietary recall or from measurements of weighed food intake obtained over an extended period of time ². Furthermore, even if the gross changes in dietary intake recommended by some modern dietary goals were fully implemented, they would make little difference to the FQ and $E_{eq_{CO_2}}$ -diet (Table 10.4). However, in many Third World countries, where the proportion of dietary energy derived from carbohydrate is high, the $E_{eq_{CO_2}}$ -diet may be up to 10% lower than that of a typical western diet (Table 10.4).

Further examples of circumstances in which $E_{eq_{CO_2}}$ -diet and FQ may deviate quite substantially from those of a typical Western diet are given below:

- a) Ethnic minorities living in 'western societies' ¹.
- b) Certain groups of athletes (US college wrestlers and 'track and field athletes' whose FQ may be close to or over 0.90 and $E_{eq_{CO_2}}$ -diet as low as 22.5 kJ/l CO_2). This however is unusual since the values for $E_{eq_{CO_2}}$ generally range from 23.0-23.7 kJ/l CO_2 (see Elia ² for further analysis).
- c) Other societies. An analysis of food available for consumption in 146 countries around the world (Food and Agricultural Organisation) shows the (alcohol free) FQ to vary from about 0.85 to 0.95 and the $E_{eq_{CO_2}}$ -diet from about 21.8 to 23.9 kJ/l CO_2 (11% range) ². Individual dietary surveys in various countries, show similar variations in FQ and $E_{eq_{CO_2}}$ -diet ^{1,2}.
- d) Hospitalised patients receiving unusual combinations of nutrients in artificial feeds. For example, the FQ of 56 commercial enteral feeds was found to range from 0.80 to 0.98,

Table 10.4

The composition of different diets and their associated food quotient (FQ) and energy equivalent of CO₂ (Eeq_{CO₂}-diet)

	Current British or 'western' type diet	NACNE recommendation		High CHO diet
		Short term	Long term	
% energy from protein	11	11	11	11
% energy from fat	38	34	30	10
% energy from alcohol	6	5	4	-
Food Quotient (FQ)	0.847	0.861	0.876	0.952
Eeq _{CO₂} -diet (kJ/l)	23.91	23.55	23.24	21.83
As % of current British diet	100	98.49	97.20	91.30

NACNE: National Advisory Committee on Nutrition Education 1983.

High CHO diet: as typically eaten in parts of India, Africa and other Third World countries (see ref ²).

and the $E_{eq_{CO_2}}$ -diet from 21.4 to more than 24.5 kJ/l CO_2 (14% range) ².

e) Infants fed exclusively on certain formulae.

10.2.2.2 Individual variation in $E_{eq_{CO_2}}$ -diet

The use of a general value for $E_{eq_{CO_2}}$ -diet of about 23.85 kJ/l CO_2 , for the 'western' type diet, was found to predict to within $\pm 5\%$ the $E_{eq_{CO_2}}$ -diet of 63 randomly-selected individuals, whose dietary intake was assessed by weighing the food eaten over a one week period (Bingham et al ⁶ and personal communication). However, it is important to note: a) that the reported alcohol intake, which affects the $E_{eq_{CO_2}}$ -diet, was low in this group of subjects (4% of the dietary intake for men and 1.2% for women); and b) that some of the subjects especially women were dieting at the time of the study and were therefore in nutrient imbalance. During dieting the $E_{eq_{CO_2}}$ -body is likely to be higher than the $E_{eq_{CO_2}}$ -diet to an extent which depends both on the degree of dietary restriction and on the composition of the diet (see below).

10.2.2.3 The effect of alcohol

Alcohol can significantly affect the $E_{eq_{CO_2}}$ -diet and FQ of the diet because its $E_{eq_{CO_2}}$ is greater than those for fat, protein and carbohydrate, since its FQ is lower. However, the extent to which $E_{eq_{CO_2}}$ -diet is affected by the presence of alcohol in the diet depends not only on the amount consumed, but also on the composition of the rest of the diet ². For example alcohol produces a greater change in the $E_{eq_{CO_2}}$ -diet when it is added to a diet rich in carbohydrate (ie a diet with a high FQ and low $E_{eq_{CO_2}}$ -diet) than when it is added to a diet rich in fat. This effect is illustrated in Table 10.5, for values of alcohol intake

Table 10.5

Effect of increasing amounts of alcohol on the dietary equivalent of CO₂ (Eeq_{CO₂}-diet) and food quotient (FQ) of four different diets

Food quotient

<u>Diet</u>	<u>% energy from alcohol</u>			
	<u>0</u>	<u>10</u>	<u>30</u>	<u>50</u>
A	0.800	0.787	0.761	0.734
B	0.850	0.832	0.795	0.759
C	0.900	0.877	0.830	0.783
D	0.950	0.921	0.864	0.807

Eeq_{CO₂}-diet (kJ/l) with % change in parentheses

<u>Diet</u>	<u>% energy from alcohol</u>			
	<u>0</u>	<u>10</u>	<u>30</u>	<u>50</u>
A	24.900	25.315	26.190	27.139
	(0.0)	(1.9)	(5.8)	(10.1)
B	23.762	24.282	25.440	26.621
	(0.0)	(2.3)	(7.1)	(12.4)
C	22.750	23.342	24.629	26.060
	(0.0)	(2.6)	(8.2)	(14.5)
D	21.845	22.529	24.020	25.719
	(0.0)	(2.9)	(9.3)	(16.5)

ranging from 10-50% of energy intake.

Alcohol intake of course varies considerably in people of different nationalities ⁷. It accounts for about 6% of energy intake of adults in Britain ⁸, but this varies considerably depending on the geographical area as well as on age, sex and socioeconomic background of the subject. Alcohol intake is particularly low in Moslem countries, and high in other countries such as France, where the mean intake per person is two-fold greater than in the U.K. ⁷. These averaged differences according to region or nationality mask much larger differences between individuals or groups of individuals, especially men, who tend to drink considerably more alcohol than women. Indeed, various dietary surveys suggest that the mean alcohol intake of selected populations ranges from 0-50% of total energy ².

A particular problem to be borne in mind in relation to adjusting the $E_{eq_{CO_2}}$ -diet for alcohol consumption is that it may often be seriously under-reported.

10.3 Adjusting $E_{eq_{CO_2}}$ for nutrient imbalance in the subject

General values for $E_{eq_{CO_2}}$ -body should not be used in subjects with major nutrient or energy imbalance. An individual who is starving or on a very low calorie diet will derive most of his energy from fat, especially after the glycogen stores are depleted. Protein will make a small contribution to energy expenditure ⁹. The $E_{eq_{CO_2}}$ -body under these circumstances may therefore be close to 27 kJ/l CO_2 , which is about 15% higher than in weight-stable subjects who maintain themselves in nutrient balance whilst ingesting a typical 'western' diet (Table 10.4).

The extent of this difference can be calculated using Equation 5, which requires knowledge of the fuels used for oxidation. If it is assumed that the energy deficit is largely

accounted for by oxidation of fat, then it can be shown ² that the energy intake (western type diet) has to be reduced by as much as 50% below energy expenditure to increase the $E_{eq_{CO_2}}$ -body by as much as 6%. This confirms the worked examples previously published by Black et al ¹.

In contrast, overfeeding (with a western type diet) to the extent of 50% above energy expenditure, will reduce the $E_{eq_{CO_2}}$ -body by only about 6% ². This degree of overfeeding is greater than that which occurs in the human neonate during the first four months of life, a period of rapid growth. During this period the metabolisable energy intake provided in milk ($E_{eq_{CO_2}}$ -milk, 24.3 kJ/l) is about 34% higher than energy expenditure ¹⁰. This extra energy is deposited predominantly as fat (1.5 kg) and to a lesser extent protein (0.4 kg) ¹⁰. Calculations based on these changes suggest that the $E_{eq_{CO_2}}$ -body will differ from the $E_{eq_{CO_2}}$ -milk by only about 3.2%. Therefore, it is clear that nutrient imbalance has to be substantial to change the $E_{eq_{CO_2}}$ -body by more than 5%. Again these estimates confirm the figures published by Black et al ¹.

Large changes in $E_{eq_{CO_2}}$ -body may also occur during rapid repletion in depleted subjects receiving hypercaloric regimens rich in carbohydrate. When the energy intake from carbohydrate is greater than energy expenditure the RQ of the body may rise to values that are persistently greater than 1.0 ¹¹, and the $E_{eq_{CO_2}}$ -body may fall to values that are below 21.1 kJ/l CO_2 . This differs by more than 15% from subjects maintaining constant body composition whilst ingesting a western type diet.

Although the assessment of $E_{eq_{CO_2}}$ -body in states of nutrient imbalance is more difficult than under normal circumstances, reasonable estimates of $E_{eq_{CO_2}}$ -body can be made by taking into account the clinical or physiological state under investigation, and the associated changes in body weight. Estimates of body composition may also be useful under certain circumstances, but

in practice it is often found that the difficulty of accurately estimating changes in body composition over the short periods used for DLW measurements are such that little advantage is gained over the use of weight changes alone.

10.4 References

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CHAPTER 11

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PRACTICAL RECOMMENDATIONS AND WORKED EXAMPLES

This section summarises the salient recommendations contained in the main body of the report and adds some practical advice for new users of the method.

11.1 Choice of two-point or multi-point technique

As discussed in Chapters 4, 5 and 9 the two generic methods of applying DLW, namely the two-point and multi-point versions, each have advantages and disadvantages. These are listed below:

11.1.1 Two-point

Advantages

- 1) Requires much less sample collection, handling and mass spectrometric analysis.

- 2) Robust to large, step changes in water turnover and CO_2 production during the measurement period (Section 9.2).
- 3) The protocol can often be arranged to permit sample collection under clinical conditions thus ensuring greater control.

Disadvantages

- 1) Provides no information on possible deviations from the original Lifson model during the measurement.
- 2) Less robust to random error in isotope enrichments and therefore yields a less precise estimate than the multi-point version unless very high doses of isotope can be afforded.
- 3) Provides no individual estimate of precision.
- 4) May yield a large error if the factor of 1.03 used to calculate the single pool is significantly different from the real physiological difference in the two pool sizes.

11.1.2 Multi-point

Advantages

- 1) Robust to random errors in isotopic enrichments and therefore provides a more precise estimate particularly at the low levels of enrichment usually employed in human studies (Section 9.3).
- 2) Inspection of residuals provides information on deviations from the Lifson model and permits exclusion of data from any measurement in which serious deviations

occur.

- 3) Provides an individual estimate of precision for every answer by propagation of error analysis.
- 4) The extent of covariance of ^2H and ^{18}O residuals provides an assessment of analytical accuracy.
- 5) Avoids potential errors inherent in the assumption of a fixed 1.03 ratio between ^2H and ^{18}O pool sizes.

Disadvantages

- 1) Involves considerably more work in sample collection, handling and analysis.
- 2) Less robust than the two-point method to step changes in water or carbon dioxide turnover. However, such changes can be detected from residual plots and the data can be recalculated using the two-point approach providing that a plateau sample has been collected.

In practice the major determinant of which method is used by different laboratories tends to be the type of mass spectrometer available. The multi-point method is impossibly time-consuming unless a fully automated instrument is used. Perhaps the major advantage of collecting data in the multi-point mode is that it can always be recalculated as two-point data (provided that the sampling regime contains time-points appropriate for the estimation of plateau enrichments). This gives maximum flexibility when it comes to the final analysis. If analytical time is at a special premium, if the investigator can accept a lower level of precision on individual estimates (as for example in large sample studies where the mean value is of interest), or when residual plots indicate significant deviation from the

Lifson model it becomes appropriate to select the two-point method. Otherwise the multi-point method is marginally preferable. The most important conclusion, however, is that given reasonable data-sets the two methods give essentially the same answer (see Table 9.6) and neither need be considered inferior.

11.2 Procedure for calculating two-point data

11.2.1 Estimating N from plateaus

The equation for calculating N from enrichments (δ) is given in Section 4.3. Most users follow Schoeller's recommendation of assuming a fixed N_p/N_o ratio of 1.03 (although Table 4.2 suggests that a marginally higher value of 1.035 might be slightly preferable). If only one space measurement is used the other should be calculated from it. If both are measured the values can be appropriately weighted.

11.2.2 Flux rates

Calculation of the elimination rate by the two point method is straightforward. Isotopic enrichments relative to baseline are calculated by taking the simple arithmetic difference. The elimination rate is then calculated as:

$$(\ln \delta_2 - \ln \delta_1) / (t_2 - t_1)$$

Except for very short times after the dose, time should be recorded to nearest hour of collection. Time for blood samples are equal to the collection time. Time for urine samples have generally been taken as the collection time, although urine is somewhat older than collection time because it is secreted into the bladder over the time between voidings. Pharmacologists

generally use the midpoint of the interval between voidings as the time, however, unlike drugs, ^{18}O and ^2H labelled water does exchange slowly across the bladder rendering even this calculation inexact. Before becoming overly concerned, however, it should be remembered that an error of even two hours is only 1% of 7 days. In general, samples should be collected at the same time of the day (to within a few hours) because of diurnal variation in energy expenditure and water flux.

One obvious problem of the two point method is that two points always make a straight line. Thus, it is impossible to detect an analytical error or a sample mislabelling. Analytical errors can be detected by repeating the isotopic analysis on a separate day. Analytical errors and labelling errors can be detected by collecting a sample near the midpoint of the metabolic period (thus making it a 3-point method). The carbon dioxide production rate can then be calculated over half the metabolic period and the entire period. Unless subjects change their expenditure dramatically, these two values should agree within 2 standard deviations of the measurement error or about 8%. If not, an error has usually been made and samples should be reanalysed.

11.3 Procedure for calculating multi-point data

Due to the extra complexities inherent in multi-point analysis this section presents worked examples using 3 of the datasets exchanged prior to the workshop. These have been selected to represent good (Subject 1), moderate (Subject 2) and indifferent (Subject 3) data.

It must again be emphasised that the choice between the 3 different fitting procedures represents a refinement of the method which need only be invoked under unusual circumstances. Readers who are new to the method should initially concentrate on

the simple log fitting procedure (or the Poisson fit if they have the computing capacity, since this represents an excellent compromise approach), and be aware that an alternative method is available to deal with data showing large residuals at the end of the measurement period.

11.3.1 Initial treatment of mass spectrometric data

The unprocessed mass spectrometric results for these subjects are presented in Table 11.1. The most convenient way of handling enrichment data is to represent all δ 's as a fraction of the initial dose given. This is achieved using the formula:

$$X = \frac{(\delta_s - \delta_p)}{(\delta_a - \delta_t)} \times \frac{18.02a}{WA}$$

where δ is the enrichment of the sample (δ_s), pre-dose baseline (δ_p), dose (δ_a) and tap water (δ_t); a is the amount of dose diluted for analysis (g); W is the amount of water used to dilute the dose (g); A is the amount of dose administered (g); and 18.02 converts g water into moles.

There are two advantages to this procedure. Firstly, the pool sizes (N_p and N_o) are derived simply as the reciprocal of the intercept (or plateau value). Similarly the intercept of the δ_o/δ_p plot is the ratio of the spaces N_p/N_o . Secondly, the procedure 'normalises' the results so that both δ_o and δ_p values can be plotted on the same scale. Table 11.1 contains the converted data for Subjects 1 - 3, and Figure 11.1 shows the untransformed plots of δ_o and δ_p .

At this stage the data can be screened for any obviously aberrant points which may be due to sample contamination, mislabelling with respect to time or faulty analysis. If the data-points in question remain outliers after re-analysis it may

Table 11.1

Mass spectrometric data for worked examples - Subject 1

Time (d)	δ (‰)		Normalised enrichment (fraction of dose $\times 10^7$)	
	^{18}O	^2H	^{18}O	^2H
0.232	177.4956	859.6359	4058	3946
0.525	169.9968	829.4619	3891	3811
1.400	153.6073	768.6668	3526	3539
2.000	140.2262	719.4943	3228	3319
3.000	128.0576	663.6164	2957	3069
4.000	111.5333	598.5746	2589	2778
5.000	101.6996	557.4485	2370	2594
6.000	90.2494	512.5227	2115	2393
7.300	80.1463	470.5026	1890	2205
8.000	73.0517	437.8699	1732	2059
9.100	65.4631	397.8614	1563	1880
10.100	55.2702	352.9356	1336	1679
11.000	51.4983	331.0315	1252	1581
12.600	42.1136	290.5759	1043	1400
12.900	40.6767	281.8590	1011	1361
13.900	36.9947	258.8373	929	1258

δ values are relative to SMOW ($^{18}\text{O}/^{16}\text{O} = 0.0020052$, $^2\text{H}/^1\text{H} = 0.00015576$)

Normalised values are calculated as described in Section 11.3.1.

Other values are: $a = 0.6785$ g, $W = 249.3215$ g, $A = 116.39$ g.

For ^{18}O : $\delta_p = -4.72$, $\delta_a = 181.55$, $\delta_e = -7.64$.

For ^2H : $\delta_p = -22.34$, $\delta_a = 895.89$, $\delta_e = -45.84$.

Table 11.1 cont.

Mass spectrometric data for worked examples - Subject 2

Time (d)	δ (‰)		Normalised enrichment (fraction of dose x 10 ⁷)	
	¹⁸ O	² H	¹⁸ O	² H
0.172	179.4361	582.7563	3811	3659
1.000	156.5229	513.5519	3336	3244
2.000	136.8417	462.8576	2928	2940
3.000	113.7356	400.3234	2446	2565
4.000	99.4571	360.9686	2153	2329
6.100	76.6886	287.0950	1681	1886
7.000	64.6290	245.9058	1431	1639
8.100	56.8144	215.0556	1269	1454
10.200	42.0535	164.3613	963	1150
11.100	36.8438	143.5166	855	1025
12.200	30.9104	123.3389	732	904
13.300	26.1831	105.8293	634	799

δ values are relative to SMOW ($^{18}\text{O}/^{16}\text{O} = 0.0020052$, $^2\text{H}/^1\text{H} = 0.00015576$)

Normalised values are calculated as described in Section 11.3.1.

