Validation of the [1,2-13C]acetate recovery factor for correction of [U-13C]palmitate oxidation rates in humans.

Validation of the [1,2-^{13}C]acetate recovery factor for correction of [U-^{13}C]palmitate oxidation rates in humans

Patrick Schrauwen, Dorien P. C. van Aggel-Leijssen, Wouter D. van Marken Lichtenbelt, Marleen A. van Baak, Annemie P. Gijsen and Anton J. M. Wagenmakers

Department of Human Biology, Maastricht University, The Netherlands

(Received 16 February 1998; accepted after revision 5 August 1998)

1. The validity of estimations of plasma fatty acid oxidation using tracers has often been questioned. The appearance of isotopic markers in breath CO_2 is delayed and incomplete. Recently suggestions have been made that substantial amounts of tracer are incorporated into products of the tricarboxylic acid cycle (e.g. glucose, glutamine and glutamate) and that an acetate correction factor can be used to correct for tracer fixation. In the present study we investigated whether the appearance of $^{13}CO_2$ during a separate infusion of [1,2-^{13}C]acetate could be used for correction of [U-^{13}C]palmitate oxidation rates in studies lasting <2 h and we quantified the appearance of tracer in the glutamine, glutamate and glucose pools of the body.

2. An infusion of either [1,2-^{13}C]acetate ($0{\cdot}104\ \mu$mol min^{-1} kg^{-1}) or [U-^{13}C]palmitate ($0{\cdot}013\ \mu$mol min^{-1} kg^{-1}) was given to eight male subjects and continued for 2 h at rest. In six subjects the infusion of [1,2-^{13}C]acetate was repeated to determine reproducibility of the acetate recovery.

3. Fractional recovery in breath from [1,2-^{13}C]acetate gradually increased during the infusion period at rest from $14{\cdot}1 \pm 0{\cdot}6$% at 60 min to $26{\cdot}5 \pm 0{\cdot}5$% at 120 min after the start of the infusion. Intersubject coefficient of variance was $8{\cdot}3 \pm 0{\cdot}6$% and intrasubject coefficient of variance of the acetate recovery tests was $4{\cdot}0 \pm 1{\cdot}5$%. After 2 h of [1,2-^{13}C]acetate infusion, $12{\cdot}4 \pm 0{\cdot}8$ and $10{\cdot}3 \pm 0{\cdot}9$% of infused ^{13}C was incorporated in the glutamine and glutamate pools, respectively.

4. In conclusion, the [1,2-^{13}C]acetate recovery factor can be used for correcting the rate of [U-^{13}C]palmitate oxidation in infusing studies of 2 h in resting conditions. Failure to use this recovery factor leads to a substantial underestimation of the rate of plasma free fatty acid oxidation. The extent of label fixation could largely be explained by accumulation of tracer carbon in glutamine and glutamate, and the accumulation in glucose is negligible.

The use of ^{13}C- and ^{14}C-fatty acid tracers to estimate the oxidation of plasma fatty acids has been questioned (Heiling *et al.* 1991). The appearance of $^{13}CO_2$ (and $^{14}CO_2$) in breath, coming from the oxidation of fatty acid tracers, is very low in the first hours of infusion, especially under resting conditions (Irving *et al.* 1983). This means that part of the label is temporarily fixed in metabolic pools in the body. When, in resting conditions, labelled $NaHCO_3$ is infused, up to 70–90% of the label is recovered implying that only a minor fraction of the tracer is entrapped in the bicarbonate pool and CO_2 fixation reactions (carboxylation and urea production) (Leijssen & Elia, 1996). Heiling *et al.* (1991) suggested that the plasma tracer first had to mix with a large intracellular fatty acid pool before it would be oxidized and that this was the main reason for the delayed and incomplete appearance of $^{13}CO_2$ ($^{14}CO_2$) in the breath. However, in contrast to Heiling *et al.* (1991), Sidossis *et al.* (1995*b*) recently showed that part of the tracer accumulated in products of the tricarboxylic acid (TCA) cycle (glucose, glutamate and glutamine; Fig. 1). It was claimed that the fixation of the tracer in these products, together with fixation in the bicarbonate pool, was the main reason for the delayed and incomplete appearance of $^{13}CO_2$ ($^{14}CO_2$) in the breath. They suggested that the amount of this label fixation can be determined by measuring the $^{13}CO_2$ production during infusion of ^{13}C- (or ^{14}C) acetate, because acetate, like palmitate, is converted to acetyl-CoA and then enters the TCA cycle before CO_2 is produced. According to Sidossis *et al.* (1995*a*) a constant value was observed for the acetate recovery factor between 3 and 4 h after the start of the

infusion and it was suggested that this steady state value should be used to correct palmitate oxidation rates. The first aim of the present study was to investigate whether the acetate recovery factor can be used during infusions lasting 2 h maximum, which would allow a shorter and less demanding infusion period for healthy volunteers and patients.

Sidossis *et al.* (1995*a*) used a simultaneous infusion of ^{14}C-acetate and ^{13}C-palmitate, so that only one experiment is needed to quantify and correct the oxidation of the palmitate tracer. However, the use of radioactive tracers is under severe limitation in many countries due to medical ethical considerations (Beckers *et al.* 1994). This implies that two separate experiments are needed, one to measure the $^{13}CO_2$ production from [U-^{13}C]palmitate and one to measure the $^{13}CO_2$ production from [1,2-^{13}C]acetate under identical conditions. However, in that case, the reproducibility of the acetate recovery factor should be high to obtain a valid estimate in two separate experiments. Therefore, the second aim of the present study was to investigate whether the acetate recovery factor has to be measured in each individual or whether the mean of a group is representative for individuals. So, we measured both the intra- and intersubject coefficient of variation of the acetate recovery factor in a group of healthy volunteers.

