Acetate stimulates flux through the tricarboxylic acid cycle in rabbit renal proximal tubules synthesizing glutamine from alanine: a ^{13}C NMR study

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Although glutamine synthesis has a major role in the control of acid–base balance and ammonia detoxification in the kidney of herbivorous species, very little is known about the regulation of this process. We therefore studied the influence of acetate, which is readily metabolized by the kidney and whose metabolism is accompanied by the production of bicarbonate, on glutamine synthesis from variously labelled [^{13}C]alanine and [^{14}C]alanine molecules in isolated rabbit renal proximal tubules. With alanine as sole exogenous substrate, glutamine and, to a smaller extent, glutamate and CO_2 , were the only significant products of the metabolism of this amino acid, which was removed at high rates. Absolute fluxes through the enzymes involved in alanine conversion into glutamine were assessed by using a novel model describing the corresponding reactions in conjunction with the ^{13}C NMR, and to a smaller extent, the radioactive and enzymic data. The presence of acetate (5 mM) led to a large stimulation

of fluxes through citrate synthase and α -oxoglutarate dehydrogenase. These effects were accompanied by increases in the removal of alanine, in the accumulation of glutamate and in flux through the anaplerotic enzyme pyruvate carboxylase. Acetate did not alter fluxes through glutamate dehydrogenase and glutamine synthetase; as a result, acetate did not change the accumulation of ammonia, which was negligible under both experimental conditions. We conclude that acetate, which seems to be an important energy-provider to the rabbit renal proximal tubule, simultaneously traps as glutamate the extra nitrogen removed as alanine, thus preventing the release of additional ammonia by the glutamate dehydrogenase reaction.

Key words: glutamine regulation, kidney metabolism, metabolic modelling.

INTRODUCTION

It has been well established that a high activity of glutamine synthetase (EC 6.3.1.2.), the enzyme that catalyses the conversion of glutamate and ammonia into glutamine, is present in the kidney of herbivorous species such as rabbits, guinea-pigs and sheep [1–4]. The activity of this enzyme in the kidney of these species is considered to be a key factor that limits the release, into both the renal vein and the urine, of the ammonia generated by the renal cells. Thus renal glutamine synthesis contributes not only to a detoxifying process that removes ammonia, a compound that is potentially toxic to the nervous system [5], but also to systemic acid–base balance regulation.

In the rabbit nephron, the functional unit of the kidney, glutamine synthetase activity has been localized in the glomerulus, the entire proximal tubule and the distal straight tubule [4]. In agreement with the latter findings, suspensions of rabbit proximal tubules have been shown to synthesize glutamine at high rates from a variety of precursors including alanine [6,7], aspartate [6–8], glutamate [9] and glucose plus ammonia [10].

However, although flux through glutamine synthetase is able to control the availability of ammonia in the kidney, only some studies have investigated the regulation of glutamine synthesis in this nephron segment. Using ^{13}C NMR spectroscopy, Jans and Willem [6] studied alanine metabolism in suspensions of proximal tubules from diabetic rabbits; they concluded that the proportion

of ^{13}C label from [$3\text{-}^{13}\text{C}$]alanine that entered the tricarboxylic acid cycle via pyruvate carboxylase relative to the proportion that entered via pyruvate dehydrogenase was no different from that obtained in tubules from normal rabbits. We have recently demonstrated that the rate of glutamine synthesis along the proximal tubule from both alanine and aspartate is regulated in a segment- and substrate concentration-dependent manner [11]. Lietz and Bryla [8] and Lietz et al. [7] have also studied the influence of glycerol, lactate and ketone bodies, which are potentially important substrates for rabbit proximal tubules, on glutamine synthesis from alanine and aspartate. They found that these compounds might either stimulate or inhibit glutamine synthesis from these two amino acids. Thus the latter results indicate that substrate interactions might greatly influence glutamine synthesis in rabbit proximal tubules.

In the present study we tested whether acetate, which represents the main volatile fatty acid in rabbit blood [12] and is metabolized at high rates by rabbit proximal tubules [9], regulates glutamine synthesis from alanine in these tubules. We therefore conducted a study involving the metabolic balance of substrate, the incorporation of label into metabolites of alanine, and the modelling of alanine metabolism (see the description of the model in the Materials and methods section). The results obtained allowed us to determine absolute fluxes through enzymes involved in alanine metabolism by rabbit renal proximal tubules. These results demonstrate that acetate slightly stimulated fluxes through

Abbreviations used: AcCoA, acetyl-CoA; Cit, citrate; CS, citrate synthase; Lac, lactate; OAA, oxaloacetate; αOG , α -oxoglutarate; αOGDH , α -oxoglutarate dehydrogenase.

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alanine aminotransferase and pyruvate carboxylase and greatly increased fluxes through citrate synthase and α -oxoglutarate dehydrogenase (α OGDH) without altering fluxes through pyruvate dehydrogenase, glutamate dehydrogenase and glutamine synthetase. These results indicate that, in isolated rabbit renal proximal tubules, acetate is an energy-provider that does not alter glutamate deamination or glutamine synthesis, the two processes controlling the availability of ammonia.

MATERIALS AND METHODS

Reagents

L-Alanine and glutaminase (grade V) were from Sigma. Other enzymes and coenzymes were purchased from Boehringer Mannheim (Meylan, France). L-[U- ^{14} C]Alanine (5.55 GBq/mmol) and L-[1- ^{14} C]alanine (1.85 GBq/mmol) were obtained from Amersham Corp. (Les Ulis, France) and NaH ^{14} CO $_3$ (2.07 GBq/mmol) was supplied by Dositek (Orsay, France).

L-[2- ^{13} C]Alanine was obtained from ICN Biomedicals (Cambridge, MA, U.S.A.). L-[3- ^{13} C]Alanine was obtained from Interchim (Montluçon, France). All ^{13} C-labelled compounds had a 99% isotopic abundance.

Rabbits

Female rabbits (1.8–2 kg; New Zealand albino strain) were obtained from the Élevage des Dombes (Châtillon-sur-Chalaronne, France) and were fed a standard diet (U. A. R., Villemoisson-sur-Orge, France).

