The involvement of pyruvate cycling in the metabolism of aspartate and glycerate by the perfused rat kidney

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The metabolism of glycerate and aspartate was investigated in perfused rat kidneys. The major pathway active for aspartate metabolism and NH_3 production was found to include transamination, and not the purine nucleotide cycle. Pyruvate cycling was identified as a means by which reducing potential is generated in the cytosol for glucose and lactate production from these substrates. Inhibition of mitochondrial pyruvate transport caused an inhibition of glucose production, accumulation of lactate and pyruvate in the perfusate, and a decrease in the [lactate]/[pyruvate] ratio in kidneys perfused with aspartate. These data indicate a role of mitochondrial pyruvate transport in the provision of cytosolic reducing potential. With either aspartate or glycerate, 3-mercaptopycolinic acid (3-MPA) suppressed glucose synthesis and caused accumulation of malate plus fumarate within the kidney. Glucose production from glycerate was much less sensitive to the presence of 3-MPA than was glucose production from aspartate, illustrating a phosphoenolpyruvate carboxykinase (PEPCK)-independent pathway for the cycling of pyruvate. In aspartate-perfused kidneys, the presence of 3-MPA, at concentrations that completely blocked glucose accumulation in the perfusate, did not affect the rate of NH_3 production and had only a minor effect on the rate of aspartate uptake. These data allow for an estimation of the rate of pyruvate formation from aspartate of about $1 \mu\text{mol}/\text{min}$ per kidney under conditions of complete PEPCK inhibition. Thus a PEPCK-independent pathway is operative for amino acid oxidation and pyruvate formation in perfused kidneys. The NADP-linked, but not the NAD-linked, 'malic' enzyme activity of the kidney cortex was found to be sufficient to catalyse this estimated rate of pyruvate formation.

INTRODUCTION

The process of gluconeogenesis requires a cytosolic source of reducing equivalents in the form of NADH for the conversion of oxaloacetate into glucose. With most glucogenic substrates, such as glutamate, serine and alanine, this requirement is met by a 'coupled' oxidation–reduction reaction in which malate efflux from mitochondria provides both the carbon and the reducing potential for glucose synthesis. Although this is also the case with pyruvate as substrate, with higher than physiological concentrations of pyruvate reduction to lactate competes with glucose formation for the available NADH. Malate, which exits from mitochondria and is oxidized for the reduction of pyruvate, ultimately re-enters mitochondria after the cytosolic conversion back into pyruvate (Rognstad & Katz, 1972; Meijer & Williamson, 1974). This substrate cycle, in which pyruvate carbon enters mitochondria as pyruvate, exits as malate, and is reconverted into pyruvate, functions as an energy-dependent shuttle of reducing equivalents (Berry, 1971; Meijer & Williamson, 1974). Pyruvate cycling has been shown to occur in liver (Friedman *et al.*, 1971; Rognstad, 1975) and in kidney cortex preparations (Rognstad & Katz, 1972; Janssens *et al.*, 1980) by a variety of isotopic methods.

In the present study, we investigated the means by

which reducing potential is generated in the renal-cortex cytosol for the conversion of aspartate and other substrates into glucose. We, as have others (Nishiitsutsuji-Uwo *et al.*, 1967; Pitts, 1971), found aspartate to be glucogenic in perfused rat kidneys. This process was re-investigated in light of subsequent observations that have illustrated the essentially unidirectional nature of the mitochondrial transmembrane transport of aspartate (LaNoue *et al.*, 1974; Tischler *et al.*, 1976). These observations suggest that, since aspartate cannot readily permeate energized mitochondria, conversion of aspartate into glucose is entirely a cytosolic process and that this conversion requires a stoichiometric supply of reducing equivalents. Because of this requirement, these conditions are not much unlike those during the metabolism of pyruvate. However, with aspartate as substrate, the mitochondrial transport of pyruvate is not directly involved in the synthesis of glucose. Thus a study of the effects of mitochondrial pyruvate transport inhibition by α -cyano-*p*-hydroxycinnamic acid (α -CHC), and thereby of pyruvate cycling, is possible with this glucogenic substrate.

