The conversion of alanine into glutamine in guinea-pig renal cortex

Essential role of pyruvate carboxylase

Martine FORISSIER and Gabriel BAVEREL

Laboratoire de Physiologie Rénale et Métabolique, Faculté de Médecine Alexis Carrel, 69008 Lyon, France

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1. The metabolism of L-alanine was studied in isolated guinea-pig kidney-cortex tubules. 2. In contrast with previous conclusions of Krebs [(1935) *Biochem. J.* **29**, 1951–1969], glutamine was found to be the main carbon and nitrogenous product of the metabolism of alanine (at 1 and 5 mM). Glutamate and ammonia were only minor products. 3. At neither concentration of alanine was there accumulation of glucose, glycogen, pyruvate, lactate, aspartate or tricarboxylic acid-cycle intermediates. 4. Carbon-balance calculations and the release of $^{14}\text{CO}_2$ from [U- ^{14}C]alanine indicate that oxidation of the alanine carbon skeleton occurred at both substrate concentrations. 5. A pathway involving alanine aminotransferase, glutamate dehydrogenase, glutamine synthetase, pyruvate dehydrogenase, pyruvate carboxylase and enzymes of the tricarboxylic acid cycle is proposed for the conversion of alanine into glutamine. 6. Strong evidence for this pathway was obtained by: (i) suppressing alanine removal by amino-oxyacetate, an inhibitor of transaminases, (ii) measuring the release of $^{14}\text{CO}_2$ from [1- ^{14}C]alanine, (iii) the use of L-methionine DL-sulphoximine, an inhibitor of glutamine synthetase, which induced a large increase in ammonia release from alanine, and (iv) the use of fluoroacetate, an inhibitor of aconitase, which inhibited glutamine synthesis with concomitant accumulation of citrate from alanine. 7. In this pathway, the central role of pyruvate carboxylase, which explains the discrepancy between our results and those of Krebs (1935), was also demonstrated.

In a previous paper (Baverel *et al.*, 1980) we have shown that alanine was synthesized by isolated guinea-pig kidney-cortex tubules metabolizing glutamate; we have also demonstrated that alanine synthesis was inhibited by amino-oxyacetate, a well-known inhibitor of alanine aminotransferase (EC 2.6.1.2), whose activity was shown to be present in guinea-pig renal cortex (Baverel *et al.*, 1980). The reversible nature of the reaction catalysed by alanine aminotransferase, which is a near-equilibrium enzyme, led us to consider that alanine could also be a substrate for the guinea-pig renal cortex. In the present study, we have examined this possibility and determined the metabolic fate of alanine in this tissue. The results obtained reveal that, in contrast with previous conclusions (Krebs, 1935), alanine may be converted into glutamine by the guinea-pig renal cortex. A pathway for this conversion is proposed, and experimental evidence for this proposal presented. In this pathway, the critical role of pyruvate carboxylase (EC 6.4.1.1), which explains the discrepancy between our results and those of Krebs (1935), is emphasized.

Materials and methods

Guinea pigs

Guinea pigs (400–600 g), of the Dunkin–Hartley albino strain, were obtained from Iffa–Credo, St. Germain-sur-l'Arbresle, France, and were fed on a standard diet (U.A.R., Villemoisson-sur-Orge, France). Unless otherwise stated, all experiments reported below were performed with kidney tubules from fed guinea pigs.

Reagents

L-Alanine, glutaminase (grade V), α -cyano-4-hydroxycinnamic acid, monofluoroacetic acid, amino-oxyacetic acid, quinolinic acid and L-methionine DL-sulphoximine were from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Other enzymes and coenzymes were supplied by Boehringer (Mannheim, Germany). L-[1- ^{14}C]Alanine (50 Ci/mol) and L-[U- ^{14}C]alanine (150 Ci/mol) were obtained from The Radiochemical Centre (Amersham, Bucks., U.K.).

Preparation of kidney-cortex tubules and incubations

Kidney tubules were prepared by collagenase treatment of renal-cortex slices as described by Baverel *et al.* (1978). Incubations other than those involving radioactive substrates were performed at 37°C in a shaking water bath, in 25 ml stoppered Erlenmeyer flasks in an atmosphere of O₂/CO₂ (19:1). The flasks contained 1 ml of the tubule suspension plus 3 ml of Krebs–Henseleit medium (Krebs & Henseleit, 1932) supplemented or not with substrates or inhibitors. In all experiments each experimental condition was performed in duplicate. Incubation was stopped by adding HClO₄ (final concn. 2%, v/v) to each flask. Metabolite assays were conducted on the neutralized supernatant. When radioactive alanine was the substrate, incubation, deproteinization, collection and measurement of the ¹⁴CO₂ formed were performed as described by Baverel & Lund (1979).