Other values are: $a = 0.5605$ g, $W = 249.4395$ g, $A = 114.33$ g.

For ^{18}O : $\delta_p = -4.4$, $\delta_a = 163.23$, $\delta_t = -7.64$.

For ^2H : $\delta_p = -27.41$, $\delta_a = 544.85$, $\delta_t = -45.84$.

Table 11.1 cont.

Mass spectrometric data for worked examples - Subject 3

<u>Time</u> (d)	<u>δ</u> (‰)		<u>Normalised enrichment</u> (fraction of dose x 10 ³)	
	¹⁸ O	² H	¹⁸ O	² H
1.000	83.2588	450.4150	5458	5039
2.000	71.5347	375.0090	4703	4264
3.000	57.5124	379.1928	3800	4307
4.000	47.2791	331.1275	3141	3813
5.000	41.1919	312.0571	2749	3617
6.000	39.0801	266.5216	2613	3149
7.000	34.1265	234.9970	2294	2825
8.000	29.0486	202.1103	1967	2487
9.000	25.9119	184.0128	1765	2301
10.000	24.3124	170.5857	1662	2163
11.000	21.1135	164.7478	1456	2103
12.000	16.8898	143.2449	1184	1882
13.000	14.2499	129.4286	1014	1740
14.000	11.4393	109.2879	833	1533

δ values are relative to SMOW (¹⁸O/¹⁶O = 0.0020052, ²H/¹H = 0.00015576)

Normalised values are calculated as described in Section 11.3.1.

Other values are: a = 0.5000 g, W = 249.5000 g, A = 70.00 g.

For ¹⁸O: δ_p = -1.496, δ_a = 77.11, δ_t = -3.00.

For ²H: δ_p = -39.87, δ_a = 487.95, δ_t = -14.00.

Table 11.2

Estimates of pool sizes, rate constants, products and ratios for the worked examples - Subject 1

	<u>Log</u>	<u>Poisson</u>	<u>Diff</u> (%)	<u>Exp'l</u>	<u>Diff</u> (%)
N_o	2450.60	2444.19	-0.26	2437.58	-0.53
(CV%)	0.71	0.59		0.52	
N_D	2527.81	2523.72	-0.16	2519.38	-0.33
(CV%)	0.56	0.50		0.45	
N_D/N_o	1.0264	1.0325	+0.59	1.0336	+0.70
k_o	0.10775	0.10818	+0.40	0.10883	+1.00
(CV%)	0.82	0.88		1.03	
k_D	0.08282	0.08307	+0.31	0.08343	+0.74
(CV%)	0.84	0.90		1.01	
$N_o k_o$	264.042	264.403	+0.14	265.282	+0.47
$N_D k_D$	209.341	209.643	+0.14	210.189	+0.41
$N_o k_o - N_D k_D$	54.702	54.760	+0.11	55.093	+0.71
k_p	0.19056				
(CV%)	0.81				
I_p ($\times 10^7$)	1.61429				
(CV%)	1.24				
k_r	0.02493				
(CV%)	1.72				
I_r	1.03152				
(CV%)	0.34				
$k_r A_r + k_p A_p$	54.700	(see Section 5.4)			

Units: N = moles; k = day⁻¹; Nk = moles.day⁻¹.
Difference columns list % offset compared to the Log fit.

Table 11.2 cont.

Estimates of pool sizes, rate constants, products and ratios for the worked examples - Subject 2

	<u>Log</u>	<u>Poisson</u>	<u>Diff</u> (%)	<u>Exp'l</u>	<u>Diff</u> (%)
N_o (CV%)	2639.91 1.02	2620.24 0.92	-0.75	2602.66 0.80	-1.41
N_D (CV%)	2711.05 0.68	2712.46 0.62	+0.05	2711.62 0.57	+0.02
N_D/N_o	1.0269	1.0352	+0.81	1.0419	+1.46
k_o (CV%)	0.13494 0.96	0.13630 1.19	+1.01	0.13824 1.48	+2.45
k_D (CV%)	0.11489 0.76	0.11477 0.90	-0.04	0.11482 1.12	-0.06
$N_o k_o$	356.230	357.139	+0.26	359.792	+1.00
$N_D k_D$	311.471	311.309	-0.05	311.348	-0.04
$N_o k_o - N_D k_D$	44.758	45.830	+2.40	48.444	+8.24
k_p (CV%)	0.24983 0.76				
I_p ($\times 10^7$) (CV%)	1.39724 1.50				
k_r (CV%)	0.02005 5.59				
I_r (CV%)	1.02694 0.88				
$k_r A_r + k_p A_p$	44.761	(see Section 5.4)			

Units: N = moles; k = day^{-1} ; Nk = moles.day^{-1} .
Difference columns list % offset compared to the Log fit.

Table 11.2 cont.

Estimates of pool sizes, rate constants, products and ratios for the worked examples - Subject 3

	<u>Log</u>	<u>Poisson</u>	<u>Diff</u> (%)	<u>Exp'l</u>	<u>Diff</u> (%)
N_o (CV%)	1708.04 3.33	1685.64 2.80	-1.31	1650.94 2.42	-3.34
N_D (CV%)	1856.41 2.16	1845.50 1.94	-0.59	1838.86 1.80	-0.95
N_D/N_o	1.0869	1.0948	+0.73	1.1138	+2.47
k_o (CV%)	0.13415 2.91	0.13580 3.14	+1.23	0.13978 3.55	+4.20
k_D (CV%)	0.08920 2.84	0.08986 2.99	+0.74	0.09028 3.33	+1.21
$N_o k_o$	229.134	228.910	-0.10	230.768	+0.71
$N_D k_D$	165.592	165.837	+0.15	166.012	+0.25
$N_o k_o - N_D k_D$	63.542	63.073	-0.74	64.756	+1.91
k_p (CV%)	0.22335 1.91				
I_p ($\times 10^7$) (CV%)	3.15376 1.24				
k_r (CV%)	0.04495 11.17				
I_r (CV%)	1.08676 4.27				
$k_r A_r + k_p A_p$	63.561	(see Section 5.4)			

Units: N = moles; k = day⁻¹; Nk = moles.day⁻¹.
Difference columns list % offset compared to the Log fit.

