Correction factors for ^{13}C-labelled substrate oxidation at whole-body and muscle level

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The oxidation of fatty acids, carbohydrates and amino acids can be measured by quantifying the rate of excretion of labelled CO_2 following administration of ^{14}C- or ^{13}C-labelled substrates at whole-body and tissue level. However, there is a theoretical need to correct the oxidation rates for the proportion of labelled CO_2 that is produced via oxidation but not excreted. Furthermore, depending on the substrate and position of the C label(s), there may also be a need to correct for labelled C from the metabolized substrate that does not appear as CO_2, but rather becomes temporarily fixed in other metabolites. The bicarbonate correction factor is used to correct for the labelled CO_2 not excreted. Recently, an acetate correction factor has been proposed for the simultaneous correction of CO_2 not excreted and label fixed in other metabolites via isotopic exchange reactions, mainly in the tricarboxylic acid cycle. Changes in metabolic rate induced, for example, by feeding, hormonal changes and physical activity, as well as infusion time, have been shown to affect both correction factors. The present paper explains the theoretical and physiological basis of these correction factors and makes recommendations as to how these correction factors should be used in various physiological conditions.

Bicarbonate: Acetate: Fatty acids: Carbohydrate: Exercise

Over the last few decades the use of radioactive and stable isotopes in metabolic studies has become increasingly popular. Many studies have measured the oxidation of substrates such as fat, carbohydrates and amino acids from the amount of labelled CO_2 produced after administration of ^{14}C- or ^{13}C-labelled substrates. Substrate oxidation at the whole-body level is estimated by quantifying the rate of excretion of labelled C in expired CO_2. At the tissue level, substrate oxidation has also been estimated by means of quantifying the labelled CO_2 produced from the tissue as a function of the labelled substrate taken up. In these experiments, whether at the whole-body or tissue level, there is a theoretical need to correct the data for the proportion of labelled CO_2 that is produced via oxidation but not excreted. Furthermore, depending on the substrate and position of the C label(s), there may also be a need to correct for labelled C from the metabolized substrate that does not appear as CO_2 but rather becomes temporarily fixed in other metabolites. The bicarbonate correction factor is used to correct for labelled CO_2 that is not excreted. Recently, an acetate correction factor has been proposed for the simultaneous correction of CO_2 not excreted and label fixed in other metabolites via isotopic exchange reactions, mainly in the tricarboxylic acid (TCA) cycle (Fig. 1). Changes in metabolic rate induced, for example, by feeding, hormonal changes and physical activity, as well as infusion time, have been shown to affect both correction factors.

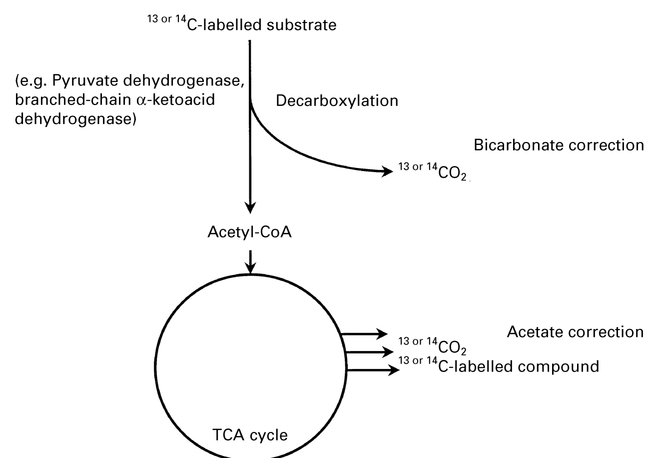

Fig. 1. Schematic representation of the fate of carbon-labelled substrates and the potential need for label retention correction. Branched-chain α-ketoacid dehydrogenase, *EC* 1.2.4.4.

Abbreviations: TCA, tricarboxylic acid; TG, triacylglycerol.
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The purpose of the present paper is to explain the theoretical and physiological basis of the bicarbonate and acetate correction factors. A discussion will be presented about how changes in metabolic rate, e.g. rest *v*. exercise, can affect the correction factors at whole-body level and across the leg. Finally, recommendations will be made as to how these correction factors can best be used in various physiological situations.

The bicarbonate correction factor

Whole-body oxidation rates of fat, carbohydrates and amino acids based on recovery of labelled CO_2 in breath of the ^{13}C- or ^{14}C-labelled substrates need to be corrected for CO_2 retention. CO_2 retention has been suggested to occur mainly within the body store(s) of bicarbonate, although some labelled CO_2 will be fixed in other metabolites via carboxylation reactions. The main bicarbonate body stores are thought to be within bone, intracellular reserves and metabolic intermediates. The extent of CO_2 retention is usually assessed from the proportion of intravenously-administered labelled bicarbonate (as a bolus or during constant infusion) that is recovered in breath. In human subjects the recovery of labelled CO_2 from administered bicarbonate varies from approximately 50 % to 100 %. This large variation has been attributed to both methodological and biological variability (for extensive review, see Leijssen & Elia, 1996).

