The Anaplerotic Substrate Alanine Stimulates Acetate Incorporation into Glutamate and Glutamine in Rabbit Kidney Tubules

A 13C NMR STUDY*

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Although acetate, the main circulating volatile fatty acid in humans and animals, is metabolized at high rates by the renal tissue, little is known about the precise fate of its carbons and about the regulation of its renal metabolism. Therefore, we studied the metabolism of variously labeled [13C]acetate and [14C]acetate molecules and its regulation by alanine, which is also readily metabolized by the kidney, in isolated rabbit renal proximal tubules. With acetate as the sole substrate, 72% of the C-1 and 49% of the C-2 of acetate were released as CO2; with acetate plus alanine, the corresponding values were decreased to 49 and 25%. The only other important products formed from the acetate carbons were glutamine, and to a smaller extent, glutamate. By combining 13C NMR and radioactive and enzymatic measurements with a novel model of acetate metabolism, fluxes through the enzymes involved were calculated. Thanks to its anaplerotic effect, alanine caused a stimulation of acetate removal and a large increase in fluxes through pyruvate carboxylase, citrate synthase, and the enzymes involved in glutamate and glutamine synthesis but not in flux through α-ketoglutarate dehydrogenase. We conclude that the anaplerotic substrate alanine not only accelerates the disposal of acetate but also prevents the wasting of the latter compound as CO2.

Acetate is the main circulating volatile fatty acid in humans and other mammalian species. Its blood concentration is low (less than 0.2 mM) in fed and starved humans and starved herbivores but may reach the millimolar range in humans after alcohol consumption and in fed herbivorous species (1–7). The sources of blood acetate are the on the one hand absorption of the acetate formed as a result of gastrointestinal bacterial fermentation, and on the other hand, the acetate formed and released by various tissues containing acetyl-CoA hydrolase activity (2, 3, 6, 8).

On the basis of experiments performed in vivo with labeled acetate, it has been shown that the turnover of circulating acetate is rapid and that, depending on the species and nutritional state, the oxidation of this compound provides from 6 to 70% of the whole body energy expenditure (3, 4, 7, 9). This means that acetate is removed and metabolized by peripheral tissues. Indeed, acetyl-CoA synthetase, the enzyme that initiates acetate degradation, has been demonstrated to be active in many tissues including the liver, kidney, heart, brain, adipose tissue, and skeletal muscle (3). It has been found that, besides the heart, the kidney contains a high activity of this enzyme (3).

In agreement with this observation, we have shown in a recent study that acetate is readily metabolized by suspensions of rabbit renal proximal tubules (10). In the same study (10), we have demonstrated that acetate significantly altered the metabolism of alanine, a major precursor of glutamine in these tissues. For this, we used 13C-labeled alanine and unlabeled acetate in combination with enzymatic and 13C NMR spectroscopy measurements to calculate metabolic fluxes related to alanine metabolism.

In an attempt to identify precisely the metabolic fate of acetate carbons and gain insight into the reciprocal effect of alanine on acetate metabolism, we have conducted concomitantly a study in which we incubated rabbit renal proximal tubules with 13C-labeled acetates in the absence and the presence of unlabeled alanine. Thanks to the development of a novel model of acetate metabolism that is of general use (see “Appendix”) and to the combination of enzymatic, radioactive, and 13C NMR measurements, we were able to estimate fluxes through enzymes involved in acetate metabolism in rabbit renal proximal tubules. We showed that the addition of alanine, which increased the removal of acetate, also stimulated its metabolism through citrate synthase but not through α-ketoglutarate dehydrogenase. We also demonstrated that acetate carbons were converted to different extents not only into CO2 but also into glutamate and glutamine, especially in the presence of the anaplerotic substrate alanine.

EXPERIMENTAL PROCEDURES

Reagents

Sodium acetate, L-alanine, and glutaminase (grade V) were from Sigma. Other enzymes and coenzymes were purchased from Roche Molecular Biochemicals. [1-13C]acetate (2.05 GBq/mmol) and [2-13C]-acetate (1.85 GBq/mmol) were obtained from the Commissariat à l’Energie Atomique (Saclay, France). [1-13C]acetate and [2-13C]acetate were obtained from the Commissariat à l’Energie Atomique and had a 90 and 99% isotopic abundance, respectively.

Rabbits

Female rabbits (1.8–2 kg; New Zealand albino strain) were obtained from the Elevage des Dombes (Châtillon-sur-Chalaronne, France) and were fed a standard diet (Usine d’Alimentation Rationnelle, Villemoisson-sur-Orge, France).

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Preparation of Kidney Tubules and Incubation

Kidney cortex tubules were prepared by the treatment of renal cortex slices with collagenase as described by Baverel et al. (11). Incubations other than those involving radioactive substrates were performed for 60 min at 37 °C in a shaking water bath in 25 ml stopped Erlenmeyer flasks in an O2/CO2 (19/1) atmosphere. The flasks contained 1 ml of the suspension plus 3 ml of Krebs-Henseleit medium (12), either supplemented or not with substrates, i.e., 5 mM (final concentration) [2-13C]acetate or [2-13C]acetate (10 mM Ba/flask), [1-13C]acetate or [2-13C]acetate in the absence and the presence of 5 mM l-alanine. These differently labeled acetates were used in an attempt to completely define the fate of the two acetate carbons. In all experiments, each experimental condition was performed in quadruplicate. Incubation was stopped by the addition of HClO4 (final concentration 2% [v/v]) to each flask. In all experiments, zero-time flasks, with or without substrates, were prepared by the addition of HClO4 before the tubules.

When radioactive acetate was present in the medium, incubation, deproteinization, collection, and measurement of the 14CO2 formed were performed as described by Baverel and Lund (13). After removal of the denaturated protein by centrifugation, the supernatant was neutralized with a mixture of 20% (v/v) ROH and 1% (v/v) H3PO4 (8 s) for metabolite determination and NMR spectroscopy.

Analytical Methods

Metabolite Assays—Lactate, pyruvate, glucose, glutamate, glutamine, ammonia, alanine, citrate, α-ketoglutarate, fumarate, malate, acetate, acetoacetate, and 3-hydroxybutyrate as well as the dry weight of tubules added to the flasks were determined as described previously (11, 13). Serine was measured by high pressure liquid chromatography with the use of the Pico-Tag method (14).

23C NMR Techniques—Perchloric acid extracts were neutralized (11), freeze-dried, and reconstituted in D2O in the presence of [2-13C]acetate or [2-13C]acetate (10 mM Ba/flask), [1-13C]acetate or [2-13C]acetate in the presence of 5 mM l-alanine. These differently labeled acetates were used in an attempt to completely define the fate of the two acetate carbons. In all experiments, each experimental condition was performed in quadruplicate. Incubation was stopped by the addition of HClO4 (final concentration 2% [v/v]) to each flask. In all experiments, zero-time flasks, with or without substrates, were prepared by the addition of HClO4 before the tubules.

