

Determinants of the acetate recovery factor: implications for estimation of ¹³C substrate oxidation

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Determinants of the acetate recovery factor: implications for estimation of [^{13}C]substrate oxidation

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A B S T R A C T

When using ^{13}C or ^{14}C tracers to study substrate metabolism, an acetate correction factor should be applied to correct for loss of label in the exchange pathways of the tricarboxylic acid cycle. We have shown recently that the [^{13}C]acetate recovery factor has a high inter-individual variability and should therefore be determined in every subject. In the present study we examined the factors that might explain some of the variability between subjects in acetate recovery factor. Data were pooled from four different studies with identical protocols, in which the acetate recovery factor was measured, prior to an intervention, to correct plasma fatty acid oxidation rates. Acetate recovery was measured after 2 h of [1,2- ^{13}C]acetate infusion at rest followed by 1 h of cycling exercise at 40–50% of maximal oxygen uptake. Inter-individual variance in acetate recovery was 12.0% at rest and 16.1% during exercise. Stepwise regression revealed that, at rest, 37.1% of the acetate recovery could be accounted for by basal metabolic rate adjusted for fat-free mass, percentage body fat and respiratory quotient (RQ). During exercise, 69.1% of the variance in acetate recovery could be accounted for by energy expenditure adjusted for fat-free mass, % body fat and RQ. In conclusion, we show that the acetate recovery factor has a high inter-individual variability, both at rest and during exercise, which can partly be accounted for by metabolic rate, RQ and % body fat. These data indicate that the acetate recovery factor needs to be determined in every subject, under similar conditions as used for the tracer-derived determination of substrate oxidation. Failure to do this might result in large under- or over-estimation of plasma substrate oxidation, and hence to artificial differences between groups.

INTRODUCTION

When using ^{13}C - or ^{14}C -labelled tracers in combination with indirect calorimetry, estimations can be made of plasma or exogenous substrate oxidation. When the ^{13}C or ^{14}C tracer is oxidized, the label should appear in the breath as $^{13}\text{CO}_2$ (or $^{14}\text{CO}_2$) and, from this, tracer oxidation can be calculated. However, the use of ^{13}C or

^{14}C tracers in studying plasma fatty acid oxidation has been questioned, since the appearance of $^{13}\text{CO}_2$ (or $^{14}\text{CO}_2$) is very low at rest [1]. This can be explained by the fact that part of the tracer will be lost in the bicarbonate pool [2,3] or in non-oxidative pathways, and will accumulate in products of the tricarboxylic acid cycle (TCA cycle). Therefore this tracer will not appear as $^{13}\text{CO}_2$ or $^{14}\text{CO}_2$ in the breath, and tracer oxidation

Key words: acetate recovery, metabolism, tracers.

Abbreviations: FFM, fat-free mass; RQ, respiratory quotient; TCA cycle, tricarboxylic acid cycle; $\dot{V}\text{CO}_2$, carbon dioxide production; $\dot{V}\text{O}_2$, oxygen consumption.

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will be underestimated. Sidossis et al. [4,5] proposed to determine this loss of label by measuring $^{13}\text{CO}_2$ production during the infusion of ^{13}C acetate. Acetate is assumed to be immediately converted into acetyl-CoA, which will then enter the TCA cycle. Therefore the recovery of ^{13}C acetate as $^{13}\text{CO}_2$ in breath can be used to calculate the loss of label in the TCA cycle, and tracer oxidation can be corrected accordingly. In their study, Sidossis et al. [4] infused ^{14}C acetate and ^{13}C palmitate simultaneously to correct plasma fatty acid oxidation for acetate recovery. However, the use of radioactive tracers is under severe limitation in many countries due to medical ethical considerations [6], and thus ^{13}C acetate recovery must be determined in a test separate from the measurement of ^{13}C substrate oxidation. Therefore we recently validated the $[1,2-^{13}\text{C}]$ acetate recovery factor in lean healthy males and found that, at rest, the recovery of acetate increases linearly with duration of isotope infusion. After 2 h at rest, the acetate recovery factor was $26.5 \pm 0.5\%$, implying an almost 4-fold underestimation of tracer oxidation when not corrected for loss of label. Furthermore, we showed that the acetate recovery factor is reproducible (intra-individual coefficient of variation 4.0%), but has a high inter-individual variability [7].