For similar reasons, the metabolism of glycerate was also investigated. With this substrate, the role of pyruvate cycling was studied through the inhibition of phosphoenolpyruvate carboxykinase (GTP) (PEPCK) by 3-mercaptopycolinic acid (3-MPA).

Abbreviations used: α -CHC, α -cyano-*p*-hydroxycinnamic acid; PEPCK, phosphoenolpyruvate carboxykinase (GTP) (EC 4.1.1.32); 3-MPA, 3-mercaptopycolinic acid.

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MATERIALS AND METHODS

Kidneys from male Wistar rats were perfused at a constant pressure of 100 mmHg as described by Scaduto & Davis (1985). In all cases, donor rats weighed between 280 and 330 g and were deprived of food, but not water, on the afternoon before perfusions.

Metabolites were determined spectrophotometrically by enzymic procedures as previously described (Lee & Davis, 1979). Glycerate was determined as described for the measurement of serine (Scaduto & Davis, 1985). Recovery of glycerate and serine as glyoxylate was 92 and 89% respectively. Although this method also includes the determination of pyruvate and threonine, the value is corrected for the pyruvate content and the amount of threonine in the perfusate relative to that of serine or glycerate is insignificant (Scaduto & Davis, 1985).

At the completion of the 60 min perfusions, kidneys were freeze-clamped with an aluminium clamp precooled with liquid N₂, without interruption of perfusate flow. Frozen tissue was stored at -80 °C until metabolite extraction. HClO₄ extraction of the frozen tissue and neutralization were as previously described (Scaduto & Davis, 1985).

For the determination of enzyme activities, the kidney cortex of rats starved overnight was homogenized in ice-cold 225 mM-D-mannitol/75 mM-sucrose/5 mM-Mops/1 mM-dithiothreitol/0.1 mM-EDTA, pH 7.4, by three passes of a Teflon-pestle/glass homogenizer. The homogenate was centrifuged for 5 min at 2000 *g*, and the supernatant was re-centrifuged for 5 min at 15000 *g* to pellet the mitochondria, and finally for 60 min at 100000 *g*_{av.} to obtain the cytosol preparation. This was used directly after dilution of the protein content to 7.5 mg/ml with the homogenization buffer. The mitochondria were washed by resuspension of the mitochondrial pellet in about 40 ml of homogenization buffer and centrifugation at 8000 *g* for 10 min. The mitochondria were then resuspended in 1–2 ml of homogenization buffer, transferred to a glass conical test tube, frozen in liquid N₂ and stored at -80 °C until use. The thawed mitochondria were then disrupted by ultrasonic treatment, as previously described (Lin & Davis, 1974). The sonicated material was centrifuged for 60 min at 100000 *g*_{av.} to obtain a clear mitosomal preparation. From a comparison of the protein content before and after centrifugation in three preparations, 46.5 ± 2.3% of the mitochondrial protein was solubilized by this procedure. Protein concentration was measured by the biuret procedure (Gornall *et al.*, 1949), with the addition of 0.33% (w/v) deoxycholate, with bovine serum albumin as standard.

The cytosolic NAD-linked 'malic' enzyme activity was estimated from the rate of ¹⁴CO₂ formation on incubation of the protein preparation with 30 mM-L-[U-¹⁴C]malate (sp. radioactivity 100 d.p.m./nmol), 1 mM-NAD⁺ and 2 mM-MnCl₂ in 100 mM-Tris/100 mM-imidazole, pH 7.0, in a total volume of 0.5 ml. Incubations were carried out in Warburg flasks at 37 °C in a shaking water bath, with 0.3 ml of 6 M-HClO₄ in the side arm and 0.25 ml of phenethylamine in the centre well. Reactions, in duplicate, were started by the addition of protein and stopped by tipping the acid into the reaction media. CO₂ was collected by continued incubation for 2 h at room temperature. The contents of

the centre well, and two washings of the well with phenethylamine, were transferred to scintillation vials. Reaction rates were calculated from the amount of ¹⁴CO₂ produced in flasks incubated for 5 and 30 min. The production of ¹⁴CO₂ was linear over this time interval.