The main TCA cycle products in which label from [1,2-^{13}C]acetate and [U-^{13}C]palmitate may accumulate are glucose formed in the liver by gluconeogenesis (Marliss *et al.* 1971), glutamate released by gut and liver and glutamine formed and released by skeletal muscle (Marliss *et al.* 1971; Darmaun *et al.* 1986; Elia *et al.* 1989). The final aim of the present study was to quantify the appearance in time in these circulating products of the TCA cycle to judge which process and tissue are most important for tracer fixation from a quantitative point of view during a 2 h infusion of acetate.

METHODS

Subjects

Eight healthy, male volunteers participated: age, 35·1 ± 1·8 years; height, 1·80 ± 0·03 m; weight, 79·5 ± 5·3 kg; percentage body fat, 22·3 ± 2·6%; with a body mass index ranging from 19 to 30 kg m^{-2}. The nature and risks of the experimental procedure were explained to the subjects, and all subjects gave their written informed consent. The study was approved by the Ethical Committee of Maastricht University.

Experimental design

The experiment consisted of two tests separated by a minimum of 1 week to prevent carry over of tracer. Subjects came to the laboratory at 08.00 h after an overnight fast. Teflon catheters were inserted in an antecubital vein for isotope infusion and retrogradely into a contralateral dorsal hand vein for sampling of arterialized venous blood. After placement of the catheters subjects rested on a bed and the hand was placed in a hot-box where air was circulated at 60 °C in order to obtain arterialized venous blood. After 30 min baseline oxygen consumption and carbon dioxide production were measured and breath and blood samples were collected. Immediately thereafter, subjects were given an intravenous dose of 0·085 mg kg^{-1} of $NaH^{13}CO_3$ to prime the bicarbonate pool. Then, at $t = 0$, a constant intravenous infusion of [U-^{13}C]palmitate (0·013 μmol min^{-1} kg^{-1}) or [1,2-^{13}C]acetate (0·104 μmol min^{-1} kg^{-1}) was started and continued for 120 min. These infusion rates led to an equal amount of ^{13}C-tracer infusion with both palmitate and acetate. The concentration of palmitate in the infusate was measured for each experiment, so that the exact infusion rate could be determined on an analytical gas chromatograph using heptadecanoic acid as internal standard (see sample analysis). The palmitate tracer (60 mg of potassium salt of [U-^{13}C]palmitate,

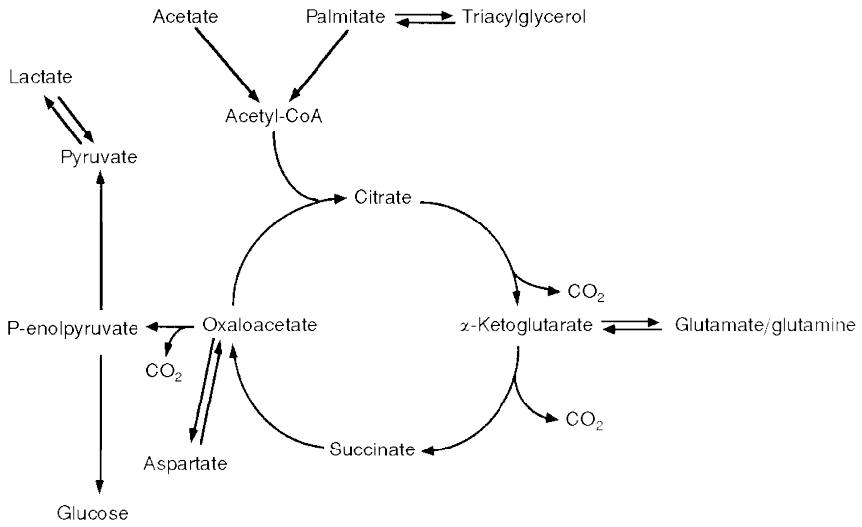

Figure 1

Pathway of acetate and palmitate and routes where label can be lost.

99% enriched; Cambridge Isotope Laboratories, Andover, MA, USA) was dissolved in heated sterile water and passed through a 0·2 μm filter into 5% warm human serum albumin to make a 0·670 mM solution. The acetate concentration was measured in each infusate with an enzymatic method (Boehringer, Mannheim, Germany). The acetate tracer (sodium salt of [1,2-^{13}C]acetate, 99% enriched; Cambridge Isotope Laboratories) was dissolved in 0·9% saline. The chemical and isotopic purity (99%) of palmitate and acetate tracers were checked by ^{1}H and ^{13}C NMR and gas chromatography–mass spectrometry (GC–MS). Blood samples were taken every 30 min, and breath samples every 10 min. Oxygen consumption ($\dot{V}_{O_2}$) and carbon dioxide production ($\dot{V}_{CO_2}$) were also measured every 10 min. In six subjects the acetate test was repeated to determine the intrasubject reproducibility of the measurements.

Oxygen consumption and carbon dioxide production were measured using open circuit spirometry (Oxycon-β, Mijnhardt, Bunnik, The Netherlands). No changes in oxygen consumption, carbon dioxide production and respiratory quotient occurred during the last hour of the resting period and therefore measurements were averaged for the last hour of the resting period (t = 60–120 min).

Diet and activity prior to testing

Subjects were asked to fill in a 3 day food intake and activity record prior to the first test. In the other tests they were asked to repeat their activity schedule and eat the same food, making sure the diet was similar in the two tests. Also, subjects were asked not to consume any products with a high natural abundance of ^{13}C (carbohydrates derived from C4 plants: maize, sugar cane) 1 week before and during the entire experimental period.

Body composition

After an overnight fast, body density was determined by underwater weighing in the fasted state. Body weight was measured with a digital balance accurate to 0·01 kg (Sauter, type E1200, Ebungen, Germany). Lung volume was measured simultaneously with the helium dilution technique using a spirometer (Volugraph 2000, Mijnhardt). Body fat percentage was calculated using the equations of Siri (1956). Fat-free mass (FFM) in kilograms was calculated by subtracting fat mass from total body mass.

