Comparison of recoveries in breath carbon dioxide of $H^{13}CO_3^-$ and $H^{14}CO_3^-$ administered simultaneously by single 6 h constant unprimed intravenous infusion

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The aim of this study was to assess the bioequivalence of $H^{13}CO_3^-$ and $H^{14}CO_3^-$, by administering both labels simultaneously by single infusion and comparing their recovery in breath CO_2 and urinary urea. Six healthy male subjects (age range 24–41 years; weight 76·7 (SD, 18·6) kg; height 1·79 (SD 0·05) m) were infused with unprimed solutions of HCO_3^- (110·0 mmol/kg) labelled with ^{13}C (0·76 mmol ^{13}C/h) and ^{14}C (48 Bq/h) at a constant rate for 6 h, in a whole-body calorimeter (1400 litres) for measurement of CO_2 production. Samples of breath were collected hourly in a Douglas bag and all urine was collected into two batches (0–4 h and 4–6 h) for estimating recovery of infused label by measurement of enrichment or specific activity. Recovery in breath CO_2 of both labels increased from about 25 % for the first hour to 88 % and above for hours 3–4 onwards. Mean recovery of ^{13}C in breath CO_2 was slightly higher than that of ^{14}C for all periods (mean difference always less than 1 % of infused label) but was significant only for the first 3 h ($P < 0·05$). Recovery of ^{14}C in urea was significantly higher ($P < 0·01$) than ^{13}C, but was confounded by substantial variability and uncertainties concerning $^{13}CO_2$ background enrichments. These results suggest that there is no compelling need to alter factors currently used for recovery of ^{14}C in breath when using ^{13}C instead, and *vice versa*.

Carbon isotopes: Bioequivalence: Bicarbonate: Recovery factors

The recovery in breath CO_2 of labelled HCO_3^- is of major importance to metabolic studies involving oxidation of C-labelled substrates, including amino acids (James *et al.* 1976; Beaufrere *et al.* 1989), fats (Issekutz *et al.* 1968) and carbohydrates (Saris *et al.* 1993; Leijssen *et al.* 1995), and to the assessment of energy expenditure using the bicarbonate (Elia *et al.* 1992) and bicarbonate-urea (Elia *et al.* 1995) methods. The choice of values appropriate for these studies for the recovery of bicarbonate in breath, depends on biological factors that lead to the incomplete recovery of label, including accumulation in the CO_2 pools of the body, especially those that have a slow turnover, and metabolic fixation into other substrates (e.g. urea) or possible recycling of tracer (Pacy *et al.* 1989). However, the situation is complex because additional label from C-labelled substrates is incorporated into citric acid and other intermediates which are linked to, or exchange label with, other pathways. Therefore, the recovery of labelled acetate may be lower than that of HCO_3^-. The acetate correction factor (fractional recovery of labelled CO_2 after infusion of acetate; Schrauwen *et al.* 1998) may be influenced by the type of labelling used (acetate labelled at C-2 results in very different correction factors from that labelled at C-1; Wolfe & Jahoor, 1990), exercise and rest (Sidossis *et al.* 1995), and the fed and fasted states (anticipated variation of flux of acetate into lipogenic substrates). It also depends on the length of infusion, which so far has been only a few hours (Wolfe & Jahoor, 1990; Sidossis *et al.* 1995). The acetate correction factor also assumes (with little direct evidence) that the tissues utilise labelled substrates and acetate in the same proportions. This would account for different fates of acetate through either the lipogenic or gluconeogenic pathways. For example, labelled citric-acid cycle intermediates can be shunted to glucose in some tissues (e.g. liver) but not others (e.g. muscle or adipose tissue which lack gluconeogenic pathways). Finally, the HCO_3^- correction factor is more appropriate than the acetate correction factor in the HCO_3^-–urea method for estimating energy expenditure through the measurement of isotopic dilution of CO_2 (Elia *et al.* 1992, 1995).

Abbreviation: PDB, Pee Dee Belemnite.