Metabolite assays

Alanine, glutamate, glutamine, aspartate, ammonia, lactate, pyruvate, glucose, glycogen, 2-oxoglutarate, fumarate, malate and the dry weight of the amount of tubules added to the flasks were determined as described previously (Baverel *et al.*, 1978, 1980).

Calculations

Net substrate utilization and product formation were calculated as the difference between the total contents of the flask (tissue + medium) at the start (zero-time flasks) and after the period of incubation. The metabolic rates, reported as means ± S.E.M., are expressed in μmol of substance removed or produced per g dry wt. of tubule fragments per unit time.

The metabolic fate of alanine was established by calculating carbon and nitrogen balance. An estimate of oxidation of alanine was obtained by carbon-balance calculation by using the following formula:

$$\text{Alanine (in } C_3 \text{ units) oxidized} = \frac{3 \text{ ALA} - 5 (\text{GLU} + \text{GLN})}{3}$$

where ALA is the rate of alanine removal and GLU and GLN are the rates of formation of glutamate and glutamine respectively.

The rates of release of ¹⁴CO₂ from L-[1-¹⁴C]-alanine and conversion of L-[U-¹⁴C]-alanine into ¹⁴CO₂ were calculated by dividing the radioactivity (c.p.m.) in ¹⁴CO₂ by the specific radioactivity of the labelled alanine determined in the zero-time samples for each experiment.

Results and discussion

Metabolic fate of alanine carbon and nitrogen

With 1 mM- or 5 mM-[1-¹⁴C]alanine as substrate, alanine utilization, which increased with the substrate concentration, was linear with time (Table 1). The high rate of alanine removal was accompanied by a high rate of glutamine formation, which increased with alanine concentration and was also linear with time over a 60 min incubation period. Only small amounts of glutamate and ammonia accumulated. No accumulation of pyruvate, lactate, glucose, glycogen, 2-oxoglutarate, fumarate, malate, citrate or aspartate was observed.

The nitrogen-balance calculations, given in Table 1, show that the amount of nitrogen found as glutamine, glutamate and ammonia is greater than the amount of nitrogen removed as alanine; this indicates that the glutamine formation from endogenous sources, which is observed in the absence of added substrate (see Table 1), still occurs, at least partly, in the presence of alanine as substrate. However, in the presence of alanine, the very large amount of glutamine formed, which exceeds several times the amount of glutamine formed from endogenous sources in the absence of alanine, clearly indicates that alanine must be the precursor of both the carbon skeleton and the nitrogen groups of the glutamine found. Neglecting the formation of glutamine from endogenous sources when alanine is the added substrate, carbon- and nitrogen-balance calculations from the data of Table 1 show that the carbon skeleton of glutamine and its nitrogenous groups accounted for about 80% and more than 90% respectively of the carbon skeleton and amino group removed as alanine.

The carbon-balance calculations (Table 1) also reveal that less than 20% of the alanine carbon removed was not accounted for by the non-volatile carbon products found, but was presumably oxidized. However, because of the formation of glutamine from endogenous sources as mentioned above, which occurs even in the presence of alanine,

the latter value (20%) is probably slightly underestimated.

The present results, which demonstrate that in guinea-pig kidney tubules glutamine is the main carbon and nitrogenous end product of alanine metabolism, contradict those obtained by Krebs (1935) and Weil-Malherbe & Krebs (1935), who concluded from their classical studies on renal glutamine synthesis that only glutamate, proline and

Table 1. *Metabolism of 1 mM- and 5 mM-L-[1-¹⁴C]alanine in guinea-pig kidney tubules*