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Calculated Net substrate utilization and product formation were calculated as the difference between the total flask contents (tissue plus medium) at the start (zero-time flasks) and after the period of incubation. The metabolic rates, reported as means ± S.E., are expressed in mmol of substances removed or produced per flask per unit time (60 min). The rates of release of 14CO2 from the 14C-labeled acetate species used were calculated by dividing the radioactivity in 14CO2 by the specific radioactivity of the labeled carbon of the acetate species of interest measured in each medium. When [13C]acetate species were the substrate, the transfer of the C-1 or C-2 of acetate to a given position in a given metabolite was calculated from (Lm - L0)/(L0 + A0 - A1), in which Lm is the amount of 13C measured in the corresponding NMR resonance, L0 is the natural abundance (1.1%) multiplied by the amount of metabolite assayed enzymatically, A0 is the total 13C abundance of the C-1 or C-2 of acetate, and A1 is the natural 13C abundance.

RESULTS

To determine the fate of acetate carbons and the metabolic pathways involved, experiments were performed in which rabbit renal proximal tubules were incubated with differently 13C- and 13C-labeled acetates with and without alanine. Substrate utilization and product formation were measured by combining enzymatic, radioactive, and 13C NMR spectroscopy measurements.

Enzymatic Measurements of Substrate Utilization and Product Formation—Substrate removal and glutamate plus glutamine accumulation and labeling were approximately linear with time over a 60-min incubation period (n = 2 in duplicate; results not shown). Table I shows that, when acetate was added as the sole exogenous substrate into the incubation medium, it was readily removed by rabbit renal proximal tubules. Although under this condition, no exogenous nitrogenous substrate was provided to the renal cells, they accumulated substantial amounts of glutamine, and to a lesser extent, of glutamate and serine. The fact that substantial amounts of glutamine were also synthesized in the absence of added substrate (Table I) means that, in the presence of acetate, these amino acids were formed, at least in part, from endogenous sources. Some acetoacetate was also formed in the presence of acetate as the sole exogenous substrate (Table I).

As shown in Table I, and in agreement with our recent results (10), alanine was also metabolized at high rates by the renal tubules when this amino acid was added as substrate together with acetate. Alanine addition caused a 32% stimulation of acetate removal and a great increase in glutamate and glutamine accumulation. In the presence of alanine, acetoacetate accumulation was also significantly reduced, and small amounts of pyruvate and lactate accumulated. Nitrogen balance calculations indicate that, in the presence of alanine, no significant room was left for glutamine and glutamate synthesis from endogenous substrates. Under none of the experimental conditions studied did we observe any substantial accumulation of glucose, ammonia, β-hydroxybutyrate, or intermediates of the tricarboxylic acid cycle.

Radioactive Measurements of CO2 Production from Acetate—Table II shows that, with acetate as the sole substrate, 72% of the C-1 and 49% of the C-2 of the acetate removed were released as CO2. This clearly indicates that, under this condition, the remainder of the C-1 and C-2 of the acetate removed was incorporated into the non-volatile carbon products found to accumulate, namely acetoacetate, glutamine, and glutamate.

The release of 13CO2 from [1-13C]acetate did not change upon the addition of alanine; under this condition, it represented only 49% of the C-1 of the acetate removed. By contrast, the presence of alanine significantly diminished the production of 14CO2 from [2-13C]acetate; under the latter condition, the production of CO2 accounted for only 25% of the C-2 of the acetate removed.

13C NMR Spectroscopy Measurements—Fig. 1, A and B, shows the 13C NMR spectra of perchloric acid extracts obtained after 60 min of incubation of renal tubules with [2-13C]acetate in the absence and the presence, respectively, of alanine. As all the C-1 and C-2 of the 14C-labeled acetates removed could not be accounted for by the production of 14CO2 (Table II), it is not surprising that a significant amount of the C-2 of [2-13C]acetate removed was recovered in glutamine and glutamate, especially in the presence of alanine. No substantial amount of lactate, acetoacetate, or serine was found to be labeled. Using these spectra and those obtained with [1-13C]acetate as substrate without and with alanine (results not shown), we calculated the amount of labeled products after correction for the 13C natural abundance (Tables III and IV).

With [2,13C]acetate as substrate (Table III), the high labeling of the C-4 of glutamate and glutamine indicates that the C-2 of acetate gave the C-2 of acetyl-CoA, and then gave the C-4 of citrate, α-ketoglutarate, glutamate, and glutamine, via the successive operation of acetyl-CoA synthetase, citrate synthase, aconitase, isocitrate dehydrogenase, alanine, or (in the absence of alanine) other amino acid aminotransferases and glutamine synthetase. The fact that virtually equal amounts of C-2 and C-3 of glutamate plus glutamine were labeled is consistent with the previous observations made by other authors with acetate and other substrates in kidney and other tissues (20–24). This is also in agreement with the view that the C-2 of acetate passed through succinate and fumarate, two symmetrical molecules, during the first tricarboxylic acid cycle turn, leading to the formation of either [2,13C]oxaloacetate or...
Kidney tubules (26.8 ± 2.1 mg of dry weight/flask) were incubated for 60 min as described under "Experimental Procedures." Results (μmol/h) are reported as means ± S.E. for four experiments. Substrate utilization and product formation, measured enzymically, are reported in Table 1. Statistical difference was measured by the paired Student’s t test against the control with acetate alone: *, p < 0.001; **, p < 0.01; ***, p < 0.001. The radioactivity and 13C NMR data corresponding to these experiments are reported in Table 2 and Tables 3 and 4, respectively.

### Table II

| Experimental condition | 13CO2 from [1-14C]acetate | 13CO2 from [2-14C]acetate |
|------------------------|---------------------------|---------------------------|
| 5 mM [14C] acetate     | 5.96 ± 0.12               | 4.05 ± 0.05               |
| 5 mM [14C] acetate + 5 mM alanine | 5.31 ± 0.11               | 2.70 ± 0.05               |

[3-13C]oxalacetate and then to either [3-13C]citrate or [2-13C]citrate and α-ketoglutarate during the second tricarboxylic acid cycle turn, yielding finally glutamate and glutamine labeled on their C-2 and C-3 after the transaminase and glutamine synthetase reactions. Consistent with the increase in the synthesis of glutamate plus glutamine shown in Table I, the addition of alanine caused an increase in the incorporation of the C-2 of acetate into the C-2, C-3, and C-4 of glutamate and glutamine (Table III).