The body stores of CO_2 are large and their effects on CO_2 transport are not well understood. The washout of bicarbonate as labelled CO_2 in the breath under resting conditions has been described by the sum of three exponential terms, implying the presence of at least three major bicarbonate pools with distinct kinetic differences. Based on the washout kinetics, a three-compartment model has been suggested with a central pool and two peripheral pools connected to the central pool but not directly to each other (Fig. 2; Irving *et al.* 1983, 1984; Barstow *et al.* 1990). There is, however, uncertainty regarding the physiological identity of these three pools in mammals. Early investigators speculated that the central pool represented vascular and extracellular bicarbonate (blood), that one peripheral pool with a fast turnover represented intracellular bicarbonate of soft tissues (among others skeletal muscle) and that the second peripheral pool with a slow turnover represented bone bicarbonate (Kornberg *et al.* 1951; Steel, 1955; Shipley *et al.* 1959). Other investigators have suggested that the central pool represented vascular and potentially interstitial bicarbonate, the fast peripheral pool represented metabolically-active tissue (heart, brain, kidney, etc.), and the slow peripheral pool primarily represented resting skeletal muscle (Slanger *et al.* 1970; Irving *et al.* 1983). At rest, tracer entry and CO_2 loss most probably occur only via the central pool. It has been shown that the compartmental dynamics of CO_2 transport and storage are very sensitive to changes in acid–base status (Leese *et al.* 1994) and changes in metabolic rate induced by exercise (Wolfe *et al.* 1984; Barstow *et al.* 1990; Tarnopolsky *et al.* 1991; Leese *et al.* 1994). Furthermore, exercise has also been shown to substantially influence CO_2 transport and storage in the post-exercise period (Wolfe *et al.* 1984; Tarnopolsky *et al.* 1991; Leese *et al.* 1994). With exercise the bicarbonate pool

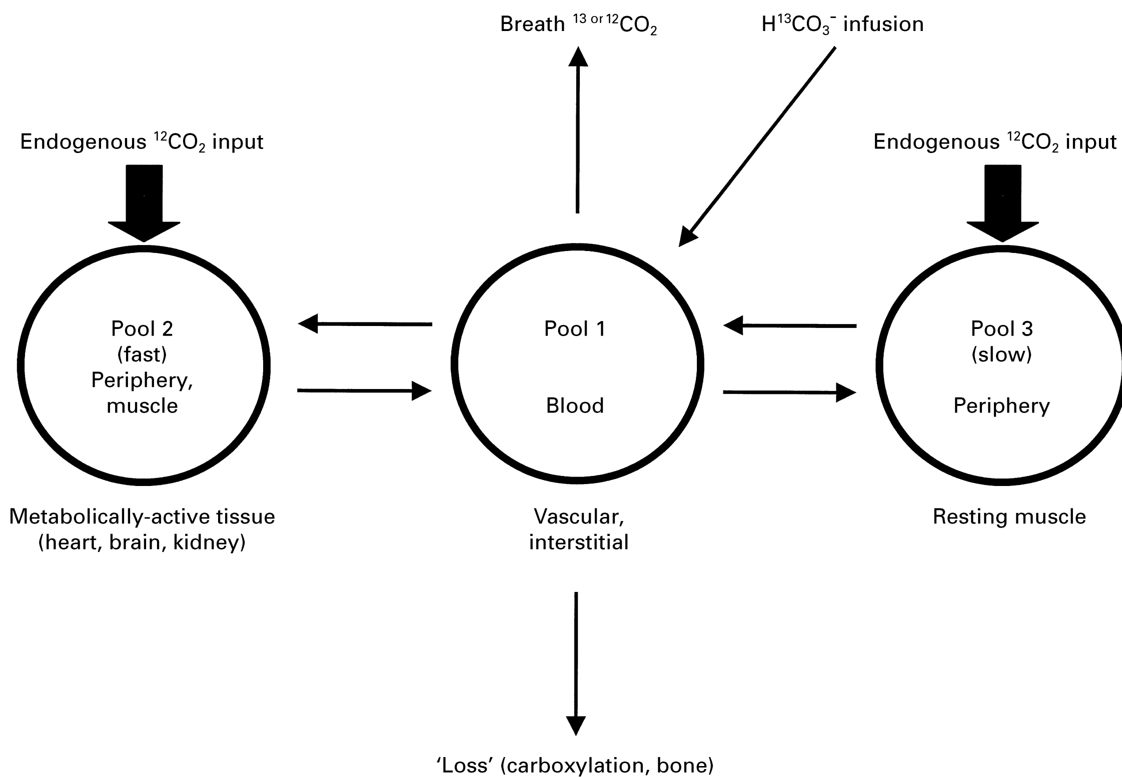

Fig. 2. Three-compartment model for the washout of $H^{13}CO_3^-$ in mammals.