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. [13]. Incubations other than those involving radioactive substrates were performed for 60 min at 37 °C in a shaking water bath, in 25 ml stoppered Erlenmeyer flasks in an O $_2$ /CO $_2$ (19/1) atmosphere. The flasks contained 1 ml of the tubule suspension plus 3 ml of Krebs–Henseleit medium [14], either supplemented or not with substrates, i.e. 5 mM (final concentration) L-[U- ^{14} C]alanine (3×10^3 Bq per flask), L-[1- ^{14} C]alanine (10^3 Bq per flask), L-[2- ^{13} C]alanine or L-[3- ^{13} C]alanine, in the absence or the presence of 5 mM (final concentration) acetate or 25 mM NaH ^{14} CO $_3$ (10^6 Bq per flask). These differently labelled alanine molecules were used in an attempt to define the fate of all the alanine carbons, which depends on the metabolic pathways involved. Moreover, ^{14} C-labelled bicarbonate was used to measure the pyruvate carboxylase-mediated incorporation of the bicarbonate carbon into C-1 of glutamate plus glutamine. In all experiments, each experimental condition was performed in quadruplicate. Incubation was stopped by the addition of HClO $_4$ [final concentration 2% (v/v)] to each flask. Metabolite assays were conducted on the neutralized supernatant. In all experiments, zero-time flasks, with and without substrates, were prepared by the addition of HClO $_4$ immediately after the tubules. When radioactive alanine or bicarbonate was present in the medium, incubation, deproteinization, collection and measurement of the ^{14} CO $_2$ formed were performed as described by Baverel and Lund [15]. After removal of the denatured protein by centrifugation, the supernatant was neutralized with a mixture of 20% (w/v) KOH and 1% (v/v) H $_3$ PO $_4$ (8 M) for metabolite determination, measurement of bicarbonate fixation and NMR spectroscopy.

Analytical methods

Metabolite assays

Glucose, glycogen, 3-glycerophosphate, lactate, pyruvate, glutamate, glutamine, alanine, aspartate, citrate, α -oxoglutarate (α OG), fumarate, malate, acetate, acetoacetate and 3-hydroxybutyrate as well as the dry weight of tubules added to the flasks were determined as described previously [13,15]. Serine was measured by HPLC with the use of the Pico-Tag method [16].

^{13} C NMR techniques

Data were recorded as described previously [9,10] at 100.6 MHz on a Bruker AM-400 WB spectrometer with a 10 mm broadband probe thermostatically controlled at 8 ± 0.5 °C. We used a standard WALTZ 16 pulse sequence for inverse-gated proton decoupling [17]. Assignments were made by comparing the chemical shifts obtained with published values [18,19].

Calculations

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 $\pm$ S.E.M., are expressed in μ mol of substances removed or produced per flask per unit time (60 min).

The rates of release of ^{14} CO $_2$ from the ^{14} C-labelled alanine species used were calculated by dividing the radioactivity in ^{14} CO $_2$ by the specific radioactivity of each labelled carbon of the alanine species of interest measured in each medium.

Fixation of ^{14} CO $_2$ from [^{14} C]bicarbonate was calculated by dividing the acid-stable radioactivity present in the incubation medium after incubation by the specific radioactivity of the total 'bicarbonate plus CO $_2$ ' pool measured in the zero-time flasks. The formation of [1- ^{14} C]glutamate plus [1- ^{14} C]glutamine was measured by the method of Squires and Brosnan [20].

When [^{13} C]alanine species were the substrate, the transfer of the C-2 or C-3 of alanine to a given position in a given metabolite was calculated from $(L_m - l_m)/(E_s - e_s)$, in which L_m is the amount of ^{13} C measured in the corresponding NMR resonance, l_m is the natural ^{13} C abundance (1.1%) multiplied by the amount of metabolite assayed enzymically, E_s is the ^{13} C enrichment of the C-2 or C-3 of alanine and e_s is the natural ^{13} C abundance.

Description of the model of alanine metabolism

Schematic representation of alanine metabolism

Scheme 1 shows the successive proportions of the metabolite conversion through the different enzymes involved in alanine metabolism: three different cycles are functioning simultaneously: (1) the citric acid cycle, (2) the 'Glu $\rightarrow$ α OG $\rightarrow$ Glu' cycle and (3) the 'Glu $\rightarrow$ Gln $\rightarrow$ Glu' cycle.

In this model, as in our previous models [21,22], the calculation of parameters (corresponding to ratios) is based on the fates of individual carbons of the substrate. These are C-1, C-2 and C-3 of the alanine molecule together with the fate of the incorporated labelled CO $_2$; they are represented in Scheme 2.

Notation

Let $[C_y \text{Met}]^{C_z \text{Ala}}$ and $[C_y \text{Met}]^{\text{Ala} + * \text{CO}_2}$ be the amount of metabolite Met formed labelled on carbon y ($1 \leq y \leq 5$) arising from alanine labelled on carbon z ($1 \leq z \leq 3$) and from alanine plus ^{14} C-labelled CO $_2$ respectively.

Scheme 1 Metabolic cycles operating during alanine metabolism

The scheme shows three cycles functioning simultaneously. At each turn of the citric acid cycle, a proportion g of OAA is resynthesized. The scheme also shows the 'Glu $\rightarrow$ α OG $\rightarrow$ Glu' and 'Glu $\rightarrow$ Gln $\rightarrow$ Glu' cycles, in which the proportion of glutamate recycled at each turn is k_1 and k_2 respectively. The proportion g takes into account the recycling of α OG through glutamate and glutamine, resulting from the functioning of these two cycles. For the formation of citrate, the proportion of alanine-derived AcCoA condensed with OAA not derived from alanine and the proportion of alanine-derived OAA condensed with AcCoA not derived from alanine are e and f respectively. Abbreviations: Ac, acetate; Lac, lactate; PEP, phosphoenolpyruvate; Pyr, pyruvate.

Let $[(C_y + C_{y'})\text{Met}]^{C_2\text{Ala}}$ and $[(C_{y,y'})\text{Met}]^{C_2\text{Ala}}$ be the sum of the amounts of $C_y\text{Met}$ plus $C_{y'}\text{Met}$ and the amount of C_y plus $C_{y'}$ doubly labelled metabolite (Met) respectively.

Let $[C_y(\text{Met1} + \text{Met2})]^{C_2\text{Ala}}$ be the amount of metabolite (Met1) labelled on carbon y plus the amount of metabolite (Met2) labelled on carbon y .

The accumulated amount of a metabolite will be indicated by a $\wedge$ sign added to the left of its name inside the square brackets.

Calculations of the parameters of the model

As indicated previously [21,22], the amount (in $\mu\text{mol/h}$) of any given intermediate or end product formed from the substrate alanine is obtained by multiplying the amount of substrate removed (X) by the successive proportions of intermediates passing through the different pathways leading to the intermediate or end product of interest. The parameters used are defined in Table 5.

Let us call g the proportion of any metabolite resynthesized after each complete turn of the citric acid cycle. Then the total

proportion of any metabolite resynthesized after an infinite number of citric acid cycle turns is given by:

$$\sum_{n=0}^{\infty} g^n = 1/(1-g) \quad (1)$$

A complete turn of the metabolic cycle is considered to have occurred as soon as the metabolite of interest in the cycle has been resynthesized once.