Sample analysis

Oxygen saturation (Hemoximeter OSM2, Copenhagen, Denmark) was determined immediately after sampling in heparinized blood. Fifteen millilitres of arterialized venous blood was sampled in tubes containing EDTA to prevent clotting and immediately centrifuged at 3000 r.p.m. (1000 g) for 10 min at 4 °C. Plasma was frozen in liquid nitrogen and stored at −80 °C until further analysis. Plasma substrates were determined using the hexokinase method for glucose (Roche, Basel, Switzerland) and the Wako NEFA C test kit for free fatty acids (Wako Chemicals, Neuss, Germany). Plasma for the analysis of glutamine and glutamate was deproteinized with 0·3 M perchloric acid (PCA) and analysed enzymatically for glutamate with glutamate dehydrogenate performed on a COBAS FARA analyser (Roche, Basel, Switzerland). Glutamine concentration was subsequently determined by converting glutamine to glutamate using glutaminase.

Breath samples were analysed for ^{13}C/^{12}C ratio using a gas chromatography–isotope ratio mass spectrometry system (GC–IRMS; Finnigan MAT 252, Bremen, Germany). Plasma glutamine and glutamate tracer/tracee ratio (TTR) was determined as the N-methyl-N-(tert-butyldimethylsilyl)-trifluoroacetamide (MTBSTFA) derivative using GC–IRMS. The resulting derivative contains twenty-three carbon atoms of which five are labelled; TTR is therefore corrected by the factor 23/5. For determination of plasma palmitate, free fatty acids (FFAs) were extracted from plasma, isolated by thin-layer chromatography, and derivatized to their methyl esters. Palmitate concentration was determined on an analytical gas chromatograph with flame ionization detection using heptadecanoic acid as internal standard and on average it comprised 24 ± 1% of total FFAs. Isotope tracer/tracee ratio of palmitate was determined using GC–IRMS and corrected for the extra methyl group in its derivative. For determination of the plasma glucose tracer/tracee ratio, the glucose was extracted with chloroform–methanol–water and derivatization occurred with butylboronic acid and acetic anhydride as described before (Pickert *et al.* 1991). The resulting derivative contains sixteen carbon atoms of which six are labelled; TTR is therefore corrected by the factor 16/6.

Calculations

^{13}C enrichment of breath CO_2 and plasma metabolites is given in the form of the tracer/tracee ratio (TTR). TTR was defined as $(^{13}C/^{12}C)_{sa} - (^{13}C/^{12}C)_{bk}$, in which sa indicates sample and bk indicates background.

Total carbohydrate and fat oxidations were calculated using stoichiometric equations (Péronnet & Masssicotte, 1991):

$$\text{Total fat oxidation} = 1{\cdot}695\dot{V}_{O_2} - 1{\cdot}701\dot{V}_{CO_2},$$

$$\text{Total carbohydrate oxidation} = 4{\cdot}585\dot{V}_{CO_2} - 3{\cdot}226\dot{V}_{O_2},$$

with $\dot{V}_{O_2}$ and $\dot{V}_{CO_2}$ in litres per minute and total fat and carbohydrate oxidations in grams per minute.

Total fatty acid oxidation was determined by converting the rate of total fat oxidation to its molar equivalent, with the assumption that the average molecular weight of triglyceride is 860 g mol^{-1}, and multiplying the molar rate of triglyceride oxidation by three because each molecule contains three fatty acid residues.

Fractional recovery of label (as a percentage) in breath CO_2, derived from the infusion of labelled palmitate or acetate was calculated as follows:

$$\text{Fractional recovery of label} = (\text{TTR}_{CO_2} \times \dot{V}_{CO_2})/F \times 100,$$

where TTR_{CO_2} is tracer/tracee ratio (TTR) in breath CO_2, $\dot{V}_{CO_2}$ is carbon dioxide production (mmol min^{-1}) and F is infusion rate (mmol min^{-1}).

Breath $^{13}CO_2$ production, corrected for acetate recovery, during [U-^{13}C]palmitate infusion was calculated as:

$$\text{Corrected } ^{13}CO_2 \text{ production} = (\text{TTR}_{CO_2} \times \dot{V}_{CO_2})/\text{ar},$$

where TTR_{CO_2} is tracer/tracee ratio (TTR) in breath CO_2, $\dot{V}_{CO_2}$ is carbon dioxide production (mmol min^{-1}) and ar is fractional acetate recovery.

The rate of [U-^{13}C]palmitate oxidation (μmol min^{-1}) was calculated as follows:

$$\text{Palmitate oxidation} = (\text{TTR}_{CO_2} \times \dot{V}_{CO_2})/(\text{TTR}_p \times \text{ar}) \times 1000,$$

where TTR_p is the tracer/tracee ratio of fatty acid carbon in plasma and ar is the fractional acetate recovery.

Total plasma fatty acid oxidation was then calculated by dividing palmitate oxidation rate by the fractional contribution of palmitate to the total FFA concentration.

Rate of appearance (R_a, μmol min^{-1}) of palmitate in plasma, which under steady state conditions is equal to the rate of disappearance (R_d) minus tracer infusion rate, was calculated as:

$$R_a = F(\mathrm{TTR_i}/\mathrm{TTR_p}),$$

where $\mathrm{TTR_i}$ is the tracer/tracee ratio of fatty acid carbon in infusion. Percentage of plasma FFA cleared from the circulation that was oxidized (%R_a oxidized) was calculated as:

$$\%\ R_a \text{ oxidized} = \text{plasma FFA oxidation}/R_a \text{ FFA}.$$

To estimate the amount of infused ^{13}C incorporated in the glutamine, glutamate and glucose pools it was assumed that the tracer/tracee ratio in plasma at the end of the infusion period was in equilibrium with the respective body pools. Using available data on the size of the glutamine and glutamate pools, the amount of infused ^{13}C incorporated in the glutamine and glutamate pools could then be calculated, by multiplying the plasma enrichment with the body pool size, as follows:

% ^{13}C incorporated in glutamine =

$$(\mathrm{TTR_{glutamine,120}} \times 5{\cdot}369 \times \mathrm{BW})/(F \times 120) \times 100,$$

and

% ^{13}C incorporated in glutamate =

$$(\mathrm{TTR_{glutamate,120}} \times 1{\cdot}247 \times \mathrm{BW})/(F \times 120) \times 100,$$

where $\mathrm{TTR_{glutamine,120}}$ and $\mathrm{TTR_{glutamate,120}}$ are tracer/tracee ratio of plasma glutamine and glutamate, respectively, at $t = 120$ min, 5·369 and 1·247 is the size of the glutamine and glutamate pools, respectively (mmol kg^{-1}) (Darmaun *et al.* 1986), BW is body weight, and F is infusion rate (mmol min^{-1}).