Since its introduction, we have determined the acetate recovery factor in all our ^{13}C palmitate tracers studies for each subject individually. The study protocols to determine the acetate recovery factor are always identical to the experimental protocol used to measure ^{13}C palmitate oxidation, i.e. a 2-h resting period followed by a 1-h exercise period. Here we report data from 89 subjects and examine the factors that might explain part of the differences in acetate recovery between subjects and populations.

METHODS

Subjects

The nature and risks of the experimental procedure were explained to the subjects, and all subjects gave their written informed consent. The studies were approved by the Medical Ethical Committee of Maastricht University. Subject characteristics are given in Table 1. Subjects were

classified as lean (body mass index $< 27 \text{ kg/m}^2$), obese (body mass index $> 27 \text{ kg/m}^2$) or suffering from type II diabetes, and were otherwise healthy.

Experimental design

Data from four different studies, in which acetate recovery was measured to correct plasma fatty acid oxidation, were included in the analysis. In study 1 the effect of diet composition on plasma fatty acid oxidation was studied in lean subjects ($n = 7$; male). In study 2 plasma fatty acid oxidation was compared in a group of lean subjects ($n = 7$; male) and a group of subjects with type II diabetes ($n = 7$; male). Study 3 was performed to investigate the effect of a 3-month training intervention in obese ($n = 51$; 31 male/20 female) and lean ($n = 7$; male) subjects. In study 4 the effect of weight reduction on plasma fatty acid oxidation in subjects with type II diabetes ($n = 10$; male) was studied. From all studies, only the pre-intervention data were used for the present analysis. Subjects were asked to refrain from physical exercise for 3 days prior to the testing day and not to consume any products with a high natural abundance of ^{13}C for 1 week before the testing day.

Isotope infusion test

On the morning of the study, after an overnight fast, subjects came to the laboratory for an acetate infusion test at 08.00 hours. A Teflon catheter was inserted in an antecubital vein for isotope infusion. After placement of the catheters, subjects rested on a bed; after 30 min, baseline oxygen consumption ($\dot{V}\text{O}_2$) and carbon dioxide production ($\dot{V}\text{CO}_2$) were measured, and breath samples were collected. Immediately thereafter, subjects were given an intravenous dose of $0.085 \text{ mg/kg NaH}^{13}\text{CO}_3$ to prime the bicarbonate pool. Then, at $t = 0$, a constant intravenous infusion of $[1,2-^{13}\text{C}]$ acetate was started (diabetic/obese subjects, $0.0496 \mu\text{mol}/\text{min} \cdot \text{kg}$; obese subjects, $0.0512 \mu\text{mol}/\text{min} \cdot \text{kg}$; lean subjects, $0.0645 \mu\text{mol}/\text{min} \cdot \text{kg}$) and continued for 120 min at rest. After the 120 min at rest, subjects cycled at 40% (study 2) or 50% of maximal $\dot{V}\text{O}_2$ for 60 min, during which the rate of infusion of acetate was doubled. The acetate concentration was measured in each infusate with an automated Cobas Farah centrifugal analyser using an enzymic

Table 1 Subject characteristics

	Men				Women
	Total ($n = 69$)	Lean ($n = 21$)	Obese ($n = 31$)	Type II diabetic ($n = 17$)	Obese ($n = 20$)
Age (years)	43.0 ± 1.3	35.3 ± 2.3	41.9 ± 1.1	54.7 ± 1.9	44.0 ± 0.8
Body fat (%)	27.5 ± 1.0	18.4 ± 1.6	30.8 ± 0.8	32.9 ± 1.3	32.7 ± 0.7
Body mass index (kg/m^2)	28.6 ± 0.6	22.1 ± 0.6	31.3 ± 0.4	31.7 ± 1.2	37.2 ± 1.8
Max. $\dot{V}\text{O}_2$ (litres/min)	2.96 ± 0.08	3.17 ± 0.16	3.22 ± 0.08	2.36 ± 0.10	2.11 ± 0.07