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size has been shown to increase several-fold (Barstow *et al.* 1990; Leese *et al.* 1994) and mean residence time of $^{13}CO_2$ in the body is decreased (Leese *et al.* 1994). In contrast, during recovery from exercise the bicarbonate pool is smaller compared with that at rest; however, mean residence time is still reduced (Leese *et al.* 1994). The net result of these kinetic changes for bicarbonate recovery at rest, during one-leg knee-extensor exercise and during recovery is shown in Fig. 3. At rest bicarbonate recovery was at steady-state in fasted subjects with a recovery of 82 %. However, at the onset of exercise bicarbonate recovery far exceeded 100 % but returned to approximately 100 % after 1 h of exercise. In contrast, the bicarbonate recovery early post-exercise was lower (52 %) than at rest before exercise. With increasing time of recovery from exercise, the bicarbonate recovery increased to an apparent steady-state of approximately 96 %. Similar results have been shown previously by other investigators during two-legged cycle exercise (Wolfe *et al.* 1984; Tarnopolsky *et al.* 1991) and recovery (Tarnopolsky *et al.* 1991). The marked changes in bicarbonate recovery during the initial period of exercise and recovery may be explained in terms of the three-pool model and the known changes in the bicarbonate pool size. The bicarbonate pool is markedly increased during exercise due to increased substrate oxidation and mobilization of CO_2 from body stores (Barstow *et al.* 1990; Leese *et al.* 1994). This increase in the total CO_2 pool is thought to occur mainly in the central bicarbonate pool, which at rest is the pool of entry and removal of label, with mobilization of CO_2 from the two peripheral pools which at rest were not available for exchange, or have a much slower turnover rate than the central pool. Furthermore, lactic acid is produced mainly during the initial period of prolonged exercise. This process causes mild acidosis that in part is buffered by bicarbonate from the body pools. Any consumption of stored bicarbonate during exercise must be replenished during recovery. If this occurs in the bicarbonate pools with a low turnover rate, label will be 'lost', resulting in lower appearance of labelled CO_2 in the breath and thus a lower bicarbonate recovery. With increased duration of recovery, the bicarbonate recovery increased to an apparent steady state of approximately 96 % (Fig. 3). This higher bicarbonate recovery compared with the pre-exercise value may originate from either the effect of exercise on bicarbonate recovery *per se* or from the infusion time. It has been shown that during prolonged bicarbonate infusion recovery gradually increased to 96 %. In that case, the slow turnover pools have most probably also reached equilibrium and a whole-body bicarbonate steady-state is achieved (Elia *et al.* 1993, 1995). Labelled CO_2 can also be retained via carboxylation reaction(s) and this process is independent of retention in the large bicarbonate pool. It is not known whether the retention of 4 % originates from labelled CO_2 fixation in carboxylation or is the consequence of a very slow bicarbonate turnover pool that has not reached equilibrium.

In order to minimize inaccuracy of substrate oxidation and to avoid changes in size and turnover rate of the bicarbonate pools, the measurements should be made in a tracer steady-state. It is clear from Fig. 3 that the bicarbonate recovery factor is changed rapidly during the first hour of exercise and recovery. After 1 h of exercise bicarbonate recovery seems to plateau at approximately 100 %, as has been observed before with the bolus and constant bicarbonate infusion methods (Bowtell *et al.* 1994; Leese *et al.* 1994).

Another consideration is whether the labelled-CO_2 washout at the start of exercise makes a substantial