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The choice of isotope ($H^{13}CO_3^-$ *v.* $H^{14}CO_3^-$) for such investigations is further confounded by considerable variability amongst the many reported values for recovery of label in breath CO_2 (reviewed by Leijssen & Elia, 1996). Although this variability may be attributable to inconsistencies in methodology between studies, it may also originate from differences in biological behaviour (bioequivalence) between $H^{13}CO_3^-$ and $H^{14}CO_3^-$, which are consistent with generally lower recoveries of ^{13}C than ^{14}C observed in breath CO_2 in studies involving either bolus administration (68·7 % *v.* 84·3 % in 194 and 13 subjects respectively) or constant infusion of label (81·7 % *v.* 87·1 % in 156 and 131 subjects respectively) (Leijssen & Elia, 1996).

Differences between ^{13}C and ^{14}C isotopes in biological systems are suggested *a priori* by the different rates of biochemical reaction observed when substrates labelled with ^{13}C are utilised in competition with the naturally most abundant ^{12}C isotope. For example, molecules containing ^{13}C are discriminated against in the conversion of carbohydrates to fatty acids, resulting in fat which is depleted by 3–5 δ ^{13}C per 10^3 compared to carbohydrate. The enrichment values for proteins and amino acids are intermediate between those of carbohydrates and fats (Klein, 1991). In plants, discrimination can also occur during photosynthesis. Those with the C_3 pathway, such as wheat and rice, discriminate against fixation of atmospheric $^{13}CO_2$ by the action of ribulose 1,5-bisphosphate carboxylase producing grain with a typical depletion of −27‰ relative to the International limestone standard Pee Dee Belemnite (PDB). In contrast, plants with the C_4 pathway, such as maize and sugar cane, produce grain with a typical value of −11‰ relative to PDB (O'Leary, 1981; Farquhar *et al*. 1989). Preferential ingestion of either C_3- (e.g. wheat, rice, sugar beet and potatoes) or C_4- (maize and sugar cane) derived carbohydrates may lead to relative differences in $^{13}CO_2$ enrichment in breath, as indicated by greater enrichment due to the more usual consumption of C_4 plants in the American diet compared to the staple C_3 plants in Europe (Wagenmakers *et al*. 1993).

However, no studies in human subjects have compared directly the bioequivalence of $H^{13}CO_3^-$ and $H^{14}CO_3^-$. Such information is important in view of the preferred use of stable isotopes (^{13}C) instead of their radioactive counterparts (^{14}C), especially for children and pregnant women and in clinical research (Pacy *et al*. 1989; Jones & Leatherdale, 1991). The purpose of this study, therefore, was to compare the recovery of ^{13}C and ^{14}C in breath following constant infusion of HCO_3^- labelled with both isotopes simultaneously, and to obtain preliminary information regarding recoveries of these isotopes in urinary urea.

Methods

Subjects

The general characteristics of the six healthy male subjects were as follows: median age 33 (range 24–41) years; mean weight 76·7 (SD 18·6) kg; height 1·79 (SD 0·05) m; BMI 24·0 (SD 5·6) kg/m^2. Subjects were studied after an overnight fast, although during infusions the drinking of water was encouraged to promote urine production.

Protocol

An unprimed constant infusion of labelled HCO_3^- was administered via an arm vein for a period of 6 h using a 'Treonic' IP3 Digital Syringe Pump (Vickers Medical, Basingstoke, Hants., UK) at a nominal rate of 8·0 ml/h ($H^{14}CO_3^-$, nominally 48 Bq/h and $H^{13}CO_3^-$, nominally 0·76 mmol ^{13}C/h). The infusate had been previously prepared under sterile conditions (Radiopharmacy, Addenbrooke's Hospital, Cambridge, UK), using $NaH^{13}CO_3$ (CK Gas Products Ltd, Finchampstead, Berks., UK) and $NaH^{14}CO_3$ (Amersham International plc, Little Chalfont, Bucks., UK), and kept frozen at −20 °C until required. The infusate was analysed for HCO_3^- concentration (110·0 mmol/kg solution), ^{13}C-enrichment (87·5 atom %) and ^{14}C-radioactivity (6 Bq/g solution). To ensure consistency between infusions and absolute accuracy, the total amount of solution infused was determined gravimetrically (change in weight of the syringe plus infusate between the beginning and end of each infusion). A constant hourly rate of infusion had been established previously by simply running the syringe pump continuously for a number of hours and collecting the delivered solution separately into pre-weighed receptacles exactly on each hour, and then re-weighing.