It should be noted that a fraction of the glutamate and glutamine molecules formed from [2-13C]acetate were simultaneously labeled on their C-3 and C-4 as revealed by the doublets that indicate 13C-13C couplings between these two glutamine and glutamate carbons (Fig. 1). However, as the spectral resolution was not sufficient to quantify these doublets in a reliable manner, no attempt was made to quantify them to determine the relative proportions of labeled and unlabeled oxalacetate and acetyl-CoA molecules contributing to the synthesis of glutamate and glutamine.

Table IV shows as expected that, with [1-13C]acetate as substrate and both in the absence and the presence of alanine, the labeling of the C-5 of glutamate and glutamine was close to the labeling of the C-4 of these two amino acids observed when [2,13C]acetate was the substrate (see Table III for comparison). The labeling of the C-1 of glutamate and glutamine is in agreement with the conversion of the C-1 of acetate into the C-4 of oxalacetate during the first tricarboxylic acid cycle turn and then the formation of the C-1 of citrate and α-ketoglutarate during the second tricarboxylic acid cycle turn before the transamination of α-ketoglutarate into glutamate followed by glutamine synthesis. The fact that the labeling of the C-1 of glutamate and glutamine when [1-13C]acetate was the substrate was smaller than the labeling of either the C-2 or the C-3 of glutamate and glutamine when [2-13C]acetate was the substrate is not surprising because, in the presence of [1-13C]acetate, the label found in the C-1 and the C-4 of oxalacetate after the first tricarboxylic acid cycle turn was lost as CO₂ during the second turn via the isocitrate dehydrogenase and the α-ketoglutarate dehydrogenase step, respectively. As already seen with the incorporation of the C-2 of acetate into the C-2, C-3, and C-4 of glutamate and glutamine (Table III), incorporation of the C-1 of acetate into the C-1 and C-5 of glutamate and glutamine was increased in the presence of alanine.

**Calculations of Proportions**—Table V shows the calculated proportions of metabolites converted into the next one(s). It should be mentioned here that, with the model used, these proportions allowed us to calculate enzymatic fluxes only when they were combined with the utilization of the substrate(s) of interest. As can be seen in Table V, some proportions could not be calculated when acetate was the sole substrate. This is because, under this condition, the 13C resonances were not great enough to allow us to quantify the corresponding conversions. The fraction of pyruvate converted into oxalacetate,
FIG. 1. $^{13}$C NMR spectra (100.62 MHz) of neutralized perchloric acid extracts obtained from rabbit kidney tubules incubated with [2-$^{13}$C]acetate in the absence (A) and the presence (B) of alanine. 1, acetate C-2 (24.10); 2, glutamine C-3 (27.10); 3, glutamate C-3 (27.83); 4, glutamine C-4 (31.70); 5, glutamate C-4 (34.16); 6, glutamine C-2 (55.10); 7, glutamate C-2 (55.53). Numbers in parentheses indicate the chemical shifts in ppm referred to tetramethylsilane. An expanded view of the C$_4$ and C$_3$ resonances of glutamate and also glutamine including the coupling between C$_4$ and C$_3$ is shown.


### TABLE III

**Effect of 5 mM alanine on the metabolism of 5 mM [2-13C]acetate in rabbit kidney tubules**

Kidney tubules (26.8 ± 2.1 mg of dry weight/flask) were incubated for 60 min as described under “Experimental Procedures.” Results (μmol/h) for 13C-labeled products accumulated are reported as means ± S.E. for four experiments performed in quadruplicate. The paired Student’s t test was used to measure the statistical difference against the control with acetate alone: *, p < 0.05; **, p < 0.01; ***, p < 0.001.

| Experimental condition        | C2       | C3       | C4       | C2       | C3       | C4       | C2       | C3       | C4       |
|------------------------------|----------|----------|----------|----------|----------|----------|----------|----------|----------|
| [2-13C]Acetate               | 0.35 ± 0.03 | 0.29 ± 0.02 | 0.53 ± 0.05 | 0.44 ± 0.02 | 0.42 ± 0.01 | 0.82 ± 0.02 |
| [2-13C]Acetate + Alanine     | 0.47 ± 0.03 | 0.41 ± 0.02 | 1.81 ± 0.08 | 0.66 ± 0.03 | 0.74 ± 0.12 | 3.06 ± 0.29 |

### TABLE IV

**Effect of 5 mM alanine on the metabolism of 5 mM [1-13C]acetate in rabbit kidney tubules**

Kidney tubules (26.8 ± 2.1 mg of dry weight flask) were incubated for 60 min as described under “Experimental Procedures.” Results (μmol/h) for 13C-labeled products accumulated are reported as means ± S.E. for four experiments performed in quadruplicate. The paired Student’s t test was used to measure the statistical difference against the control with acetate alone: *, p < 0.05; **, p < 0.01; ***, p < 0.001.

| Experimental condition        | C1       | C5       | C1       | C5       |
|------------------------------|----------|----------|----------|----------|
| [1-13C]Acetate               | 0.10 ± 0.01 | 0.36 ± 0.07 | 0.16 ± 0.02 | 0.64 ± 0.06 |
| [1-13C]Acetate + Alanine     | 0.29 ± 0.01 | 1.98 ± 0.11 | 0.43 ± 0.01 | 2.90 ± 0.28 |

### TABLE V

**Various proportions through pathways of acetate metabolism in the absence or in the presence of 5 mM alanine in rabbit kidney tubules**

Values, given as means ± S.E. for four experiments, were calculated from those of Tables 2–4. Lowercase italic letter symbols indicate the proportion of a given intermediate metabolized at a given step. The symbols of the various proportions are shown in scheme I. Note that (u) takes into account αKG recycling through Glu and Gln. The paired Student’s t test was used to measure the statistical difference against the control with acetate as sole substrate: *, p < 0.05; **, p < 0.01; ***, p < 0.001.

| Proportion of converted to | Parameter notation | Parameter value With alanine | Parameter value Without alanine |
|---------------------------|--------------------|------------------------------|-------------------------------|
| OAA                       | Cit                | 0.94 ± 0.01                 | 0.91 ± 0.01                  |
| OAA                       | PEP                | 0.06 ± 0.01                 | 0.01 ± 0.01                  |
| PEP                       | Pyr                | 0.06 ± 0.12                 | 0.00 ± 0.03                  |
| Pyr                       | OAA                | 0.99 ± 0.03                 | 0.99 ± 0.03                  |
| Pyr                       | AcCoA              | 0.35 ± 0.04**               | 0.35 ± 0.04**               |
| AcCoA                     | Cit                | 0.99 ± 0.03                 | 0.99 ± 0.03                  |
| AcCoA                     | AcAc               | 0.01 ± 0.01***              | 0.01 ± 0.01***              |
| αKG                       | OAA                | 0.05 ± 0.01                 | 0.05 ± 0.01                 |
| αKG                       | accumulated Glx    | 0.45 ± 0.01***              | 0.45 ± 0.01***              |

| Proportion^b              | Parameter notation | Parameter value With alanine | Parameter value Without alanine |
|---------------------------|--------------------|------------------------------|-------------------------------|
| In TCA cycle              |                    | 0.51 ± 0.02                  | 0.51 ± 0.02                  |
| in OAA → PEP → Pyr → AcCoA → OAA cycle |                    | 0.01 ± 0.01                  | 0.01 ± 0.01                  |
| in OAA → PEP → Pyr → OAA cycle |                    | 0.02 ± 0.01                  | 0.02 ± 0.01                  |
| Equivalent recycling factor^d |                    | 0.61 ± 0.02                  | 0.61 ± 0.02                  |

*a* PEP, P-enolpyruvate.