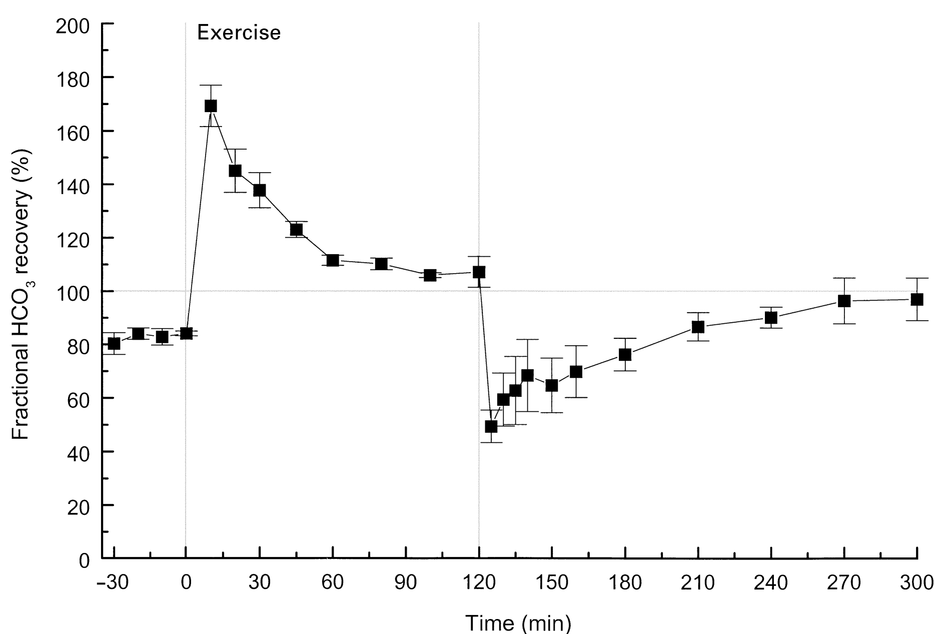

Fig. 3. Whole-body bicarbonate recovery during rest, exercise and recovery. Exercise consisted of 2 h of one-leg knee extension at 60 % of their maximal leg workload. Bicarbonate was infused via a forearm vein at a known constant rate. Recovery (%) was calculated as: enrichment $^{12 \text{ or } 13}CO_{2breath} \times CO_{2breath}$ / infusion rate × 100. Values are means and standard deviations represented by vertical bars for four subjects.

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contribution to the amount of labelled CO_2 originating from substrate oxidation. Substrate oxidation may increase substantially with exercise, e.g. in the case of [^{13}C]glucose, and in that case the quantitative contribution of labelled CO_2 from washout may be minimal. In contrast, if the oxidation of a substrate is only marginally increased with exercise, e.g. in case of the amino acid leucine, then the washout may contribute substantially and omission of the bicarbonate recovery factor would lead to an overestimation of the rate of oxidation during exercise. Thus, it seems appropriate only to use the bicarbonate recovery factor when an acceptable steady-state has been reached, i.e. in the second hour of exercise and after 2 h recovery. In most cases this would mean that during exercise bicarbonate recovery is 100 %, implying that no correction has to be made for substrate oxidation (Bowtell *et al*. 1994, 1998).

The retention of bicarbonate in the leg at rest and during exercise and recovery seems to be low. With constant bicarbonate infusion little difference was observed in the amount of $^{13}CO_2$ present in femoral arterial *v*. venous blood at rest, during exercise and in the second and third hour of recovery (Fig. 4). The large changes seen in bicarbonate recovery at the whole-body level during the initial phase of exercise and recovery were much smaller across the exercising leg. However, other tissues may contribute much more to the whole-body changes seen during exercise. During one-leg knee-extensor exercise the bicarbonate recovery across the resting leg resembled the whole-body bicarbonate recovery as shown in Fig. 3 (G van Hall, unpublished results). This finding implies that the recommendations regarding the best period over which to make reliable estimates of substrate oxidation are the same as those for studies at whole-body level.

The acetate correction factor

^{14}C- and ^{13}C-labelled free fatty acids have been used to estimate plasma fatty acid oxidation in an attempt to differentiate between the oxidation of fatty acids derived from blood and the oxidation of fatty acids originating from the breakdown of triacylglycerol (TG) in blood (chylomicron TG and VLDL-TG) and of intracellular TG. These studies suggested a large contribution (up to 70 %) of circulating and intracellular stored TG (Havel *et al*. 1967; Hagenfeldt & Wahren, 1968; Dagenais *et al*. 1976; Coyle *et al*. 1997). However, the validity of the tracer estimates of plasma fatty acid oxidation has been questioned, based on the observation that very little label was converted to $^{13 \text{ or } 14}CO_2$ in the first hours after the start of the tracer infusion. Originally it was suggested that the major reason for this low $^{13 \text{ or } 14}CO_2$ production was the consequence of rapid esterification of the plasma fatty acids and disappearance into the TG pool rather than being oxidized (Dagenais *et al*. 1976; Heiling *et al*. 1991). The fatty acids that were oxidized would originate from the intracellular TG pool, and as this pool is large it would take many hours before that pool reached equilibrium. As a result, the enrichment of plasma free fatty acids would be much higher than that of the intracellular free fatty acids, and plasma free fatty acid oxidation would be underestimated. Recently, however, an alternative explanation has been put forward to explain a major part of the low $^{13 \text{ or } 14}CO_2$ production observed when labelled fatty acids are infused. Sidossis *et al*. (1995*a*) suggested that the C label(s) of fatty acids are fixed via isotopic exchange reactions in the TCA cycle (Fig. 1). A labelled acetate infusion was used to correct for label fixation in the TCA cycle. Acetate is converted to acetyl-CoA, and thereafter it behaves like acetyl-CoA originating