Figure 11.1

Untransformed data plots

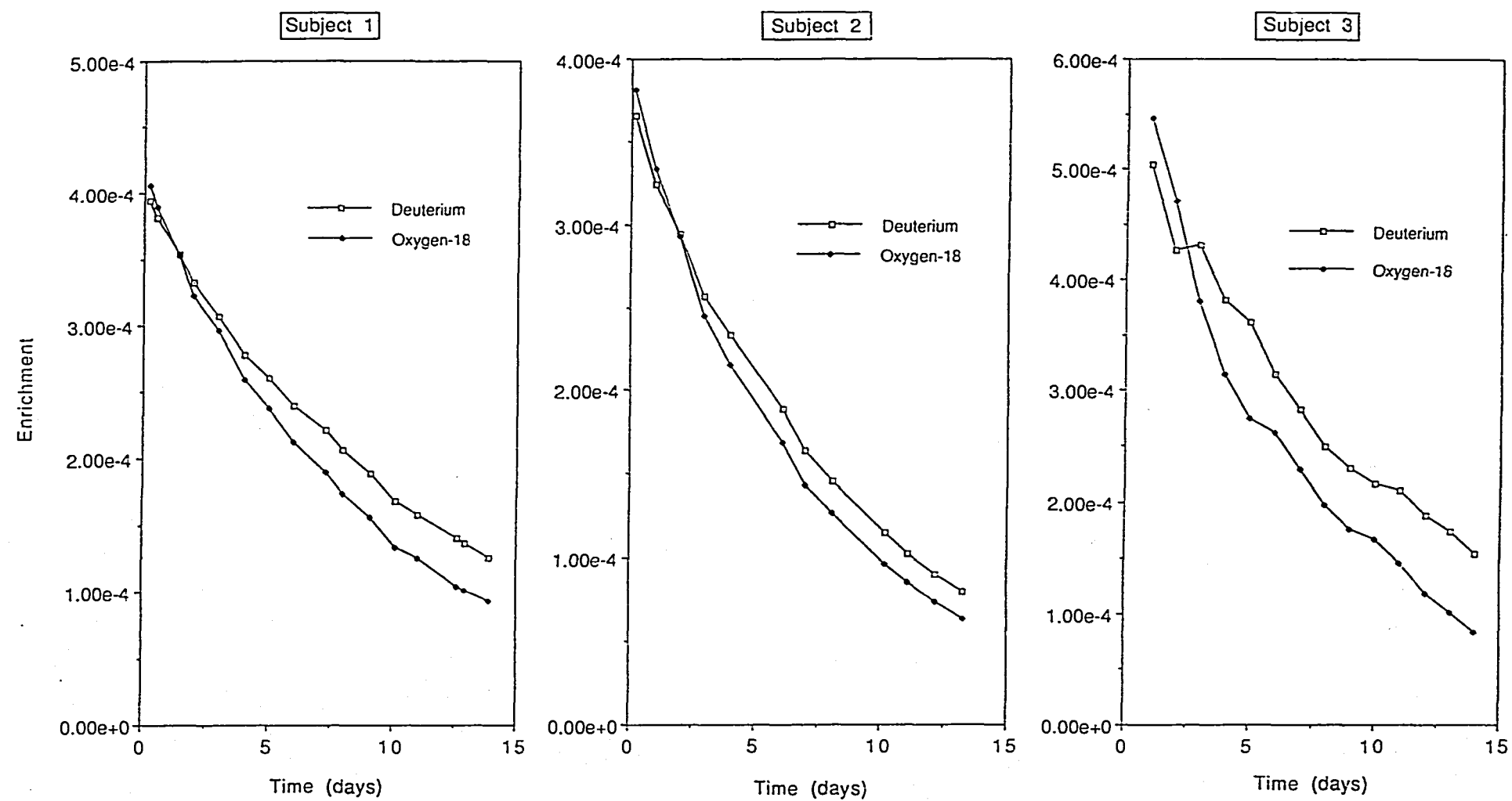


Figure 11.2

Log transformed data plots

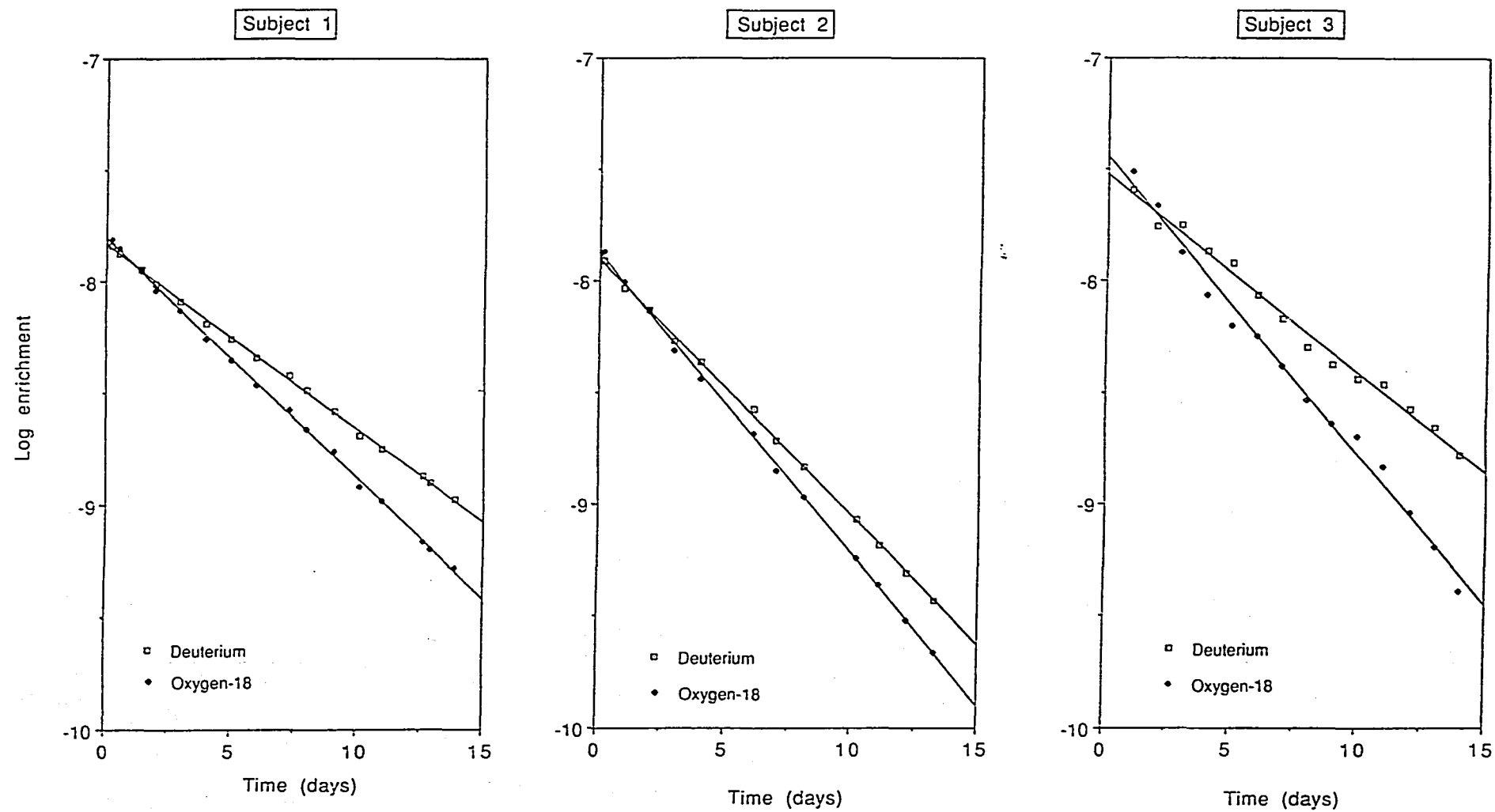


Figure 11.3

Residuals from log fit

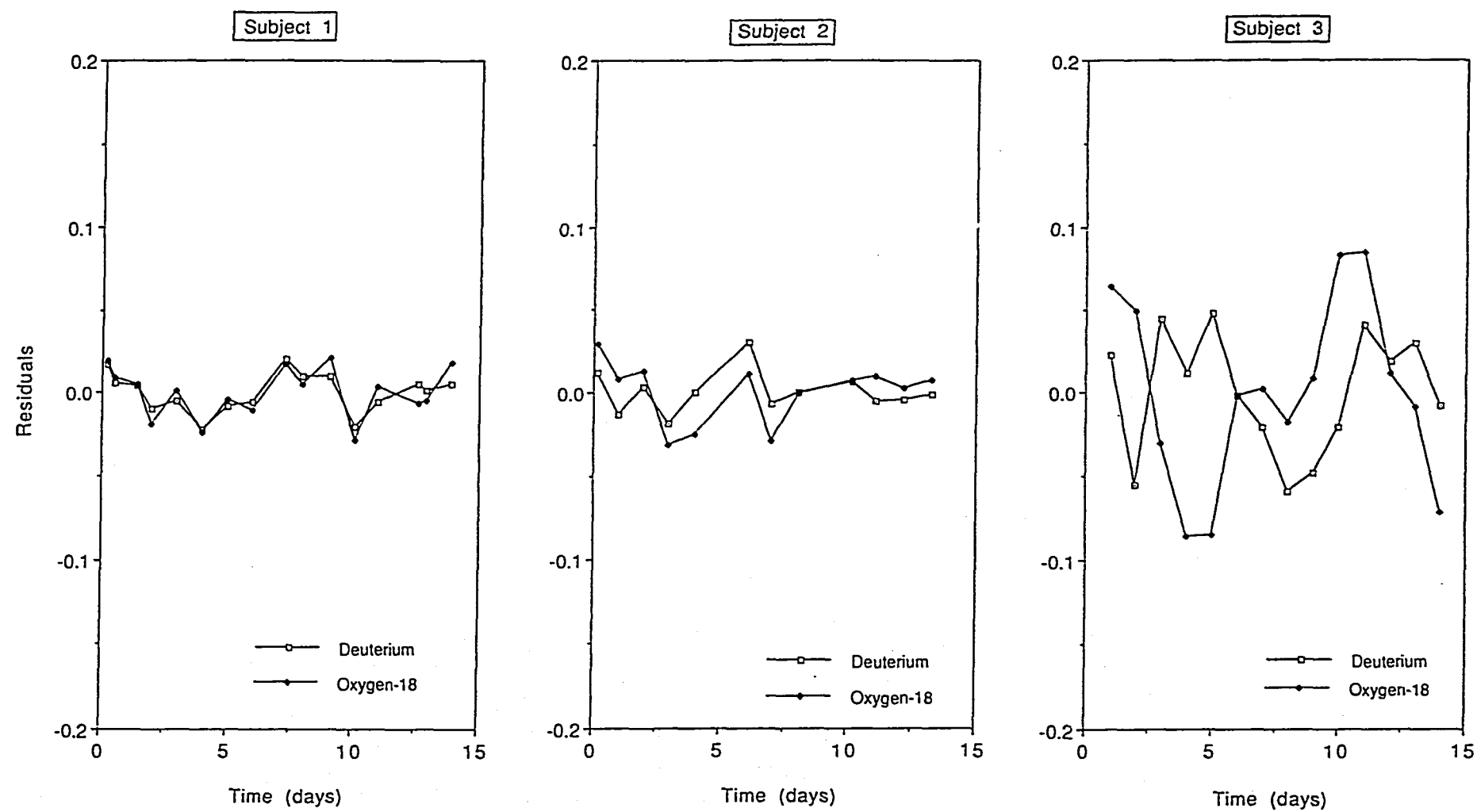


Figure 11.4

Residuals from Poisson fit

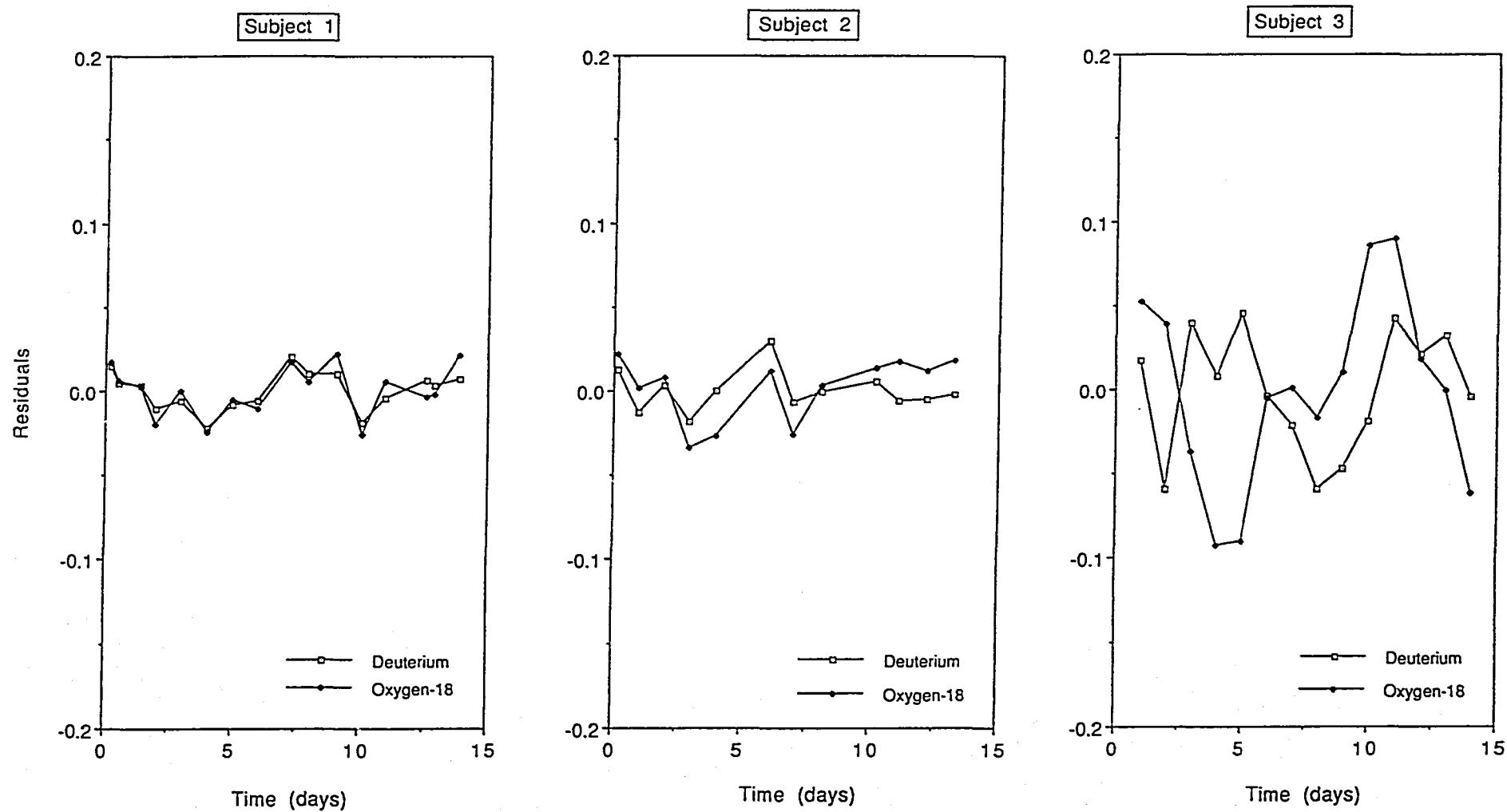


Figure 11.5 Residuals from exponential fit

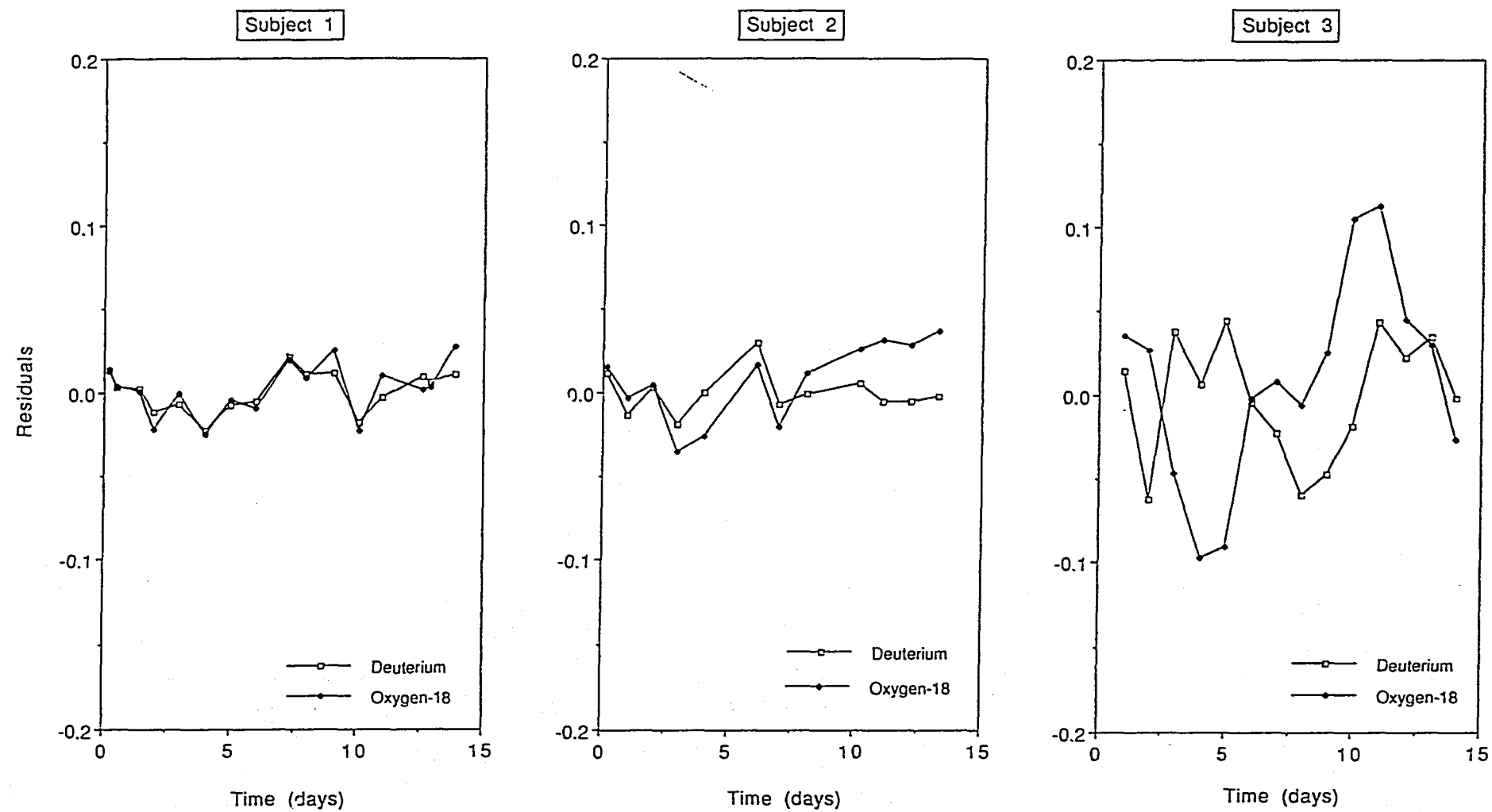


Figure 11.6 Log transformed ratio plots

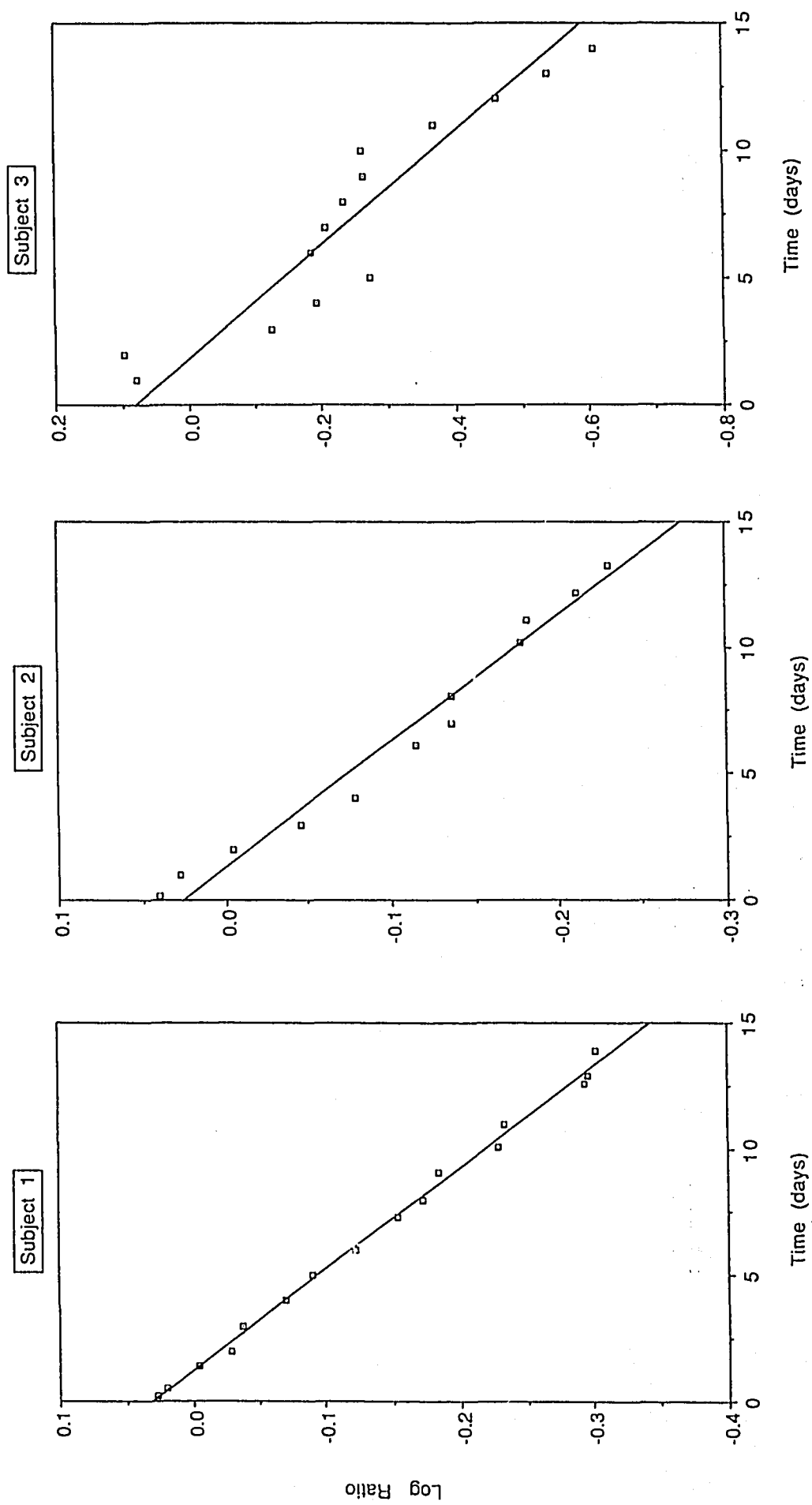


Figure 11.7

Log transformed product plots

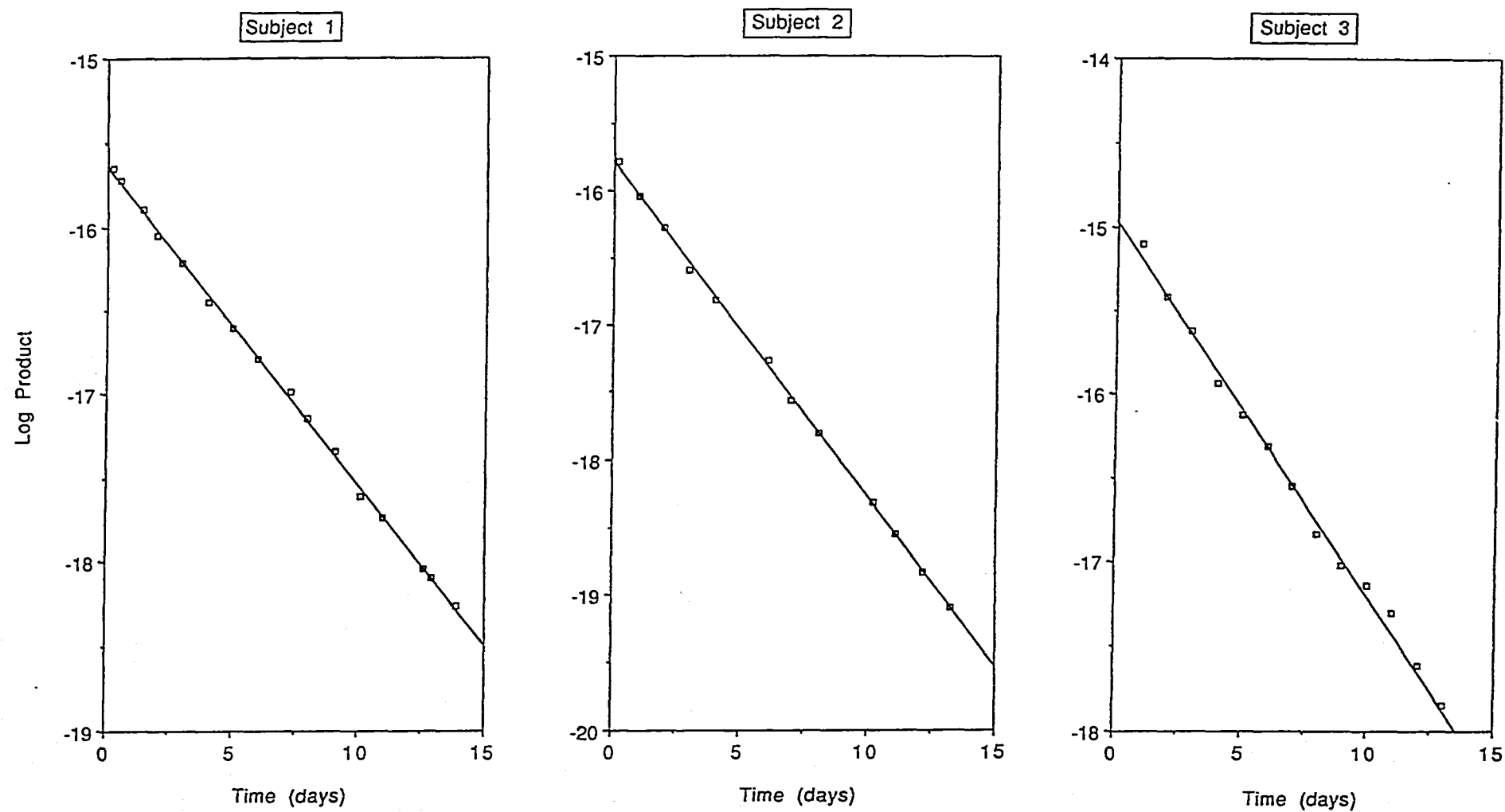
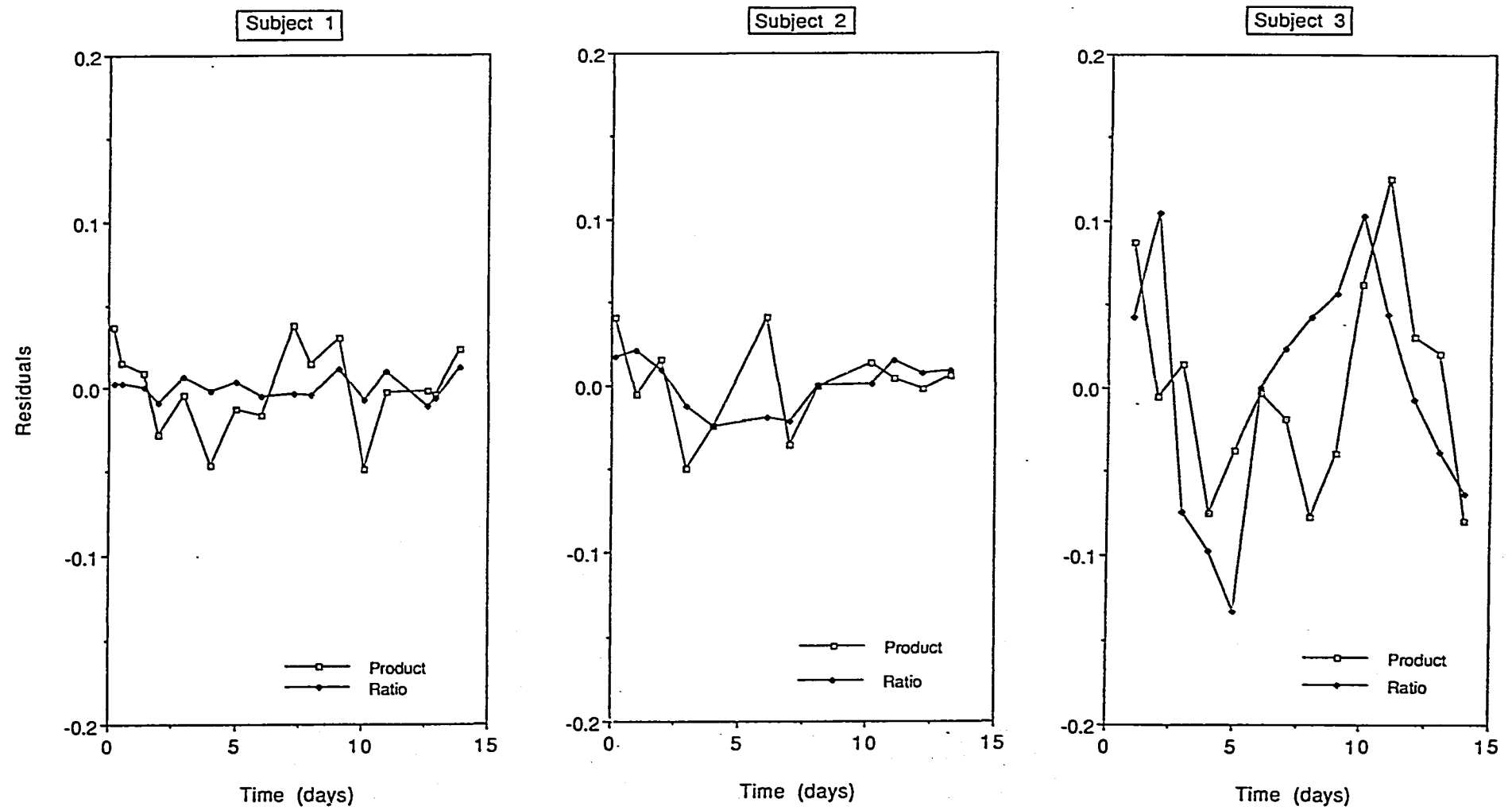


Figure 11.8

Residuals from log transformed ratio and product plots



be acceptable to exclude them from the analysis always remembering that the fewer the number of data points the less justifiable this procedure becomes. In practice untransformed plots need not be drawn since the same use can be made of log transformed plots (see below).