This model also integrates the α OG recycling through glutamate and glutamine, which is denoted by a double prime ($''$) (see Scheme 2).

Scheme 2 also shows that g , the recycling factor of the citric acid cycle, is:

$$g = as'' \quad (2)$$

Therefore g takes into account the recycling of α OG through glutamate and glutamine.

The other parameters of the model were calculated by using the following equations derived from Schemes 1 and 2:

$$Xl = [C_3\text{Lac}]^{C_3\text{Ala}} \quad (3)$$

(in which Lac stands for lactate) and

$$l = [C_3\text{Lac}]^{C_3\text{Ala}}/X \quad (4)$$

while

$$c + d = 1 - l \quad (5)$$

$$[C_2\text{Ser}]^{C_3\text{Ala}} = [C_2\text{OAA}]^{C_3\text{Ala}}bj$$

and

$$[C_2\text{Lac}]^{C_3\text{Ala}} = [C_2\text{OAA}]^{C_3\text{Ala}}brl$$

Thus

$$rl/j = [C_2\text{Lac}]^{C_3\text{Ala}}/[C_2\text{Ser}]^{C_3\text{Ala}}.$$

Because no formation of $C_2\text{Lac}$ from $C_3\text{Ala}$ was found and eqn. (4) yields $l \neq 0$, it follows that $r = 0$. Moreover, no labelled glucose was found from $C_3\text{Ala}$, indicating that serine is the only product accumulated from alanine-derived phosphoenolpyruvate; therefore $j = 1$.

$$\begin{aligned} [C_2\text{OAA}]^{C_3\text{Ala}} &= X(\frac{1}{2}ds'' + ci) \sum_{n=0}^{\infty} (\frac{1}{2}g)^n \\ &= X(\frac{1}{2}ds'' + ci)/(1 - \frac{1}{2}g) \end{aligned}$$

and finally:

$$[C_2\text{OAA}]^{C_3\text{Ala}} = Xds''/(2-g) + Xci/(1 - \frac{1}{2}g) \quad (6)$$

$$[C_3\text{OAA}]^{C_3\text{Ala}} = X[\frac{1}{2}ds'' + c(1-i)] + \frac{1}{2}g[C_2\text{OAA}]^{C_3\text{Ala}}$$

Thus:

$$[C_3\text{OAA}]^{C_3\text{Ala}} = Xds''/(2-g) + Xc[1-i(1-g)/(1-\frac{1}{2}g)] \quad (7)$$

Then:

$$[C_3\text{OAA}]^{C_3\text{Ala}} - [C_2\text{OAA}]^{C_3\text{Ala}} = Xc(1-2i) \quad (8)$$

$$[C_4\text{OAA}]^{C_1\text{Ala}} = Xci$$

Similarly, the amount of glutamate plus glutamine accumulated, which was derived from acetyl-CoA (AcCoA), denoted $_{AcCoA \rightarrow} [Glx]^{Ala}$, can be expressed as:

$$_{AcCoA \rightarrow} [Glx]^{Ala} = [AcCoA]^{Ala}(1-s'') \quad (16)$$

Eqns. (8) and (15) yield:

$$[C_2Glx]^{C_3Ala} - [C_3Glx]^{C_3Ala} = Xc(1-2i)a(1-s'') \quad (17)$$

Similarly, $[C_3Glx]^{C_2Ala} - [C_2Glx]^{C_2Ala} = Xc(1-2i)a(1-s'')$.

Since X and i are known, eqns. (2) and (17) yield:

$$ca(1-s'') = c(a-g) \quad (18)$$

$$= ([C_2Glx]^{C_3Ala} - [C_3Glx]^{C_3Ala})/[X(1-2i)]$$

$$ca/d = ca(1-s'')/d(1-s'') \quad (19)$$

[see eqns. (14) and (18)]

and $[*CO_2]^{(C_2+C_3)Ala}$

$$= [C_1OAA]^{(C_2+C_3)Ala}a + [C_4OAA]^{(C_2+C_3)Ala}(b+g).$$

Moreover, $[C_1OAA]^{(C_2+C_3)Ala} = [C_4OAA]^{(C_2+C_3)Ala}$ and $a+b=1$.

Therefore:

$$[*CO_2]^{(C_2+C_3)Ala} = [C_4OAA]^{(C_2+C_3)Ala}(1+g) \quad (20)$$

Eqns. (2) and (12) yield $[C_1Glx]^{(C_2+C_3)Ala} = [C_4OAA]^{(C_2+C_3)Ala}(a-g)$.

Thus:

$$(1+g)/(a-g) = [*CO_2]^{(C_2+C_3)Ala}/[C_1Glx]^{(C_2+C_3)Ala} \quad (21)$$

$$[C_1Glx]^{C_1Ala} = Xci(a-g), \text{ and } [C_1Ser]^{C_1Ala} = Xc(1-i)b.$$

Thus:

$$(1-i)b/[i(a-g)] = [C_1Ser]^{C_1Ala}/[C_1Glx]^{C_1Ala} \quad (22)$$

Then:

$$b/(a-g) = [i/(1-i)][C_1Ser]^{C_1Ala}/[C_1Glx]^{C_1Ala} \quad (23)$$

From Scheme 1, one can deduce that:

$$b = 1 - a \quad (24)$$

$$a/b = a/(1-a) = \frac{1}{2}[(a-g)/b + (1+g)/b - 1] \quad (25)$$

[see eqns. (23) and (24)], and

$$a = [a/(1-a)]/(1+[a/(1-a)]) \quad (26)$$

Then:

$$c/d = (ca/d)/a \quad (27)$$

[see eqn. (19)]. Eqns. (19), (5) and (27) yield:

$$d = (c+d)/(1+c/d) \quad (28)$$

and

$$c = (c+d) - d \quad (29)$$

Eqns. (19), (26) and (29) yield:

$$s'' = 1 - ca(1-s'')/ca \quad (30)$$

From Scheme 2 one can deduce that:

$$q/t\beta = [C_2Glu]^{C_3Ala}/[C_2Gln]^{C_3Ala} \quad (31)$$

$$[C_3C_4Cit]^{C_3Ala} = (1-e)[C_2AcCoA]^{C_3Ala}[C_2OAA]^{C_3Ala}/[OAA]^{Ala}$$

(in which Cit stands for citrate) and

$$(1-e) = [OAA]^{Ala}a(1-s'')$$

$$[C_3C_4Glx]^{C_3Ala}/([C_4Glx]^{C_3Ala}[C_4Glx]^{C_3Ala}) \quad (32)$$

$$[OAA]^{Ala} = X(c+des'')/(1-g) \quad (33)$$

or

$$[OAA]^{Ala} = X(c+ds'')/(1-fg) \quad (34)$$

$$[C_2OAA]^{C_3Ala} = X(ic + \frac{1}{2}ds'')/(1 - \frac{1}{2}g) \quad (35)$$

and

$$[AcCoA]^{Ala} = [C_2AcCoA]^{C_3Ala} = Xd \quad (36)$$

To calculate $[OAA]^{Ala}$ and $[AcCoA]^{Ala}$, e is given successive arbitrary values until the calculated value of $[C_3C_4Glx]^{C_3Ala}$ fits with its experimental value.