During the infusion, the subject was confined to a purpose-built portable 'tent' indirect calorimeter of about 1400 litres capacity, fitted over a bed (Crisp & Murgatroyd, 1985) in which limited movement was permitted. The difference between CO_2 concentrations of ingoing ambient air and outgoing calorimeter air, measured with an infra-red CO_2 analyser, was used to assess CO_2 production rates under conditions of standardised temperature and pressure (Elia & Livesey, 1992).

At hourly intervals, beginning 1 h before the start of infusion, the subject collected end expiratory air in a Douglas bag of about 12 litres capacity. After emptying the bladder and discarding the urine produced overnight, all subsequent urine production was collected into the following three batches: (1) 2 h immediately before the beginning of infusion (for background measures); (2) 0–4 h of infusion; (3) 4–6 h of infusion.

Measurements

Infusate. HCO_3^- concentration of the infusate was measured in triplicate using an established colorimetric method (Fuller & Elia, 1989; Elia *et al*. 1992). Standard reference solutions were prepared using BDH analar quality K_2CO_3 and $KHCO_3$ (BDH, Merck Ltd, Poole, Dorset, UK), adjusted for percentage purity claimed by the manufacturer, or were obtained commercially (Sigma, Carbonate/Chloride standards; Sigma Diagnostics, Sigma Chemical Company, Poole, Dorset, UK). Radioactivity of the infusate (dpm/g) was measured in triplicate by weight (accurate to the nearest 0·0001 g) of a small amount of solution (nominally between 0·5 and 1 ml) in a scintillation vial (Elia *et al*. 1992, 1995). The ^{13}C fractional abundance (atom %) of labelled HCO_3^- in the infusate was determined by reverse isotope dilution with combustion isotope-ratio MS using a Carlo Erba 1500 Nitrogen Analyser (Carlo Erba, Milan, Italy) interfaced to a

Sira 10 gas isotope-ratio MS. The mass balance equation was as follows (Spear *et al*. 1995):

$$f_{mix} = ((f_{std} \times n_{std}) + (f_{inf} \times n_{inf}))/(n_{inf} + n_{std}),$$

therefore:

$$f_{inf} = (f_{mix}(n_{std} + n_{inf}) - (f_{std} \times n_{std}))/n_{inf},$$

where n_{inf} and n_{std} are C (μmol) in the infusate and Na_2CO_3 standard respectively, and f_{mix} and f_{std} are the ^{13}C fractional abundances in the diluted infusate and Na_2CO_3 standard respectively (measured by gas isotope-ratio MS).

Breath. Specific activity of breath CO_2 was measured by passing the expired gas collected in the Douglas bag through an accurately weighed (nominally 3 ml) amount of hyamine–methanol trapping agent (hyamine hydroxide in methanol, nominal concentration about 560–580 mmol/kg, titrated gravimetrically against 0·2 mol/litre HCl (volumetric 'analar' reagent (BDH) adjusted for density) using specifically designed apparatus as previously described (Elia *et al*. 1995). Scintillation counting was carried out as before (Elia *et al*. 1992, 1995) for calculation of specific activity of CO_2. Enrichment of ^{13}C in breath CO_2 was measured in samples of expired gas obtained by connecting a syringe to the outlet of the Douglas bag, drawing up 11 ml and then expelling it (in total, to prevent fractionation) into an 11 ml evacuated container (Exetainers; Labco Ltd, High Wycombe, Bucks., UK). These samples were taken before and after the ^{14}C sampling to ensure that no fractionation had occurred. $^{13}C:{}^{12}C$ ratios were measured for each breath sample, after cryogenic purification of CO_2, using a gas isotope-ratio MS (Sira 10; Micromass, Wythenshawe, UK), internal precision ≤ 0·01 ‰. Results were expressed as $\delta\ ^{13}C$ per 10^3 relative to PDB (Craig, 1957; Schoeller *et al*. 1980), where:

$$\delta\ ^{13}C = ((R_S/R_{PDB} - 1)) \times 10^3,$$

$$R_S = {}^{13}C/{}^{12}C, \text{ of the sample,}$$

and

$$R_{PDB} = {}^{13}C/{}^{12}C, \text{ of PDB} = 0{\cdot}0112372.$$

This was then converted to atom % excess, for calculation of recoveries (Klein, 1991).

Urea. Specific activity and enrichment of urinary urea were determined on the same sample, by the urease method, essentially as described previously (Fuller & Elia, 1989; Elia *et al*. 1995) but with certain additional precautions to minimise possible fractionation effects. The urease reaction was allowed to proceed to completion but no vial containing hyamine–methanol trapping agent was present at this stage. Instead, the flask was isolated by a three-way luer adaptor tap connected to the side arm. Once the reaction had reached completion, the flask air containing the CO_2 that had been produced was released through the tap into a 1 litre Douglas bag. CO_2-free water (degassed using a vacuum pump) was added to the flask via the third arm of the tap in 50 ml increments until all air had been displaced into the Douglas bag. Two 1 ml samples of gas in the Douglas bag were then collected for measurement of $^{13}CO_2$ enrichment as described earlier. Specific activity of CO_2 in the Douglas bag was assessed as for breath CO_2 (see earlier). A further 1 ml sample was taken from the gas remaining in the Douglas bag for measurement of enrichment, again to ensure that fractionation due to sampling had not occurred.

Calculation of recovery

Recovery of infused label as breath CO_2 was calculated as the product of the mean specific activity (SA) or atom % enrichment (E) of label (corrected for background) measured in samples taken at the beginning and at the end of each time period, and total CO_2 production for that period, relative to the amount of label infused :

$$\text{Percentage of label recovered} = (((SA_a + SA_b)/2) \times CO_2 \text{ produced}_{(\text{between a and b})}) \times 100/\text{amount of label infused}_{(\text{between a and b})}, \text{ or:}$$

$$(((E_a + E_b)/2) \times CO_2 \text{ produced}_{(\text{between a and b})}) \times 100/\text{amount of label infused}_{(\text{between a and b})},$$

where suffixes a and b indicate the beginning and end of that particular time period. The estimations were made assuming a linear change in specific activity or enrichment of CO_2 over that time (an assumed exponential change in specific activity or enrichment over the same time resulted in similar recoveries; see also Elia *et al*. 1992). The area under the curve was used to estimate total recovery for the whole 6 h time course. Calculation of the recovery of label in urea was similar to that of breath, the difference being that the time periods (a–b) were either 0–4 h or 4–6 h:

$$\text{Percentage of label recovered} = (((SA_a + SA_b)/2) \times \text{urea excreted}_{(\text{between a and b})}) \times 100/\text{amount of label infused}_{(\text{between a and b})},$$

or:

$$(((E_a + E_b)/2) \times \text{urea excreted}_{(\text{between a and b})}) \times 100/\text{amount of label infused}_{(\text{between a and b})}.$$

Statistics

Mean and standard deviation were used as the descriptive statistics. Paired *t* test and the Pearson correlation coefficient (*r*) were used to test for differences or associations between values. CV was estimated as the standard deviation of differences (d) between repeated measurements as a percentage of the overall mean and adjusted by $\sqrt{2}$ to account for variability due to each individual measurement.

Ethical approval

Ethical approval for the study was obtained from the Ethical Committees of the Dunn Clinical Nutrition Centre and Addenbrooke's Hospital, Cambridge, UK. Signed informed consent was obtained from all subjects.

Results

The CV for repeated measurements was 0·7 % for radioactivity in CO_2, 0·3 % for enrichment in CO_2, and 1·1 % for HCO_3^- concentration and 1·0 % for specific activity in both samples and the infusate.