11.3.2 Data transformation and curve fitting

Figure 11.2 illustrates plots of the log transformed enrichments and fitted regression lines. Similar plots could be drawn using the exponential or Poisson fits (Chapters 4 and 5) if preferred. Whichever method is used, the rate constants (k_p and k_o) are represented by the slopes of the regression, and the pool sizes (N_p and N_o) are represented by the reciprocal of the intercepts. These are listed in Table 11.2.

From Subject 1 to Subject 3 the data are progressively less tidy, and although covariance between the δ_o and δ_p plots is evident for Subject 1 and Subject 2, this is not the case for Subject 3.

11.3.3 Checking pool space ratios

As discussed in Sections 4.3 and 9.6, N_p/N_o ratios lying outside the range 1.015 - 1.060 should be treated with scepticism and most probably indicate analytical or dosing error (the latter will only hold true if the doses are administered separately). Table 11.2 shows that Subjects 1 and 2 have acceptable pool space ratios irrespective of which fitting procedure is used. Subject 3, on the other hand, has unacceptably high ratios by all methods of calculation (ranging from 1.087 - 1.114), and according to the IDECG guidelines should be rejected.

11.3.4 Residual plots

The next step is to calculate the residuals (i.e. the difference between the model and each of the experimental points). These are plotted for each of the 3 models in Figures 11.3 - 11.5. The residual plots are extremely useful in three respects.

Firstly, they highlight deviations from the Lifson model. For instance, positive residuals at each end of the measurement with negative residuals in the middle, or vice versa, would indicate curvature due to changing flux or pool size.

Secondly, they provide information about the error structure exhibited by the data and hence about which fitting procedure is preferable. For instance, if the residuals from a log plot increase through the experiment it is an indication that errors are not proportional to δ_o or δ_p values and that an exponential fit may be more appropriate.

Thirdly, they provide the optimum way of assessing covariance between the ^2H and ^{18}O data. On the log scale used, a value of +0.1 indicates that the observed value is 10% higher than the fitted value. For Subject 1 the residuals are small and highly covariant; for Subject 2 the residuals are larger but usually covariant; but for Subject 3 the residuals are both large and frequently not covariant. This provides clear evidence that the data from Subject 3 is far from ideal. The poor level of covariance suggests possible analytical errors which should be checked by re-analysing all of the samples. If it persists then other explanations, such as gross changes in water flux, must be sought. Whatever the explanation, the inspection of residuals has identified the data from this subject as problematical and needing cautious interpretation or outright rejection especially when considered together with the pool space anomaly (Section 11.3.3).

An examination of residuals for early time points is also informative. If these are not close to zero, it indicates that isotope distribution spaces calculated from the intercepts would differ from those calculated from the early time points. This is certainly true for the theoretical situations described in Chapter 9. In the present examples Subjects 2 and 3 show differences but these are relatively small, and in the same direction for both isotopes. (Note that there was no early data for Subject 3.)

It should be stressed that comparison of the results of the three fitting procedures gives little information on which is the appropriate choice or on the 'quality' of the data. As discussed in Chapter 9 only the residuals can be used to decide on the appropriate fit. Even with no measurement error the 3 fitting methods will give rise to different estimates for the parameters. In general, the Poisson fit will give parameter values intermediate between the log and exponential estimates. This will always hold for the rate constants, almost always for the intercept, pool sizes and r_{H_2O} , and for r_{CO_2} in most cases. Thus any of the 3 fitting procedures can be used to obtain a residual plot before deciding which is the best for the final analysis.

11.3.4 Product and ratio plots

Figure 11.6 shows plots of δ_o/δ_p ratios, and Figure 11.8 shows their residuals. (Note that Ratios and Products can also be fitted using Poisson or exponential procedures, but to save space these are not illustrated.) A good fit with small residuals indicates relatively constant rates of CO_2 production (irrespective of possible variations in water turnover) combined with adequate analytical technique. Poor fit indicates the opposite.

A further point about δ_o/δ_p plots is that their intercept on the Y axis directly indicates the N_p/N_o ratio. Thus an inter-

cept of +0.03 on the log scale is equivalent to $N_b/N_o = 1.03$. The lines in Figure 11.6 indicate ratios of 1.031, 1.026 and 1.083 respectively. Once again Subjects 1 and 2 are within the range considered acceptable by IDECG, but Subject 3 is well outside this range. This generates a danger of bias in the CO_2 production estimate as indicated in Section 5.7.

Similarly, deviation of the residuals for early time points away from zero indicates that ratios of volume calculated from intercepts are different from those calculated from early points. If this is the case, then curvature leading to bias may be the cause.

Finally, Product plots of $\delta_o \cdot \delta_b$ and their residuals (Figs 11.7 and 11.8) provide information about the constancy of water turnover. Again, good fits and small residuals indicate the absence of such fluctuations. These need not be important if CO_2 production is relatively constant, as indicated by the δ_o/δ_b plot. However, there is the possibility that curvature on the δ_o and δ_b plots, even if it is covariant, may produce biased estimates of N_o and N_b from the intercepts of the fitted lines. This is the possibility considered in Table 9.2 and discussed in Chapter 9.

In Figure 11.8 residuals for time points early in the disappearance curve for Subjects 1 and 2 indicate that $N_o \cdot N_b$ calculated from the early time points is 4% smaller than $N_o \cdot N_b$ calculated from the intercept (residual = +0.04). Provided N_b/N_o is near the expected value of 1.03 this represents a difference of 2% between values for CO_2 production calculated from intercept isotope distribution spaces and calculated using early values for N_o and N_b . This degree of bias is small enough to be of no concern, but users of the methodology should always check for bias of this type in particular groups of subjects or particular experimental protocols associated with them, in case it leads to erroneous conclusions. In other words some bias on individual

subjects is to be expected, but the bias should be randomly distributed about zero for groups of subjects.

11.3.5 Further calculation of results

Table 11.2 contains further intermediate results for the 3 worked examples. Pool spaces (moles) are calculated as the reciprocal of the zero-time intercept derived from the chosen fitting procedure. Rate constants (d^{-1}) are the slope of the disappearance curves. The percentage differences between results from the different fitting procedures are listed. Considering each subject separately, the following observations can be made:

Subject 1

The data are well-behaved in all respects. Using simple fitting procedures (ie not using the product-ratio method), the standard errors for estimates of pool spaces average about 0.5%, and for rate constants about 0.8 - 1.0%. None of the pool sizes or rate constants differ by more than 1% when calculated using the 3 different fitting procedures, and $N_o k_o - N_p k_p$ (ie $2 \cdot r'_{co_2}$) differs by only 0.71% between the 3 procedures. Note that the estimate of $k_r A_r + k_p A_p$ obtained from the product-ratio method (equivalent to $N_o k_o - N_p k_p$) is always the same as that obtained from the log plot with the exception of minor rounding-error differences. The variance calculated from the product-ratio method is $\pm 1.3\%$.

Taken together with the tight residual plots, almost perfect covariance between the two isotopes and acceptable N_p/N_o value of close to 1.03, the results represent a model case and can be considered very secure.

Subject 2

The data are moderately well-behaved. Using simple fitting procedures, the standard errors for estimates of pool spaces are higher than for Subject 1 and are 0.6 - 1.0%. The same is true for the errors on the rate constants which are between 0.8 and 1.5%. The answers obtained from the different fitting procedures are quite similar, the largest difference being 2.45%. However, when calculated through to $N_0k_0 - N_bk_b$, the log and Poisson methods differ by 2.40%, but the exponential and log methods differ by 8.24%. The variance calculated from the product-ratio method is $\pm 3.98\%$.

The wider standard errors on the estimates of pool size and rate constants propagate through to the higher final estimate of error of about ± 4 . This is still quite acceptable and is backed up by the existence of an acceptable N_b/N_0 ratio of about 1.035, and respectable residual plots. The only cause for concern is that the exponential fit gives a higher answer (+ 6-8%) than the other two fitting procedures. However, the residuals give no *a priori* evidence that an exponential fit is required, and the good agreement between the other two fitting procedures suggest that they are preferable the Poisson fit would represent a sensible compromise solution.

Subject 3

Inspection of any of the data plots (Figs 11.1 - 11.8) immediately shows that the data from this subject are very variable. Using the simple fitting procedures the standard errors for estimates of pool spaces vary between 1.8 and 3.3%, and for rate constants between 2.9 and 3.6%. The pool sizes and rate constants differ by up to 4.2% when calculated using the 3 different fitting procedures, but $N_0k_0 - N_bk_b$ differs by only 1.91% between the 3 procedures. This contrasts with the 8.24% difference for this parameter in Subject 2 and serves to

illustrate that a difference in derived parameters between the 3 fitting methods is not a reliable indicator of data quality. The variance calculated from the product-ratio method is $\pm 7.8\%$.

When interpreting the results of this analysis it is important to consider them in the context of the experiment. In Subject 3 the starting enrichment of ^{18}O was lower than that for Subjects 1 and 2. This in itself would give rise to greater variance on the derived parameters. Also, Subject 3 was an athlete in training who exhibited large daily changes in both r_{CO_2} and $r_{\text{H}_2\text{O}}$ when these were assessed by independent measurements. Therefore the standard errors on the derived parameters and a variance of $\pm 7.8\%$ on r_{CO_2} may well be reasonable. Indeed this estimate is within the recommended cut-off of 8% for the two-point method (Section 11.2.2). However, the magnitude of the residuals from the ratio plot and, more importantly the large pool size ratio, indicate that there may be an analytical problem with this data, and that the samples and the dose should be reanalysed before proceeding.

11.3.6 Incorporation of fractionation corrections and conversion of r_{CO_2} into energy expenditure

Full guidance for converting r'_{CO_2} into r_{CO_2} is provided in Chapters 4, 5 and 6. Further conversion into energy expenditure is described in Chapter 9.

11.4 Practical hints

11.4.1 Administration of dose

The importance of the dosing procedure cannot be over-emphasised. When using a pre-prepared mixture of $^3\text{H}_2\text{O}$ and H_2^{18}O

any errors in calculating the dose given or any spillages or incomplete transfer of the dose to the subject will produce an equivalent percentage error in the estimate of pool spaces and therefore in the final estimate of energy expenditure. If the doses are administered separately an error in the administration of just one of the doses could potentially lead to an even greater error.

If isotope is spilled during administration it is vital not to pretend that this has not occurred. The estimate of energy expenditure will be wrong and this will only serve to devalue the whole of your data-set. When it has taken days to recruit the subject, explain the procedures, obtain informed consent and arrange the dosing appointment, and with the prospect of wasting expensive isotope it is very tempting to ignore a less than perfect dosing. This temptation must be avoided, and the run should be terminated immediately in order not to waste any further effort on an invalid measurement. It should be noted that if the subject is redosed before their enrichment has returned to background another pre-dose must be taken to calculate dilution spaces.

Adults

Dosing adults presents no real practical difficulties. The main requirements are:

- 1) Most investigators require that subjects should be fasted for a minimum of 4 hours and preferably overnight.
- 2) A pre-dose urine sample must be collected to determine the subject's background enrichment. This also ensures that the subjects have voided prior to dosing.
- 3) An accurate body weight (corrected to nude weight) is required.

- 4) Care must be taken to avoid any spillage.
- 5) The dose should be kept sealed for as long as possible to avoid evaporation and fractionation.
- 6) The exact quantity of dose delivered must be obtained by weighing the dose container before and after administration (accuracy $\pm 0.2\%$ or better is desirable).
- 7) An aliquot of dose should be reserved for dilution and analysis alongside the samples.
- 8) A small 'chaser' of unlabelled water (50 - 100 ml) is probably advisable to wash the dose into the stomach and avoid immediate evaporation from the mouth or oesophagus.
- 9) Early protocols insisted that subjects should remain fasted during the 3 - 6 hour equilibration period. The theoretical concern about excessive fluid ingestion during the equilibration period is that it will slightly expand the dilution space. However, the converse can be argued if no fluid is allowed, and recent studies have often permitted moderate food and fluid intake.
- 10) Collection and analysis of all urine voided during the equilibration period can be performed in order to correct the estimate of dose given by subtraction of dose voided. In practice, however, this correction is usually trivial and can be dispensed with.

Babies and infants

This is much more difficult than dosing adults and many obvious errors (such as estimates of isotope dilution spaces

which appear to be greater than the entire body volume) can be traced back to problems with dosing. The actual dosing technique is an art, and success rates increase with experience. Whenever possible it is therefore best to allow a single investigator to develop and practice their technique, and to be responsible for all pediatric dosing. The optimal way to dose very young babies is probably to trickle the dose into the back of the mouth using a fine tube attached to a syringe. Older infants can successfully be dosed by diluting the dose with juice or cordial in a feeding cup. If this is done the entire dose must be consumed and any residue should be washed into the child with a small refill of the cup.

The requirements listed above for adults are all applicable to young children, but the pre-dose fast and equilibration periods can be shorter. Pre-dose fasts as short as 2 hours appear to be satisfactory in children less than 2 years old. Equilibration periods of 2 - 4 hours are usually used. It is often impossible to keep a young infant fasted during the equilibration period, and in practice small comfort feeds have to be tolerated.

In order to avoid having to give a large volume of dose to infants it is best to purchase $H_2^{18}O$ with at least 10% enrichment and preferably about 15%.

Quantity of dose

The most important constraint when deciding how much dose to give is the cost of ^{18}O . This almost always means that measurements are made using the minimum possible dose. Schoeller¹ has summarised the theoretical basis for determining the amount of dose to give and the reader is referred to his paper for a full derivation of the optimal doses to be employed. In practice the decision is always a 3 way compromise between cost, the need to make a sufficiently long measurement and errors incurred as

the final samples approach background enrichment levels. In adult studies many laboratories employ doses of 0.05 g $^2\text{H}_2\text{O}$ /kg and 0.15 g H_2^{18}O /kg. Irrespective of the absolute amount given it is important to keep the ratio of the two isotopes close to this in most circumstances since, as Schoeller has pointed out (Section 8.4), this minimises the effects of fluctuations in background enrichment by taking full advantage of the natural covariance in such changes.

11.4.2 Choice of physiological fluid to be sampled

Isotope enrichments can be determined in any physiological fluid provided that the same fluid is sampled throughout. Unless there is a specific interest in studying the short-term dynamics of isotope mixing after dosing there is no advantage in using plasma, and there are obvious disadvantages. The choice is therefore between urine and saliva. Urine is usually chosen for adult studies. The main precaution which it is necessary to take is to ensure that the urine has not been collecting in the bladder over a long period since it will then be impossible to ascribe an accurate time to the sample. Saliva is more commonly used in studies of infants since they will not provide a urine sample on demand. Clearly the temporal definition is superior with saliva sampling. However, Schoeller argues that saliva should not be used (especially for the two-point method) since different degrees of fractionation may be present at different sampling times.

11.4.3 Collection and storage of samples

Most adults can be trusted to collect their own samples if provided with a series of numbered universal bottles (or similar), and a record sheet on which to record the time and date of each sample. However, the experimenter must beware of a minority of subjects who confuse the order of the bottles, fill

several bottles with aliquots of the same urine sample (presumably because they have forgotten to collect samples on a number of previous days) or even fill the bottles with water!

Collection of saliva samples from infants can conveniently be achieved by swabbing the mouth with a small sponge or some absorbent cotton wool on the end of an orange stick. When saturated this is immediately transferred to a 1ml syringe in order to express the saliva into a small sampling tube using the plunger.

All sampling and storage procedures should observe the following rules to avoid isotopic fractionation: a) the sample should only be exposed to the atmosphere for the minimum possible time; b) containers should be absolutely air-tight; c) there should be a minimum of air-space above the sample to minimise the possibility of isotopic exchange with any trapped atmospheric moisture; d) although some mass spectrometer procedures only require microlitre samples it is prudent to collect several ml of urine and at least 0.5 ml of saliva in order to minimise the chances of significant fractionation during pre-analytical manipulations such as transfer to auto-sampler vials.