*b* Of recycling at each turn.

*TCA, tricarboxylic acid.

^d* Introduced to explain total OAA formation.

which was close to unity in the absence of alanine, was significantly decreased in the presence of alanine, whereas a significant fraction of pyruvate was converted into acetyl-CoA only in the presence of alanine.

The decrease in the fraction of acetyl-CoA converted into acetooacetate caused by the addition of alanine was fully compensated by an increase in the acetyl-CoA converted into citrate. The addition of alanine also significantly increased the proportion of α-ketoglutarate converted into glutamate plus glutamine at the expense of the proportion of α-ketoglutarate converted into oxalacetate (Table V). Table V also shows that the proportions of the oxalacetate recycled at each turn of the three different cycles could be quantified only in the presence of alanine.

### Enzymatic Fluxes—Table VI shows the absolute values of fluxes through enzymes involved in acetate metabolism both in the absence and the presence of alanine. Fluxes through acetyl-CoA synthetase were identical to the values of acetate removal reported in Table I. Fluxes through 3-ketothiolase were also identical to the acetocetate accumulations presented in Table I because there was no evidence of β-hydroxybutyrate accumulation. On the addition of alanine, flux through acetyl-CoA synthetase was stimulated, whereas that through 3-ketothiolase was inhibited. In the absence of alanine, the minimum flux through pyruvate carboxylase (1.35 μmol/h; in Table VI, see [PC] – [PEPCK])^3 logically matches the exit of α-ketoglutarate from the tricarboxylic acid cycle to form glutamate and glutamine (in Table VI, see Glx accumulated). It should be mentioned that the small 13C labeling data obtained did not provide evidence for the existence of the OAA → P-enolpyruvate → Pyr

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^3* The abbreviations used are: PC, pyruvate carboxylase; PEPCK, phosphoenolpyruvate carboxykinase; OAA, oxalacetate; Met, metabolite; PK, pyruvate kinase; αKG, α-ketoglutarate; αKGDH, α-ketoglutarate dehydrogenase.
Effect of 5 mM alanine on fluxes through pathways of acetate metabolism in rabbit kidney tubules

Values, given as means ± S.E. for four experiments, were calculated from those of Tables I–V; fluxes are defined in the Appendix. Fluxes are expressed in μmol/h of C₃ unit equivalents. The paired Student’s t test was used to measure the statistical difference against the control with acetate as sole substrate: *, p < 0.05; **, p < 0.01; ***, p < 0.001.

Table VI

| Experimental condition | Acetyl-CoA synthetase | 3-Keto thiolase | [PC] | [PEPCK] | (PC) – (PEPCK) | (PK) | (PC) – [PK] |
|------------------------|-----------------------|----------------|------|---------|---------------|------|------------|
| 5 mM acetate           | 8.26 ± 0.30           | 0.45 ± 0.02    | 1.35 ± 0.64 |
| 5 mM acetate + 5 mM alanine | 10.91 ± 0.44         | 0.13 ± 0.01    | 7.97 ± 0.40 |

Table VI also shows the effects of alanine on acetate metabolism. Alanine is a carbon substrate that can be used for the synthesis of glutamate and glutamine. The addition of alanine increased the flux through pyruvate dehydrogenase and phosphoenolpyruvate carboxykinase, indicating that these enzymes are stimulated by alanine. The increased fluxes through these enzymes were accompanied by an increased production of glutamate and glutamine, as seen in Table I. This is in agreement with the results published by others (3, 7, 25) and suggests that the renal proximal tubule is capable of metabolizing acetate in vivo.

DISCUSSION

Thanks to enzymatic, radioactive, and 13C NMR measurements in combination with an original model of acetate metabolism that is of general use, this study not only establishes the fate of acetate in rabbit renal proximal tubules in the absence and presence of alanine but also provides a precise quantification of fluxes through the enzymes related to acetate metabolism.

Fate of Acetate—Confirming previous results (10, 16), acetate was avidly metabolized by rabbit kidney tubules. Carbon balance calculations, comparing on the one hand the removal of acetate (Table I) and on the other hand the sum of the 14CO₂ formation (Table II) and the 13C incorporated into glutamate plus glutamine (Tables III and IV), indicate that most, if not all, of the acetate metabolized could be accounted for by the products measured. The small discrepancies observed may be due to small experimental errors in the determination of substrate removal or product formation. In this respect, it is also probable that small amounts of acetoacetate were labeled with carbon 13 during the incubations but were lost during the sample preparation for 13C NMR measurements because of the well established instability of this ketone body.

With acetate as the sole exogenous substrate, most (72%) of the C-1 and only about half (49%) of the C-2 of acetate was recovered as CO₂. This clearly means that a substantial fraction of acetate was metabolized beyond the first tricarboxylic acid cycle turn because the acetyl-CoA carbons incorporated into citrate cannot be released as CO₂ during the first tricarboxylic acid cycle turn. This observation means that part of the acetate carbons removed was incorporated into non-volatile compounds. Our 13C NMR measurements revealed that these compounds were glutamine and, to a lesser extent, glutamate. This finding implies that an amount of oxaloacetate equivalent to the accumulation of labeled glutamate and glutamine was formed from endogenous sources to replenish the tricarboxylic acid cycle. The diversion of α-ketoglutarate carbons from the tricarboxylic acid cycle to convert them into non-volatile compounds also explains why the two carbons of acetate were not converted into CO₂ at the same rates. Indeed, at the end of the first tricarboxylic acid cycle turn, the C-1 of acetate was recovered either as the C-1 or the C-2 of oxaloacetate, whereas the C-2 of acetate was recovered only as the C-2 or the C-3 of oxaloacetate. The C-1 and C-4, but not the C-2 and C-3, of oxaloacetate could be released as CO₂ during the next tricarboxylic acid cycle turn, the probability to recover the C-1 of acetate did not increase, and that of the C-2 significantly diminished. This was due to an increased incorporation of both acetate carbons into glutamate and glutamine as revealed by the 13C NMR data (Tables III and IV).