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% NaCl. The chemical and isotopic purity (99%) of acetate tracers were checked by ^1H and ^{13}C NMR and GC/MS. Breath samples were taken at $t = 0, 90, 100, 110$ and 120 min (at rest), and at $t = 150, 160, 170$ and 180 min (during exercise). At rest, $\dot{V}\text{O}_2$ and $\dot{V}\text{CO}_2$ were measured continuously during the first 90 min using open-circuit spirometry (Oxycon- β ; Mijnhardt). During exercise, $\dot{V}\text{O}_2$ and $\dot{V}\text{CO}_2$ were measured immediately before the measurement of breath $^{13}\text{CO}_2$ enrichment.

Procedures

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). Lung volume was measured simultaneously with the helium dilution technique using a spirometer (Volugraph 2000; Mijnhardt). In the subjects of study 2, body density was measured using skinfold callipers. Skinfold measurement locations were subscapular, supra-iliac, and over the biceps and triceps of the upper arm. The percentage of body fat was calculated using the equations of Siri [8]. Fat-free mass (FFM) in kg was calculated by subtracting fat mass from total body mass.

Sample analysis

Breath samples were analysed for the $^{13}\text{C}/^{12}\text{C}$ ratio using a gas chromatography-isotope ratio MS system (Finnigan MAT 252, Bremen, Germany).

Calculations

Basal metabolic rate and energy expenditure during exercise were calculated from $\dot{V}\text{O}_2$ and $\dot{V}\text{CO}_2$ using stoichiometric equations [9]. ^{13}C enrichment of breath CO_2 is given as the tracer/tracee ratio (TTR), defined as:

$$\text{TTR} = (^{13}\text{C}/^{12}\text{C})_{\text{sa}} - (^{13}\text{C}/^{12}\text{C})_{\text{bk}}$$

in which sa denotes sample and bk denotes background.

Fractional recovery of label in breath CO_2 , derived from the infusion of labelled acetate, was calculated as follows:

$$\text{Fractional recovery of label (\%)} = \frac{\text{TTRCO}_2 \times \dot{V}\text{CO}_2}{F} \times 100$$

where TTRCO_2 is tracer/tracee ratio in breath CO_2 , $\dot{V}\text{CO}_2$ is carbon dioxide production (mmol/min) and F is the rate of infusion of ^{13}C (mmol/min).

Statistical analysis

All data are presented as means \pm S. E. M., and the level of significance was set at $P < 0.05$. The acetate recovery

factor at rest and during exercise was calculated as the area under the curve of the relationship between time and acetate recovery for $t = 90$ – 120 min (rest) and $t = 150$ – 180 min (exercise), and expressed as a percentage. Pearson correlation coefficients were calculated to determine the relationship between selected variables. Basal metabolic rate and energy expenditure during exercise were adjusted for their major determinant, FFM, as described previously [10]. Using stepwise regression, the contributions of selected variables to the variance in acetate recovery factor were determined. Covariance analysis was used to test whether acetate recovery was different between subject groups. Equality of slopes was determined using an F -test. Coefficient of variance was calculated as (S.D./mean) \times 100%.

RESULTS

Overall, the average acetate recovery factor at rest was $23.3 \pm 0.3\%$ (mean \pm S.E.M.), with an inter-individual coefficient of variance of 12.0%. During exercise the average acetate recovery factor was $69.0 \pm 1.2\%$, with an inter-individual coefficient of variance of 16.1%.