No significant fractionation was evident in consecutive samples taken by continuous sampling of ^{14}C into the hyamine–methanol trapping agent or in the 11 ml spot samples for enrichment of ^{13}C taken either side of the continuous sampling.

Mean background enrichment of breath CO_2 for the six subjects (–25·61 (SD 1·19) δ ^{13}C per 10^3, relative to PDB) was significantly lower ($P < 0·001$; paired t test) than that of urinary urea (–22·61 (SD 1·09) δ ^{13}C per 10^3), although there was a strong relationship (r 0·90) between them. Enrichment of breath CO_2 reached a mean of 72·57 (SD 11·15) δ ^{13}C per 10^3 (equivalent to a change of 98·18 δ ^{13}C per 10^3) at the end of the 6 h infusion, whereas urea enrichment reached a mean of only –7·95 (SD 4·92) δ ^{13}C per 10^3 (equivalent to a change of 14·66 δ ^{13}C per 10^3) in the 4–6 h collection.

Table 1 shows the CO_2 production rates for each hour of the 6 h infusion (these values comprise measured CO_2 production rates plus estimates of CO_2 collected in the Douglas bag intended for the sampling of enrichment and specific activity of CO_2). In addition, mean enrichments and specific activities of CO_2 for each of the hourly periods are presented in order to illustrate the increasing appearance of label in breath CO_2 over the course of the infusion.

Recovery of infused label (for both ^{13}C and ^{14}C) as breath CO_2 was found to rise rapidly due to the infusion, reaching values close to 90 % for 3–6 h (Table 2). Inter-individual variation in recovery was small (generally about 2–5 %) at each hour (Table 2). The area under the time-recovery curve of $^{13}CO_2$ in breath was slightly higher (< 1 %) than that for $^{14}CO_2$, but was not statistically significant (Table 2). However, recoveries of $^{13}CO_2$ in breath were significantly higher than for $^{14}CO_2$ for the first 3 h (time periods 0–1, 1–2 and 2–3 h, $P < 0·05$, $< 0·01$ and $< 0·05$ respectively), despite the small mean differences. From 4 h onwards, no significant difference was detectable even though $^{13}CO_2$ recovery remained higher.

The mean recovery of infused ^{13}C in urinary urea was 0·12 (SD 0·07) % for the period 0–4 h and 0·50 (SD 0·22) % for the 4–6 h period following the start of the infusion. The recovery of infused ^{14}C was significantly higher ($P < 0·01$; paired t test) for both periods; 0·15 (SD 0·07) % and 0·54 (SD 0·22) % respectively.

Discussion

Considering the importance of CO_2 recovery to intermediary metabolism and energy expenditure and the current preference for using ^{13}C, it is perhaps surprising that there

Table 1. Carbon dioxide production rates and increases in enrichment and specific activity of breath carbon dioxide for each hourly period of a 6 h continuous simultaneous infusion of $H^{13}CO_3^-$ and $H^{14}CO_3^-$*

(Mean values and standard deviations for six subjects)

Time period (h)	CO_2 production rates† (mmol/h)		CO_2 production rates† (ml/min)		Enrichment (δ relative to PDB)		Specific activity (dpm/mmol)	
	Mean	SD	Mean	SD	Mean	SD	Mean	SD
0	–	–	–	–	–25·61	1·19	–	–
0–1	626	88	234	33	2·09	3·61	1133	163
1–2	647	86	242	32	40·75	7·00	2710	310
2–3	654	100	244	37	57·58	8·38	3400	365
3–4	669	85	250	32	66·58	10·04	3777	434
4–5	673	98	251	36	70·25	10·58	3929	462
5–6	641	82	239	31	71·68	10·81	3988	479

PDB, Pee Dee Belemnite.

* For details of subjects and procedures see pp. 270–271.

† Values comprise measured CO_2 production rates plus estimates of CO_2 collected in the Douglas Bag intended for the sampling of enrichment and specific activity of CO_2.