Samples can be stored indefinitely and should preferably be frozen although this is not an absolute requirement under difficult field conditions. There is no objection to samples being frozen, defrosted (for example in transit) and then refrozen.

11.4.4 Sampling regimes

The pros and cons of various sampling regimes have been discussed fully in the body of the report. The basic rule governing the duration of a measurement is that it should last between 2 and 3 biological half-lives of each isotope. This tends

to yield measurement periods of; 6-9 days in babies and children; 12-16 days in normal sedentary adults in temperate climates; 8-12 days in exceptionally active adults or people living in tropical climates where high rates of water turnover reduce the half-lives; and 16-20 days in elderly subjects.

In the two-point method the critical issue relating to sample timing is the most appropriate choice of the equilibration period in order to ensure that the sample obtained is on the plateau.

In the multi-point method it has been shown in Section 5.5 that the most efficient sampling regime uses a cluster of samples at each end of the measurement period. However, this demonstration is based on random errors and does not account for possible step changes in flow rates. If no samples are collected in the middle of the measurement period the process of inspecting residuals for deviations from the Lifson model becomes much less sensitive, and it is more difficult to decide which data should be rejected.

11.4.5 Collection of subsidiary data

Environmental conditions

Although it is impossible to make accurate predictions of the fractionated proportion 'x' (see Chapter 6) the working group recommended that information should be provided on average temperature and humidity encountered during each study. This will provide the reader with at least some means of assessing whether they endorse the fractionation assumptions selected by the investigators.

Fractionation corrections

In most circumstances it is probably futile to attempt to collect direct information on the appropriate value for 'x'. The variables required are: total water turnover, respiratory water losses and non-sweating insensible water losses. The first of these is known (r_{H_2O}) and the others cannot be determined in free-living people. Measurement of urinary output is not helpful in this respect. Figure 6.3 contains data on insensible water losses measured under cool conditions (ie with the children assumed not to be sweating) in a whole-body respiration chamber. This data is useful in indicating that 'x' is likely to be very low, but the insensible losses were measured in sleeping children and may not be entirely representative of 24-hour values in real life.

Changes in isotopic background

If substantial changes in isotopic background are anticipated it may be advisable to enrol an undosed control group in order to quantify the average change by analysis of serial samples. Examples of such a situation may be when subjects change their primary water source (eg Arctic explorers), or when the enrichment of the main water source may change (eg during seasonal recharge of drinking water wells in the tropics). As discussed in Chapter 8 any changes in isotopic background have to be quite severe before they become a major concern to DLW particularly if Schoeller's advice concerning isotope ratios in the loading dose is adhered to.

Food quotients

As a very minimum it is necessary to have some qualitative knowledge of the fat:carbohydrate ratio in the diet in order to

decide what RQ to assume when ascribing an energy equivalence to the estimate of carbon dioxide production. The reader is referred to Chapter 9 and the publication of Black et al ² for methods of calculating the energy equivalent of CO₂.

Assessing changes in body composition

It would be useful to have an accurate assessment of changes in body composition during a measurement period for the following reasons: a) to assess possible isotope sequestration (see Chapter 7); b) to correct the food quotient by allowing for endogenous fat oxidation or fat deposition; and c) to assess agreement between simultaneous estimates of energy intake and expenditure. In theory this could be achieved by making a second estimate of total body water at the end of the measurement (using ²H₂O for economy). However, in practice this is of limited value since, even if total body water can be assessed to within $\pm 0.5\%$ at the beginning and end of the run this feeds through to an uncertainty in the region of ± 1 MJ/day with regard to fat oxidation or deposition. Changes in body weight therefore tend to be as useful as any refined attempts to assess fat store changes.

Additional physiological measures

Measurement of basal metabolic rate in subjects studied by DLW allows calculation of the Physical Activity Level (PAL) as TEE/BMR. An alternative way of expressing the same information is to calculate the energy cost of activity-plus-thermogenesis as TEE - BMR. Both of these are extremely valuable indices. It therefore seems false economy ever to make a DLW measurement which is not matched by an estimate of BMR particularly in view of the large investment inherent in DLW measurements.

11.5 References

1. Schoeller DA (1983) Energy expenditure from doubly-labelled water: some fundamental considerations in humans. *Am J Clin Nutr*; 38: 999-1005.
2. Black AE, Prentice AM & Coward WA (1986) Use of food quotients to predict respiratory quotients for the doubly-labelled water method for measuring energy expenditure. *Human Nutr: Clin Nutr*; 40C: 381-391.

CHAPTER 12

RECOMMENDATIONS FOR DATA PRESENTATION IN PUBLICATIONS

The working group recommended that future publications on DLW should contain as much methodological information and detail of the intermediary steps in data reduction as possible. A good paper should contain the following:

Methods

Explicit description of:

- 1) The dosing procedure - including mode of administration; timing; physiological state of the subjects; amount of dose given; equilibration period; any corrections for isotope losses during equilibration and any precautions taken to ensure completeness of isotope ingestion (particularly in studies of babies and young children).
- 2) Mass spectrometric procedures - including instrumentation; standards; estimates of analytical precision for both ^2H and ^{18}O within the ranges used; and an

assurance that the dose enrichment had been measured (and not assumed from the manufacturer's value).

- 3) Calculations - including details of formula used; exact description of how plateaus, intercepts and slopes were derived; method of deriving a single pool space if this was used; calculations of precision where appropriate and details of any cut-off used to screen out unacceptable estimates; details of inspection of residual plots and extent of covariance in ^2H and ^{18}O values.
- 4) Fractionation corrections - including method of estimating fractionated proportion 'x'; statement of whether individual or group mean estimates were used; a statement of the mean value derived for 'x' if individual predictions were used; details of any environmental measurements pertinent to the determination of 'x' (ie ambient temperature, humidity and working conditions); listing of the exact values assumed for f_1 , f_2 and f_3 .
- 5) RQ assumptions - including details of specific derivations from dietary assessment with or without corrections for energy imbalance during the measurement period; justification for group mean estimates of RQ if used.

Results

- 1) Tabulated 'raw' data - including (unless the data set is very large) the individual estimates of N_p and N_o , k_p and k_o , $r_{\text{H}_2\text{O}}$ and r_{CO_2} ; individual values for N_p/N_o if the separate estimates of volumes are not listed; individual estimates of precision (where calculated).
- 2) Tabulated final data - including individual estimates of

total energy expenditure and BMR wherever possible.

Adoption of these recommendations will ensure a clear and comprehensive presentation of DLW results. It will allow readers to assess the quality of the data being presented, for instance by examining the values of N_p/N_o and the estimates of precision. In certain circumstances it may also permit them to recalculate the results using their preferred formula in order to make a more direct comparison with their own data. It is appreciated that certain journals may be resistant to the concept of publishing intermediate data from which the final estimates of expenditure are derived. However workers in this field can exert considerable influence on editors as reviewers. Reference to the recommendations contained in this document may also help to persuade editors of the value of explicit and complete data presentation.

CHAPTER 13

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USE OF THE DOUBLY-LABELLED WATER METHOD UNDER DIFFICULT CIRCUMSTANCES

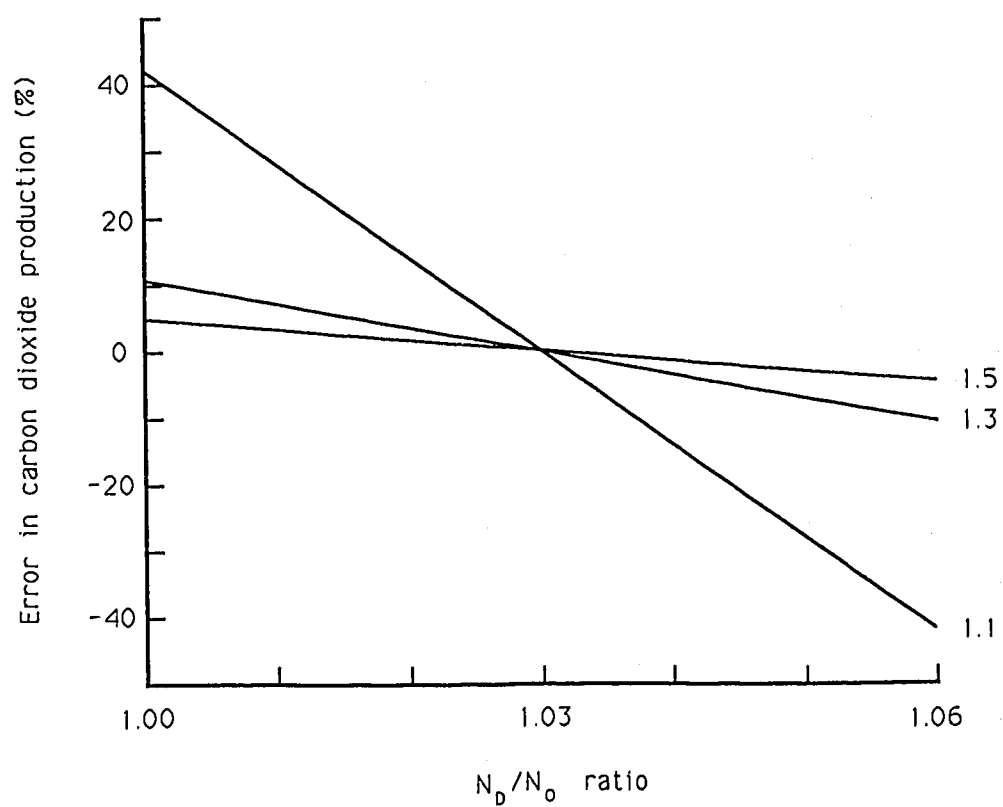
13.1 General considerations

The single most important factor in determining the potential for error in doubly-labelled water measurements is the difference between the rates of ^2H and ^{18}O disappearance. In subjects such as healthy adults, the difference between the two disappearance rates is relatively large. However, in certain groups of subjects who have either very high rates of water intake and/or low metabolic rates in relation to body size, the difference between the two disappearance rates can be reduced substantially. In these cases, there is a much greater potential for error generated by various aspects of the method, in particular the precision of measurements of rate constants and intercepts, and factors such as the mathematical correction for isotopic fractionation.

The magnification of error associated with a low k_o/k_p ratio is illustrated in Figures 13.1 and 13.2, which show the error associated with measurement of the two dilution spaces (N_o and

Figure 13.1

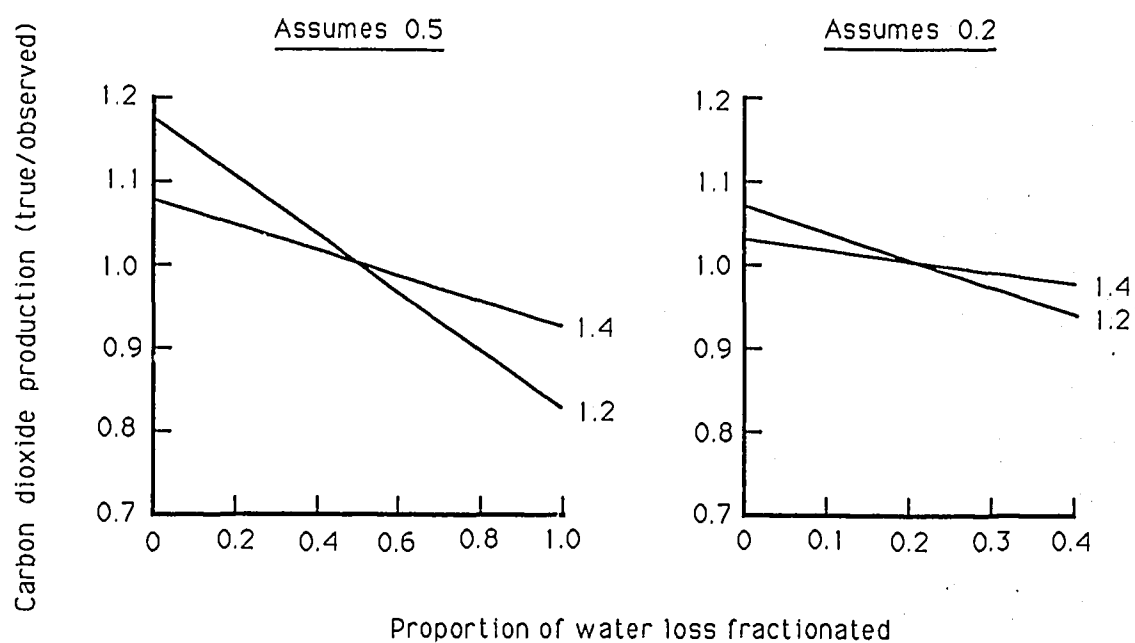
Interaction between the k_o/k_p ratio and deviation of the true N_p/N_o ratio from an assumed value of 1.03



Labels on the 3 lines indicate the k_o/k_p ratio.

Figure 13.2

Effect of an error in the fractionation assumption on the final estimate of r_{CO_2}

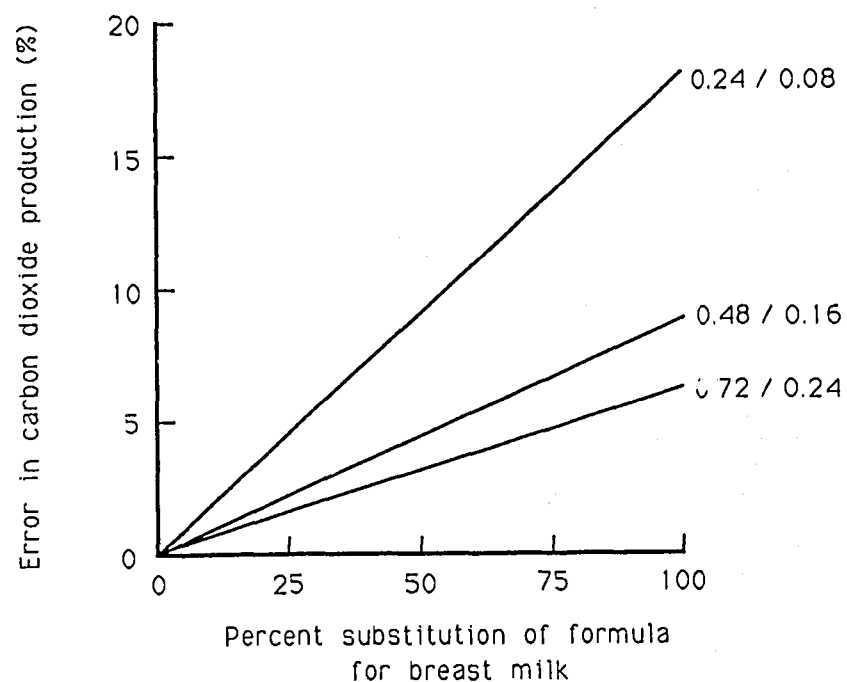


The two panels illustrate the effect of error around initial assumptions of 0.5 (i.e. Lifson's value) and 0.2 (closer to the value anticipated in children).

Labels on the 4 lines indicate the k_o/k_p ratio.

Figure 13.3

Effect of weaning on the accuracy of doubly-labelled water at different dosing levels



Labels on the 3 lines are g H_2^{18}O / $^2\text{H}_2\text{O}$ per kg TBW.

N_D) and the correction for isotopic fractionation, for k_o/k_b ratios ranging from 1.5 (the upper limit of values for adult athletes) to 1.1 (the lower limit of the range for infants). It can be seen that the k_o/k_b ratio has a major influence on the potential for error. For example, incorrect measurement (or assumption) of a ratio of N_D/N_o of 1.03 for an individual with a value of 1.06 will result in a difference in the calculated CO_2 production rate of 6% when k_o/k_b is 1.5 compared to 43% when k_o/k_b is 1.1 (Figure 13.1).

Similarly, concerning the correction for isotopic fractionation (Figure 13.2), the maximum potential error associated with assuming that isotopic fractionation occurs in 50% of water output would affect the calculated CO_2 production rate by 3% when k_o/k_b is 1.5 compared to 45% when k_o/k_b is 1.1. However, in this case, it is important to note that although the potential for error is great when k_o/k_b is low, the actual error generated is much less than this. This is because the probable range of water loss subject to isotopic fractionation is reduced when total water turnover is high (the usual cause of a low k_o/k_b ratio) ¹. For example, when expressing fractionated water output as a percentage of total water output (x), the probable range for adults is 20-50% (giving a maximum error when using the mid-point of approximately 3%), whereas the range 10-20% is more usual in infants (giving a maximum error when using the mid-point of approximately 6%).

In subjects in whom there are no stepwise changes in water intake or carbon dioxide production during the doubly-labelled water study, compensation for a low k_o/k_b ratio can be achieved by increasing the number of data points used to determine outflow rates. A corollary of this statement is that the measurement of CO_2 production rate in a subject with a low k_o/k_b ratio will be substantially less precise than a comparable measurement in a subject with a high k_o/k_b ratio unless more data points are used to determine the outflow rates of the isotopes. An additional means

by which the precision of doubly-labelled water measurements can be enhanced during the study of difficult subjects with a low k_o/k_d ratio is to increase the doses of the isotopes given at the start of the study, while at the same time ensuring that the ratio of isotopes given is appropriate for the study population (see Chapter 11).