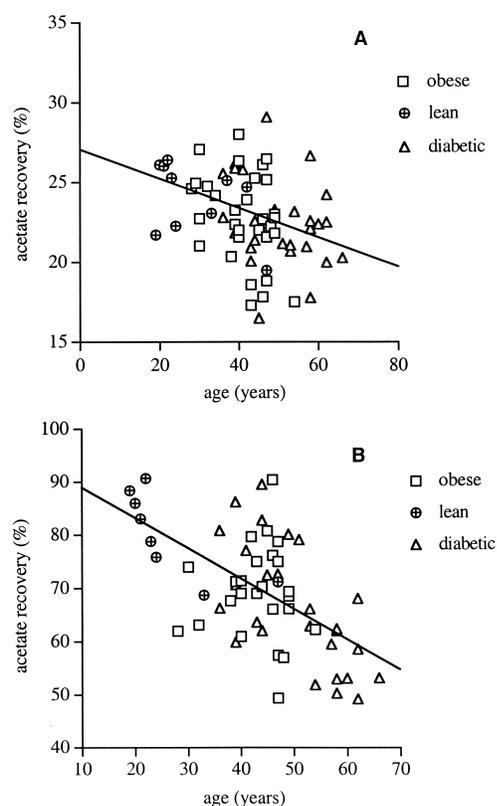


Figure 1 Relationship between age and acetate recovery at rest (A) and during exercise (B) in male subjects (A) $r = -0.347$, $P = 0.004$; (B) $r = -0.593$, $P = 0.0001$.

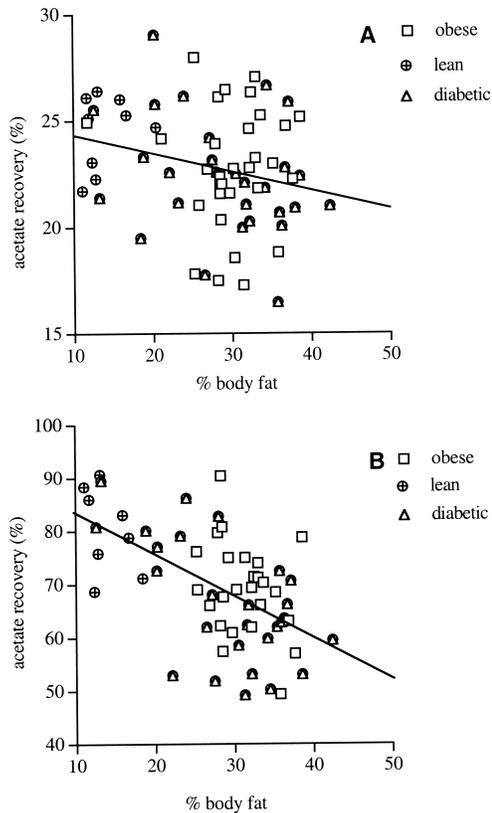


Figure 2 Relationship between % body fat and acetate recovery at rest (A) and during exercise (B) in male subjects (A) $r = -0.261$, $P = 0.032$; (B) $r = -0.585$, $P = 0.0001$.