Table 2. Recovery of label in breath carbon dioxide for each hourly period of a 6 h continuous simultaneous infusion of $H^{13}CO_3^-$ and $H^{14}CO_3^-$*

(Mean values with their standard deviations for six subjects)

Time period (h)	Recovery of label (%)						Significance of difference
	^{13}C		^{14}C		Difference		
	Mean	SD	Mean	SD	Mean	SD	
0–1	24·9	1·8	24·7	1·9	0·2	0·2	$P < 0·05$
1–2	62·0	4·7	61·4	4·8	0·6	0·4	$P < 0·01$
2–3	78·4	5·9	77·6	5·8	0·8	0·7	$P < 0·05$
3–4	88·9	2·3	88·3	2·3	0·6	0·8	NS
4–5	92·7	3·0	92·1	3·3	0·6	0·9	NS
5–6	90·0	5·8	89·3	4·9	0·7	1·2	NS
0–6*†	72·2	2·8	72·8	2·8	0·7	0·7	NS

* For details of subjects and procedures see pp. 270–271.

† Mean cumulative recovery, equivalent to the recovery calculated using the area under the time–recovery curve.

has been no previous attempt to compare directly recoveries of $H^{13}CO_3^-$ and $H^{14}CO_3^-$ (Leijssen & Elia, 1996). This study has established for the first time that there is very little difference between ^{13}C and ^{14}C recovered in breath CO_2 (overall the total recoveries for the 6 h period were not significantly different, 72·2 % *v.* 72·8 % respectively) and, therefore, there is no real practical basis for altering recovery factors determined for ^{14}C in breath when using ^{13}C instead for energy expenditure estimation. However, these findings do not suggest that the acetate correction factor is equally applicable to both isotopes when measuring substrate oxidation (see later).

HCO_3^- The consistency of results in this study was achieved by careful attention to detail in all methodological procedures. Wherever possible, the same protocol was adopted for both isotopes. For example, a single intravenous infusion of $H^{13}CO_3^-$ and $H^{14}CO_3^-$ enabled these isotopes to be administered simultaneously over the same period of time. Furthermore, all measurements of ^{13}C and ^{14}C were obtained using the same sample of breath CO_2 or urinary urea. In addition, the same measured values of CO_2 or urea production rate were applied to the calculation of recovery for both isotopes in breath and urine respectively (see p. 271). Methodological precautions were also taken to eliminate or at least minimise the possibility of fractionation or the isotopic exchange of labelled sample CO_2 with ambient CO_2. Therefore, the only uncertainties in methodology were apparently limited to possible errors in the measurement of HCO_3^- concentration, ^{13}C enrichment and ^{14}C radioactivity. However, certain precautions were taken to ensure that these particular measurements were as accurate and precise as possible, including that: (1) all critical measurements were obtained gravimetrically (to 0·0001 g), rather than volumetrically; (2) the concentrations of acid labile CO_2 in the HCO_3^- standards were virtually identical, whether they were prepared in house or were commercially available; and (3) appropriate corrections were applied for density of solutions where necessary (see pp. 270–271).

Although the results suggest that for practical purposes the same recovery factor can be used for both isotopes when calculating oxidation of C-labelled substrates and energy expenditure, they do not exclude the possibility that enzymic reactions may still discriminate between ^{13}C- and ^{14}C-labelled substrates (Pacy *et al.* 1989) to produce slightly different quantities of $^{13}CO_2$ and $^{14}CO_2$. Measuring the recovery of labelled CO_2 in breath is too insensitive to be of value in assessing isotopic fractionation with respect to metabolic fixation of CO_2 or its entry into body CO_2 pools of slow turnover. This is because only a small proportion of the isotope is retained in the body after the first 3 h of infusion; for example, a small discrepancy of 1 %, nominally between 90 % and 91 %, of label recovered in breath CO_2 implies a difference of 11 % in the quantity of label retained or excreted via alternative routes. A more direct measurement of labelled CO_2 retained in body pools may be more sensitive and provide greater insight into metabolic bioequivalence of ^{13}C- and ^{14}C-isotopes. This may be especially relevant in view of the importance of the acetate correction factor to substrate oxidation (Wolfe and Jahoor, 1990; Sidossis *et al.* 1995; Schrauwen *et al.* 1998). However, there is no indication from this study of the need, or otherwise, to change this particular factor.