It is important to mention that, although the addition of alanine stimulated acetate utilization, the release as CO₂ of the C-1 of acetate did not increase, and that of the C-2 significantly diminished. This was due to an increased incorporation of both acetate carbons into glutamate and glutamine as revealed by the 13C NMR data (Tables III and IV). Note that in the presence of alanine, only 49% of the C-1 and 25% of the C-2 of acetate were released as CO₂ (Tables I and II). Thus, depending on the intensity of the diversion of α-ketoglutarate carbon from the tricarboxylic acid cycle, which is necessarily matched by an equivalent anaplerotic activity, the proportions of the acetate carbons released as CO₂ may greatly vary. This observation is of general importance for all in vivo studies in which acetate oxidation is studied (3, 7, 25) or in which acetate is used as a labeled precursor for measuring metabolic fluxes (26–28).
It should be emphasized that the measurement of the differential yield in labeled CO₂ from acetate may seem at first sight confirmatory of a concept established for a long time. In fact, it is a key measurement allowing us to validate our calculations of the proportion of α-ketoglutarate converted into oxalacetate both in the absence and in the presence of alanine. Indeed, the alanine-induced decrease in this proportion (Table V), which reflects the recycling in the tricarboxylic acid cycle, is in excellent agreement with the finding reported in Table II that alanine reduced the CO₂ from the C-2 of acetate but not that from the C-1 of acetate.

Fluxes through Enzymes of Acetate Metabolism—The values of fluxes presented in Table VI, which are mainly derived from 13C NMR data (see the equations of the model under “Appendix”), are consistent with the enzymatic and radioactive measurements shown in Tables I and II. As expected in the presence of acetate as the sole exogenous substrate, most of the acetyl-CoA formed from acetate was metabolized by the citrate synthase reaction, and most of the α-ketoglutarate derived from citrate was metabolized through α-ketoglutarate dehydrogenase. Because no exogenous nitrogenous substrate was present in the incubation medium in the absence of alanine, the formation of glutamate occurred by transamination of α-ketoglutarate with endogenous amino acids derived from proteolysis. Some of the latter glutamate was probably oxidatively deaminated by glutamate dehydrogenase, which is very active in rabbit kidney tubules (29), to provide the ammonia needed for the synthesis of the glutamine accumulated. Note that the minimum flux through the anaplerotic enzyme, pyruvate carboxylase, corresponds to the net conversion of α-ketoglutarate into glutamate (Table VI) and that in the presence of acetate as the sole exogenous substrate, the pyruvate metabolized by the pyruvate carboxylase reaction was formed exclusively from endogenous substrates.

The fact that alanine addition, which caused an increased synthesis of oxalacetate by pyruvate carboxylase, stimulated the flux through acetyl-CoA synthetase by 32% strongly suggests that the availability of oxalacetate was limiting for acetate utilization. The diminution of flux through 3-ketoliase probably resulted from a diversion of the acetyl-CoA synthesized from acetate to citrate formation.

The very large stimulation of the flux through citrate synthase caused by alanine addition resulted not only from an increased availability of oxalacetate derived from alanine but also from an increased formation of acetyl-CoA also derived from alanine thanks to the pyruvate dehydrogenase reaction, which functioned at a high rate despite the presence of acetate. It is of interest to observe that the stimulation of the flux through citrate synthase was not accompanied by a statistically significant increase in flux through α-ketoglutarate dehydrogenase but rather by a considerable stimulation of the conversion of α-ketoglutarate into glutamate and glutamine. The increased synthesis of glutamate can be explained by the increased transamination of α-ketoglutarate with alanine, leading to stoichiometric increases in flux through both glutamate dehydrogenase and glutamine synthetase (Table VI).

It should be emphasized that the appearance of tracer carbons from acetate in glutamine represented an unidirectional conversion of acetate carbons into glutamine carbons thanks to the glutamine synthetase reaction and not a substantial exchange of carbons as a result of the concomitant action of glutamine synthetase and glutaminase, two enzymes that function unidirectionally. This view is supported by the following observations. (i) The amount of glutamine present at the start of incubation (in zero-time flasks) was 0.12 ± 0.01 μmol. (ii) Nitrogen balance calculations indicate that glutamine was progressively synthesized from endogenous sources during the incubation period in the absence of exogenous substrate and in the presence of acetate as the sole exogenous substrate but not in the presence of acetate plus alanine (see “Results”). In the presence of acetate as the sole exogenous substrate, the concentration of glutamine at the end of the incubation period was 0.4 mM. Given that the production of glutamine was linear with time, one can calculate from results obtained previously in this laboratory (30) that, in our rabbit kidney tubules, flux through glutaminase, whose activity is low (31), was negligible when compared with that through glutamine synthetase, whose activity is high (31, 32). It is also interesting to underline that neither at zero time nor at the end of the incubation period did we observe any glucose accumulation; therefore, no exchange of labeled acetate carbons could occur with glucose.

It is important to mention that the values of the enzymatic fluxes obtained in the present study with 13C-labeled acetates plus unlabeled alanine are very close to those found with 13C-alanines plus unlabeled acetate (10). In our opinion, this represents a strong support not only for the validity of our results obtained with differently labeled substrates but also for the validity of our methodological approach. This approach combines experimental results derived mainly from 13C NMR data with new mathematical models of the metabolic pathways involved when acetate and alanine are concomitant substrates of the rabbit kidney tubules. It is worthwhile to emphasize that such an approach is directly applicable to any cell that has the capacity to metabolize both acetate and alanine and contains significant activities of the enzymes involved in our study.

Physiological Importance—As shown in previous works (10, 16) and in this study, the rabbit proximal tubule has the capacity in vitro to avidly metabolize not only acetate, the most concentrated volatile fatty acid in the rabbit blood (6), but also alanine, an important precursor of glutamine (10, 33–35). It is very likely that in vivo, the rabbit kidney also metabolizes acetate and then, besides providing energy for the renal transport of mineral and organic solutes, contributes to the addition of bicarbonate to the urine, which is alkaline in this herbivorous species (36). In terms of acid base balance equilibrium, it is of interest to note that the alanine-induced stimulation of the removal of acetate, an anion, was balanced by an increased accumulation of glutamate and lactate, two other anions (Table I). Thus, the addition of alanine did not increase the production of bicarbonate as a result of an increased acetate metabolism and, therefore, did not alter the acid base balance equilibrium. Concomitantly, alanine greatly stimulated the synthesis of glutamine, which incorporated a significant fraction of the acetate carbons used. Thus, the stimulation of acetate metabolism by alanine, if it occurs in vivo, might appear as a salvage mechanism that does not increase the loss of acetate carbons as CO₂ but rather incorporates them in the form of glutamine, an amino acid reabsorbed by the kidney and made available to other tissues.