Substrate added	Incubation (min)	Metabolite removal or production					Carbon balance (C ₃ units)	Nitrogen balance
		Alanine	Glutamate	Glutamine	Ammonia	¹⁴ CO ₂		
1- ¹⁴ C Alanine (1 mM)	30	-267.4 ± 21.5	+8.1 ± 2.7	+129.3 ± 9.0	+20.0 ± 2.8	+244.5 ± 14.6	-38.4	+19.3
1- ¹⁴ C Alanine (1 mM)	60	-473.8 ± 38.4	+6.1 ± 2.1	+229.9 ± 26.4	+40.5 ± 7.1	+388.7 ± 35.5	-80.5	+32.6
None	30	-0.5 ± 0.6	+2.1 ± 2.1	+42.0 ± 3.7	+4.5 ± 2.0	—	+73.0	+90.1
None	60	+2.6 ± 1.5	-2.2 ± 2.1	+74.3 ± 13.4	+15.2 ± 4.7	—	+121.0	+164.2
1- ¹⁴ C Alanine (5 mM)	30	-347.3 ± 11.3	+15.9 ± 1.6	+159.4 ± 4.8	+30.2 ± 2.3	+272.8 ± 10.1	-55.1	+17.6
1- ¹⁴ C Alanine (5 mM)	60	-674.0 ± 34.0	+26.8 ± 4.4	+333.0 ± 17.2	+52.7 ± 2.1	+548.1 ± 28.7	-74.3	+71.5

Kidney tubules were incubated as described in the Materials and methods section. Values (μmol/g dry wt.) for metabolite removal (—) or production (+) are reported as means ± S.E.M. for four and five experiments in the 1 mM-alanine and 5 mM-alanine series respectively. Each flask contained 7.6 ± 1.5 (for 1 mM-alanine) and 21.7 ± 0.8 mg dry wt. of tissue (for 5 mM-alanine). Carbon and nitrogen balances were calculated as the sum of the carbon or nitrogenous substances removed minus the sum of those produced (in these calculations a — sign indicates a deficit and a + sign an excess of carbon skeletons or nitrogen found as compared with those removed).

hydroxyproline, but not alanine, could give rise to glutamine formation in renal-cortex slices of the guinea pig.

Routes for the formation of glutamine from alanine

The finding of the present study that alanine (an amino acid whose molecule contains three carbon atoms and one of nitrogen) is virtually quantitatively converted into glutamine (an amino acid whose molecule contains five carbon atoms and two nitrogen atoms) raises the question of which pathways are involved in the metabolism of both the amino group and the carbon skeleton of alanine.

Role and intracellular distribution of alanine aminotransferase. The almost complete inhibition of alanine removal by guinea-pig kidney tubules in the presence of 0.5 mM-amino-oxyacetate (results not shown), an inhibitor of transaminases (Braunstein, 1964), demonstrates that transamination of alanine with 2-oxoglutarate to form pyruvate and glutamate represents the only initial step in alanine metabolism by this tissue. That most of the alanine metabolized is transaminated after prior transport into the mitochondria is strongly suggested by the modest inhibition of alanine removal by α-cyano-4-hydroxycinnamate (Table 2), a potent inhibitor of pyruvate transport across the mitochondrial membrane (Halestrap & Denton, 1974), used at a concentration of 2.5 mM, which causes 85% inhibition of pyruvate removal by the tubules (Table 2). The effects of α-cyano-4-hydroxycinnamate observed here are in agreement with the demonstration by De Rosa & Swick (1975) that, in guinea-pig renal cortex, most of the alanine aminotransferase activity is located within the mitochondria.

Role of glutamate dehydrogenase. Since the glutamine molecule contains two nitrogen atoms, two alanine molecules must be metabolized for each glutamine molecule formed from alanine. In this process, it is also clear that the amide nitrogen of glutamine must pass through the stage of ammonia and its amino group through the stage of glutamate. Of the two glutamate molecules formed by the transamination of two alanine molecules, one can be converted into glutamine by glutamine synthetase, which is very active in guinea-pig renal cortex (Krebs, 1935; Baverel *et al.*, 1980). Evidence is presented in Table 3 that glutamate dehydrogenase, which plays a major role in glutamate metabolism in guinea-pig tubules (Baverel *et al.*, 1980), provides from the second glutamate molecule available the ammonia needed for the formation of the glutamine amide nitrogen. The suppression of glutamine synthesis by L-methionine DL-sulphoximine, an inhibitor of glutamine synthetase (Meister, 1968), was accompanied by a large accumulation of the ammonia released by glutamate dehydrogenase,

Table 2. *Effect of α -cyano-4-hydroxycinnamate on the removal of alanine and pyruvate by guinea-pig kidney tubules* Kidney tubules (23.5 ± 1.9 and 10.1 ± 0.7 mg dry wt. per flask in the experiments with alanine and pyruvate respectively) were incubated for 60 min as described in the Materials and methods section. Results (in $\mu\text{mol/g}$ dry wt.) are reported as means $\pm$ S.E.M. for four experiments in both series. Statistical difference was measured by the paired Student's *t* test against the control without α -cyano-4-hydroxycinnamate: *P < 0.05; **P < 0.01; ***P < 0.001.