Mitochondrial NAD-linked 'malic' enzyme activity was measured spectrophotometrically as previously described (Lin & Davis, 1974). These determinations were done with 30 mM-malate, 1 mM-NAD⁺, 2 mM-arsenite, 2 mM-MnCl₂ and 0.1 M-Tris/HCl, pH 7.2. The NADP-linked activity in both preparations was measured spectrophotometrically with 30 mM-2-malate, 1 mM-NADP⁺, 2 mM-arsenite, 2 mM-MnCl₂ and 0.1 M-Tris/HCl, pH 7.4. Lactate dehydrogenase activity was determined with 2 mM-pyruvate, 2 mM-arsenite, 0.12 mM-NADH and 0.1 M-Tris/HCl, pH 7.2. Glutamate dehydrogenase activity was determined as described by Schmidt (1974).

Lactate dehydrogenase and glutamate dehydrogenase activities were used to correct for the cross-contamination of protein in these two cellular fractions (Elbers *et al.*, 1974). The NAD- and NADP-linked activities of 'malic' enzyme(s) were corrected for cross-contamination by assuming that the total activity was localized to these two compartments. For this calculation, the cytosolic NAD-linked activity was corrected to that expected at 30 °C, by assuming a *Q*₁₀ of 2.0. Total activity of 'malic' enzyme in the kidney cortex was estimated by comparison of the corrected activity and that of the appropriate marker enzyme to the total amount of marker enzyme per g of cortex.

The rates of metabolite release and substrate consumption were determined from the amount of metabolite present in the perfusate at the time of sampling. This calculation is based on the metabolite concentration in the perfusate and the circulating volume after appropriate corrections for the changes owing to sampling. The release of some metabolites, such as lactate and pyruvate, was expressed as the amount released during 60 min of perfusion, rather than as a rate, since their release was often non-linear.

All data have been expressed as the mean of replicate experiments ± 1 S.E.M. Student's *t* test was used for statistical evaluation of results: *P* < 0.05 was considered significant.

All chemicals were obtained from Sigma Chemical Co. (St. Louis, MO, U.S.A.), with the following exceptions. D-Glycerate (hemicalcium salt) was a product of Calbiochem (La Jolla, CA, U.S.A.). Just before use, the required amount was converted into the sodium salt by passage through a Dowex 1 × 8 resin column (H⁺ form) and neutralization of the effluent with NaOH. α-CHC was a product of Aldrich Chemical Co. (Milwaukee, WI, U.S.A.). Hadacidin and 3-MPA were generously given by Dr. Hans Gadebusch of the Merck Institute and Dr. H. L. Saunders of Smith, Kline and French Laboratories respectively. Hadacidin was recrystallized from ethanol/water before use.

RESULTS AND DISCUSSION

Unlike in the perfused rat liver (Ross *et al.*, 1967), aspartate is rapidly metabolized to glucose and NH₃ by the perfused rat kidney (Nishiitsutsuji-Uwo *et al.*, 1967; Pitts, 1971; Scaduto & Davis, 1985). Although the major site of renal NH₃ production from amino nitrogen is

generally considered to be due to glutamate dehydrogenase activity (Tannen, 1978), the possible contribution of the purine nucleotide cycle, especially during aspartate metabolism, is controversial (Bogusky *et al.*, 1983; Strzelecki *et al.*, 1983). We investigated the source of NH_3 production under conditions of renal aspartate metabolism, since this bears considerable significance as to the mechanism for the provision of cytosolic reducing potential for the conversion of this substrate into glucose. Glucose production from aspartate via the purine nucleotide cycle would not require efflux of reducing potential from mitochondria, whereas glucose production via the coupling of cytosolic aspartate aminotransferase and mitochondrial glutamate dehydrogenase would have this requirement.

We reported (Scaduto & Davis, 1985) that 0.3 mM-amino-oxyacetate, a transaminase inhibitor (Hopper & Segal, 1962), inhibited the consumption of aspartate and the release of glutamate, serine and glucose by 89, 87, 88 and 93% respectively. In addition, the release of aspartate-derived NH_3 was suppressed by 89%. However, if glutamate was the sole substrate, its metabolism was not diminished by the transaminase inhibitor. These results were taken to show that the main, if not the only, route of aspartate metabolism is initiated by transamination, coupled to glutamate dehydrogenase activity. In the present work, these studies were extended in two types of experiments. In the first, kidneys were perfused with either glutamate or aspartate as sole substrate, and approximately half-way through the perfusion period amino-oxyacetate was added. With aspartate as substrate, amino-oxyacetate strongly suppressed NH_3 production and virtually abolished net glucose synthesis (Fig. 1). On the other hand, with glutamate as substrate, glucose

synthesis was affected only marginally, and NH_3 release was even increased.