For glucose there is inconsistency about the true volume of distribution. Therefore, a minimal and maximal estimation of the amount of infused ^{13}C incorporated in the glucose pools was calculated as follows:

% ^{13}C incorporated in glucose =

$$(\mathrm{TTR_{glucose,120}} \times 40 \times \mathrm{Conc_{glucose}} \times \mathrm{BW})/(F \times 120) \times 100,$$

and

% ^{13}C incorporated in glucose =

$$(\mathrm{TTR_{glucose,120}} \times 200 \times \mathrm{Conc_{glucose}} \times \mathrm{BW})/(F \times 120) \times 100,$$

where $\mathrm{TTR_{glucose,120}}$ is tracer/tracee ratio of plasma glucose at $t = 120$ min, 40 and 200 are the minimum and maximum volume

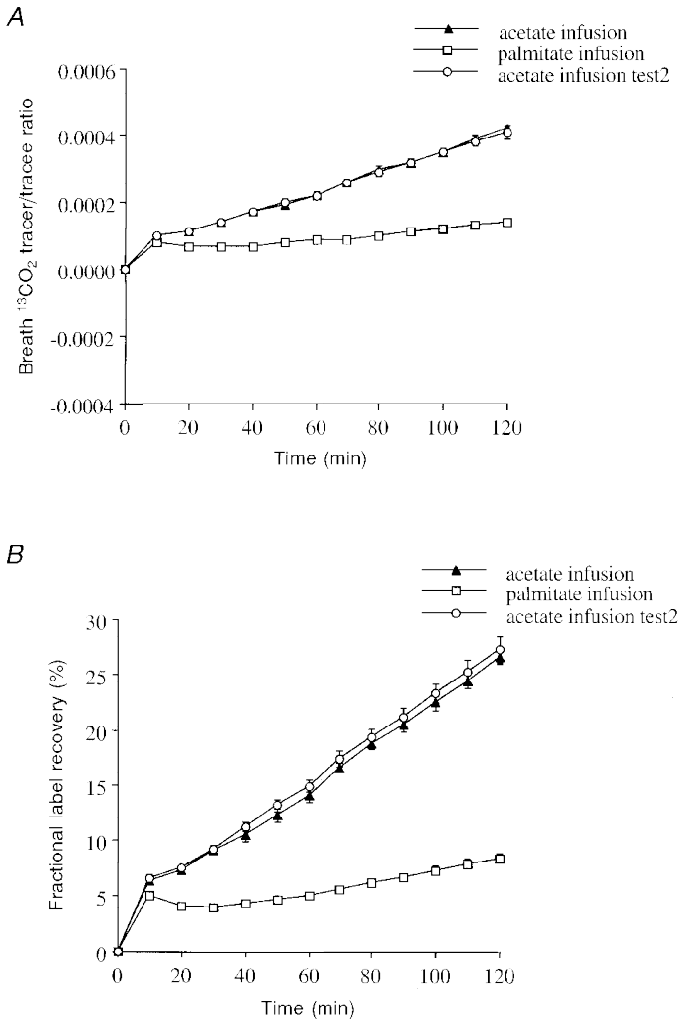

Figure 2

Breath $^{13}CO_2$ tracer/tracee ratio (*A*) and fractional label recovery (%) in breath CO_2 (*B*) when infusing either [1,2-^{13}C]acetate or [U-^{13}C]palmitate.

Table 1. Plasma (tracers) and total (indirect calorimetry) fatty acid kinetics for the last hour of [U-^{13}C]palmitate infusion

	Plasma fatty acid oxidation (μmol kg^{-1} min^{-1})	R_a (μmol kg^{-1} min^{-1})	Percentage R_a oxidized (%)	Total fatty acid oxidation (μmol kg^{-1} min^{-1})
No correction	0·56 ± 0·05	7·32 ± 0·31	7·6 ± 0·4	3·83 ± 0·28
Acetate correction	2·43 ± 0·17	7·32 ± 0·31	33·1 ± 1·6	3·83 ± 0·28

Values are means ± S.E.M. for 8 volunteers.

of distribution of glucose (ml kg^{-1}), $Conc_{glucose}$ is the average glucose concentration (t = 90–120 min, mmol l^{-1}), BW is body weight and F is infusion rate (mmol min^{-1}).

Statistical analysis

To examine if there were individual differences in increase in acetate recovery factor, linear regression lines were determined for the period of t = 60 to t = 120 min and slopes and intercepts were tested using analysis of covariance (ANCOVA). The coefficient of variance (CV) was calculated as S.D./mean × 100. All data are presented as means ± S.E.M. and $P < 0·05$ is considered as significance level.

RESULTS

Breath $^{13}CO_2$ tracer/tracee ratio gradually increased during the infusion period both when infusing palmitate and when infusing acetate (Fig. 2*A*). No plateau was reached at the end of the 2 h infusion period.