Finally, this study also illustrates the utmost importance of anaplerosis for the renal metabolism of compounds which, like acetate, provide only two carbon units to intermediary metabolism and are potentially incorporated into non-volatile compounds such as glutamate and glutamine. The accumulation of glutamate and glutamine reflects, at least in part, a physiological cataplerosis that constantly depletes the concentration of citric acid cycle intermediates. Therefore, as for liver and cardiac cells (37, 38), for the renal cells to continue generating energy, a constant anaplerosis was necessary in the present study. This anaplerosis was made possible by metabolizing alanine carbons through pyruvate carboxylase.
arising from acetate labeled on its carbon z, where z is equal to 1 or 2 because we used [1-13C]acetate, [1-14C]acetate, [2-13C]acetate, and [2-14C]acetate as labeled substrates. In the condition where unlabelled alanine is also added in the medium, the exponent C\textsubscript{Ac} is replaced by C\textsubscript{Ac} + Ala. If the calculation procedure is the same for [C\textsubscript{Met}]\textsuperscript{Ac} and [C\textsubscript{Met}]\textsuperscript{Ac} + Ala, we present only one equation by writing simply [C\textsubscript{Met}]\textsuperscript{Ac+Ala}.

Similarly, [Met]\textsuperscript{Ac} and [Met]\textsuperscript{Ac+Ala} represent the total amount of the metabolite (Met) formed when the added substrate is acetate and acetate plus alanine, respectively. For simplicity, we write [Met] instead of [Met]\textsuperscript{Ac+Ala} when the calculation procedures are the same. This principle is also applied to accumulated metabolites and enzymatic fluxes. The accumulated amount of a metabolite is indicated by a ′′ emblem added on the left side of its name inside the square brackets. An enzymatic flux is represented by the abbreviated name of the enzyme placed inside braces.

Calculations of the Parameters of the Model—The amount (in μmol/h) of any given intermediate or end product formed from the substrate acetate is obtained by multiplying the amount of the substrate removed [Y] by the successive proportions of intermediates passing through the different pathways leading to the intermediate or end product of interest. Each individual parameter is defined in Table 1.

This model takes into account the \(\alpha\)-ketoglutarate recycling through glutamate and glutamine; the corresponding proportion is noted (\(\alpha\)). Let us call \(g\), \(h\), and \(z\) the proportions of any metabolite resynthesized after each complete turn of the citric acid cycle, the OAA \rightarrow P-enolpyruvate \rightarrow Pyr \rightarrow OAA, and OAA \rightarrow P-enolpyruvate \rightarrow Pyr \rightarrow AcCoA \rightarrow OAA cycle, respectively. Oxalacetate, the common intermediate to these three cycles, is either converted into citrate or phosphoenolpyruvate with the proportion \(a\) and \(b\), respectively. Also, because at each bifurcation, the sum of all proportions adds up to one, the result is as shown in Eq. 1,

\[
b = 1 - a \tag{Eq. 1}
\]

Similarly, as shown in Eq. 2,

\[
j = 1 - r \tag{Eq. 2}
\]

since phosphoenolpyruvate yields either pyruvate or serine, whereas pyruvate can be converted to oxalacetate, acetyl-CoA, lactate, or simply accumulated with proportions \(c\), \(d\), \(l\), or \(p\), respectively. Thus, as shown in Eq. 3,

\[
p = 1 - c - d - l \tag{Eq. 3}
\]

All the acetate utilized is converted into acetyl-CoA, which in turn yields either acetoacetate or citrate; let us call the corresponding proportions \(u\) and \((1 - u)\), respectively. Recycling factors \(g\), \(h\), and \(z\) can be calculated from Schemes 1 and 2, as shown in Eqs. 4–6,

\[
h = (1 - a)^{\alpha} \tag{Eq. 4}
\]

\[
g = a s' \tag{Eq. 5}
\]

\[
z = (1 - a) r q (1 - u) \cdot s'^{\alpha} \tag{Eq. 6}
\]

where \(s'\) is the proportion of citrate-derived \(\alpha\)-ketoglutarate that has been converted into oxalacetate after partial Recycling through glutamate and glutamine. Therefore, \(g\) takes into account the recycling of \(\alpha\)-ketoglutarate through glutamate and glutamine.

Let \(Y\) be the amount of Ac utilization. Schemes 1 and 2 indicate that citrate formed from Ac-derived AcCoA is equal to \(Y(1 - u) s'^{\alpha}\), corresponding to the beginning of the first multicycle turn for AcCoA carbons.

**APPENDIX**

Scheme 1 shows that acetate metabolism consists of a multicycle made of five different cycles operating simultaneously: (i) the citric acid cycle, (ii) the OAA \rightarrow P-enolpyruvate \rightarrow Pyr \rightarrow OAA, and OAA \rightarrow P-enolpyruvate \rightarrow Pyr \rightarrow AcCoA \rightarrow OAA cycles, the proportion of metabolite resynthesized at each turn is \(g\), \(h\), and \(z\), respectively. \(k_1\) and \(k_2\) are the recycling proportions in the Glu \rightarrow \(\alpha\)-KG \rightarrow Glu and Glu \rightarrow Glu \rightarrow Glu cycles, respectively. The proportions \(g\) and \(z\) take into account the recycling of \(\alpha\)-ketoglutarate through these two latter cycles. Scheme 1 also shows that oxalacetate is an important metabolite common to three cycles. The oxalacetate that condensed to acetyl-CoA to yield citrate was partially resynthesized in the multicycle; it originated mainly from endogenous precursors in the presence of acetate as the sole added substrate or from alanine in the presence of acetate plus alanine.

**SCHEME 1. Metabolic cycles operating during acetate metabolism.** This scheme shows five cycles functioning simultaneously. At each turn of this multicycle, each carbon atom undergoes successive shifts of its position inside metabolite molecules. In the citric acid, the OAA \rightarrow P-enolpyruvate \rightarrow Pyr \rightarrow OAA, and OAA \rightarrow P-enolpyruvate \rightarrow Pyr \rightarrow AcCoA \rightarrow OAA cycles, the proportion of metabolite resynthesized at each turn is \(g\), \(h\), and \(z\), respectively. \(k_1\) and \(k_2\) are the recycling proportions in the Glu \rightarrow \(\alpha\)-KG \rightarrow Glu and Glu \rightarrow Glu \rightarrow Glu cycles, respectively. The proportions \(g\) and \(z\) take into account the recycling of \(\alpha\)-ketoglutarate through these two latter cycles. Scheme 1 also shows that oxalacetate is an important metabolite common to three cycles. The oxalacetate that condensed to acetyl-CoA to yield citrate was partially resynthesized in the multicycle; it originated mainly from endogenous precursors in the presence of acetate as the sole added substrate or from alanine in the presence of acetate plus alanine.