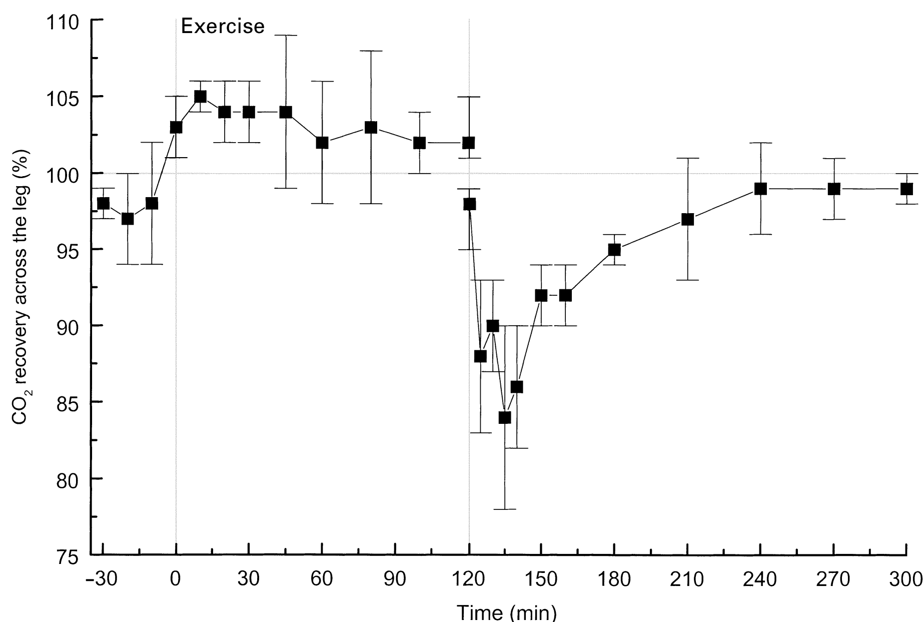

Fig. 4. Leg bicarbonate recovery during rest, exercise and recovery. Exercise consisted of 2 h of one-leg knee extension at 60 % of their maximal leg workload. Bicarbonate was infused via a forearm vein at a known constant rate. Acetate recovery (%) was calculated as: (enrichment $^{12 \text{ or } 13}CO_{2venous} \times CO_{2venous}$)/(enrichment $^{12 \text{ or } 13}CO_{2arterial}) \times CO_{2arterial}$) × 100. A recovery lower than 100 % represents net $^{13}CO_2$ retention. Values are means and standard deviations represented by vertical bars for four subjects.

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from fatty acids. Thus, where the bicarbonate recovery factor is used to correct only for labelled-CO_2 retention, the acetate recovery factor corrects both for labelled-CO_2 retention and for label 'lost' via fixation in isotopic exchange reactions in the TCA cycle (Figs. 1 and 5). Although the acetate correction factor was originally introduced to correct tracer estimations of plasma fatty acid oxidation (Sidossis *et al.* 1995*a*), theoretically an acetate correction factor also has to be applied for estimations of glucose and amino acid tracer oxidation as long as some of the labelled C enters the TCA cycle. Indeed, it has been shown that the estimation of glucose oxidation from indirect calorimetry and [U-^{13}C]glucose gave identical results when the appropriate corrections for $^{13}CO_2$ recovery in breath were applied, which included the acetate recovery factor (Tounian *et al.* 1996).

Label fixation via TCA cycle exchange reactions can occur in many tissues and organs. Many tissues convert α-ketoglutarate to glutamate and glutamine (Fig. 5). Oxaloacetate, via gluconeogenesis in liver and kidney, is also converted to glucose. However, label fixation in glutamate and glutamine is quantitatively much larger than that in glucose (Schrauwen *et al.* 1998). Thus, although the substrate is in principle completely oxidized, its label does not appear as CO_2 in breath since the label accumulates in the glutamate and glutamine pools. This factor then leads to an underestimation of the true substrate oxidation rate. The label is only temporarily fixed, since glutamate and glutamine will re-enter the oxidative pathways at a later point in time. The pools of these compounds are unlabelled at the start of the labelled substrate infusion, but more and more label from the substrate will accumulate with increasing infusion time. However, a steady-state is unlikely to be reached within hours in these pools due to the fact that these pools are so large. Breath CO_2 enrichment increases linearly with time for several hours (Fig. 6; Sidossis *et al.* 1995*b*; Schrauwen *et al.* 1998). A recent study showed that approximately 12 h of acetate infusion led to a plateau in breath CO_2 enrichment (Mittendorfer *et al.* 1998).