Fractional label recovery (as a percentage) in breath CO_2 of [U-^{13}C]palmitate increased in a linear way (slope, 0·056 % min^{-1}; r^2, 0·99) from 5·1 ± 0·2 % at t = 60 min to 8·4 ± 0·4 % at t = 120 min. Fractional label recovery in breath CO_2 from [1,2-^{13}C]acetate also increased in a linear way (slope, 0·20 % min^{-1}; r^2, 0·99) during the infusion period from 14·1 ± 0·6 % at t = 60 min to 26·5 ± 0·5 % at t = 120 min (Fig. 2*B*). With acetate, there were no significant differences in slopes of the linear regression lines between subjects, but the intercept was significantly different between subjects ($P < 0·05$).

Percentage difference (percentage of maximal value) in the fractional recovery in breath CO_2 from [1,2-^{13}C]acetate between subjects ranged from 15·1 to 32·9 % at different time points. This resulted in a mean intersubject coefficient of variation of 8·3 ± 0·6 %.

Reproducibility

In six subjects, the determination of the acetate recovery factor was repeated after 2–4 weeks. There were no significant differences in fractional acetate recovery between the first and second test at any time point. Intraclass correlation coefficient between test 1 and test 2 was 0·985 (r^2 = 0·97, $P < 0·0001$). Percentage difference (percentage of maximal value) in the fractional recovery in breath CO_2 from [1,2-^{13}C]acetate between two tests in the same subject ranged from 0·1 to 14·0 %, with an average of 5·4 %. Mean intrasubject coefficient of variation over the whole infusion period was 4·0 ± 1·5 %.

Plasma substrate tracer/tracee ratio

Plasma palmitate tracer/tracee ratio reached a plateau after 30 min of rest (Fig. 4*A*) but $^{13}CO_2$ production, corrected for

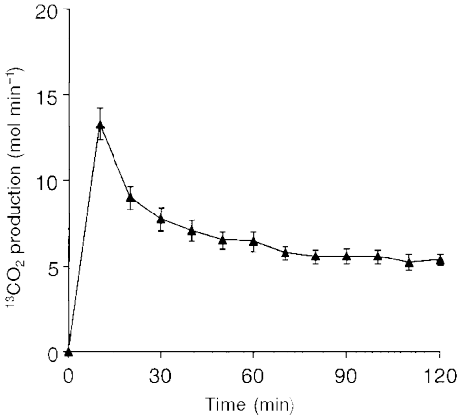

Figure 3

Breath $^{13}CO_2$ production corrected for recovery when infusing either [1,2-^{13}C]acetate or [U-^{13}C]palmitate.

recovery, reached a plateau during the last hour of infusion (Fig. 3). Therefore, substrate kinetics were averaged over the last hour. Plasma FFA concentration was $416{\cdot}8 \pm 26{\cdot}4$ μmol l^{-1} and plasma palmitate concentration was $100{\cdot}5 \pm 8{\cdot}1$ μmol l^{-1} (24 ± 1 % of FFA).

Isotopically determined plasma FFA oxidation (Fig. 4*B*), rate of appearance (R_a) and percentage of rate of appearance oxidized are given in Table 1, both with and without using the acetate correction factor. Using the acetate correction factor, R_a of FFA was $7{\cdot}32 \pm 0{\cdot}31$ μmol kg^{-1} min^{-1}. Plasma FFA oxidation was $2{\cdot}43 \pm 0{\cdot}17$ μmol kg^{-1} min^{-1} ($= 33{\cdot}1 \pm 1{\cdot}6$ % of R_a).

Plasma glutamine concentration was 539 ± 13 and 531 ± 14 μmol l^{-1} when given palmitate and acetate, respectively. Glutamine tracer/tracee ratio gradually increased during both palmitate and acetate infusion and did not reach a plateau after the 120 min of rest. The TTR at $t = 120$ min was 0·000101 and 0·000544 after palmitate and acetate infusion, respectively (Fig. 5*A*).

Plasma glutamate concentration was 83 ± 9 and 76 ± 6 μmol l^{-1} after palmitate and acetate infusion, respectively. Glutamate tracer/tracee ratio gradually increased during both palmitate and acetate infusion and did not reach a plateau after the 120 min of rest. The TTR at $t = 120$ min was 0·000386 and 0·001946 after palmitate and acetate infusion, respectively (Fig. 5*B*).

Plasma glucose concentration was $4{\cdot}9 \pm 0{\cdot}1$ and $5{\cdot}1 \pm 0{\cdot}1$ mmol l^{-1} after palmitate and acetate infusion, respectively. Glucose tracer/tracee ratio gradually increased during both palmitate and acetate infusion and did not reach a plateau after 120 min of rest. The TTR at $t = 120$ min was $0{\cdot}0000214 \pm 0{\cdot}0000017$ and $0{\cdot}0000928 \pm 0{\cdot}0000084$ after palmitate and acetate infusion, respectively (Fig. 5*C*).

DISCUSSION

The results of the present study demonstrate that the acetate recovery factor can be used to correct palmitate oxidation rates in infusion studies of 2 h or less. However, to correct palmitate oxidation rates, the acetate recovery factor needs to be determined for every time point, because the fractional label recovery in breath CO_2 of both [U-^{13}C]palmitate and [1,2-^{13}C]acetate increased in a linear way.

The present study demonstrates a good reproducibility for the acetate recovery factor. Intrasubject coefficient of variation was 4·0 %. However, this does not guarantee that in any given individual the acetate recovery factor can be