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An equal amount of OAA is necessary to form these citrate molecules, but simultaneously, a fraction \((1 - \alpha)\alpha\) (Scheme 1) of this amount of OAA is utilized through another pathway. Thus, even at the beginning of the multicycle, it is obvious that not all of the OAA participating in the metabolism can be seen through acetate metabolism.

Note that in the condition with alanine plus acetate, the total OAA formed can be obtained from the fate of alanine carbons (10). In a classical representation, if OAA was reprocessed only in the citric acid cycle with a proportion \(g\) at each turn, the total amount of OAA formed over a theoretically infinite number of turns would be given by the OAA, noted \([\text{OAA}]_0\), formed at the beginning of the first cycle turn multiplied by Eq. 7.

\[
\sum_{n=0}^{\infty} g^n = \frac{1}{1-g} \quad \text{(Eq. 7)}
\]

In this study, the total amount of OAA formed is expressed as a function of \([\text{AcCoA}]_0\), the AcCoA utilized at the beginning of the first multicycle turn, as expressed in Eq. 8 (see Schemes 1 and 2).

\[
[\text{OAA}] = [\text{AcCoA}]_0 \alpha' \left(1 - h - F - z\right)
= Y(1-u)\alpha' \left(1 - h - F - z\right) \quad \text{(Eq. 8)}
\]

It should be stressed that, especially in the presence of added alanine, a significant part of OAA does not require AcCoA to be formed but is derived from endogenous or exogenous OAA precursors such as alanine. Therefore, to explain the total OAA formation, it was necessary to replace \(g\), the tricarboxylic acid cycle recycling factor, by \(F\), which is an equivalent recycling factor. Note that the higher the ratio of AcCoA utilized to OAA formed, the lower the ratio \(F/g\).

Note also that, during this metabolism, a part of the AcCoA can be formed from OAA and thus, gives rise to successive turns of the OAA \(\rightarrow\) P-enolpyruvate \(\rightarrow\) Pyr \(\rightarrow\) AcCoA \(\rightarrow\) OAA cycle, the other part being obtained from the added acetate.

From Schemes 1 and 2, one can demonstrate the following equations (Eqs. 9–15).

\[
[^{13}C\text{Glx}]_{\text{AcCoA}(+\text{Ala})} = [^{13}C\text{Glx}]_{\text{OAA}(+\text{Ala})}(1 - s') \quad \text{(Eq. 9)}
\]

\[
(1 - s') = (1 - s) \cdot \left(1 + \frac{q}{k_2}\right) \quad \text{(Eq. 10)}
\]

\[
(1 - s') = \frac{1}{1 - (1 - k_1 - k_2)} \quad \text{(Eq. 11)}
\]

\[
(1 - s') = \frac{1}{1 - k_1} \cdot \left(1 - \frac{k_2}{1 - s}\right) \quad \text{(Eq. 12)}
\]

\[
[^{13}C\text{Glx}]_{\text{AcCoA}(+\text{Ala})} = [^{13}C\text{Glx}]_{\text{AcCoA}(+\text{Ala})}(1 - a) \cdot \left(1 - u\right)(1 - s') \quad \text{(Eq. 14)}
\]

\[
[^{13}C\text{Glx}]_{\text{OAA}(+\text{Ala})} = [^{13}C\text{Glx}]_{\text{AcCoA}(+\text{Ala})}(1 - a) \cdot \left(1 - u\right)(1 - s') \quad \text{(Eq. 15)}
\]

Moreover, Eq. 16 shows,

\[
[^{13}C\text{Glx}]_{\text{AcCoA}(+\text{Ala})} = [^{13}C\text{Glx}]_{\text{AcCoA}(+\text{Ala})} \left(1 - \frac{u}{1 - \left(1 - s'\right)}\right) \quad \text{(Eq. 16)}
\]

thus, as shown in Eq. 17,

\[
[^{13}C\text{Glx}]_{\text{AcCoA}(+\text{Ala})} = [^{13}C\text{Glx}]_{\text{AcCoA}(+\text{Ala})} \left(1 - \frac{u}{1 - \left(1 - s'\right)}\right) \quad \text{(Eq. 17)}
\]

Knowing \(Y\), the amount of acetate utilized, Eq. 17 allows us to...
Thus, as shown in Eq. 21,
\[ [\text{AcCoA}]^{\text{Ac}+\text{Ala}} = Y + ([\text{OAA}]^{-r} + [\text{Pyr}]_z)^{\text{Ac}+\text{Ala}}d \]  
(Eq. 21)
In the absence of alanine, the difference between the acetate-derived AcCoA and the OAA available to form citrate is so important that virtually no OAA or OAA precursor is available to form AcCoA, as shown in Eq. 22,
\[ ([\text{OAA}]^{-r} + [\text{Pyr}]_z)^{\text{Ac}+\text{Ala}}d = 0 \]  
(Eq. 22)
Combining Eq. 18 either to Eq. 21 or Eq. 23, we obtain Eqs. 24 and 25,
\[ \left[^{13}\text{Glx}\right]^{\text{Ac}+\text{Ala}} = Y(1 - u)(1 - s') \]  
(Eq. 23)
Then, as shown in Eq. 20,
\[ \left[^{13}\text{Glx}\right] = Y(1 - u)(1 - s') \]  
(Eq. 24)
\[ \left[^{13}\text{Glx}\right] = Y(1 - u)(1 - s') \]  
(Eq. 25)
where \(^{13}\text{Glx}\)^{Ac+Ala} but not \(^{13}\text{Glx}\)^Ac, represents the total amount of Glx accumulated.

Indeed, in the presence of alanine, the accumulation of Glx not derived from citrate is considered to be negligible, whereas we demonstrate using Eq. 17 that it is not negligible in the absence of alanine. Once \(^{13}\text{Glx}\)^Ac and \(^{13}\text{Glx}\)^Ac+Ala are obtained from Eq. 17 and enzymatic measurement, respectively, \([\text{AcCoA}]^{\text{Ac}+\text{Ala}}\) and \([\text{AcCoA}]^{\text{Ac}+\text{Ala}}\) can be calculated using Eq. 18.