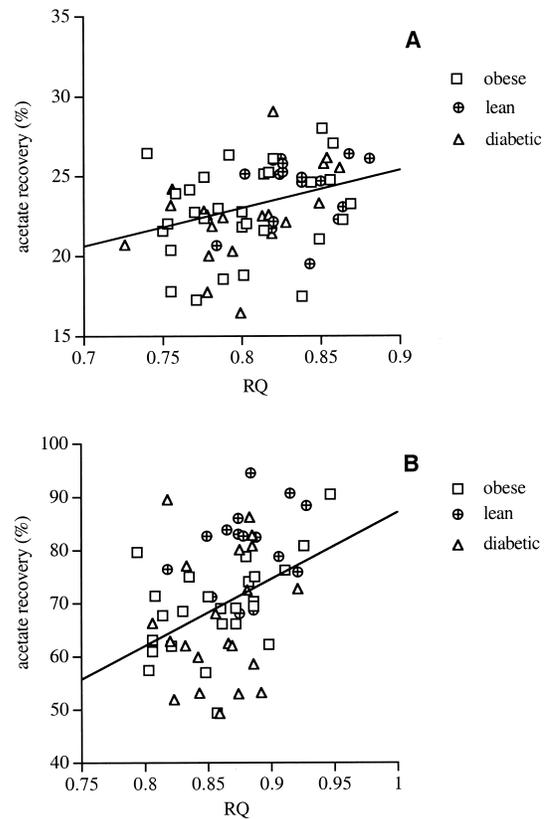


Figure 3 Relationship between RQ and acetate recovery at rest (A) and during exercise (B) in male subjects (A) $r = 0.346$, $P = 0.003$; (B) $r = 0.361$, $P = 0.003$.

The acetate recovery factor at rest was significantly lower in men than in women ($22.9 \pm 0.3\%$ and $24.6 \pm 0.6\%$ respectively; $P = 0.0186$). During exercise the opposite was the case, with higher acetate recovery in men ($70.7 \pm 1.4\%$) than in women ($63.2 \pm 1.9\%$; $P = 0.0061$). Using covariance analysis, we found that, after correction for energy expenditure adjusted for FFM and respiratory quotient (RQ), acetate recovery during exercise was no longer significantly different between men and women. However, at rest, the difference in acetate recovery between men and women persisted after correction for basal metabolic rate adjusted for FFM and RQ. Furthermore, the slope of the relationship between % body fat and acetate recovery was significantly different between men and women [F -test (1,48, 0.05): 4.51]. Therefore, in the remainder of the analysis data from men and women were analysed separately.

In men, the acetate recovery factor at rest was significantly correlated with age ($r = -0.347$, $P = 0.0038$) (Figure 1), % body fat ($r = -0.261$, $P = 0.0315$) (Figure 2), RQ ($r = 0.346$, $P = 0.0025$) (Figure 3) and basal metabolic rate corrected for FFM ($r = 0.359$, $P = 0.0027$) (Figure 4). Similarly, during exercise the acetate recovery factor was significantly correlated with age ($r = -0.593$, $P = 0.0001$), % body fat ($r = -0.585$, $P =$

0.0001), RQ ($r = 0.361$, $P = 0.0029$) and energy expenditure corrected for FFM ($r = 0.799$, $P = 0.0001$) (Figures 1–4).

Stepwise regression analysis revealed that, at rest, 12.9% of the variance in acetate recovery could be accounted for by basal metabolic rate corrected for FFM. Adding % body fat accounted for an additional 15.4%, and RQ could account for 8.8% of the variance. Thus these three parameters accounted for 37.1% of the variance in acetate recovery at rest, whereas age did not contribute significantly. During exercise, energy expenditure corrected for FFM (63.2%) and RQ (5.9%) contributed significantly to the variance in acetate recovery factor, and accounted for 69.1% of the variance.

We then assessed whether the acetate recovery factor differed significantly in obese, lean and diabetic subjects. At rest, acetate recovery tended to differ between diabetic ($22.0 \pm 0.6\%$), obese ($22.9 \pm 0.5\%$) and lean ($24.0 \pm 0.5\%$) subjects ($P = 0.053$). During exercise, acetate recovery was significantly different between all groups ($P = 0.0001$). Acetate recovery was highest in lean subjects ($78.5 \pm 2.1\%$), followed by obese ($69.9 \pm 1.7\%$) and diabetic ($59.2 \pm 2.1\%$) subjects. Using covariance analysis, we found that, after correction for % body fat, the difference in acetate recovery at rest between lean,