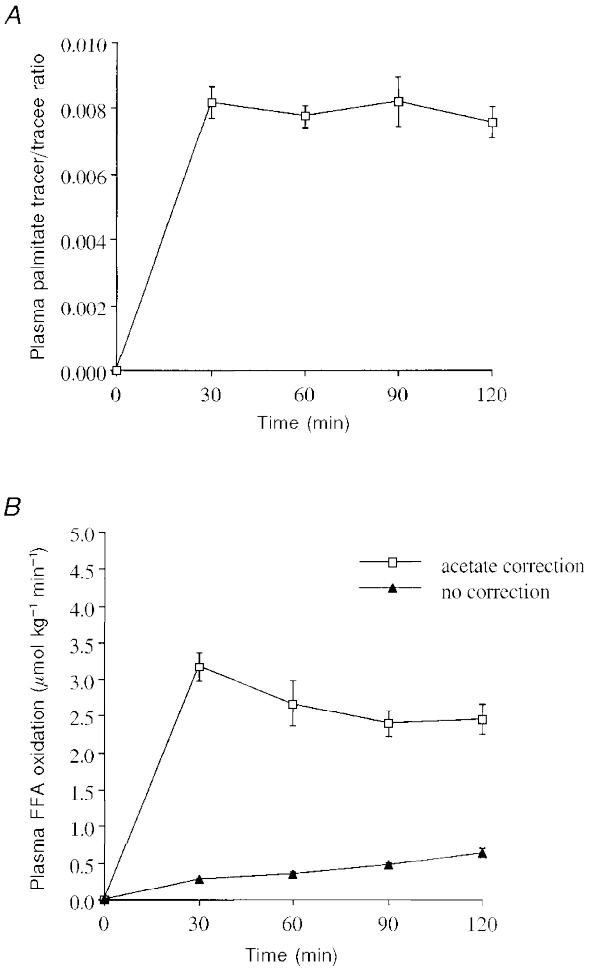

Figure 4
^{13}C-palmitate tracer/tracee ratio (*A*) and plasma FFA oxidation calculated using acetate correction factor (*B*) when infusing [U-^{13}C]palmitate.

determined in a separate experiment. Therefore the range in percentage difference in individual subjects between the two tests at different time points is more important. The maximum difference found within one subject between the two tests at any time point was 14·0%. The mean difference between the two tests was 5·4%. These results show that it is valid to estimate the fractional recovery in a separate experiment, making it possible to use ^{13}C-labelled acetate to determine the correction factor needed to correct the plasma FFA oxidation data.

To study whether it is necessary to determine the acetate correction factor in each individual the intersubject coefficient of variance was calculated. The intersubject coefficient of variance was $8{\cdot}3 \pm 0{\cdot}6$%, with a percentage difference between subjects ranging from 15·1 to 32·9% at different time points. This means that an individual estimation of the recovery of ^{13}C-acetate is needed. Using one average recovery factor for a group as a whole can lead to over- or under-estimation of fatty acid oxidation of more than 30% in individual subjects.

One possible pathway for label fixation is the loss of ^{13}C from the TCA cycle in the form of glutamate and glutamine. The label is then stored in the glutamine and glutamate pools, which are in equilibrium with plasma glutamine and glutamate. We found a gradual increase in both glutamine and glutamate tracer/tracee ratio in plasma. This implies that the tracer/tracee ratio of the intracellular glutamine and glutamate pools will also gradually increase. Once the

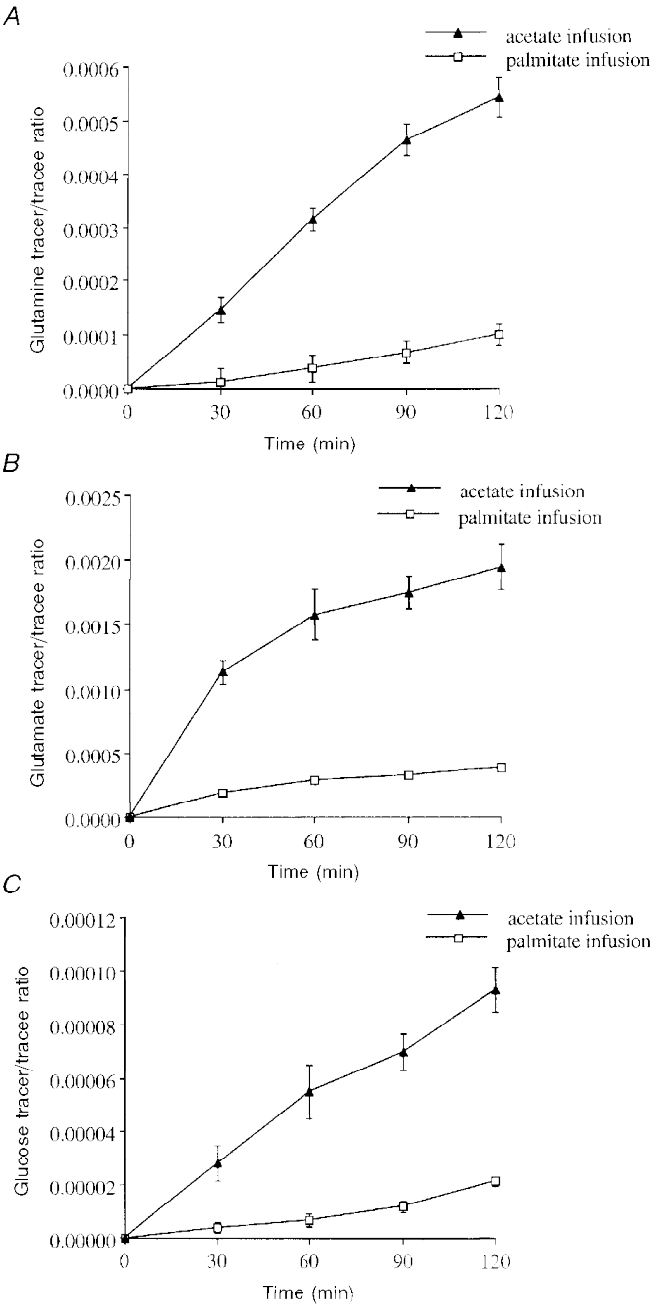

Figure 5
^{13}C glutamine tracer/tracee ratio (*A*), ^{13}C glutamate tracer/tracee ratio (*B*) and ^{13}C glucose tracer/tracee ratio (*C*) when infusing either [1,2-^{13}C]acetate or [U-^{13}C]palmitate.