Thus:

$$[OAA]^{Ala}a(1-f) = [AcCoA]^{Ala}(1-e) \quad (37)$$

Then $(1-f)$ and f can be calculated:

$$(1-f) = [AcCoA]^{Ala}(1-e)/(a[OAA]^{Ala}) \quad (38)$$

$$(1-e) = [OAA]^{Ala}/([OAA]^{Ala} + [OAA]^{non-Ala}) \quad (39)$$

$$(1-f) = [AcCoA]^{Ala}/([AcCoA]^{Ala} + [AcCoA]^{non-Ala}) \quad (40)$$

Thus $[OAA]^{non-Ala} = [OAA]^{Ala}e/(1-e)$ and $[AcCoA]^{non-Ala} = [AcCoA]^{Ala}f/(1-f)$.

Calculations of the enzymic fluxes

The alanine aminotransferase flux is given by the amount X of alanine utilized measured enzymically or by ¹³C NMR: $[OAA]^{Ala}$ = pyruvate carboxylase flux + α OG dehydrogenase flux.

The phosphoenolpyruvate carboxykinase and phosphoserine aminotransferase fluxes given by $[OAA]^{Ala}b$ and $[OAA]^{Ala}bj$ respectively are equal in this particular case because $j=1$ (see Table 5).

The lactate dehydrogenase flux, obtained by multiplying the pyruvate kinase flux by l , is equal to $[OAA]^{Ala}brl$.

The flux through pyruvate dehydrogenase, which is equal to $[AcCoA]^{Ala}$, can be derived from Scheme 1: $[AcCoA]^{Ala} = [OAA]^{Ala}brd$.

The flux through citrate synthase, {CS}, is given by the amount of citrate formed, as shown in Scheme 1: $\{CS\} = [Cit]^{Ala} = [OAA]^{Ala}a + [AcCoA]^{Ala}e$.

The net flux of glutamate transamination into α OG is given by the sole formation of serine.

The net flux through glutamate dehydrogenase is equal to the net flux of glutamate conversion into α OG minus the net flux through phosphoserine aminotransferase.

The net flux through glutamine synthetase is equal to the amount of glutamine accumulated, $[Gln]^{Ala}$, and represents the difference between the glutamine synthetase flux, {GS}, and the glutaminase flux, {Glnase}.

The α OGDH flux, $\{\alpha$ OGDH $\}$, is given by the amount of α OG converted into succinyl-CoA (SucCoA) and subsequently into OAA (see Scheme 1):

$$\{\alpha$$
OGDH $\} = [SucCoA]^{Ala} = s''[Cit]^{Ala}$

Because OAA is produced by either pyruvate carboxylase or α OGDH, the pyruvate carboxylase flux is equal to $[\text{OAA}]^{\text{Ala}}$ minus the α OGDH flux (see Scheme 1).

RESULTS

To quantify fluxes through the main enzymes involved in the conversion of alanine into glutamine, we performed a series of experiments in which we combined enzymic, radioactive and ^{13}C NMR spectroscopy measurements. We used specifically or uniformly ^{14}C -labelled and/or ^{13}C -labelled alanine in the absence and the presence of acetate.

Enzymic measurements of substrate utilization and product formation

Table 1 shows that, in the presence of alanine as the sole substrate, rabbit renal proximal tubules utilized alanine and synthesized glutamine at high rates. Alanine removal and glutamine synthesis were linear with time over a 60 min incubation period (results not shown). Only small amounts of glutamate and serine accumulated and no significant accumulation of pyruvate, lactate, glucose, glycogen, 3-glycerophosphate, intermediates of the tricarboxylic acid cycle, aspartate, ammonia or ketone bodies was observed (Table 1).

When added to the incubation medium, acetate, which was also utilized by the tubules at high rates, led to a small but significant stimulation of alanine removal and to a large increase in glutamate accumulation (Table 1). Surprisingly, because glutamine synthesis from alanine plus glycerol has been shown to be inhibited by the presence of ketone bodies in rabbit renal proximal tubules [8], acetate did not significantly change glutamine production or ammonia accumulation (Table 1).

Considering that each alanine, glutamate and serine molecule contains one nitrogen atom and that each glutamine molecule contains two nitrogen atoms, nitrogen balance calculations indicate that the nitrogen removed as alanine could be fully accounted for by the nitrogen found as glutamine, glutamate and

serine. These calculations also suggest that the production of glutamine from endogenous substrates observed in the absence of exogenous substrate (see Table 1) was virtually suppressed in the presence of alanine in both the presence and the absence of acetate.

Radioactive measurements

Table 2 shows that, in the presence of alanine alone, most of the CO_2 released from alanine was accounted for by C-1 of this amino acid. This indicates that C-2 and C-3 of alanine mainly contributed to the carbons of the glutamine and glutamate found and were only to a small extent metabolized through α OGDH during the first Krebs cycle turn, to give rise to CO_2 during subsequent turns of this cycle. The large release of C-1 of alanine as CO_2 is in agreement with significant fluxes of the alanine carbon skeleton through pyruvate dehydrogenase and isocitrate dehydrogenase; indeed, the former enzyme releases C-1 of pyruvate and the latter releases C-6 of isocitrate arising from C-1 of the OAA formed by the pyruvate carboxylase reaction (see Scheme 2). Most but not all the glutamine plus glutamate found (Table 1) was labelled on C-1 when the tubules were incubated in the presence of unlabelled alanine plus $\text{NaH}^{14}\text{CO}_3$ (Table 2). This shows that the OAA formed by the pyruvate carboxylase reaction underwent equilibration with fumarate to only a small extent.

The presence of acetate did not significantly change the release of $^{14}\text{CO}_2$ from $[1\text{-}^{14}\text{C}]\text{alanine}$ or the labelling of C-1 of glutamate plus glutamine after incubation in the presence of alanine plus $\text{NaH}^{14}\text{CO}_3$ (Table 2). In contrast, acetate stimulated the release of $^{14}\text{CO}_2$ from $[\text{U-}^{14}\text{C}]\text{alanine}$; this means that acetate increased the release of C-2 and C-3 of alanine as CO_2 and therefore that flux through α OGDH was stimulated.