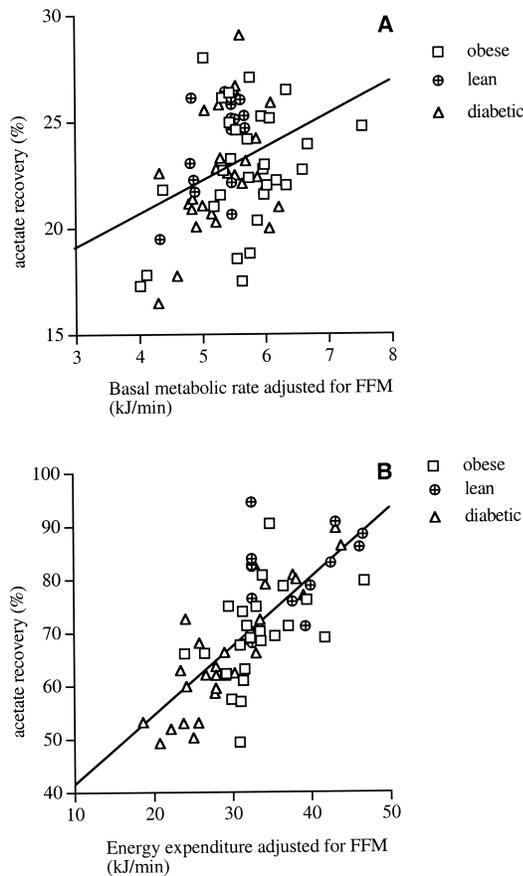


Figure 4 Relationships between acetate recovery and (A) basal metabolic rate adjusted for FFM at rest and (B) energy expenditure adjusted for FFM during exercise in male subjects (A) $r = 0.359$, $P = 0.003$; (B) $r = 0.799$, $P = 0.0001$.

obese and diabetic subjects disappeared. However, during exercise, acetate recovery tended to be lower in diabetic subjects ($P = 0.08$) even after correction for energy expenditure, RQ and % body fat.

In women, acetate recovery at rest was significantly correlated with % body fat ($r = -0.557$, $P = 0.008$). During exercise, acetate recovery was correlated with energy expenditure adjusted for FFM ($r = 0.85$, $P = 0.0001$) and age ($r = -0.542$, $P = 0.011$), whereas RQ showed a (non-significant) tendency towards correlation ($r = 0.381$, $P = 0.09$). Stepwise regression revealed that 73% of the variance in acetate recovery during exercise could be explained by energy expenditure adjusted for FFM, whereas RQ explained an additional 8.4%.

DISCUSSION

Since the introduction of the acetate correction factor by Sidossis et al. [4], estimations of plasma substrate oxidation using tracers can be corrected for the loss of tracer in the TCA cycle and bicarbonate pools by applying this correction factor. The recovery of acetate at rest is low

and increases with infusion time, whereas acetate recovery during exercise is much higher. In the present study we found an average acetate recovery between 90 and 120 min after the start of the acetate infusion of approx. 23%. This value is in accordance with our previous results [7], and illustrates that omission of this correction factor when studying substrate metabolism would result in an approx. 4-fold underestimation of substrate oxidation. Furthermore, we confirm here that the recovery of acetate has a high inter-individual coefficient of variance, both at rest (12%) and during exercise (16%). Together with the range in values obtained for acetate recovery (16.5–33.0 at rest; 49.3–94.5 during exercise), this strongly suggests that, when measuring tracer-derived plasma substrate oxidation, an acetate recovery factor should be determined for every individual, under similar conditions as employed for the tracer-derived determination of plasma substrate oxidation. Our previous finding that the acetate recovery factor has a low intra-individual coefficient of variation (4%) makes it possible to determine the [^{13}C]acetate recovery in a separate test from the determination of [^{13}C]substrate oxidation [7].