Knowing \([\text{AcCoA}]\) and \(Y\), Eq. 21 allows the calculation of the flux through pyruvate dehydrogenase, as shown in Eq. 26.
\[ \left[\text{PDH}\right] = ([\text{OAA}]^{-r} + [\text{Pyr}]_z)d \]  
(Eq. 26)
The proportion \(u\) of AcCoA converted into AcAc is given by Eq. 27.
\[ u = [\text{AcAc}]/[\text{AcCoA}] \]  
(Eq. 27)
Knowing \(Y\), the latter equation and Eq. 17 allow the calculation of \(s'\) and, using Eq. 20, the flux through citrate synthase, can be calculated in Eq. 18, and since \(s'\) is calculated. The \(\alpha\)-ketoglutarate dehydrogenase flux can be calculated as follows in Eq. 28.
\[ \left[\text{oKGDH}\right] = ([\text{CS}]^{-r} - [\text{OAA}]^{-r}s = [\text{OAA}]^{-r}g \]  
(Eq. 28)
We have obtained \([\text{OAA}]^{-r}\), but subsequent calculations from experimental data cannot yield a precise value for \([\text{OAA}]^{-r}\). However, in the presence of alanine, the proportion \(g\) has already been obtained by studying the fate of the alanine carbons (10). Then, Eq. 28 allows the calculation of \([\text{OAA}]^{\text{Ac}+\text{Ala}}\), and Eq. 5 allows the calculation of \(a\) from \(g\).

OAA is formed either from pyruvate carboxylase or from \(\alpha\)-ketoglutarate dehydrogenase, and thus, pyruvate carboxylase flux can be calculated as shown in Eq. 29.
\[ [\text{PC}]^\gamma = [\text{OAA}]^{-r} - [\text{oKGDH}] \]  
(Eq. 29)
Moreover, as shown in Eq. 30, Schemes 1 and 2 show,
\[ [\text{PC}] = ([\text{OAA}]^{-r} + [\text{Pyr}]_z)c \]  
(Eq. 30)
Note that, as indicated above, when acetate is added as the sole substrate, one cannot obtain the value of the parameter \(g\). Therefore, Eq. 28 cannot be utilized to calculate \([\text{OAA}]^{\text{Ac}}\). It follows that fluxes through pyruvate carboxylase, phosphoenolpyruvate carboxykinase, and pyruvate kinase cannot be obtained separately.

The net flux of oxalacetate formation, \([\text{PC}] - [\text{PEPCK}]\), is equal to the net output of \(\alpha\)-ketoglutarate, noted \(\text{net}\left[\text{aKG} \rightarrow \text{Glu}\right]\), that is also given by the amount of glutamate plus glutamine accumulated, noted \(^{13}\text{Glx}\). Moreover, \([\text{PK}] = [\text{PEPCK}]^{-r}\) (Scheme 1). Then, as shown in Eqs. 31 and 32,
\[ [\text{PK}]^{\gamma} = [\text{PEPCK}]^{\gamma} \]  
(Eq. 31)
and
\[ [\text{PC}]^{\gamma} - [\text{PEPCK}]^{\gamma} = [\text{PC}]^{\gamma} - [\text{PK}]^{\gamma} \]  
(Eq. 32)
With acetate as the sole substrate, no serine was found to accumulate; thus \(r = 1\) (Scheme 1). From Schemes 1 and 2, one can deduce that, as shown in Eqs. 33–35,
\[ [\text{LDH}] = ([^{13}\text{Glu}])^r \]  
(Eq. 33)
\[ [^{13}\text{Pyr}] = ([^{13}\text{OAA}]^{-r} + [^{13}\text{Pyr}]_z)(1 - c - d - l) \]  
(Eq. 34)
\[ [^{13}\text{Ser}] = ([^{13}\text{OAA}]^{-r} + [^{13}\text{Pyr}]_z)^c \]  
(Eq. 35)
From Schemes 1 and 2 and Eqs. 3, 26, 30, 33, and 34, we obtain the equation shown in Eq. 36.
\[ [^{13}\text{Pyr}] = ([^{13}\text{OAA}]^{-r} + [^{13}\text{Pyr}]_z) \]  
(Eq. 36)
Then, \(c\) and \(d\) can be calculated using Eqs. 30 and 36 and Eqs. 26 and 36, respectively, as shown in Eq. 37.
\[ \text{and, as shown in Eq. 38.} \]  
(Eq. 38)
\[ \text{From Scheme 1 and Eqs. 2, 35, and 36, we have Eq. 39.} \]  
(Eq. 39)

Knowing \([\text{OAA}]^{\text{Ac}+\text{Ala}}\) and \(a\), Eq. 39 allows us to calculate \([\text{Pyr}]_0^{\text{Ac}+\text{Ala}}\), which allows us to use Eq. 36 to calculate the flux through pyruvate kinase, \([\text{PK}] = [\text{OAA}]^{\text{Ac}+\text{Ala}}(1 - a)^r\), and since \(d\) is known (see Eq. 38), one can calculate the flux through pyruvate dehydrogenase as \([\text{PDH}] = [\text{OAA}]^{\text{Ac}+\text{Ala}}(1 - a)rd\).

From the previous equations one can easily calculate the following parameters: \(r; l = \left[^{13}\text{La}\right]/[\text{Pyr}]; p = 1 - c - d - l = \left[^{13}\text{Pyr}\right]/[\text{Pyr}], \text{the proportion of pyruvate formed that accumulated. The proportions } h \text{ and } z \text{ are calculated using the Eqs. 4 and 6, respectively. Eq. 8 yields (1 - h - F - z) = Y(1 - u) \cdot s'/[\text{OAA}], whereas from } Y, u, \text{ and } s', \text{ one can calculate } Y(1 - u) \cdot s', \text{ and since } h \text{ and } z \text{ are already known, } F \text{ can be obtained.} \]

After its formation through citrate synthase, a part of the \(\alpha\)-ketoglutarate is decarboxylated in the citric acid cycle by \(\alpha\)-ketoglutarate dehydrogenase, whereas the other part corresponding to the net flux of \(\alpha\)-ketoglutarate to glutamate, \(\text{net}\left[\text{aKG} \rightarrow \text{Glu}\right]\), leaves the citric acid cycle. Thus, \(\text{net}[\text{aKG} \rightarrow \text{Glu}] = [\text{CS}] - [\text{oKGDH}]\).

Note also that: \(\text{net}[\text{aKG} \rightarrow \text{Glu}] = \left[^{13}\text{Glx}\right] = \left[^{13}\text{GS}\right] + \left[^{13}\text{Glu}\right]. \)
Since the glutamate and the glutamate plus glutamine accumulated that can be explained by acetate metabolism are represented by [\textsuperscript{13}C\textsuperscript{Glu}] and [\textsuperscript{13}C\textsuperscript{Glx}], respectively, flux through glutamine synthetase, [GS], can be calculated.

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