The quantitative contribution of label fixation depends on whether the label from the substrate enters the TCA cycle as the C-1 or C-2 of acetyl-CoA (Fig. 5). If the label enters at the C-2 position then the likelihood of fixation is higher. The theoretical reason for this difference is shown in Fig. 5. The first turn of the TCA cycle does not lead to the release of labelled CO_2 derived from the label in either label position C-1 or C-2, and thus the probability of fixation of the label in both positions via glutamate or glutamine is equal. Furthermore, the label in both positions ends up in oxaloacetate, a symmetrical molecule where the C-1 and C-4 positions and the C-2 and C-3 positions are identical, resulting in equilibration of label. Theoretically, half the oxaloactetate molecules are labelled in C-1 and C-2 positions and the other half in the C-3 and C-4 positions. In the second turn of the TCA cycle, assuming that a non-labelled acetyl-CoA is entering the cycle, half the remaining C-1 position label is lost before and the other half after the possibility for fixation in glutamate or glutamine. All the original C-2 position label is still within the TCA cycle, and thus can undergo fixation. In the third turn of the TCA cycle, for the first time label from the C-2 position is liberated as CO_2, but label still remains in the cycle.

In agreement with the theoretical prediction, Wolfe & Jahoor (1990) clearly demonstrated that after 4 h of infusion of [1-^{13}C]acetate, 81 % of the label was recovered compared with only 53 % following infusion of [2-^{13}C]acetate.

During exercise, label recovery from acetate is much higher than at rest (Fig. 6), as shown earlier (Sidossis *et al.* 1995*a*). With exercise the TCA cycle activity has to increase several-fold. Assuming that during exercise the rate of exchange reactions (glutamate or glutamine) is the same or perhaps lower (Sidossis *et al.* 1995*b*), then the rate of the TCA cycle accelerates in comparison with the exchange reactions, and thus less label would be fixed via the exchange reactions, leading to higher $^{13 \text{ or } 14}CO_2$ recovery. Sidossis *et al.* (1995*a*) observed that the $^{14}CO_2$ recovery from [1-^{14}C]acetate increased from 66 % at an O_2 consumption of 10 ml/kg per min to 94 % at an O_2 consumption of 40 ml/kg per min. Recently, it has been observed that acetate recovery is 100 % at a cycle intensity of ≥ 75 % maximum workload in trained subjects (Van Loon *et al.* 1999). Fig. 6 shows that even intense one-leg knee-extensor exercise, where only the *m. quadriceps* is engaged in exercise (approximately 2·5 kg muscle), may lead to an approximately 85 % recovery of [1,2-^{13}C]acetate. Another important observation was that during exercise, a plateau in breath CO_2 enrichment is reached after 30 min, whereas it takes more than 12 h before a plateau is reached at rest (Fig. 6; Van Loon *et al.* 1999). Thus, whereas the acetate correction factor in resting conditions is extremely important and is dependent on infusion time, during exercise acetate correction is of minor importance and far less dependent on infusion time.

The accuracy of the acetate correction factor depends on two major assumptions. The first assumption is that the acetate infused is fully oxidized via the TCA cycle and is not used for lipogenesis and ketogenesis. In the post-absorptive state (overnight fasted) lipogenesis is low. However, during lipogenic situations, as with glucose infusion or following ingestion of a carbohydrate meal, some labelled acetate may be incorporated into fatty acids (Hellerstein *et al.* 1991*a*,*b*) rather than oxidized. Ketogenesis, on the other hand, is an active pathway of acetate metabolism and increases substantially with prolonged fasting (Balasse, 1970). However, it has been proposed that this increase does not affect the acetate recovery factor since the major fate of ketone bodies is oxidation (Sidossis *et al.* 1995*b*). The second major assumption is that acetate is metabolized in the same tissue and proportionally to the same extent as the substrate. Under resting conditions, acetate has been suggested to be mainly oxidized in liver, but also to some extent in muscle (Bleiberg *et al.* 1992; Pouteau *et al.* 1996), which is in contrast to most substrates which are mainly oxidized in the periphery. So far only one study has quantified labelled-C recovery from acetate across tissues (Mittendorfer *et al.* 1998). The leg acetate recovery was found to be similar to the whole-body label recovery in the breath, suggesting that whole-body acetate recoveries can be used to correct for muscle substrate oxidation. Additional research is needed to verify whether whole-body acetate recoveries can be used to correct for substrate oxidation by