In a second type of experiment, hadacidin (*N*-formyl-*N*-hydroxyglycine), an inhibitor of adenylosuccinate synthase (and therefore of the purine nucleotide cycle; Shigeura & Gordon, 1962), was added to perfusions with aspartate as substrate. In a series of perfusions with 3 mM-aspartate ($n = 4$), 1 mM-hadacidin (on average) tended slightly to decrease aspartate consumption as well as the release of NH_3 , glucose and glutamate (results not shown). Since hadacidin is a competitive inhibitor with respect to aspartate, at the concentrations used in these perfusions, it would have been expected (Shigeura & Gordon, 1962) to have inhibited flux through the purine nucleotide cycle (i.e. regeneration of AMP from IMP) $> 92\%$, assuming that these concentrations reflect the tissue concentrations. Also, hadacidin was without effect on either the adenine nucleotide pool or the ratios of the individual nucleotides ($P > 0.5$; results not shown). The possibility remains that hadacidin did not reach its intracellular site of inhibition. This possibility seems to us extremely unlikely, in view of the unique capacity of the kidney to extract virtually all solutes, including cations, anions and neutral molecules such as glucose. Hadacidin inhibits the purine nucleotide cycle specifically in exercising perfused muscle (Aragon & Lowenstein, 1980).

The present data on the specificity of amino-oxyacetate as an inhibitor of NH_3 and glucose production from aspartate, but not from glutamate, together with the lack of effects by hadacidin, provide rather convincing evidence that the pathway of aspartate metabolism involves transamination, and not the purine nucleotide cycle. This conclusion is supported by the report by Strzelecki *et al.* (1983), who used a completely independent experimental approach to this question.

Provision of cytosolic reducing potential for the synthesis of glucose and lactate

Since aspartate is unable directly to enter energized mitochondria (LaNoue *et al.*, 1974; Tischler *et al.*, 1976), glucose production from aspartate via the cytosolic aspartate aminotransferase represents a pathway in which carbon transfer from aspartate to glucose is completely a cytosolic process. This pathway is essentially gluconeogenesis from cytosolic oxaloacetate, but the NADH required for this reductive process must come from mitochondrial substrate oxidations.

One source of NADH for this conversion was identified as serine production from glycolytic intermediates (Scaduto & Davis, 1985). Serine synthesis from aspartate (or glutamate) is the result of the activity of either the 'phosphorylated' and/or 'non-phosphorylated' pathway(s). Since both pathways involve a cytosolic NAD-linked oxidation, this process can be coupled to glucose synthesis through redox reactions. Such a pathway is illustrated in Scheme 1, assuming that serine is synthesized via the 'phosphorylated' pathway, which is the more likely of the two (Scaduto & Davis, 1985). As shown, the stoichiometry of this couple must be 2 equivalents of serine synthesized per equivalent of glucose for serine synthesis to provide sufficient reducing potential for the quantity of glucose formed from aspartate. However, kidneys perfused with 3 mM-aspartate released serine and glucose at approximately equal rates (Scaduto & Davis, 1985). Therefore, serine

Fig. 1. Effect of amino-oxyacetate on the release of NH_3 and glucose by the isolated kidney perfused with 5 mM-glutamate or with 10 mM-aspartate

Just after withdrawal of the 27 min perfusate sample, indicated by the arrow, amino-oxyacetate was added to the perfusate reservoir to give a concentration of 0.3 mM. Δ , $\blacktriangle$, Aspartate as substrate; $\circ$, $\bullet$, glutamate as substrate; $\triangle$, $\circ$, glucose release; $\blacktriangle$, $\bullet$, NH_3 release.

Scheme 1. A scheme for the coupling of glucose and serine synthesis from aspartate

This scheme illustrates a mechanism whereby serine synthesis can provide reducing potential for glucose synthesis from aspartate through the dismutation of 3-phosphoglycerate.

synthesis at most could have provided half of the reducing potential for the observed rate of glucose production. Considering that some of the glucose released by the cortex is oxidized in other portions of the kidney (Hems & Gaja, 1972; Scaduto & Davis, 1985), this value is less than one-half.