^{13}C NMR spectroscopy measurements

Figures 1 shows the ^{13}C NMR spectra of HClO_4 extracts obtained after 60 min of incubation of rabbit tubules with $[3\text{-}^{13}\text{C}]\text{alanine}$

Table 1 Effect of 5 mM acetate on the metabolism of 5 mM L-alanine in rabbit kidney tubules

Kidney tubules (26.8 ± 2.1 mg dry wt. per flask) were incubated for 60 min as described in the Materials and methods section. Results for metabolite removal (—) or production are reported as means $\pm$ S.E.M. for four experiments performed in quadruplicate. A paired Student's *t* test was used to measure the statistical difference from the control with alanine alone: *P < 0.05; **P < 0.01; ***P < 0.001. The radioactivity and ^{13}C NMR results corresponding to these experiments are shown in Tables 2, 3 and 4.

Experimental condition	Metabolite removal (—) or production ($\mu\text{mol/h}$)							
	Alanine	Acetate	Glutamine	Glutamate	Lactate	Pyruvate	Serine	Ammonia
Alanine (5 mM)	-10.86 ± 0.76	-0.01 ± 0.03	5.10 ± 0.33	0.68 ± 0.04	-0.09 ± 0.04	0.06 ± 0.01	0.33 ± 0.19	0.08 ± 0.01
Alanine (5 mM) + acetate (5 mM)	$-12.54 \pm 0.55^*$	$-10.52 \pm 0.43^{***}$	4.65 ± 0.34	$2.40 \pm 0.14^{***}$	$0.33 \pm 0.05^{**}$	$0.12 \pm 0.04^{**}$	0.35 ± 0.20	0.01 ± 0.01
No added substrate	0.15 ± 0.23	-0.02 ± 0.01	2.32 ± 0.01	-0.07 ± 0.01	-0.15 ± 0.02	0.02 ± 0.23	-0.02 ± 0.01	-0.05 ± 0.03

Table 2 Effect of 5 mM acetate on the release of $^{14}\text{CO}_2$ from 5 mM $[1\text{-}^{14}\text{C}]\text{alanine}$ and $[\text{U-}^{14}\text{C}]\text{alanine}$ and on the accumulation of $[1\text{-}^{14}\text{C}]\text{glutamate}$ plus $[1\text{-}^{14}\text{C}]\text{glutamine}$ as a result of $^{14}\text{CO}_2$ fixation during the metabolism of 5 mM alanine in rabbit kidney tubules

Kidney tubules (26.8 ± 2.1 mg dry wt. per flask) were incubated for 60 min as described in the Materials and methods section. Results are reported as means $\pm$ S.E.M. for four experiments. Substrate utilization and product formation, measured enzymically, are shown in Table 1. Statistical difference was measured by a paired Student's *t* test from the control without acetate: *P < 0.01.

Experimental condition	$^{14}\text{CO}_2$ from $[1\text{-}^{14}\text{C}]\text{Ala}$ ($\mu\text{mol/h}$)	$^{14}\text{CO}_2$ from $[\text{U-}^{14}\text{C}]\text{Ala}$ ($\mu\text{mol/h}$)	$[1\text{-}^{14}\text{C}]\text{Glx}$ from $\text{Ala} + \text{NaH}^{14}\text{CO}_3$ ($\mu\text{mol/h}$)
$[^{14}\text{C}]\text{Alanine}$ (5 mM)	9.28 ± 0.34	11.95 ± 0.53	5.18 ± 0.46
$[^{14}\text{C}]\text{Alanine}$ (5 mM) + acetate (5 mM)	10.35 ± 0.71	$14.99 \pm 1.41^*$	6.49 ± 0.67

Figure 1 ^{13}C NMR spectra (100.62 MHz) of neutralized perchloric acid extracts obtained from rabbit kidney tubules incubated with $[3\text{-}^{13}\text{C}]$ alanine in the absence (top) and the presence (bottom) of acetate

Peaks shown are: 1, alanine C-3 (17.01 p.p.m.); 2, lactate C-3 (20.97 p.p.m.); 3, glutamine C-3 (27.10 p.p.m.); 4, glutamate C-3 (27.83 p.p.m.); 5, glutamine C-4 (31.70 p.p.m.); 6, glutamate C-4 (34.16 p.p.m.); 7, glutamine C-2 (55.10 p.p.m.); 8, glutamate C-2 (55.53 p.p.m.); 9, serine C-3 (61.17 p.p.m.); 10, glutamine C-1 (175.80 p.p.m.); 11, glutamate C-1 (176.25 p.p.m.) (numbers in parentheses are the chemical shifts referred to tetramethylsilane). An expanded view of the C-4 and C-3 resonances of glutamate and glutamine including the coupling between C-4 and C-3 is shown.

in the absence and the presence of acetate. As expected, the main labelled non-volatile carbon products of alanine metabolism were glutamine and, to a smaller extent, glutamate. Small amounts of labelled lactate and serine were also found.

From these spectra and from those obtained when $[2\text{-}^{13}\text{C}]$ alanine was present with and without acetate (results not

shown), we calculated the quantities of labelled products after correction for the natural abundance of ^{13}C as described in the Materials and methods section (see Tables 3 and 4). In agreement with the results presented in Table 1 showing a stimulation of alanine removal by acetate, this compound diminished the amount of labelled alanine remaining in the incubation medium

Table 3 Effect of 5 mM acetate on the metabolism of 5 mM [3-¹³C]alanine in rabbit kidney tubules

Kidney tubules (26.8 ± 2.1 mg dry wt. per flask) were incubated for 60 min as described in the Materials and methods section. Results for ¹³C-labelled products accumulated are reported as means ± S.E.M. for four experiments performed in quadruplicate. A paired Student's *t* test was used to measure the statistical difference from the control with alanine alone: *P < 0.05; **P < 0.01; ***P < 0.001.

Experimental condition	Amount of labelled product (μmol/h)					
	Glutamate				Alanine	Lactate
	C1	C2	C3	C4	C3	C3
[3- ¹³ C]Ala	0.02 ± 0.01	0.61 ± 0.06	0.24 ± 0.02	0.52 ± 0.05	9.47 ± 0.76	0.13 ± 0.04
[3- ¹³ C]Ala + acetate	0.09 ± 0.07**	1.19 ± 0.14**	0.72 ± 0.07**	0.67 ± 0.09	7.56 ± 0.73***	0.67 ± 0.12**

Experimental condition	Amount of labelled product (μmol/h)					
	Glutamine				Serine	
	C1	C2	C3	C4	C2	C3
[3- ¹³ C]Ala	0.09 ± 0.01	3.22 ± 0.35	0.88 ± 0.15	2.75 ± 0.38	0.06 ± 0.01	0.36 ± 0.05
[3- ¹³ C]Ala + acetate	0.14 ± 0.02	2.59 ± 0.30	1.41 ± 0.15**	1.28 ± 0.20*	0.00 ± 0.01**	0.14 ± 0.01*

Table 4 Effect of 5 mM acetate on the metabolism of 5 mM [2-¹³C]alanine in rabbit kidney tubules

Kidney tubules (26.8 ± 2.1 mg dry wt. per flask) were incubated for 60 min as described in the Materials and methods section. Results for ¹³C-labelled products accumulated are reported as means ± S.E.M. for four experiments performed in quadruplicate. A paired Student's *t* test was used to measure the statistical difference from the control with alanine alone: *P < 0.05; **P < 0.01; ***P < 0.001.