13.2 Use of the doubly-labelled water method in infants

There are several considerations that need to be addressed when using the doubly-labelled water method in infants:

- 1) In particular, infants have high rates of water intake in relation to body size and carbon dioxide production, leading to the increased potential for error discussed above. Mean values for k_o/k_d in premature infants are 1.13, with individual values being as low as 1.10², while in older infants a mean of 1.20 has been reported³. Thus, the potential for error is approximately 4-fold greater in infants than in adults.
- 2) In addition, rapid growth during doubly-labelled water measurements is common in infants. Premature infants can increase in weight by as much as 14% during a doubly-labelled water study, compared to 4% or less in normally growing 3-month infants and in adults in moderately severe positive or negative energy balance (Table 7.2). The increase in the isotope dilution space occurring during growth needs to be taken into consideration to avoid an under-estimation of CO₂ production rate (see Appendix 3).
- 3) When growth rate is extremely rapid, as in the case of premature infants, the necessary adjustment to the calculation of outflow rates and CO₂ production involves use of study means for the isotope dilution spaces (rather than

individual measurements for the dilution spaces that are made at the start of the determination) in the calculation of outflow rates. In their study of premature infants Roberts *et al* ² used weighted study means (assuming an exponential change in water during the study) for each dilution space in the calculation of F_{H_2O} and $F_{H_2O+CO_2}$. The weighted means were calculated using the general equation:

$$N = (N_1 - N_2) / \{\ln(N_1/N_2)\}$$

where N is the mean dilution space during the study, and N_1 and N_2 are the dilution space volumes at the beginning and end of the doubly-labelled water study. The dilution space at the end of the study was determined from body weight, assuming proportional increases in dilution spaces and weight during the measurement. This procedure gives results that are similar to those obtained using a simple average for the study period that is based on linear weight change. By contrast, use of the initial dilution spaces measured at the start of the study results in a significant under-estimation of carbon dioxide production (by an average of 10% in the case of the infants studied by Roberts *et al* ²).

- 4) When growth rates are not extremely rapid, as in the case of older infants, and in adults undergoing moderately severe positive or negative energy balance, the correction for weight change during the measurement makes only a very small difference to the calculated CO_2 production rate. The correction described above for premature infants may be used.
- 5) The final theoretical factor that needs to be taken into consideration in doubly-labelled water studies in infants is the possibility that isotopic backgrounds may change significantly during the measurement period. This is because infants undergo major dietary changes during the normal

course of infancy, and the different diets that are used may have very different ^2H and ^{18}O contents, as described in Chapter 8. Changes in isotopic backgrounds in the body will accompany changes in isotopic intakes. If such changes occur during a doubly-labelled water measurement, they can have a significant effect on the accuracy of the determination of CO_2 production, the magnitude of which will be determined by the isotope dose intake and the duration of the doubly-labelled water study ⁴. For a study period of 7 days (equivalent to 3 half-lives for ^2H disappearance), complete weaning from breast milk to infant formula during a doubly-labelled water study can result in an under-estimation of CO_2 production rate ranging from 18% if a relatively low isotope dose is given (0.24 g/kg H_2^{18}O and 0.08 g/kg $^2\text{H}_2\text{O}$), to 6% if a high isotope dose is given (0.72 g/kg H_2^{18}O and 0.24 g/kg $^2\text{H}_2\text{O}$). (Figure 13.3). The implication of these calculations is that doubly-labelled water studies should be avoided during rapid weaning. If this is not possible, use of a large isotope dose will reduce the potential for error. In addition, control subjects who receive no isotope can be studied to quantify changes in backgrounds, which can then be used to correct the raw isotope data.

- 6) The ratio of ^{18}O to ^2H in dose water influences the accuracy of energy expenditure data. The optimal dose ratio for infants and young children exceeds the optimal ratio for most older people since rates of CO_2 production per kg body weight are greater in the younger population. Schoeller ⁵ has discussed this issue in detail.

Some practical concerns include:

- 7) Discrepancies between weighed and administered doses can occur because of spitting or drooling during dose administration, or because of post-dose regurgitation. The effects of losses can be reduced by using doses with low

percent enrichments. Doses may be administered by trickling the dose deep into the back of the mouth by pointing the syringe directly toward the uvula and repeatedly squirting small quantities which can be swallowed easily. In some infants, this is easier if they are crying. Swallowing can be encouraged by quickly blowing into the infant's face. Alternatively, a small diameter polythene tube can be attached to the syringe and placed in the back of the throat. This procedure may cause some infants to gag. A simple and worthwhile step is to test both dosing procedures using unlabelled water in each infant prior to the study. To quantify losses, a pre-weighed tissue can be sealed in a plastic bag, used to absorb drops lost by spitting, and reweighed to adjust the weight of dose administered.

- 8) The potential for regurgitation after feedings and requirements for more frequent feedings in comparison to protocols used in studies of older people necessitate special procedures in studies of infants and young children during the 3 to 5 hour interim between dosing and isotopic equilibration. Most adult studies using isotope dilution to measure total body water have withheld food and water from the subjects during the interim between dosing and completion of the urine sample protocol. Isotope losses due to post-dose regurgitation may be reduced by delaying the post-dose feeding until isotopic equilibration has been achieved. However, since this interval may exceed the recommended period of fasting for infants, a feeding is usually offered at 0.5 to 1 hour post-dose. Since this additional water must equilibrate with the dose and body water, it effectively extends the equilibration period. Isotopic equilibration between diet water, body water and dose water should be evidenced by the finding of no biologically significant difference between isotopic enrichment in the urine used to calculate total body water volume (ie 3 to 5 hours post-dose) and the urine sample

which preceded it. A reasonable cut-off for the difference is 2 to 4%, depending on the application of the data. Although diet volume is small (<5%) in comparison to total body water, some investigators subtract the volume ingested from the dilution space to calculate total body water volume. A related problem may be that the exact amount of dietary intake is unknown, particularly when infants are breastfed - mothers may elect to suckle their infants during the equilibration period. The investigator needs to be aware in advance of the dosing if an infant is being breastfed, and should attempt to weigh the infant before and after the feeding to estimate the volume ingested.

- 9) In clinical studies of infants, doses may be administered by oral or nasal gavage. This procedure requires a wash or "chaser" to rinse isotope from the tubing and has the disadvantage that some tracer may adhere to the sides of the tube as it is withdrawn from the infant.

13.3 APPLICATION OF THE DOUBLY-LABELLED WATER METHOD AT HIGH ACTIVITY LEVELS

The doubly-labelled water method is ideal for measuring the energy cost of heavy work since it does not interfere with the performance of the subject. However, when planning observations at high activity levels there are some extra precautions and assumptions to be made compared with observations in the "normal" sedentary adult.

1) Length of the observation period

For maximal precision, the observation period should be one to three biological half-lives of the isotopes ⁵. During heavy sustained exercise the half-lives of ¹⁸O and ²H decrease to 2-4 days, about one third of the values in the

"normal" adult ⁶. Consequently, depending on the expected activity level, the observation period should be shortened. As a rule of thumb, daily energy expenditure is 1.5 times basal metabolic rate for sedentary people and 4-5 times basal metabolic rate at maximum performance. The length of the observation period should be adjusted accordingly.

2) Isotope fractionation corrections

At high activity levels the rate of water loss via fractionating gaseous routes increases as does the rate of carbon dioxide production. Breath water vapour is proportional to the ventilation volume. Transcutaneous water loss increases proportionally more than carbon dioxide production ⁷ but here the major part is unfractionated sweat. These effects therefore have a tendency to cancel, and there may be no need to apply different fractionation corrections at high and low activity levels. However, each situation should be critically assessed with the aid of information provided in Chapter 6.

3) The energy equivalent of CO₂

At high activity levels people tend to have a different diet from the general population. Therefore it is preferable to measure dietary intake simultaneously with carbon dioxide production when the latter data have to be converted to energy expenditure. In The Netherlands the FQ for the average subject is close to 0.85 while for instance endurance athletes have FQ values generally higher than 0.90 ⁸ due to a higher carbohydrate intake. Calculating the RQ, one has to know the FQ and the change in body composition over the observation interval. The latter is not really feasible over intervals with the length of a DLW run so the best that can be done is to measure body mass and body water

volume at the start and end of the observation period and translate any changes in the energy equivalent of CO_2 .

4) Potential accuracy

The potential error in the calculated rate of carbon dioxide production decreases with increasing activity level. Subjects at a high activity level have a relatively higher r_{CO_2} than $r_{\text{H}_2\text{O}}$ compared with sedentary subjects, as derived from the ratio between k_o and k_d (Table 13.1).

In conclusion, there are extra precautions and assumptions to be made when one uses the doubly-labelled water method at high activity levels. The single validation study performed at high activity indicates that the method has a similar precision at low- and high-activity levels ⁷.

13.4 Application of DLW under tropical conditions

There is an inevitable loss of precision when DLW is used under conditions where high ambient temperatures cause high rates of water turnover with a consequent reduction in the k_o/k_d ratio. In studies in The Gambia ^{9,10} precision was about 3 times worse than similar measurements in temperate climates. There is little that can be done about this except to increase dose levels and pay extra attention to analytical precision.

13.5 Application of DLW in non-compliant subjects

One of the major advantages of DLW is limited level of compliance which is necessary. Young children represent one group in which compliance is low and yet in which the technique has been very successfully applied. Studies have also been performed

in a group of highly non-compliant elderly, mental patients with good results ¹¹.

13.6 Application of DLW in clinical situations

The major constraint of the method in most clinical circumstances is the need to study several half-lives. This means that rapidly changing clinical states cannot be successfully studied.

The method has been used in children treated for burn injury ¹². Depending upon the type of treatment given fractionation may be high due to high levels of water loss across the burn. This generates the greatest methodological problem which must be assessed on an individual basis.

Table 13.1

k_d/k_o in subjects at low and high activity levels

<u>Activity level</u>	<u>n</u>	<u>TEE/BMR</u>	<u>k_d/k_o</u>
low	5	1.4 ± 0.09	0.78 ± 0.02
high	8	2.6 ± 0.25	0.74 ± 0.03
very high	15	4.9 ± 0.57	0.74 ± 0.04

Values are means \pm sd.

13.7 References

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APPENDIX 1

Contributor: Andy Coward

A BRIEF INTRODUCTION TO KINETIC STUDIES WITH TRACERS

App 1.1 Single pool systems

Figure App 1.1 illustrates a single pool of water with volume N and input and output rates equal at a value F per unit time. The fraction of the pool being replaced per unit time is thus F/N and we call this value the rate constant for the system (k).

If a quantity (Q_0) of a tracer is added to this at time zero, calculus shows that:

$$Q_t = Q_0 e^{-kt}$$

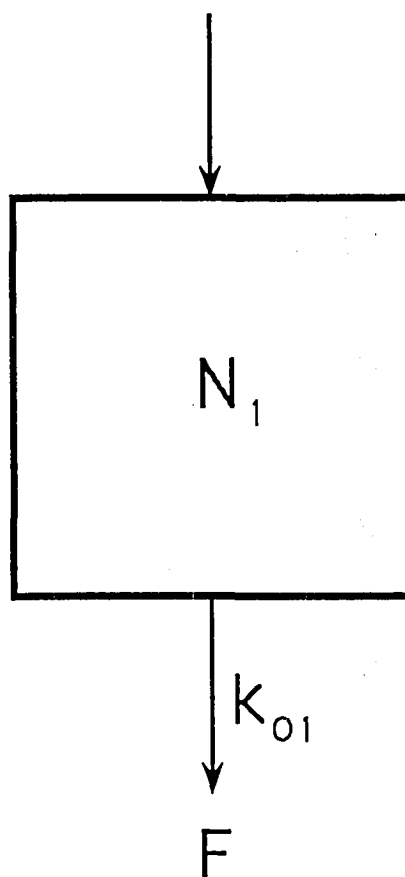
If N remains constant we can convert this equation for quantity of tracer into one for tracer concentration (C):

$$\frac{Q_t}{N} = \frac{Q_0}{N} e^{-kt}$$

$$C_t = C_0 e^{-kt}$$

Figure App 1.1

Single pool system with a single outflow



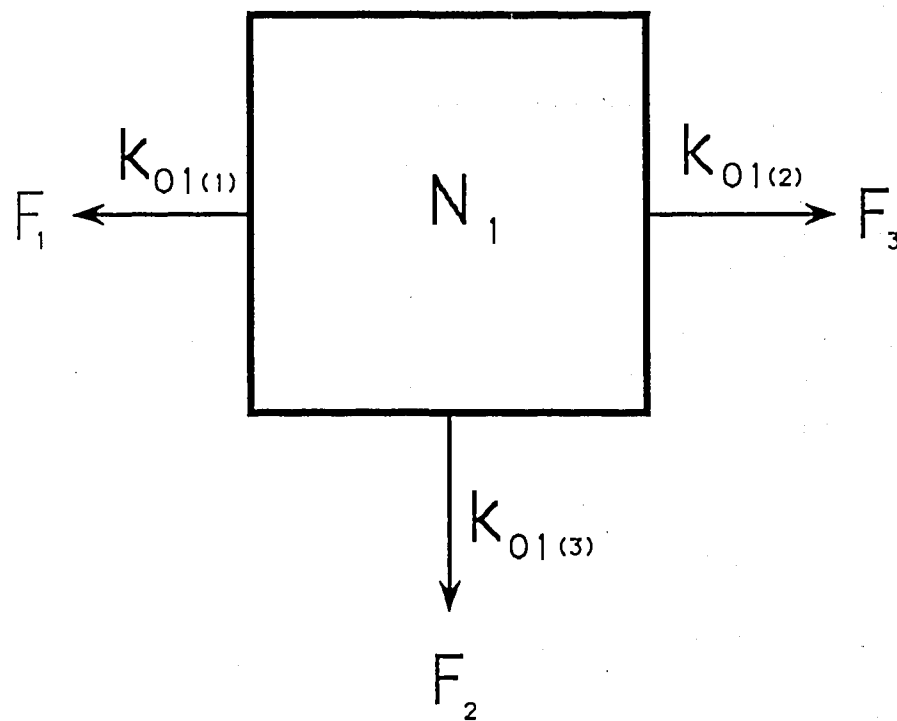
N = pool space

F = flow

k = rate constant where subscript 01 indicates to the outside (0) from compartment 1.

Figure App 1.2

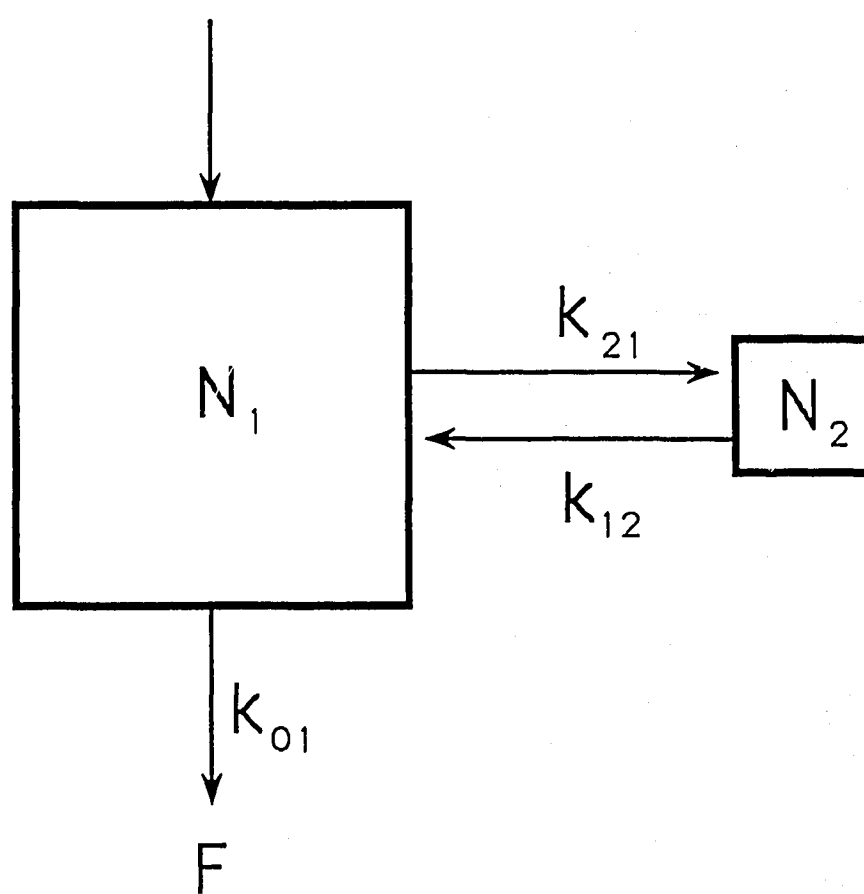
Single pool system with several outflows



Symbols are as in Figure App1.1.

Figure App 1.3

Two pool system



Symbols are as in Figure App 1.1.

Thus, the slope of a plot of $\log C_t$ against time has the value k and the time zero intercept is C_0 . The quantity of tracer added to the system Q_0 is the dose given (A) in the context we are discussing here so we can calculate N as A/C_0 .

The single pool system we have described has only one exit to the outside. If we consider other exits, as illustrated in Figure App 1.2, it is important to realise that the value k represents the sum of all the fractional losses ($k = k_1 + k_2 + k_3$) and cannot be used to obtain values for individual exits. These would need to be directly investigated. This leads us to an important concept in analysis of this type, and that is, that the precise nature of the system cannot be deduced unambiguously from tracer data and only if a model is correct will values calculated from tracer data be correct.

App 1.2 Systems with two or more pools

Figure App 1.3 illustrates a simple two compartment system. Let us suppose that N_1 is body-water and N_2 is some other pool with which the ^2H or ^{18}O in water exchanges. In this case if enough observations were made on the isotopic concentration in body-water it would be seen that the disappearance curve consists of two exponential components that add together to produce the observed curve. However, the slopes of these exponentials are not directly equivalent to k_{01} or any of the other rate constants in the model system. Similarly, the intercepts of the individual exponentials do not directly give the values of N_1 , N_2 or $(N_1 + N_2)$. N_1 is in fact given by the sums of the intercepts.