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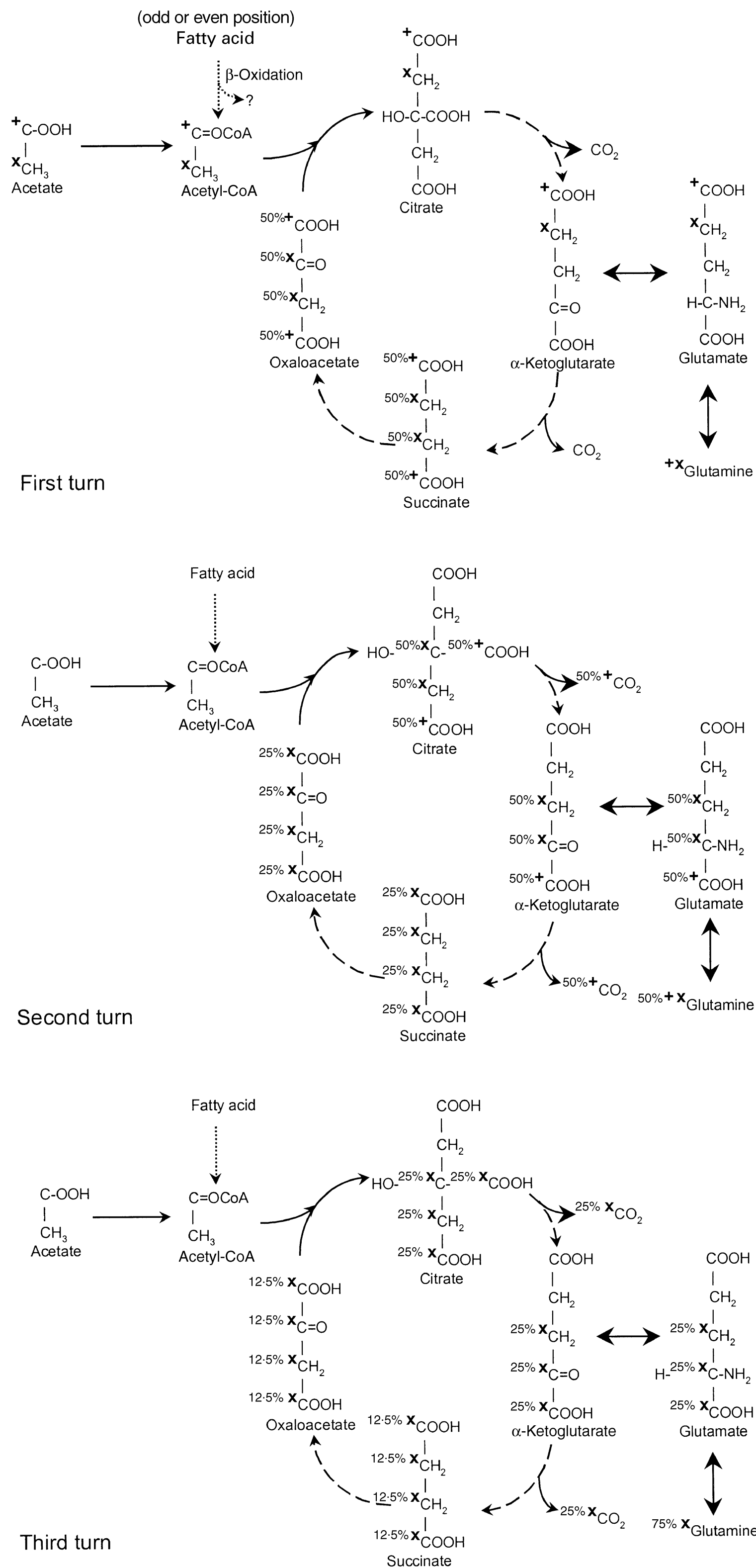

Fig. 5. Schematic diagram giving the fate of labelled carbon in the C-1 ([1-^{13}C]acetate; +) or C-2 ([2-^{13}C]acetate; x) positions of acetyl CoA after entering the tricarboxylic acid (TCA) cycle. The label originating from acetate or fatty acids entering into the TCA cycle in the first turn of the TCA cycle is labelled in the C-1 and C-2 positions. In the second and third turn no labelled acetyl-CoA enters the cycle. Furthermore, since oxaloacetate is a symmetrical molecule, the label in C-1 and C-4 positions and the C-2 and C-3 positions are identical. Theoretically, half the oxaloacetate molecules are labelled in C-1 and C-2 positions, and the other half in the C-3 and C-4 positions.