Experimental condition	Amount of labelled product (μmol/h)					
	Glutamate				Alanine	Lactate
	C1	C2	C3	C5	C2	C2
[2- ¹³ C]Ala	0.03 ± 0.01	0.18 ± 0.02	0.59 ± 0.03	0.50 ± 0.04	9.55 ± 0.71	0.12 ± 0.07
[2- ¹³ C]Ala + acetate	0.15 ± 0.01**	0.71 ± 0.05**	1.19 ± 0.09**	0.86 ± 0.05**	7.49 ± 0.50*	0.76 ± 0.17**

Experimental condition	Amount of labelled product (μmol/h)					
	Glutamine				Serine	
	C1	C2	C3	C5	C2	C3
[2- ¹³ C]Ala	0.16 ± 0.03	0.91 ± 0.13	3.27 ± 0.32	2.97 ± 0.35	0.25 ± 0.02	0.09 ± 0.01
[2- ¹³ C]Ala + acetate	0.28 ± 0.03**	1.40 ± 0.19*	2.58 ± 0.23	1.50 ± 0.14*	0.11 ± 0.02***	0.07 ± 0.02

at the end of the incubation period (Tables 3 and 4). No carbon of alanine other than that labelled at the start of the incubation was found to be labelled after 60 min of incubation; this means that no resynthesis of alanine occurred.

With [3-¹³C]alanine as substrate (Table 3), the labelling of C-4 of glutamate and glutamine indicates that the pyruvate formed from alanine by the alanine aminotransferase reaction passed through pyruvate dehydrogenase to give [2-¹³C]AcCoA and then [4-¹³C]citrate. The subsequent metabolism gave [4-¹³C]αOG via the tricarboxylic acid cycle and then [4-¹³C]glutamate and [4-¹³C]glutamine by the successive operation of alanine aminotransferase and glutamine synthetase. The fact that, from [3-¹³C]alanine, more [2-¹³C]glutamate plus [2-¹³C]glutamine was formed than [3-¹³C]glutamate plus [3-¹³C]glutamine (Table 3) is

in agreement with a limited equilibration with fumarate of the [3-¹³C]OAA formed by pyruvate carboxylase. It is also coherent with a limited flux through αOGDH of the [2-¹³C]αOG formed during the first turn of the tricarboxylic acid cycle. Further evidence of a limited flux through αOGDH is the observation that only very small amounts of C-1 of glutamate and glutamine were labelled from [3-¹³C]alanine. Indeed, such labelling occurs when the [2-¹³C]αOG formed during the first Krebs cycle turn is metabolized through αOGDH and then passes through succinate and fumarate, two symmetrical molecules, to give [4-¹³C]OAA. Then, during the second turn of the Krebs cycle, [1-¹³C]αOG yields [1-¹³C]glutamate and [1-¹³C]glutamine by the successive operation of alanine aminotransferase and glutamine synthetase. The addition of acetate led to an increased labelling of C-2 of

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

Values, given as means $\pm$ S.E.M. for four experiments, were calculated from Tables 2–4. Lowercase italic letters indicate the proportion of a given intermediate metabolized at a given step. The symbols of the various proportions are shown in Scheme 1. A paired Student's *t* test was used to measure the statistical difference from the control with alanine as sole substrate: *P < 0.05; **P < 0.01; ***P < 0.001.

Proportion of	Converted to	Parameter notation	Parameter value	
			Without acetate	With acetate
OAA	Citrate	<i>a</i>	0.99 $\pm$ 0.01	0.99 $\pm$ 0.01
OAA	Phosphoenolpyruvate	<i>b</i>	0.01 $\pm$ 0.01	0.01 $\pm$ 0.01
Phosphoenolpyruvate	Pyruvate	<i>r</i>	0.00 $\pm$ 0.01	0.00 $\pm$ 0.01
Phosphoenolpyruvate	Ser	<i>j</i>	1.00 $\pm$ 0.01	1.00 $\pm$ 0.01
Pyruvate	OAA	<i>c</i>	0.53 $\pm$ 0.01	0.59 $\pm$ 0.01*
Pyruvate	AcCoA	<i>d</i>	0.46 $\pm$ 0.01	0.35 $\pm$ 0.01***
Pyruvate	Lactate	<i>l</i>	0.01 $\pm$ 0.01	0.06 $\pm$ 0.01**
α -OG	OAA	<i>s''</i>	0.33 $\pm$ 0.02	0.52 $\pm$ 0.02*
α -OG	Accumulated Glx	1 – <i>s''</i>	0.67 $\pm$ 0.02	0.48 $\pm$ 0.02*

Other parameters	Parameter notation	Parameter value	
		Without acetate	With acetate
Glu accumulated/Gln accumulated	<i>q/t</i> β	0.19 $\pm$ 0.01	0.52 $\pm$ 0.03***
Inversion	<i>i</i>	0.14 $\pm$ 0.01	0.27 $\pm$ 0.01**
Citric acid cycle recycling	<i>g</i> = <i>as''</i>	0.33 $\pm$ 0.02	0.51 $\pm$ 0.02*
Ala-derived AcCoA condensed with OAA not derived from Ala	<i>e</i>	0.16 $\pm$ 0.05	0.01 $\pm$ 0.02*
Ala-derived OAA condensed with AcCoA not derived from Ala	<i>f</i>	0.52 $\pm$ 0.04	0.70 $\pm$ 0.02*

glutamate, of C-3 of glutamate and glutamine and of C-3 of lactate (Table 3). In contrast, acetate decreased the labelling of C-4 of glutamine and of C-3 of serine.

A comparison of the results obtained with [3-¹³C]alanine and [2-¹³C]alanine shown in Tables 3 and 4 reveals that, as expected, C-2 and the C-3 of alanine behaved in a symmetrical way in both the absence and the presence of acetate. The labelling of C-5 of glutamate and glutamine from [2-¹³C]alanine resulted from the successive formation of [2-¹³C]pyruvate, [1-¹³C]AcCoA and then [5-¹³C] α OG, which was transaminated with alanine. Table 4 also shows that the small labelling of C-1 of glutamate and glutamine formed from [2-¹³C]alanine was greater in the presence than in the absence of acetate; this is in agreement with the acetate-induced increase in flux through α OGDH mentioned above.