Concn. of α -cyano-4-hydroxycinnamate (mM)	Substrate ...	5 mM-L-Alanine		5 mM-Pyruvate	
		Alanine removed	Pyruvate removed	Glucose found	
0		641.7 ± 29.3	911.9 ± 76.4	82.1 ± 3.2	
0.25		$602.5 \pm 22.0^*$	$300.8 \pm 32.3^{**}$	$4.4 \pm 3.3^{**}$	
0.5		$592.6 \pm 36.1^*$	$273.2 \pm 5.2^{**}$	$1.9 \pm 1.9^{***}$	
1.0		$571.0 \pm 41.0^*$	$215.4 \pm 12.7^{**}$	0***	
2.5		$543.5 \pm 38.4^{**}$	$137.7 \pm 6.5^{**}$	0***	

Table 3. *Effect of L-methionine DL-sulphoximine on the metabolism of 5 mM-alanine in guinea-pig kidney tubules* Kidney tubules (25.5 ± 2.2 mg dry wt. per flask) were incubated for 60 min as described in the Materials and methods section. Results ($\mu\text{mol/g}$ dry wt.) for metabolite removal (–) or production (+) are reported as means $\pm$ S.E.M. for four experiments. Statistical difference was measured by the paired Student's *t* test against the control without L-methionine DL-sulphoximine: *P < 0.02; **P < 0.01; ***P < 0.001.

Experimental condition	Metabolite removal or production				
	Alanine	Glutamate	Glutamine	Ammonia	$^{14}\text{CO}_2$
[1- ^{14}C]Alanine (5 mM)	-667.4 ± 46.0	$+19.6 \pm 3.3$	$+337.7 \pm 26.0$	$+46.0 \pm 2.4$	$+541.1 \pm 55.5$
[1- ^{14}C]Alanine (5 mM) + L-methionine DL-sulphoximine (5 mM)	$-512.8 \pm 28.6^{**}$	$+146.7 \pm 11.7^{***}$	$-2.3 \pm 9.3^{***}$	$+398.8 \pm 27.1^{***}$	$+417.7 \pm 35.1^*$

Table 4. *Effects of NH_4Cl and quinolinate on the metabolism of 5 mM-alanine in guinea-pig kidney tubules* Kidney tubules (20.2 ± 0.9 mg dry wt. per flask) were incubated for 60 min as described in the Materials and methods section. Results ($\mu\text{mol/g}$ dry wt.) for metabolite removal (–) or production (+) are reported as means $\pm$ S.E.M. for four experiments. No aspartate accumulated in the presence of quinolinate. Statistical difference was measured by the paired Student's *t* test against the control without NH_4Cl or quinolinate: *P < 0.05; **P < 0.02; ***P < 0.01; N.S., not significant (*P* > 0.05).

Experimental condition	Metabolite removal or production				
	Alanine	Glutamate	Glutamine	Ammonia	$^{14}\text{CO}_2$
[1- ^{14}C]Alanine (5 mM)	-617.4 ± 52.2	$+35.5 \pm 4.0$	$+304.6 \pm 20.5$	$+53.3 \pm 4.3$	$+474.3 \pm 37.6$
[1- ^{14}C]Alanine (5 mM) + NH_4Cl (5 mM)	$-410.1 \pm 36.9^{**}$	$+37.2 \pm 2.4$ (N.S.)	$+186.3 \pm 16.3^{***}$	$+56.5 \pm 5.0$ (N.S.)	$+319.1 \pm 23.7^*$
[1- ^{14}C]Alanine (5 mM) + quinolinate (5 mM)	-664.2 ± 35.0 (N.S.)	$+32.8 \pm 4.1$ (N.S.)	$+318.1 \pm 11.8$ (N.S.)	$+35.1 \pm 4.2$ (N.S.)	$+462.9 \pm 19.9$ (N.S.)