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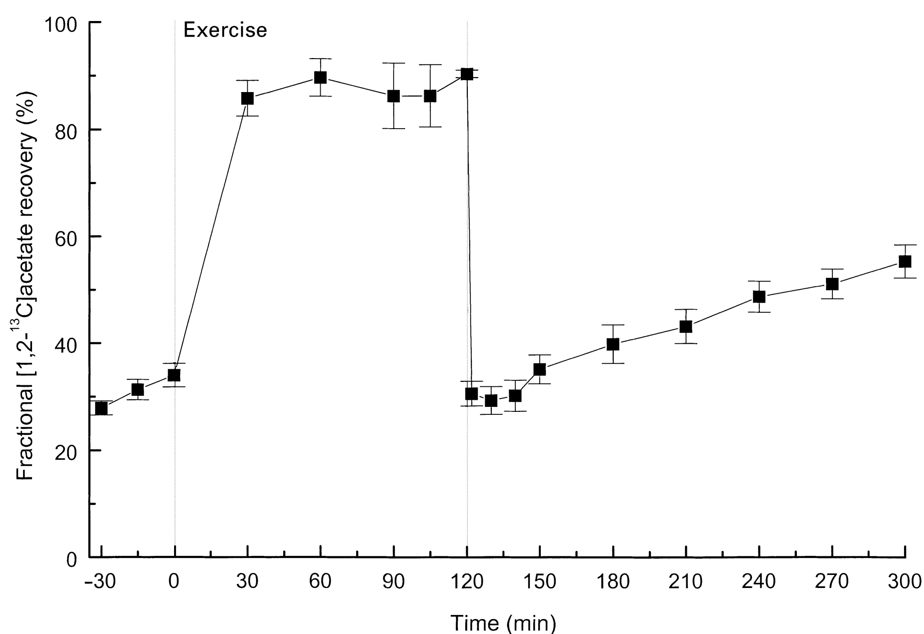

Fig. 6. Whole-body acetate recovery at rest, during exercise and recovery. Exercise consisted of 2 h of one-leg knee extension at 60 % of their maximal leg workload. [1,2-^{13}C]acetate was infused via a forearm vein at a known constant rate. Recovery (%) was calculated as: enrichment $^{12 \text{ or } 13}CO_{2breath} \times CO_{2breath}$ /(infusion rate / 2) × 100. Values are means and standard deviations represented by vertical bars for five subjects.

skeletal muscle at rest and during exercise. The assumptions included in the acetate recovery factor are more likely to hold during exercise at reasonable work intensities, since most of the acetate is directed to the active muscles, and acetate oxidation rates are high and recoveries are 80–100 % (Fig. 6; Sidossis *et al.* 1995*a*; Van Loon *et al.* 1999).

In summary, the amount of labelled CO_2 produced after administration of C-labelled substrates should be corrected for label loss, especially during resting conditions. When the C label is lost via decarboxylation reactions, the oxidation rate has to be corrected by applying the bicarbonate correction factor. However, if C label enters the TCA cycle the oxidation rate has to be corrected by applying the acetate correction factor (Fig. 1). Depending on the position of the C label within the substrate, [1-^{13}C]-, [2-^{13}C]- or [1,2-^{13}C]acetate must be used to determine the acetate correction factor. At rest, bicarbonate recovery is usually around 80 %; however, the actual value may depend to some extent on time of infusion, nutritional status, previous physical activity and biological variation. During exercise, and recovery from exercise, it seems appropriate to use only the bicarbonate recovery factor when an acceptable steady-state has been reached, i.e. in the second hour of exercise and after 2 h recovery. In most cases this would mean that during exercise bicarbonate recovery is 100 %, implying that no bicarbonate correction has to be made for substrate oxidation. More research is needed to draw conclusions as to the bicarbonate recovery factor in limbs and tissues. At rest, acetate recovery at the whole-body level increases with the duration of infusion. It is essential, therefore, that the acetate recovery is measured for the same period of infusion as the substrate for each oxidation measurement, and preferentially each substrate measured on an individual basis (Schrauwen *et al.* 1998). More research is needed to establish acetate recovery factors of limbs and tissues, and to investigate whether acetate handling by tissues at rest is comparable with that of the substrate. During exercise, the acetate recovery reached a plateau after approximately 30 min and was ≥ 70 % at intensities of 40 % maximum O_2 consumption. With exercise intensities of 75 % maximum workland in trained subjects, acetate recovery was 100 % and thus there was no need to apply an acetate recovery factor. Additional research is required before it can be confirmed that this situation also holds for subjects other than trained individuals. However, it is clear that the impact of the acetate correction factor is minimal only at moderately-high exercise intensities.

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