Figure 1 also shows doublets that correspond to ¹³C–¹³C couplings between C-3 and C-4 of glutamate and glutamine when [3-¹³C]alanine was the substrate. These couplings originate from glutamate and glutamine molecules that were simultaneously labelled on C-3 and C-4. As indicated in the Model description section, the quantification of these doublets allowed us to determine the proportions (1 – *e*) and (1 – *f*) of labelled OAA and labelled AcCoA respectively.

Calculations of proportions

Table 5 shows the calculated proportions of metabolites converted into the next one(s). It should be emphasized that, although these proportions constitute the basis of our model, they do not give direct access to fluxes that also take into account the formation of the substrate of interest. Table 5 shows that, in both the absence and the presence of acetate, the proportion of the OAA synthesized that was converted into citrate was close to unity and a very small proportion of this OAA was converted

into phosphoenolpyruvate by phosphoenolpyruvate carboxykinase. Table 5 also shows that the phosphoenolpyruvate synthesized did not yield pyruvate but was converted solely into serine.

Of the pyruvate synthesized in the presence of alanine alone, only a very small fraction yielded lactate, whereas about equal proportions were metabolized through pyruvate dehydrogenase and pyruvate carboxylase. The addition of acetate increased the proportions of pyruvate converted into lactate and OAA at the expense of that converted into AcCoA (Table 5).

As can be seen in Table 5, under the control conditions without acetate, approximately one-third and two-thirds of the α OG synthesized were converted into OAA and accumulated as glutamate plus glutamine respectively. The presence of acetate significantly changed these proportions because about half the α OG synthesized was converted into OAA and the other half was recovered as accumulated glutamate plus glutamine.

As expected from the labelling patterns observed in Figure 1 and those shown in Tables 3 and 4 when alanine was the sole substrate, a small but significant proportion of the OAA synthesized by the pyruvate carboxylase reaction was inverted. This inversion results from an equilibration with fumarate owing to the reversible part of the tricarboxylic acid cycle catalysed by malate dehydrogenase and fumarase. This proportion was nearly doubled in the presence of acetate (Table 5).

It can also be seen in Table 5 that, on the addition of acetate, the proportion (*g*) of the OAA recycled at each turn of the tricarboxylic acid cycle was greatly augmented. Acetate also led to a large decrease in the proportion of the alanine-derived AcCoA that condensed with OAA that originated not from alanine but from endogenous sources. Note also that acetate led to a large increase in the proportion of alanine-derived OAA that condensed with AcCoA not derived from alanine. The latter

Table 6 Effect of 5 mM acetate on fluxes through pathways of alanine metabolism in rabbit kidney tubules

Values, reported as means $\pm$ S.E.M. for four experiments, were calculated from Tables 1–5. A paired Student's *t* test was used to measure the statistical difference from the control with alanine as sole substrate: *P < 0.05; **P < 0.01.

Experimental condition	Flux (μ mol/h of C ₃ -unit equivalents)				
	Alanine aminotransferase	Lactate dehydrogenase	Pyruvate dehydrogenase	Pyruvate carboxylase	Phosphoenolpyruvate carboxykinase
Alanine (5 mM)	10.86 $\pm$ 0.78	0.12 $\pm$ 0.04	5.05 $\pm$ 0.46	5.69 $\pm$ 0.30	0.07 $\pm$ 0.01
Alanine (5 mM) + acetate (5 mM)	12.54 $\pm$ 0.55*	0.69 $\pm$ 0.11**	4.45 $\pm$ 0.27	7.40 $\pm$ 0.27*	0.14 $\pm$ 0.02**

Experimental condition	Flux (μ mol/h of C ₃ -unit equivalents)				
	Phosphoserine aminotransferase	Citrate synthase	α OGDH	Glutamate dehydrogenase (Glu $\rightarrow$ α OG)	Glutamine synthetase
Alanine (5 mM)	0.07 $\pm$ 0.01	9.75 $\pm$ 0.88	3.29 $\pm$ 0.47	4.33 $\pm$ 0.48	5.42 $\pm$ 0.36
Alanine (5 mM) + acetate (5 mM)	0.14 $\pm$ 0.02**	15.11 $\pm$ 0.94*	7.85 $\pm$ 0.74*	5.28 $\pm$ 0.35	4.78 $\pm$ 0.23

AcCoA was probably formed from fatty acids released by the degradation of endogenous fatty acids when alanine was added as sole exogenous substrate and from acetate when this compound was added to the incubation medium. Finally, as expected from the results in Table 1, acetate increased the glutamate accumulated relative to the glutamine accumulated.

Calculations of the enzymic fluxes

Table 6 shows the absolute values of fluxes through enzymes involved in alanine metabolism in the absence and the presence of acetate. Because there was no evidence of alanine resynthesis, the flux through alanine aminotransferase was identical with the removal of alanine measured enzymically (see Table 1); this flux was stimulated by acetate. The increased synthesis of glutamate resulting from the stimulation of alanine utilization by acetate was not accompanied by increased fluxes through glutamate dehydrogenase and glutamine synthetase (which explains why glutamate accumulated and no ammonia accumulated) (see Table 1). The increase in flux through lactate dehydrogenase caused by the addition of acetate was also expected from the results in Table 1 and the labelling patterns of lactate seen in Figure 1 and calculated in Tables 3 and 4. Acetate did not alter the flux through pyruvate dehydrogenase but significantly stimulated the flux through pyruvate carboxylase. The resulting increase in OAA availability together with the increased availability of AcCoA owing to acetate utilization by AcCoA synthetase led to large stimulations of flux through CS and α OGDH (Table 6), which are in agreement with the results in Table 2.

Table 6 also reveals that the increased synthesis of OAA caused by acetate was accompanied by a stimulation of fluxes through phosphoenolpyruvate carboxykinase and phosphoserine aminotransferase. However, this stimulation is not consistent with the results in Tables 3 and 4 for serine, for which ^{13}C resonances decreased; this is probably due to the fact that small resonances are difficult to measure accurately, leading to uncertainties in the determination of small fluxes.

DISCUSSION

The present study shows that, as in guinea-pig renal proximal tubules [23], glutamine and, to a smaller extent, glutamate are the

only important non-volatile carbon products of alanine metabolism. This was suggested in a Figure in the paper by Jans and Willem [6], who studied the metabolism of 5 mM [^{13}C]alanine in rabbit renal proximal tubules. However, the information on the quantitative importance of the resonances contained in that Figure should be interpreted with caution. Indeed, Jans and Willem were not able to quantify the ^{13}C enrichment of the different carbons of glutamate and glutamine because they used short relaxation times. The rate of glutamine synthesis from 5 mM alanine found in our study was approx. 1.8-fold greater than that found by Lietz et al. [7]. This is probably because our tubules utilized alanine at a rate that was more than twice that found in the other laboratory [24].