Table 5. *Effect of monofluoroacetate on the metabolism of 5 mM-[U- ^{14}C]alanine in guinea-pig kidney tubules* Kidney tubules (20.2 ± 2.8 mg dry wt. per flask) were incubated for 60 min as described in the Materials and methods section. Results ($\mu\text{mol/g}$ dry wt.) for metabolite removal (–) or production (+) are means $\pm$ S.E.M. for four experiments. Statistical difference was measured by the paired Student's *t* test against the control without fluoroacetate: *P < 0.01; **P < 0.001.

Experimental condition	Metabolite removal or production					Conversion of [U- ^{14}C]alanine into $^{14}\text{CO}_2$
	Alanine	Glutamate	Glutamine	Ammonia	Citrate	
[U- ^{14}C]Alanine (5 mM)	-671.0	$+22.2$	$+343.8$	$+30.0$	$+1.4$	$+211.9$
	± 22.6	± 4.7	± 11.2	± 1.1	± 1.6	± 9.2
[U- ^{14}C]Alanine (5 mM) + monofluoroacetate (1 mM)	-196.0	$+10.0$	$+63.6$	$+179.5$	$+48.8$	$+63.5$
	$\pm 19.6^{**}$	$\pm 3.3^*$	$\pm 3.5^{**}$	$\pm 5.2^{**}$	$\pm 4.9^*$	$\pm 2.1^{**}$

whose role was masked by the very efficient operation of glutamine synthetase.

The addition of alanine (5 mM) plus NH_4Cl (5 mM) did not increase glutamine synthesis, as has been observed when glutamate was the substrate (Baverel *et al.*, 1980), and no ammonia was removed (Table 4); on the contrary, both glutamine synthesis and alanine removal were inhibited. This probably resulted from the end-product inhibition of glutamate dehydrogenase, which catalyses a central reaction in alanine metabolism, by high concentrations of ammonia, as previously demonstrated by Baverel *et al.* (1980).

Conversion of pyruvate into α -oxoglutarate. For the synthesis of the glutamine carbon skeleton from alanine, glutamate must first be formed. The provision of ammonia required for the synthesis of the glutamine amide group by glutamate dehydrogenase implies that glutamate dehydrogenase cannot at the same time function in the direction of reductive amination of 2-oxoglutarate to form the glutamate needed for glutamine synthesis and in the direction of oxidative deamination. Thus the only alternative route to form glutamate appears to be the alanine aminotransferase reaction. In this reaction, one molecule of 2-oxoglutarate is required for each molecule of alanine transaminated. Since two alanine molecules are metabolized to form one glutamine molecule, two molecules of 2-oxoglutarate are also needed. Thanks to the glutamate dehydrogenase reaction, one is re-formed in a cyclic way. Therefore another molecule of 2-oxoglutarate must be synthesized from alanine, which is the only exogenous carbon source. Since two molecules of pyruvate are formed during the transamination of two molecules of alanine, the absence of accumulation of pyruvate, lactate, glucose or glycogen led us to consider pyruvate as the possible source of the second molecule of 2-oxoglutarate needed for glutamine synthesis. The only pathway for the formation of 2-oxoglutarate from pyruvate appeared to involve the operation of the pyruvate dehydrogenase complex and pyruvate carboxylase (EC 6.4.1.1), allowing citrate to be formed via citrate synthase (EC 4.1.3.7), as well as the operation of the enzymes of the tricarboxylic acid cycle responsible for the conversion of citrate into α -oxoglutarate.

The sequence of reactions of the complete pathway proposed for the conversion of alanine into glutamine can be summarized as follows (cofactors omitted).

(1) Release of NH_3 via glutamate dehydrogenase:

(2) Synthesis of glutamine:

(3) Conversion of pyruvate into α -oxoglutarate:

Such a pathway, in which citrate formation is the only way to remove the pyruvate formed from alanine, implies that the rates of utilization of pyruvate through pyruvate dehydrogenase and through pyruvate carboxylase are identical. The high rate of $^{14}\text{CO}_2$ release from $[1\text{-}^{14}\text{C}]$ alanine (Table 1) clearly indicates that pyruvate dehydrogenase was involved in the decarboxylation of the $[1\text{-}^{14}\text{C}]$ pyruvate formed by alanine aminotransferase. But the fact that the rate of $^{14}\text{CO}_2$ release was higher than half the rate of $[1\text{-}^{14}\text{C}]$ alanine utilization (and therefore of $[1\text{-}^{14}\text{C}]$ pyruvate formation) indicates that some reaction additional to that catalysed by pyruvate dehydrogenase was responsible for part of the $^{14}\text{CO}_2$ release. Two possibilities were then considered. The first is the conversion of pyruvate into oxaloacetate and the release of the label by the phosphoenolpyruvate carboxykinase (EC 4.1.1.32) reaction after equilibration of the oxaloacetate formed with fumarate via the malate dehydrogenase and fumarase reactions. This possibility was ruled out by the use of 5 mM-quinolinate (Table 4), which failed to affect the release of $^{14}\text{CO}_2$ from $[1\text{-}^{14}\text{C}]$ alanine, despite its established inhibitory effect on phosphoenolpyruvate carboxykinase in guinea-pig renal cortex (see Baverel *et al.*, 1980). The second possibility, which seems therefore more likely, is the release of $^{14}\text{CO}_2$ at the level of isocitrate dehydrogenase (EC 1.1.1.41) after incorporation of $[1\text{-}^{14}\text{C}]$ pyruvate into oxaloacetate and the subsequent formation of citrate, *cis*-aconitate and isocitrate. Thus the high rate of $^{14}\text{CO}_2$ release from $[1\text{-}^{14}\text{C}]$ alanine, together with the absence of effect of quinolinate on this release, strongly favours the view that pyruvate dehydrogenase, pyruvate carboxylase and isocitrate dehydrogenase are involved in the conversion of alanine into glutamine.

Effect of fluoroacetate. Addition of monofluoroacetate, which causes an inhibition of aconitase (Lotspeich *et al.*, 1952), completely suppressed glutamine synthesis from alanine (Table 5). As expected, inhibition of glutamine synthesis was accompanied by an accumulation of citrate, which was maximum at the concentration of fluoroacetate used. These data provide further evidence that citrate and aconitase are on the pathway of alanine conversion into glutamine.

Table 6. *Effect of bicarbonate on the metabolism of 5 mM-[1-¹⁴C]alanine in guinea-pig kidney tubules incubated in phosphate/saline buffer*
 Kidney tubules (24.5 ± 4.3 mg dry wt. per flask), previously washed three times and resuspended in phosphate/saline buffer, pH 7.4 (Krebs, 1933), were incubated in 4 ml of the same buffer in an atmosphere of pure O₂. When NaHCO₃ was added to the phosphate/saline buffer, NaCl concentration was decreased to keep the osmolality of the medium constant, and the buffer as well as the flasks were gassed with a mixture of O₂ and CO₂ in appropriate concentrations to maintain pH 7.4 under all conditions. Results (μ mol/g dry wt.) for metabolite removal (—) or production (+) are reported as means $\pm$ S.E.M. for four experiments. Statistical difference was measured by the paired Student's *t* test against the control without bicarbonate: *P < 0.05; **P < 0.01; ***P < 0.001.

Buffer system	Substrate added	Metabolite removal or production					
		Alanine	Glutamate	Glutamine	Ammonia	Lactate	¹⁴ CO ₂
Phosphate/saline; pure O ₂	[1- ¹⁴ C]Alanine (5 mM)	-219.6 $\pm$ 5.1	+10.9 $\pm$ 3.5	+71.8 $\pm$ 6.1	+190.3 $\pm$ 13.3	+33.3 $\pm$ 8.3	+190.0 $\pm$ 8.4
Phosphate/saline + 10.5 mM-HCO ₃ ⁻ ;	[1- ¹⁴ C]Alanine (5 mM)	-440.0 $\pm$ 11.4***	+34.2 $\pm$ 8.7*	+178.4 $\pm$ 19.2**	+157.8 $\pm$ 24.0	+16.9 $\pm$ 8.3**	+383.9 $\pm$ 20.5***
2.1% CO ₂ in O ₂							
Phosphate/saline + 25 mM-HCO ₃ ⁻ ;	[1- ¹⁴ C]Alanine (5 mM)	-560.4 $\pm$ 10.1***	+51.6 $\pm$ 14.7*	+253.4 $\pm$ 16.8***	+115.8 $\pm$ 16.1***	+7.8 $\pm$ 4.3*	+492.1 $\pm$ 18.2***
5% CO ₂ in O ₂							
Phosphate/saline; pure O ₂	None	—	+9.7 $\pm$ 2.6	+58.4 $\pm$ 3.2	+66.5 $\pm$ 10.2	—	—
Phosphate/saline + 10.5 mM-HCO ₃ ⁻ ;	None	—	+23.7 $\pm$ 8.5	+48.3 $\pm$ 6.7	+45.0 $\pm$ 8.0**	—	—
2.1% CO ₂ in O ₂							
Phosphate/saline + 25 mM-HCO ₃ ⁻ ;	None	—	+22.3 $\pm$ 10.9	+42.4 $\pm$ 3.7*	+46.8 $\pm$ 5.8	—	—
5% CO ₂ in O ₂							