intracellular glutamine and glutamate pools increase in tracer/tracee ratio, more tracer will be recovered after oxidation of these pools. Indeed, in our experiment, we did not reach a steady state in breath $^{13}CO_2$ tracer/tracee ratio at the end of the 2 h infusion period. $^{13}CO_2$ tracer/tracee ratio increased in a linear way. As long as the glutamine/glutamate tracer/tracee ratio has not reached a plateau, the amount of recycling of ^{13}C will increase and therefore breath $^{13}CO_2$ tracer/tracee ratio will increase. Considering the large glutamine/glutamate pools in muscle, it is not to be expected that during a 2 h infusion period an isotopic steady state can be derived. Furthermore, Van Acker *et al.* (1996) showed that no isotopic steady state in both plasma and muscle glutamine tracer/tracee ratio was obtained after 11 h of infusion of labelled glutamine. Wolfe & Jahoor (1990) also showed a gradually increasing $^{13}CO_2$ production for a period of 4 h. In contrast, Sidossis *et al.* (1995*a*) reported an apparent plateau in breath $^{13}CO_2$ tracer/tracee ratio at the end of 3 h of infusion at rest. However, they also reported an increase in glutamine tracer/tracee ratio which had not reached a plateau at the end of the infusion period. The extracellular glutamine pool has been estimated to be 110 μmol kg^{-1}, the intracellular muscle glutamine pool to be 5200 μmol kg^{-1} and the intracellular liver glutamine pool to be 39 μmol kg^{-1} (Darmaun *et al.* 1986). Using these figures, and assuming that the plasma glutamine tracer/tracee ratio at the end of the 2 h infusion period is in equilibrium with the muscle glutamine tracer/tracee ratio, it can be estimated that 12·4 $\pm$ 0·8 % of infused ^{13}C was incorporated in the glutamine pools after 2 h of [1,2-^{13}C]acetate infusion. The same estimation can be performed for the glutamate pools, considering an extracellular glutamate pool of 7 μmol kg^{-1}, an intracellular liver free glutamate pool of 40 μmol kg^{-1} and intracellular muscle glutamate pool of 1200 μmol kg^{-1} (Darmaun *et al.* 1986), resulting in 10·3 $\pm$ 0·9 % of infused ^{13}C incorporated in the glutamate pools. It should be kept in mind that these calculations reflect the minimal amount of tracer that is incorporated in the glutamine and glutamate pools as it may well be possible that after 2 h of infusion the ^{13}C tracer/tracee ratio of glutamine and glutamate at the endogenous production site is higher than the plasma tracer/tracee ratio. Thus the loss of ^{13}C from the TCA cycle in the form of glutamate and glutamine contributes significantly to the low recovery of acetate at rest.

Another possible pathway for label fixation is the loss of ^{13}C from the TCA cycle to glucose in gluconeogenic pathways. In our experiment the glucose tracer/tracee ratio gradually increased. When only the plasma volume (40 ml kg^{-1}) is considered as the volume of distribution for glucose, it can be calculated that 0·06 $\pm$ 0·09 % of infused ^{13}C is incorporated in the glucose pools. When the total extracellular fluid is assumed to be 0·2 l kg^{-1} (Darmaun *et al.* 1986) these figures become 0·32 $\pm$ 0·04 %. There is some discrepancy about the true distribution volume of glucose, but the true value will be somewhere between these two figures. Thus it can be concluded that the loss of ^{13}C to glucose is negligible. This also means that gluconeogenesis contributes only little to label fixation and forms a minor component of the acetate recovery factor in post-absorptive subjects in resting conditions. Label fixation in glutamine and glutamate from a quantitative point of view is more important.

In our experiment, recovery of uniformly labelled acetate was $\pm$27 % at the end of the 2 h infusion period. These figures are far less than the results obtained by Sidossis *et al.* (1995*a*). They found an acetate recovery of 56 % at the end of a 3 h infusion period at rest. In our experiment, the recovery increased in a linear way and extrapolation to 3 h results in an acetate recovery of 38 %. This is still less then the recovery found in the study of Sidossis *et al.* (1995*a*). However, Sidossis *et al.* (1995*a*) used ^{14}C-acetate, which was labelled in the (C-1) position, while in our experiment acetate was uniformly labelled (C-1 and C-2). The effect of the position of the labelled carbon atom on percentage recovered label was studied by Wolfe & Jahoor (1990). After a 4 h infusion period 81 % of infused [1-^{13}C]acetate was recovered, while only 53 % of [2-^{13}C]acetate was recovered. The difference in tracer position therefore may explain a major part of the difference in acetate recovery found between our study and the study of Sidossis *et al.* (1995*a*).

The acetate correction factor had to be determined using uniformly labelled acetate because it was used to correct the oxidation rate of [U-^{13}C]palmitate. A major reason for use of [U-^{13}C]palmitate is that only one-sixteenth of the dose of [1-^{13}C]palmitate tracer is required to get an equivalent amount of $^{13}CO_2$ production. A major practical limitation with the use of palmitate tracers is the low solubility in aqueous solutions. For this reason palmitate is infused bound to human albumin. In case of the [1-^{13}C]palmitate tracer the amount of albumin infused is so large that infusion periods maximally may last 1–2 h (to prevent unacceptable osmotic shifts in the plasma). This period can be substantially lengthened when a [U-^{13}C]palmitate tracer is used. The amount of fixation of tracer in TCA cycle products will be larger with [U-^{13}C]palmitate than with [1-^{13}C]palmitate but can be corrected by measuring the breath $^{13}CO_2$ recovery from [1,2-^{13}C]acetate.

The impact of the acetate correction factor is shown in Table 1. In this table plasma FFA oxidation is shown with and without applying an acetate correction factor. In our study, without the correction factor plasma FFA oxidation at rest is underestimated 4-fold. Using the acetate correction factor, the calculated plasma fatty acid oxidation was approximately 66 % of total fatty acid oxidation. The contribution of other fatty acids than those derived from plasma to energy expenditure in the resting state is controversial. In their study, Sidossis *et al.* (1995*b*) suggest that, at rest, nearly all fatty acids used for oxidation are plasma-borne fatty acids. However in another study by Sidossis *et al.* (1996) plasma fatty acid oxidation is only responsible for 80 % of total FFA oxidation, leaving 20 % for other tissues (intramuscular oxidation, liver oxidation or very-low-density lipoprotein (VLDL) oxidation).