It has been shown previously that acetate recovery is much higher during exercise than in the resting state, and that the recovery of acetate increases with exercise intensity [4]. Our finding that basal metabolic rate and energy expenditure adjusted for FFM were the major determinants of acetate recovery at rest and during exercise respectively confirm these findings. Both with and without (results not shown) adjustment for FFM, metabolic rate was strongly correlated with acetate recovery. When the metabolic rate is high, the probability that label is lost in the exchange reactions of the TCA cycle is decreased, which will result in increased acetate recovery. The fact that this relationship also holds true at rest, when the variation in metabolic rate between subjects is much lower than during exercise, demonstrates that the acetate recovery factor is increased under circumstances of increased metabolic rate. This implies that, under experimental conditions where the metabolic rate is increased (such as stimulation of the sympathetic nervous system), the acetate correction factor should be determined under the same conditions, to avoid serious error of estimation.

During exercise, we found a linear relationship between acetate recovery and energy expenditure. Although in our study all subjects exercised at $\sim 50\%$ of maximal $\dot{V}\text{O}_2$, there was a range in absolute workload from 35 W to 169 W, and therefore also in energy expended during exercise. Thus absolute workload, rather than intensity, seems to determine acetate recovery during exercise. Furthermore, in contrast with the suggestion of Sidossis et al. [4], acetate recovery did not plateau when energy expenditure was increased to (sub)maximal levels during exercise. Rather, we hypothesize that it may increase

above 100% when there is a significant contribution of anaerobic glycolysis to energy delivery, due to emptying of the pre-labelled bicarbonate pools. Therefore, as with all techniques involving indirect calorimetry, the acetate correction factor should be interpreted with caution when the respiratory exchange ratio is near 1.0.

In the present study we found that the acetate recovery factor was highest in lean subjects and lowest in subjects with type II diabetes. When all male subjects were considered, we found a negative correlation between % body fat and acetate recovery, and this correlation was independent of basal metabolic rate. Thus the difference in % body fat between lean, obese and type II diabetic subjects explained most of the difference in acetate recovery between these groups. However, during exercise there was still a tendency for a lower acetate recovery factor in subjects with type II diabetes, even after correction for % body fat, RQ and metabolic rate. It could be suggested that an increased rate of gluconeogenesis in the diabetic state would lead to increased loss of label and thus lower acetate recovery. However, future studies are necessary to examine whether acetate metabolism (and recovery) is different in subjects with type II diabetes compared with control subjects matched for body composition and age.

In addition to metabolic rate and % body fat, RQ also had an effect on acetate recovery. With increasing RQ (increased glucose oxidation), acetate recovery increased. This might be explained by differences in TCA cycle activity, since glucose oxidation produces 21.8 kJ (5.2 kcal)/litre of CO₂, whereas for fat oxidation this value is 28.9 kJ (6.9 kcal)/litre of CO₂. Thus, for a certain amount of energy, less CO₂ is produced when the energy is derived from fat oxidation compared with glucose oxidation. With high rates of glucose oxidation (high RQ), there would, in theory, be less chance of label being lost in TCA cycle exchange pathways, resulting in higher acetate recovery. The finding that RQ and acetate recovery are positively correlated is in accordance with the finding that bicarbonate recovery is higher in the fed (high RQ) compared with the fasting (low RQ) state [3,11], and illustrates that differences in feeding pattern and thus substrate oxidation between subjects and/or populations can influence the recovery of acetate, and should thus be taken into consideration when using ¹³C tracer methodology.

The acetate recovery factor should not be limited to fat metabolism, but should always be applied when studying substrate oxidation, through the TCA cycle, using ¹³C tracers. Tounian et al. [12] showed that using a bicarbonate correction factor of 0.8 [13], instead of applying the acetate recovery factor, resulted in underestimation of total glucose oxidation and overestimation

of endogenous glucose oxidation. Thus failure to apply the acetate recovery factor might result in erroneous conclusions regarding substrate metabolism.

In conclusion, we show that the acetate recovery has a high inter-individual variability, both at rest and during exercise. Part of this variance can be explained by differences in metabolic rate, RQ and % body fat. These data indicate that the acetate recovery factor needs to be determined in every subject under similar conditions as are used for the tracer-derived determination of substrate oxidation. Failure to do this might result in large under- or over-estimations of plasma substrate oxidation.

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