As far as the present application of the tracer methodology is concerned we have therefore to consider what is likely to happen if a single-compartment solution is applied to a system that is in reality a two-compartment one. For example, in the system in Figure App 1.3 we can assign values of 0.1 and

0.005/day for k_{01} and k_{21} respectively and vary k_{12} from 5 to 0.00005, calculate the characteristics of the curve and then fit a single compartment model to it using early concentration values from Days 0 to 14. With this sampling regime there would be no convincing evidence that a two compartment system existed. Table App 1.1 shows the results. For rapid recycling of isotope predicted volume (N_1) and rate constant (k_{01}) are close to true values and inversely related so that little error is incurred in the measurement of flow (F) as the product (k_{01})(N_1). However, as the degree of recycling becomes smaller errors in k_{01} increase because we reach the stage when the estimate of N_1 is correct (recycling is so slow that N_1 cannot see N_2), but k_{01} is overestimated by 5% as a consequence of the virtually unrecycled losses as k_{21} . The latter case is what is likely to happen to some extent when ^2H is sequestered as fat (see Chapter 7). Elsewhere (Chapter 4) it is suggested that rapid recycling of ^2H explains the differences between volumes obtained with ^{18}O and ^2H .

Table App 1.1

Effect of different degrees of isotope recycling in a two-compartment system when a one-compartment solution is used

k_{12}	N_1	k_{01}	F
5	1.0011	.09986	.09997
0.5	1.0066	.09960	.10026
0.05	1.0051	.10284	.10336
0.005	1.0007	.10472	.10479
0.0005	1.0000	.10499	.10499
0.00005	1.0000	.10499	.10499

Assumptions used:

$$k_{01} = 0.1$$

$$k_{21} = 0.005$$

$$k_{12} = \text{variable}$$

$$N_1 = 1$$

$$F_{\text{True}} = (k_{01})(N_1) = 0.1$$

APPENDIX 2

Contributor: Andy Coward

DEVELOPMENT OF EQUATIONS FOR CALCULATING POOL SIZES FROM ISOTOPE DILUTION

In much of the literature (see Chapter 4 & ref¹) the relationship for calculating total body water from the enrichment of ²H or ¹⁸O in a urine, saliva or breath sample takes the form:

$$N(\text{kg}) = \frac{A}{\text{Mwt}_a} \times \frac{\text{APE}_a}{100} \times \frac{18.02f}{(\delta_s - \delta_p)R_{st}} \quad \dots\dots\dots 1$$

where f is a fractionation factor for the biological material analysed and APE_a is the atom % excess of the dose given. R_{st} is the absolute isotopic ratio in the standard against which the enrichment δ (‰) of post-dose and pre-dose samples are measured. Converting to moles we obtain:

$$N(\text{moles}) = \frac{A}{\text{Mwt}_a} \times \frac{\text{APE}_a}{100} \times \frac{1000f}{(\delta_s - \delta_p)R_{st}} \quad \dots\dots\dots 2$$

In contrast, for work with man, we recommend a relationship

similar to that described by Halliday and Miller ² namely:

$$N(\text{moles}) = \frac{WA}{18.02a} \times \frac{(\delta_a - \delta_t)f}{(\delta_a - \delta_p)} \dots\dots\dots 3$$

That these equations are equivalent or even appropriate may not be immediately obvious. Equation 2 is somewhat confusing because no less than three types of unit are used to describe isotopic content, namely APE, δ and R. Furthermore, an investigator new to the field is given no indication of how APE_a or MWT_a is to be calculated and the temptation must be to read these values off the label on the bottle of isotope. Such a manoeuvre is fraught with danger because it assumes that both the isotope manufacturer and the investigator can get the same value for an analysis. This may or may not be the case. For these reasons the procedure we describe insists that the isotope content of the dose given is measured and, for clarity all the units used for expressing isotopic content are the same.

The principle of any dilution procedure for measuring the size of a single pool is that the increase in the amount of isotope within the pool following dose administration is equal to the amount of isotope given. We need therefore, to measure each of these quantities.

Increase of isotope within the pool is equal to the peak isotopic enrichment found after the dose is given minus the amount that was there as background before the dose was given. The peak enrichment is the intercept determined from back-extrapolation of the isotope curve to time zero in the case of the slope-intercept method, and the isotope enrichment of the plateau measurement in the case of the 2-point technique.

If we assume that the amount of dose is small relative to pool size we can write:

$$NC_a - NC_p$$

$$\text{or } N(C_a - C_p) \dots\dots\dots 4$$

for the amount of isotope gained, where C is fractional abundance (see Section 3.3.3).

To derive a value for the amount of isotope given the isotope concentration in the dose should also be measured. This can be conveniently done by diluting a small weight of dose in a large weight of water in the same way that body-water dilutes the dose given. Making the assumption that the amount of dose diluted (a, grams) is small relative to the amount of water used for the dilution (W, grams of molecular weight 18.02) we can therefore write:

$$\frac{WC_a}{18.02} - \frac{C_t}{18.02} = \frac{W}{18.02} \times (C_a - C_t) \dots\dots\dots 5$$

for the amount of isotope derived from (a) of dose. Thus the amount of isotope given is:

$$\frac{W}{18.02} \times \frac{(C_a - C_t)}{(a/MWt_a)} \times A/MWt_a \dots\dots\dots 6$$

The molecular weight terms cancel giving:

$$\frac{WA}{18.02a} \times (C_a - C_t) \dots\dots\dots 7$$

Putting Equation 4 equal to Equation 7 (since dose found equals dose given) and re-arranging we derive:

$$N = \frac{WA}{18.02a} \times \frac{(C_a - C_t)}{(C_a - C_p)} \dots\dots\dots 8$$

and if the sample analysed is fractionated relative to body water then:

$$N = \frac{WA}{18.02a} \times \frac{(C_a - C_t)}{(C_s - C_p)} \times f \quad \dots\dots\dots 9$$

in which the term $(C_a - C_t)/(C_s - C_p)$ can be expressed in terms of isotopic ratios (R). For 2H the general substitution is:

$$^2C = \frac{^2R}{1 + ^2R} \quad \dots\dots(\text{see Section 3.3.3}) \quad \dots\dots\dots 10$$

and this gives:

$$\frac{^2C_a - ^2C_t}{^2C_s - ^2C_p} = \frac{(^2R_a - ^2R_t)}{(^2R_s - ^2R_p)} \times \frac{(^2R_s + 1)}{(^2R_a + 1)} \times \frac{(^2R_p + 1)}{(^2R_t + 1)} \quad \dots\dots\dots 11$$

or:

$$\frac{^2C_a - 2C_t}{^2C_s - 2C_p} = \frac{^2R_a - ^2R_t}{^2R_s - ^2R_p} \quad \dots\dots\dots 12$$

when:

$$\frac{(^2R_s + 1)}{(^2R_a + 1)} \times \frac{(^2R_p + 1)}{(^2R_t + 1)} = 1 \quad \dots\dots\dots 13$$

Substitution of typical values for R_s, R_p, R_a, R_t justifies this approximation.

Similarly, for ^{18}O where:

$$^{18}O = \frac{^{18}R}{^{18}R + ^{17}R + 1} \quad \dots\dots\dots 14$$

it can be shown that:

$$\frac{{}^{18}\text{C}_a - {}^{18}\text{C}_t}{{}^{18}\text{C}_s - {}^{18}\text{C}_p} = \frac{[{}^{18}\text{R}_a(1 + {}^{17}\text{R}_t) - {}^{18}\text{R}_t(1 + {}^{17}\text{R}_a)]({}^{18}\text{R}_s + {}^{17}\text{R}_s + 1)({}^{18}\text{R}_p + {}^{17}\text{R}_p + 1)}{[{}^{18}\text{R}_s(1 + {}^{17}\text{R}_p) - {}^{18}\text{R}_p(1 + {}^{17}\text{R}_s)]({}^{18}\text{R}_a + {}^{17}\text{R}_a + 1)({}^{18}\text{R}_t + {}^{17}\text{R}_t + 1)}$$

.....15

Again, substitution of typical values justifies:

$$\frac{{}^{18}\text{C}_a - {}^{18}\text{C}_t}{{}^{18}\text{C}_s - {}^{18}\text{C}_p} = \frac{{}^{18}\text{R}_a - {}^{18}\text{R}_t}{{}^{18}\text{R}_s - {}^{18}\text{R}_p}$$

.....16

To convert isotope ratios to relative delta per mil values the following substitution can be applied (see Section 3.3.3):

$$R = R_{\text{std}} \frac{(\delta + 1)}{1000}$$

.....17

In which case, for both ${}^2\text{H}$ and ${}^{18}\text{O}$:

$$\frac{R_a - R_t}{R_s - R_p} = \frac{\delta_a - \delta_t}{\delta_s - \delta_p}$$

.....18

giving the equation for total body water described earlier as Equation 3:

$$N(\text{moles}) = \frac{\text{WA}}{18.02a} \times \frac{(\delta_a - \delta_t)f}{(\delta_s - \delta_p)}$$

.....3

The reader, now familiar with the relationships between δ , R and C will be able to understand the origin of both Equation 2 and Equation 3. Equation 3 is justified if the ranges of enrichments measured are sufficiently small to replace all

measurements of change in isotope concentration with change in isotope ratio. A similar approximation exists in Equation 2 because the amount of isotope found in the subject after dose administration is calculated as:

$$\frac{N(\delta_s - \delta_p)R_{st}}{1000f} \dots\dots\dots 19$$

which is the same as $N(R_s - R_p)/f$ rather than $N(C_s - C_p)/f$ and this value is divided into the amount of dose given expressed in units of isotope concentration.

Users of the multi-point methodology will find it convenient to use the reciprocal of Equation 3 for all values of isotopic enrichment obtained during the measurement period. This measurement is equivalent to isotopic concentration (mole/mole body water) expressed as fraction of the dose given and the intercept of the log-linear plot of such values is $1/N$ (see Chapter 11 for examples).

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APPENDIX 3

Contributor: Mike Franklin

DERIVATION OF A GENERAL EQUATION TO COPE WITH CHANGES IN POOL SPACE

App 3.1 Basic derivation

In this appendix we outline some of the mathematics underlying the DLW technique. We will attempt to keep the presentation simple and refer readers to the original work of Lifson and McClintock ¹ and Coward et al ² for a more formal development.

A useful starting point is to assume that all the Lifson and McClintock assumptions hold except that neither the flow rates nor the pool sizes are necessarily constant. The change of deuterium tracer enrichment during any interval is affected by the relative quantity of water leaving the pool, the enrichment and any change in pool size. The relationship can be expressed by the differential equation:

$$\delta'_d(t) = \frac{r_{H_2O}(t) \cdot \delta_d(t)}{N_d(t)} - \frac{N'_d(t) \cdot \delta_d(t)}{N_d(t)} \dots\dots\dots 1$$

where $N(t)$ denotes the pool size at time t , $r_{H_2O}(t)$ and $\delta(t)$ denote the water flow rate and enrichment; $\delta'(t)$, $N'(t)$ denote the rates of change of $\delta(t)$ and $N(t)$ respectively (ie $\delta'(t)$ denotes $d\delta(t)/dt$); the suffix D denotes we are referring to the deuterium pool. A useful way to reformulate Equation 1 is to write it as:

$$\frac{N_D(t) \cdot d\log\delta_D(t)}{dt} = -r_{H_2O} - N'_D(t) \quad \dots\dots\dots 2$$

which when all the assumptions hold simplifies to

$$\frac{N_D d\log\delta_D(t)}{dt} = -r_{H_2O} \quad \dots\dots\dots 2a$$

This differential equation can be solved to give:

$$N_D \log\delta_D(t) = N_D \log\delta_D - r_{H_2O} t$$

or:

$$\delta_D(t) = \delta_D e^{-kt}$$

where E_D is the enrichment at time 0 and $k = r_{H_2O} / N_D$ is the rate constant.

Similar equations can be derived for the change in oxygen-18 enrichment by noting it is lost through both water and CO_2 .

$$\delta'_O(t) = \frac{-r_{H_2O}(t) \cdot \delta_O(t)}{N_O(t)} - \frac{2r_{CO_2}(t) \cdot \delta_O(t)}{N_O(t)} - \frac{N'_O(t) \cdot \delta_O(t)}{N_O(t)} \quad \dots\dots\dots 3$$

where $r_{CO_2}(t)$ denotes the flow rate of CO_2 and the suffix O denotes oxygen. As before we can reformulate this equation as:

$$\frac{N_o d\log\delta_o(t)}{dt} = - r_{H_2O}(t) - 2r_{CO_2}(t) - N'_D(t) \quad \dots\dots\dots 4$$

which when all assumptions hold simplifies to:

$$\frac{N_o d\log\delta_o(t)}{dt} = - r_{H_2O} - 2r_{CO_2} \quad \dots\dots\dots 4a$$

from Equations 2 and 4 we get:

$$2r_{CO_2}(t) = \frac{N_D(t) \cdot d\log\delta_D(t)}{dt} - \frac{N_O(t) \cdot d\log\delta_O(t)}{dt} + N'_D(t) - N'_O(t) \quad \dots\dots\dots 5$$

Note that $d\log\delta_D(t)/dt$ is the slope of the \log_e transformed enrichment curve at time t . Thus we can obtain the flow rate (ie production rate of CO_2) at any instant if we know the oxygen and deuterium pool sizes and the slopes of the \log_e enrichment curves. By integrating 5 between the two times t_1 , t_2 say we can derive the total CO_2 production over the period:

$$2r_{CO_2} = \int_{t_1}^{t_2} \frac{N_D(t) \cdot d\log\delta_D(t) \cdot dt}{dt} - \int_{t_1}^{t_2} \frac{N_O(t) \cdot d\log\delta_O(t) \cdot dt}{dt} + [N_D(t_2) - N_D(t_1) - N_O(t_2) + N_O(t_1)] \quad \dots\dots\dots 6$$

which if all assumptions hold simplifies to:

$$2r_{CO_2} = N_O \log(\delta_{O1}/\delta_{O2}) - N_D \log(\delta_{D1}/\delta_{D2}) \quad \dots\dots\dots 6a$$

The final term in Equation 6 involves the changes in the two pool sizes and is equal to zero if the pool sizes are equal. The problem we usually encounter is the evaluation of the two integrals. Integration is a simple matter in certain special cases (eg when pool sizes are constant) but this does not help us

understand what happens in general. To do this we look at Equation 2 and see what happens under conditions which will be approximately true at most times.

App 3.2 A general solution to Equation 2

Consider a short time period t_1, t_2 say, in which the water output flow is in constant ratio with the input flow and both are directly related to the pool size. We may express this fairly generally as:

$$f_i(t) = x_i(yN(t))^z \quad f_o(t) = X_o(yN(t))^z \quad \dots\dots\dots 7$$

where $f_i(t), f_o(t)$ denote the input and output flows and x_i, x_o and z are constants, x_i, x_o and y are greater than 0, but no constraint is placed on z . It can be shown that if Equation 7 is true then:

$$r = \frac{N_1 - N_2}{\log_e(N_1/N_2)} \cdot \log(\delta_1/\delta_2) + N_1 - N_2 \quad \dots\dots\dots 8$$

where r is the total flux in the period t_1, t_2 ; δ_1 and δ_2 are the enrichments at times t_1, t_2 . This is the equation originally derived by Coward et al¹.

The wide range of conditions for which 8 holds true make it a very important equation underlying DLW work. We may consider three special cases:

- (i) $z = 0$, here the pool size varies but the flow rates are constant.
- (ii) $z = 1$, here the relative flow rates are constant although the pool sizes and flow rates vary.

(iii) $d = +\infty$, to understand this write $q = z^{-1}$ and write:

$$N(t) = y^{-1}(F_1(t)/x_1)^q = y^{-1}(F_0(t)/x_0)^q$$

and let q tend to zero. Thus we see the equation hold if the flow rates vary and the pool size is constant. In this case Equation 6 further simplifies to:

$$r = N \log(\delta_1/\delta_2) = N(\log \delta_1 - \log \delta_2) \quad \dots\dots\dots 9$$

where N denotes the constant pool size.

The importance of Equation 8 lies not only in its generality but in its simplicity. All that needs to be known are the pool sizes and the enrichments at the beginning and end of the period. In the event that even greater simplicity is required then the expression $(N_1 - N_2)/\log(N_1/N_2)$ is equal to the mean pool size when the pool size N has changed exponentially over the period (t_1, t_2) . It can be approximated by the mean of N_1 and N_2 but the geometric mean provides an even better approximation, differing from N by less than 1% for a 20% difference in N_1 and N_2 .

App 3.3 Estimating CO₂ production

We return to Equation 6. Firstly we observe that the third term will normally contribute very little for the two pools are of very similar size and any change will have virtually identical effects on both. In quantitative terms let the two pool sizes be in constant ratio such that $p = N_o(t)/N_o(t)$ and let the oxygen pool increase from N_o to $N_o(1+m)$ then the third term reduces to yield a change equal to $(p-1)mN_o$. Typically $p = 1.03$ and in a period with 5% growth $m = 0.05$ yielding a change equal to $0.0015 N_o$. The problem then reduces to determining the difference between the two integrals.

App 3.4 The shape of the enrichment curve

If the enrichment of deuterium in the urine is studied from the time of introducing the tracer, there is a short stabilisation period up to time t_0 , say, followed by a period in which the enrichment decays to zero. As an illustration we may seek the shape of the curve for the general case described in Section App 3.2. We can show that:

$$\frac{d \log \delta(t)}{dt} = \frac{-k_i}{N_0^{-\alpha} - \alpha(k_i - k_0)t} \quad \dots\dots\dots 10$$

where α and $\alpha^{-1} \neq 0$ so that the slope of the log enrichment curve is constant only in certain special cases. The principal special case is when $K(t) = F(t)/N(t)$ is constant. Note from Equation 8 that when the enrichment curve is not straight the principal parameters of interest are the values at the extremes of the range of interest.