The need to correct oxidation for the recovery of infused label not only concerns fat metabolism. Because loss of label occurs in the TCA cycle, the fraction of label which is recovered should also be determined when studying carbohydrate and protein metabolism. Recently, it was shown that during glucose infusion exogenous glucose oxidation (determined by tracer) accounted for all glucose oxidation (determined by indirect calorimetry) when the acetate recovery factor was applied, whereas when only using a bicarbonate correction factor (i.e. 0·8; Bonadonna *et al.* 1990) endogenous glycogen oxidation contributed substantially to total glucose oxidation (Tounian *et al.* 1996). These studies indicate the importance of the recovery factor in studying oxidation rates of ^{13}C-enriched substrates especially at rest.

In conclusion, the [1,2-^{13}C]acetate recovery factor can be used for correcting the rate of [U-^{13}C]palmitate oxidation in infusing studies of 2 h in resting conditions. Intrasubject coefficient of variation was small, which makes it possible to determine the acetate recovery factor in a second separate experiment using [^{13}C]acetate in one experiment and [^{13}C]palmitate in the other. However, the acetate recovery factor should be determined in each individual and for every time point, because the acetate recovery increases with time. From a quantitative point of view accumulation of tracer carbon in glutamine and glutamate seems much more important to explain the label fixation than accumulation in glucose released by the liver. Failure to correct for the low acetate recovery under these conditions leads to dramatic underestimation of the oxidation rate of plasma fatty acid tracers. Therefore, most previous estimates of plasma fatty acid oxidation published in the literature are major underestimations.

Beckers, E. J., Halliday, D. & Wagenmakers, A. J. M. (1994). Glucose metabolism and radioactive labeling: what are the real dangers? *Medicine and Science in Sports and Exercise* **26**, 1316–1318.

Bonadonna, R. C., Groop, L. C., Zych, K., Shank, M. & DeFronzo, R. A. (1990). Dose-dependent effect of insulin on plasma free fatty acid turnover and oxidation in humans. *American Journal of Physiology* **259**, E736–750.

Darmaun, D., Matthews, D. E. & Bier, D. M. (1986). Glutamine and glutamate kinetics in humans. *American Journal of Physiology* **251**, E117–126.

Elia, M., Schlatmann, A., Goren, A. & Austin, S. (1989). Amino acid metabolism in muscle and in the whole body of man before and after ingestion of a single mixed meal. *American Journal of Clinical Nutrition* **49**, 1203–1210.

Heiling, V. J., Miles, J. M. & Jensen, M. D. (1991). How valid are isotopic measurements of fatty acid oxidation? *American Journal of Physiology* **261**, E572–577.

Irving, C. S., Wong, W. W., Shulman, R. J., Smith, E. O. & Klein, P. D. (1983). ^{13}C bicarbonate kinetics in humans: intra- vs. interindividual variations. *American Journal of Physiology* **245**, R190–202.

Leijssen, D. P. C. & Elia, M. (1996). Recovery of $^{13}CO_2$ and $^{14}CO_2$ in human bicarbonate studies: a critical review with original data. *Clinical Science* **91**, 665–677.

Marliss, E. B., Aoki, T. T., Pozefsky, T., Most, A. S. & Cahill, G. F. (1971). Muscle and splanchnic glutamine and glutamate metabolism in postabsorptive and starved man. *Journal of Clinical Investigation* **50**, 814–817.

Péronnet, F. & Masssicotte, D. (1991). Table of nonprotein respiratory quotient: an update. *Canadian Journal of Sports Science* **16**, 23–29.

Pickert, A., Overkamp, D., Renn, W., Liebich, H. & Eggstein, M. (1991). Selected ion monitoring gas chromatography/mass spectrometry using uniformly labelled (^{13}C)-glucose for determination of glucose turnover in man. *Biological Mass Spectrometry* **20**, 203–209.

Sidossis, L. S., Coggan, A. R., Gastaldelli, A. & Wolfe, R. R. (1995*a*). A new correction factor for use in tracer estimations of plasma fatty acid oxidation. *American Journal of Physiology* **269**, E649–656.

Sidossis, L. S., Coggan, A. R., Gastaldelli, A. & Wolfe, R. R. (1995*b*). Pathway of free fatty acid oxidation in human subjects: implications for tracer studies. *Journal of Clinical Investigation* **95**, 278–284.

Sidossis, L. S., Stuart, C. A., Shulman, G. I., Lopaschuk, G. D. & Wolfe, R. R. (1996). Glucose plus insulin regulate fat oxidation by controling the rate of fatty acid entry into the mitochondria. *Journal of Clinical Investigation* **98**, 2244–2250.

Siri, W. E. (1956). The gross composition of the body. *Advances in Biological and Medical Physiology* **4**, 239–280.

Tounian, P., Schneiter, P., Henry, S. & Tappy, L. (1996). Effects of infused glucose on glycogen metabolism in healthy humans. *Clinical Physiology* **16**, 403–416.

Van Acker, B. A. C., Hulsewe, K. W. E., Wagenmakers, A. J. M., Deutz, N. E., Van Kreel, B. K., Soeters, P. B. & Van Meyenfeld, M. F. (1996). Measurement of glutamine metabolism in gastrointestinal cancer patients. *Clinical Nutrition* **15**, 1.

Wolfe, R. R. & Jahoor, F. (1990). Recovery of labelled CO_2 during the infusion of C-1- vs C-2-labeled acetate: implications for tracer studies of substrate oxidation. *American Journal of Clinical Nutrition* **51**, 248–252.

Corresponding author

P. Schrauwen: Department of Human Biology, Maastricht University, PO Box 616, 6200 MD Maastricht, The Netherlands.

Email: P.Schrauwen@HB.Unimaas.NL