That part of the glutamine found in the presence of alanine as substrate was formed from endogenous sources (as revealed by nitrogen-balance calculation; see Table 1) probably explains why the oxidation of alanine estimated by carbon balance calculations is lower than that obtained by measuring the conversion of [U- ^{14}C]alanine into $^{14}\text{CO}_2$ (Table 5).

By comparing the data with [1- ^{14}C]- and [U- ^{14}C]-alanine in Tables 1 and 5, it can be seen that the conversion of [U- ^{14}C]alanine into $^{14}\text{CO}_2$ is slightly higher than the release of $^{14}\text{CO}_2$ from [1- ^{14}C]alanine divided by 3. This suggests that the alanine degraded beyond the stage of C-1 as well as complete oxidation of alanine are very limited. This also indicates that most of the alanine carbon used is being removed from the tricarboxylic acid cycle before the α -oxoglutarate dehydrogenase step. It should be noted that, although it does not represent an important fraction of alanine metabolism, complete oxidation, together with partial oxidation of alanine at the level of glutamate dehydrogenase, pyruvate dehydrogenase and isocitrate dehydrogenase, is sufficient to account for the ATP needed by the glutamine synthetase and pyruvate carboxylase reactions.

Role of pyruvate carboxylase. Table 6 presents results of experiments designed to identify the reasons why Krebs (1935) failed to demonstrate the synthesis of glutamine from alanine by guinea-pig renal-cortex slices. When guinea-pig kidney tubules were incubated under the conditions used by Krebs (1935) [phosphate medium with an atmosphere of pure O_2 (Krebs, 1933)], glutamine synthesis in the presence of alanine was in the same range as that in the absence of exogenous substrate. Under these conditions, ammonia accumulated at a rate close to that of alanine utilization; this indicates that the alanine nitrogen transferred to 2-oxoglutarate by alanine aminotransferase was not incorporated as glutamate into glutamine, but that it was only released as free ammonia via glutamate dehydrogenase. Table 6 also shows that a large and progressive increase in glutamine synthesis occurred when progressively increased concentrations of bicarbonate were added to the medium. This observation demonstrates the critical role of CO_2 fixation in the conversion of alanine into glutamine. Since 'malic' enzyme (EC 1.1.1.40), whose activity is low in guinea-pig renal cortex (Baverel *et al.*, 1980; Watford *et al.*, 1980), does not readily function in the direction of CO_2 fixation, pyruvate carboxylase, which has been shown to be present in this tissue by Robinson (1976), appears to be the only possible enzyme responsible for CO_2 fixation, on the pyruvate formed from alanine. Thus our data provide a satisfactory explanation of the discrepancy

between our results and those of Krebs (1935), who was unable to demonstrate glutamine formation from alanine under his conditions. They also show that, in the absence of a sufficient bicarbonate concentration, the CO_2 released by pyruvate dehydrogenase is not available for pyruvate carboxylase.

The mechanism by which the pyruvate formed by transamination of alanine is totally directed, in a co-ordinated and stoichiometric manner, to pyruvate dehydrogenase and pyruvate carboxylase for the subsequent synthesis of citrate remains to be elucidated. In this respect, it should be emphasized that, despite the presence of pyruvate carboxylase in guinea-pig renal cortex, a tissue that forms glucose from pyruvate (see Table 2), no glucose was formed from alanine (even after 48 h without food; results not shown); in view of the fact that alanine is considered to be the main gluconeogenic amino acid in the liver (Ross *et al.*, 1967; Exton & Park, 1967), such an observation is surprising and deserves further investigation.

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