Absolute values of fluxes through enzymes involved in alanine metabolism

As in previous studies, in which we used glucose [10,23] or glutamate [9,24] as the substrate in isolated rabbit renal proximal tubules, we developed an original model of alanine metabolism. This model is based on previous knowledge of the pathway of glutamate and glutamine synthesis from alanine that requires the utilization of two alanine molecules for each glutamate or glutamine found [23]. Our model, in combination with the measurement of alanine removal and ^{13}C NMR and ^{14}C radioactivity measurements, allowed us to measure absolute fluxes through enzymes related to alanine metabolism in these tubules. Such information has not previously been available.

This model, which was specifically designed for the study of alanine metabolism, is based on the same principles as the models used to quantify fluxes related to glucose and glutamate metabolism [21,22]. The advantages of using ^{13}C NMR spectroscopy have been described in detail in our previous papers [9,10].

It should be emphasized that the values of the fluxes in Table 6, which were derived mainly from ^{13}C NMR measurements, are consistent with the enzymic and radioactive measurements presented in Tables 1 and 2, as mentioned in the Results section. Note also that the flux through alanine aminotransferase minus the sum of the fluxes through glutamate dehydrogenase and glutamine synthetase (Table 6) is in excellent agreement with the glutamate accumulated (Table 1).

The fact that, with alanine as sole substrate, the flux through pyruvate dehydrogenase was much smaller than the flux through pyruvate carboxylase might seem surprising at first sight (Table 6); this simply indicates that the degradation of fatty acids derived from endogenous triglycerides provided the missing AcCoA needed to synthesize citrate. It is of interest that, in our study when alanine was the sole substrate, the mean flux ratio of pyruvate carboxylase to pyruvate dehydrogenase was 1.13:1. Note that this ratio is lower than the 1.55:1 found by Jans and Willem [6]; the reason for the difference is unclear. It should be emphasized that they did not measure absolute values of fluxes through the above enzymes but only used the ratios of ¹³C enrichment of specific carbons of glutamate and glutamine to calculate this ratio. In addition, because they used short relaxation times for obtaining their spectra, they had to employ correction factors to compare the different ¹³C resonances, thus introducing potential errors. Although Jans and Willem [6] did not mention whether their rabbits were fed or starved, it is unlikely that the higher value of this ratio that they found was due to the use of starved rabbits; indeed, they found negligible ¹³C resonances of glucose, which cannot explain a higher flux through pyruvate carboxylase.

Effect of acetate

The most important effects of acetate were stimulations of flux through CS and α OGDH and, to a smaller extent, through alanine aminotransferase and pyruvate carboxylase; as a result of the latter effects, the addition of acetate also increased the accumulation of glutamate.

The stimulation of alanine removal induced by acetate can be explained both by an increased availability of α OG resulting from the increased flux through CS and by an accelerated removal of pyruvate by pyruvate carboxylase. At first sight, it might be found surprising that the increased removal of alanine nitrogen was fully accounted for by an increased accumulation of glutamate without any increase in the accumulation of either ammonia or glutamine. Indeed, one might have expected that an increased synthesis of glutamate by alanine aminotransferase would stimulate flux through glutamate dehydrogenase, resulting in an increased availability of ammonia and therefore in a stimulation of flux through glutamine synthetase. Clearly, the increased accumulation of glutamate in the presence of acetate suggests that glutamate dehydrogenase, an NAD⁺-dependent enzyme, was rate-limiting. This was probably due to a shift of the mitochondrial NAD⁺-to-NADH ratio to a more reduced state as a result of acetate oxidation. Consistent with this view are (1) the increased flux through key enzymes of the tricarboxylic acid cycle (see Table 6), (2) the increased equilibration of OAA with fumarate (see Table 5) probably resulting from a stimulation of the malate dehydrogenase reaction in the backwards direction secondary to a greater availability of NADH, and (3) the demonstration that such a shift occurs when rabbit renal proximal tubules oxidize fatty acids [25].

The stimulation of the flux through pyruvate carboxylase (see Table 6), which logically matched the increase in the accumulation of glutamate (see Table 1), can be explained by the increased provision of acetate-derived AcCoA, a potent activator of pyruvate carboxylase [26] (Table 1). It should be stressed here that, although acetate was the main AcCoA provider for the CS reaction, the flux through pyruvate dehydrogenase was not significantly altered in the presence of this fatty acid. Finally, the increased flux through α OGDH might be explained simply by the increased synthesis of α OG resulting from the acetate-induced stimulation of citrate synthesis.

Physiological significance

It is known that the rabbit kidney, which produces an alkaline urine [2], contains a high activity of glutamine synthetase [1–3] located in the proximal tubule [4] and does not readily use glutamine as substrate [10,27] because its glutaminase activity is low [2]. Accordingly, in the rabbit, the urinary excretion of ammonium ions is low and the high rate of renal glutamine synthesis known to occur *in vitro* [1,7–11] seems to be an important process that detoxifies the great quantities of ammonia produced by the very active glutamate dehydrogenase reaction in this organ. The rabbit renal proximal tubule also has a high capacity to metabolize acetate [9] (Table 1), the most concentrated volatile fatty acid in the rabbit blood [12]. It is therefore very likely that, in the rabbit kidney *in vivo*, acetate metabolism interacts with the metabolism of glutamine precursors such as alanine.

Because, in rabbit proximal tubules, the carbons of acetate (an anion because it is added as the sodium salt) are recovered mainly as CO₂ or glutamine, two neutral compounds, acetate metabolism leads to the production of bicarbonate ions. Therefore, under conditions *in vivo*, renal acetate metabolism is liable to increase the need for excreting bicarbonate and, as a result, for diminishing the renal excretion and production of ammonia. It is therefore logical from a physiological point of view that, despite an increased removal of nitrogen as alanine observed in the present study in the presence of acetate, there was no increased release of ammonia. Under these conditions, the observation that acetate caused an increased accumulation of glutamate, another anion, is in agreement with a limitation of the production of bicarbonate. It is likely that the main function of acetate, if it is utilized at high rates by the rabbit kidney *in vivo*, is to provide energy for the renal transport work. As its utilization might lead, as suggested by the present study, to an increased removal of amino acid nitrogen, it is of great importance to note that its metabolism is organized in such a way that it prevents the release of this nitrogen as ammonia, the need for which is concomitantly decreased. Clearly, studies *in vivo* are needed to test whether or not this view holds *in vivo*.

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Received 16 December 1998/11 May 1999; accepted 15 June 1999