App 3.5 References

1. Lifson N & McClintock R (1966) Theory of the use of the turnover rate of body water for measuring energy and material balance. *J Theoret Biol*; 12: 46-74.
2. Coward WA, Cole TJ, Gerber H, Roberts SB and Fleet, I (1982) Water turnover and the measurement of milk intake. *Pfleugers Archiv*; 393: 344-347.

APPENDIX 4

Contributor: Mike Franklin

FURTHER COMMENTS ON ESTIMATING WATER FLUX AND CO₂ PRODUCTION

App 4.1 Modelling enrichment data

This Appendix expands on some of the points raised in Chapter 5 and may be helpful in providing a different perspective on the issues.

In order to model the enrichment data it is usual to assume that the observed values arose from some 'true' value plus an error component, that we can model the true component and that the error component is random with mean value zero. Differences arise between the assumptions made relating to the model and to the errors. The target, however, in all the methods is the same, namely to provide an estimate of:

$$\log \delta_1 - \log \delta_2$$

where $\delta_1 = \delta(t_1)$ etc

The two-point method is a useful starting point for it can

be justified in (at least) two ways:

- a) make no assumptions about model or errors, simply take observations at t_1 and t_2 and use these to provide the estimates of the true values for $\delta(t_1)$ and $\delta(t_2)$;
- b) plot the two values $\log\delta(t_1)$, $\log\delta(t_2)$ and assume the function is a line passing through these points with no error.

Clearly the strength of the method is that it does not require assumptions about a model. Its weaknesses are its failure to use known information about the model and its failure to recognise the presence of errors.

In general the decline of the log enrichment curve with multi-point data is not perfectly linear but in some special cases it may be so. For example, if all the Lifson and McClintock assumptions hold perfectly then the decline is linear, and in practice there are very many cases where the decline is seen to be nearly linear even though all the assumptions are unlikely to have held throughout the period. In these cases it may be desirable to assume a linear model. If the enrichment curve is replaced by a straight line then the slope of this line is equal to the relative flow rate or rate constant k and the value $\log\delta_1 - \log\delta_2$ is replaced by $k(t_2 - t_1)$, i.e. the observed values $\log\delta_1$, $\log\delta_2$ are replaced by the values predicted from the straight line. Indeed whenever we try to model the enrichment curve one of the objectives is to obtain estimates for $\log\delta_1$ and $\log\delta_2$. The quality of these estimates is clearly dependent on the quality of the model.

When the log enrichment curve departs from linearity the use of the linear regression slope to estimate the rate constant, k , is a fairly robust procedure if the data points are symmetrically spread about \bar{t} . On the other hand, under the same conditions, the

use of the intercept to derive the pool size may lead to substantial errors. The problem then arises as to how to use the observations and the curve drawn through these observations to estimate the initial enrichment. The answer will normally be best obtained through knowledge of the methodology used and intelligent study of the data. One solution, considered appropriate for data produced under conditions similar to those used at Cambridge, is presented in Section 5.7. Essentially it involves adjusting a reliable observation taken near time zero. This type of trade-off between early observations and the value predicted by the curve is likely to be a widely applicable procedure.

App 4.2 The effect of auto-correlated errors on linear regression

In calculating the flux by either the two-point or multi-point method, it is common to assume that the errors are independent (ie the errors on one day are unrelated to those on the next day). This is rarely the case for a sudden large water intake say, may influence the measured concentrations for several successive days. Plotting residuals from many data sets shows a tendency for residuals on neighbouring days to be correlated. This type of correlation is known as 'auto-correlation'.

Auto-correlation is important when data points are close together but less so when they are distant. Thus the multi-point method is more affected than the two-point method, and the effect of auto-correlation is to reduce the advantage of the multi-point method. To illustrate the effect of this auto-correlation we present, in Table App 4.1, the relative variances that would be obtained from a study lasting twelve days (ie 13 assessments) if measurements were obtained daily or at 2,3,6 day intervals or at the beginning and end only. The variances are given for various degrees of auto-correlation. (The level of correlation will be

affected by the nature of the investigation and the experimental procedure).

Perhaps the best illustration of the effect of serious autocorrelation is seen for $p = 0.75$ where using 13-points is of little advantage over using two and probably inferior to using 3. The reason for this apparent reversal is that standard linear regression procedures are inefficient when there is a high degree of autocorrelation. Because autocorrelation between errors may arise naturally or may arise from attempting to fit an incorrect model, we recommend that if there are ten or more equally-spaced data points the autocorrelation coefficient for the residuals is obtained. A high absolute value may help to identify wrongly specified models.

Table App 4.1

The relative variance of the estimated slope (k) and the mean value (y) using standard linear regression when the data exhibit selected levels of auto-correlation between successive days

A. Variance (k)

<u>Correlation</u>	<u>No of sampling days</u>				
	<u>13</u>	<u>7</u>	<u>5</u>	<u>3</u>	<u>2</u>
p = 0.00	.0055	.0089	.0111	.0139	.0139
0.25	.0080	.0096	.0112	.0139	.0139
0.50	.0116	.0116	.0121	.0139	.0139
0.75	.0150	.0140	.0135	.0134	.0134
1.00	.0000	.0000	.0000	.0000	.0000

B. Variance (y)

<u>Correlation</u>	<u>No of sampling days</u>				
	<u>13</u>	<u>7</u>	<u>5</u>	<u>3</u>	<u>2</u>
p = 0.00	.077	.143	.200	.333	.500
0.25	.123	.159	.205	.333	.500
0.50	.207	.220	.244	.340	.500
0.75	.400	.392	.392	.419	.516
1.00	1.000	1.000	1.000	1.000	1.000

Notes on Table App 4.1

The columns denote the number of (equally spaced) sampling occasions with the first occasion on day 0 and the last on day 12.

The values were generated on the assumption that the errors obey a first order auto-regressive model.

The variance of the intercept a at $t=0$ is $V(a) = V(y) + 36V(k)$, and the covariance $(a,k) = 6V(k)$. These can be used to calculate the relative variances of the estimated pool size and flux rates for selected values of a and b .

Observe that at very high levels of autocorrelation (towards $p=1$) the slopes are precisely estimated but the intercepts are not. Also note for $p = 0.75$ the multi-point method may be inferior to the two-point method (because the wrong model is being fitted).

APPENDIX 5

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Tim Cole

DETERMINATION OF OPTIMAL DOSING RATIOS

App 5.1 Background

The consequences of changing isotopic backgrounds have been discussed in Chapter 8. Strictly speaking the background values to be subtracted from post-dose enrichment values to determine isotope disappearance rates should be those to which the exponentials are decaying at infinite time. These values are assumed to be the same as those existing at the start of the experiment and this assumption may not be correct. If it is not correct error will be introduced. However, the background changes for ^2H and ^{18}O are likely to be covariant (see Chapter 8) and the slope of background variation may have a slope similar to that of the meteoric water line ($\delta^2\text{H} = 8\delta^{18}\text{O} + 10$). Intuitively, one would expect the magnitude of the error in $k_o - k_d$ caused by the assumption of an incorrect background to depend on the size of the doses given and the length of time over which measurements are made. Large doses will minimise the effect of background changes, but if experiments continue for a long time background effects will be more significant. There is however another important consideration. Since background changes are likely to

be covariant it ought to be possible to minimise the effects of background variation by giving appropriate amounts of dose as first suggested by Schoeller ¹.

App 5.2 Derivation of a formula to predict optimal dose ratios

$$\begin{aligned}\text{Let } C_r(t) &= C_o(t) / C_d(t) \\ &= [C_o'(t) - \delta^{18}O] / [C_d'(t) - \delta^2H]\end{aligned}$$

where C' is the absolute enrichment, C is the enrichment net of background and (t) indicates time t . Assume that the background enrichments of ^{18}O and 2H vary along the line given by the equation:

$$\delta^2H = S.\delta^{18}O + K$$

where for the meteoric water line $S = 8$ and $K = 10$. The differential of $C_r(t)$ with respect to $\delta^{18}O$ is given by:

$$[S.C_r(t) - 1] / C_d(t)$$

indicating dependence on S but not K .

$$\begin{aligned}\text{Now } C_o(t) &= C_o(0).e^{-k_o.t}, C_d(t) = C_d(0).e^{-k_d.t} \text{ and} \\ C_r(t) &= C_r(0).e^{-k_r.t}\end{aligned}$$

where $k_r = k_o - k_d$ and (0) indicates time zero.

So $e^{k_r.t} = C_r(0) / C_r(t)$ and the differential of $e^{k_r.t}$ with respect to $\delta^{18}O$ is given by:

$$C_r(t) / C_d(0) [(e^{k_o.t} - 1) - (e^{k_d.t} - 1).S.C_r(0)]$$

For $e^{k_r.t}$ and hence k_r to be least affected by $\delta^{18}O$ its

differential should be zero. Setting it to zero and solving for $C_r(0)$ gives the optimal ratio of post-dose concentrations:

$$C_r(0) = (e^{k_o \cdot t} - 1) / S \cdot (e^{k_d \cdot t} - 1)$$

If t is chosen to be n half-lives for 2H and p is the ratio k_o/k_d , then the optimal ratio of 2H to ^{18}O , as measured by the increment in enrichment immediately post dose, is the reciprocal of $C_r(0)$ and is given by:

$$S \frac{(2^n - 1)}{(2^{pn} - 1)}$$

App 5.3 Practical consequences

In practice n will usually be in the range 2 - 3 and p is unlikely to be smaller than 1.1 or greater than 1.3. For a value of $S = 8$ (the same as the meteoric water line) these ranges produce the optimal ratios shown in Table 1. Using dose regimes that produce these initial enrichments will provide protection against both random and unidirectional changes in background during the experiments.

The advantages of using appropriate dose regimes is illustrated in Fig App 5.1. Here, the correct background values are -4 ($\delta^{18}O$) and -22 (δ^2H) and the correct $k_o - k_d$ difference is 0.02 ($k_o = 0.12$ and $k_d = .10$). Other values are incorrect but covariantly so. Thus, for example, if pre-dose values were -6 and -38 but in reality the subject was equilibrating to backgrounds of -4 and -22 errors of -3.3 and +7.5% would be produced for initial ratios of 6.68 and 4.02 respectively (Curves D and E). Clearly inappropriately tailored doses such as those producing ratios of 11.22 and 2.81 (Curves B and C) are dangerous to use compared to the ideal ratio of 5.61 (Curve A). Fig App 5.2 shows the expected effect of generally increasing dose levels but

maintaining a variety of ratios. If that is done errors are reduced but the general shape of the curves remains the same.

Unfortunately, although adopted here for simplicity, these are not the only considerations. The relative measurement precisions for ^{18}O and ^2H are important and, if it is necessary to increase ^2H enrichment in order to improve analytical precision, maintaining an appropriate ratio could mean that ^{18}O costs limit the amount of work that can be done. Clearly balances need to be found but gross deviations away from ideal dose regimes are not advisable.

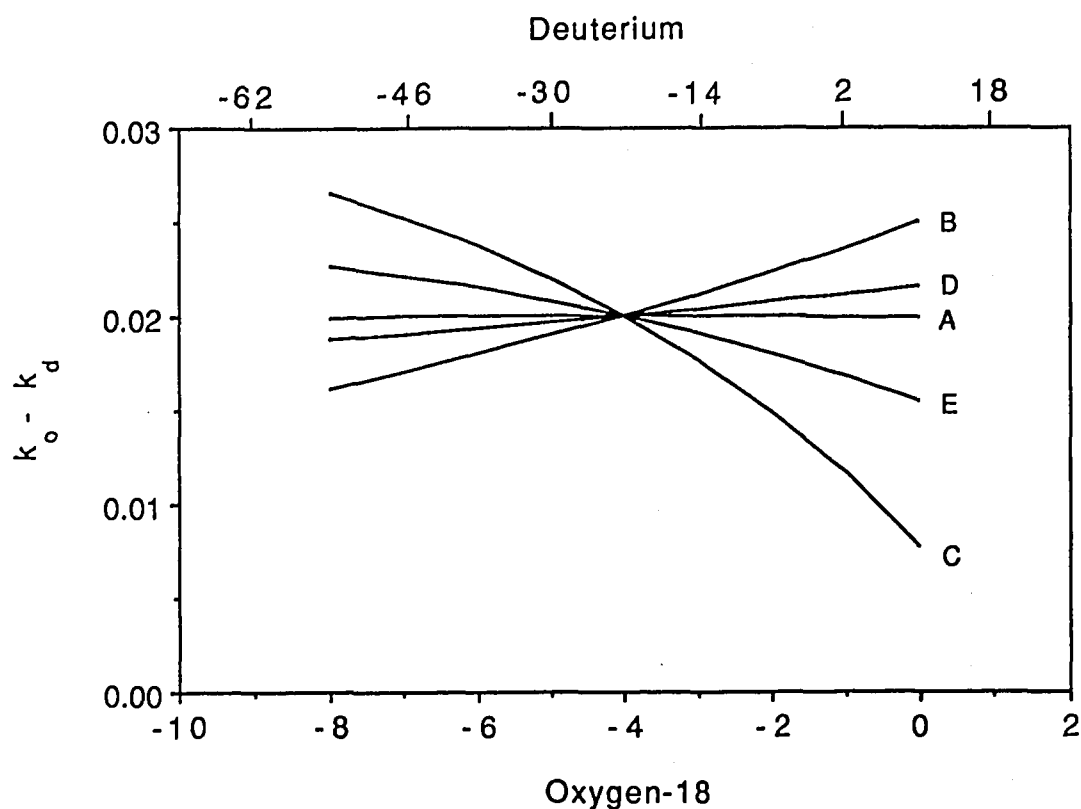
Table App 5.1

Optimal initial isotopic ratios ($\delta^2\text{H}/\delta^{18}\text{O}$, net of background) for different numbers of ^2H half-lives and k_o/k_d ratios

		k_o/k_d		
		1.1	1.2	1.3
<u>Number of half-lives</u>	2	6.68	5.61	4.74
	3	6.32	5.03	4.02

Figure App 5.1

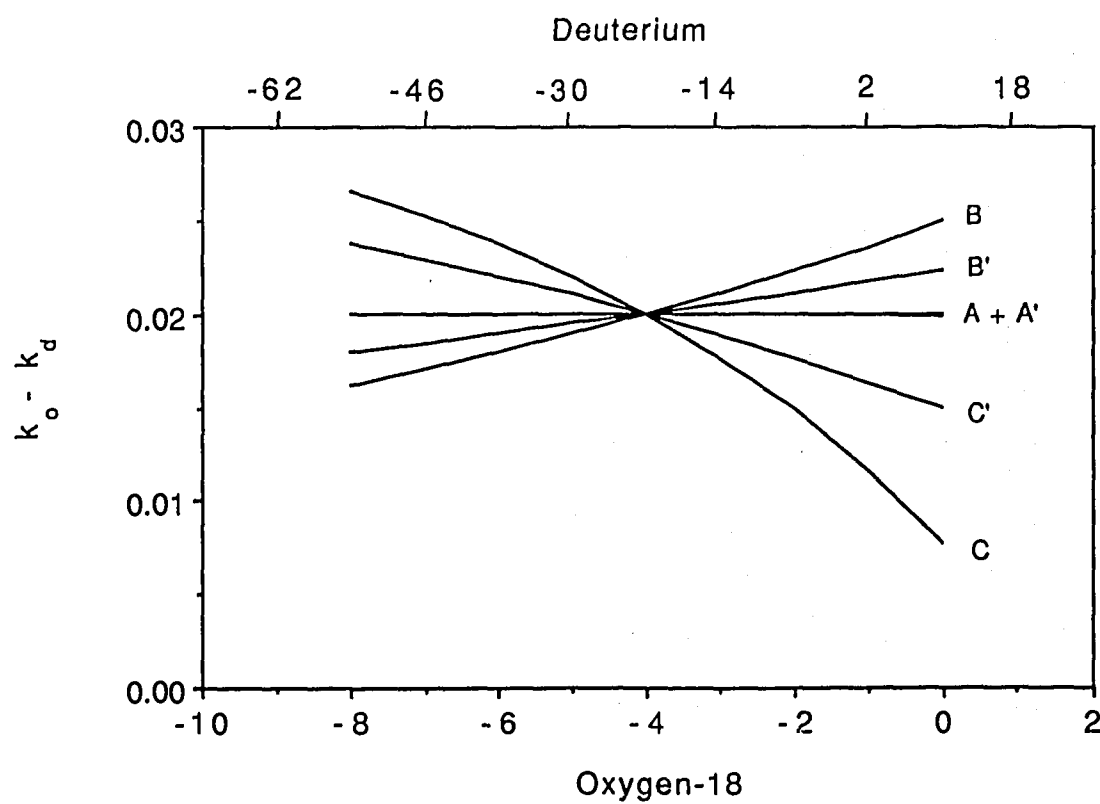
Effect of background variations on estimates of $k_o - k_d$ for true k_o and k_d values of 0.12 and 0.10



Initial enrichment for ^{18}O relative to SMOW (net of background) was 142.59‰ and $\delta^2\text{H}/\delta^{18}\text{O}$ ratios are 5.61 (Curve A), 11.22 (Curve B), 2.81 (Curve C), 6.68 (Curve D) and 4.02 (Curve E). The figure assumes covariant background changes along the meteoric water line and that the duration of the experiment was 2 half-lives for ^3H .

Figure App 5.2

Effect of background variations on estimates of $k_o - k_d$ for true k_o and k_d values of 0.12 and 0.10



Curves A, B and C and other assumptions are the same as in Fig App 5.1. A', B' and C' ratios correspond to A, B and C but initial enrichments net of background have been doubled.

App 5.4 Reference

1. Schoeller DA (1983) Energy expenditure from doubly labelled water: some fundamental considerations in humans. *Am J Clin Nutr*; 38: 999-1005.