Assessment of label in urinary urea potentially offers a simple way of obtaining such insight into metabolic fixation of CO_2. However, this possibility may be confounded by two related factors. First, sensitivity may be compromised as loss of label via this route accounts for only a very small fraction of the administered label compared to the proportion recovered in breath CO_2. Second, the recovery of ^{14}C in urinary urea was significantly greater than that of ^{13}C, which is in contrast with their respective recoveries in breath CO_2. The lower recovery of ^{13}C in urea is consistent with its greater recovery in breath. However, there was a significant difference between the background enrichment of ^{13}C in urinary urea and that in breath CO_2 (see p. 272). Although background enrichment of each individual was specifically used in the calculation of recovery of ^{13}C appropriately in either breath or urine (pp. 270–271), any change in background levels over the 6 h course of the study would not have been taken into account thus compromising the integrity of any comparison with ^{14}C. However, preliminary work undertaken in similar conditions (M Harding, unpublished results) suggested that any such change in enrichment of breath CO_2 would be small (maximum individual variability (SD) for breath CO_2 was only 0·40 δ ^{13}C per 10^3 compared to the peak group mean change in the study subjects of 98·18 δ ^{13}C per 10^3 at 6 h). This would have a negligible effect on recovery of ^{13}C in breath CO_2 and could, therefore, be safely ignored. In contrast, variability in urea background would have a greater effect on recovery of ^{13}C because of the relatively lower change in enrichment of urea over the 6 h study period; if it is assumed that variability for urea background is 0·89 δ ^{13}C per 10^3 (the observed value) compared to the peak group mean change of 14·66 δ ^{13}C per 10^3 in the 4–6 h period, recoveries of ^{13}C would be slightly lower than those reported here (0·11 % *v.* 0·12 % for the 0–4 h period and 0·47 % *v.* 0·50 % for the 4–6 h period) resulting in greater differences between ^{13}C and ^{14}C, but with ^{14}C recovery still higher than ^{13}C. It is of interest that the background enrichment of urea was higher than that for breath CO_2, which is consistent with the greater recovery of ^{14}C than ^{13}C in urea, and may suggest that lighter isotopes are preferentially excreted in breath (i.e. rate of excretion, $^{12}C > {}^{13}C > {}^{14}C$) possibly making heavier isotopes more available for use in the liver, and would explain the greater excretion of the heavier isotopes in urinary urea despite possible discrimination against them by the enzymes of the urea cycle. Alternatively, the CO_2 that is used to form urea in the liver may have a different enrichment to CO_2 in breath because the liver may oxidise fuels with different C-enrichments to the other organs of the body. Furthermore, faster turnover of the circulating CO_2 pool than that of the urea pool implies that CO_2 excreted in breath may not have been produced at the same time as that utilised in the urea cycle. Furthermore, for a near steady-state to be achieved with an unprimed infusion requires considerably longer than the 6 h of this study. Such infusions indicate that about 2 % of the administered label is excreted in urea (Fuller & Elia, 1989; Elia *et al.* 1995), compared to only 0·5 % between 4–6 h observed here. Therefore, prolonged infusions that achieve higher and

near steady-state enrichments and specific activities are indicated for use in future studies.

In summary, the results of this study suggest that, for practical purposes, the same recovery factors for $^{14}CO_2$ can also be used for $^{13}CO_2$ when assessing energy expenditure using the bicarbonate or bicarbonate–urea methods. However, they do not exclude the possibility of fractionation due to enzymatic discrimination within metabolic pathways, such as the urea cycle. In addition, as the possible preferential fixation of label into the citric-acid cycle was not evaluated, no conclusion can be drawn concerning the effect of the different isotopes on the acetate correction factor for use in assessing oxidation of C-labelled substrates.

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