Cycling of pyruvate through vectorial movements across the mitochondrial membrane and involving the following transformations constitutes an energy-dependent translocation of reducing equivalents from mitochondria to cytosol: cytosolic pyruvate $\rightarrow$ mitochondrial pyruvate $\rightarrow$ mitochondrial oxaloacetate $\rightarrow$ mitochondrial malate $\rightarrow$ cytosolic malate $\rightarrow$ cytosolic pyruvate. Studies with perfused liver and isolated hepatocytes have provided evidence that such a cycle is operative in the synthesis of glucose and lactate from pyruvate (e.g. Friedman *et al.*, 1971; Rognstad, 1975; Mapes & Harris, 1976). This concept has been extended in studies with kidney tubule fragments oxidizing pyruvate (Saggerson, 1978). The final step in this cycling process (cytosolic malate $\rightarrow$ cytosolic pyruvate) could theoretically proceed through any of at least three processes: (a) malate $\rightarrow$ oxaloacetate $\rightarrow$ phosphoenolpyruvate $\rightarrow$ pyruvate, involving cytoplasmic malate dehydrogenase (EC 1.1.1.37), PEPCK (EC 4.1.1.32) and pyruvate kinase (EC 2.7.1.40); (b) malate $\rightarrow$ pyruvate + CO_2 , involving the NADP^+ -linked (EC 1.1.1.40) and/or the NAD^+ -linked (EC 1.1.1.39) 'malic' enzyme; and (c) malate $\rightarrow$ oxaloacetate $\rightarrow$ pyruvate + CO_2 , involving cytoplasmic malate dehydrogenase and an oxaloacetate decarboxylase. There is apparently a consensus that pathway (c) is inoperative, based on the fact that the cytoplasmic free $[\text{NAD}^+]/[\text{NADH}]$ ratio is very high (about 10^3), and that no oxaloacetate-decarboxylating enzyme has been found which can act at a significant rate on oxaloacetate at the concentration present in cells. In the present study we have evaluated gluconeogenesis and formation of other products from aspartate and D-glycerate, both of which require an additional (mitochondrially derived) source of

reducing equivalents for synthesis of glucose and lactate. The results are contrasted with those obtained with glutamate or malate as substrate, both of which provide glucose precursors coupled to a source of cytoplasmic NADH. α -CHC, a powerful inhibitor of mitochondrial pyruvate transport (Halestrap & Denton, 1974), was employed to evaluate the necessity for pyruvate recycling. These experiments are summarized in Table 1. With either aspartate or D-glycerate as sole substrate, α -CHC strongly suppressed glucose appearance, caused accumulation of lactate plus pyruvate, and markedly lowered the $[\text{lactate}]/[\text{pyruvate}]$ ratio. This result shows that pyruvate transport was inhibited (partially or completely) and that this was associated with oxidation of the cytosolic NAD^+/NADH couple and suppression of gluconeogenesis. This inhibition could have arisen from two effects: inhibition of generation of cytoplasmic reducing potential, as well as inhibition of ATP synthesis by blocking entry of precursor-derived substrate for mitochondrial oxidations. With this possibility in mind, kidneys were perfused with D-glycerate, plus octanoate (the latter to provide additional oxidizable substrate). Under this condition, glucose synthesis and glycerate usage were restored, lactate plus pyruvate accumulation increased about 7-fold, but the $[\text{lactate}]/[\text{pyruvate}]$ ratio was still diminished (Table 1). As a first consideration it would not necessarily be predicted that octanoate would restore gluconeogenesis from glycerate. However, the probable explanation is as follows: α -CHC elevated the concentration of pyruvate more than 10-fold and, at the concentration used, α -CHC does not suppress pyruvate transport completely (Vaartjes *et al.*, 1979; Rognstad, 1983). Therefore, pyruvate uptake was partially restored when its concentration was greatly elevated. That this process was still partially uncompensated is evidenced by the finding that the $[\text{lactate}]/[\text{pyruvate}]$ ratio was not restored to the uninhibited value. It has been shown that, when pyruvate is in the physiological concentration range, octanoate stimulates carboxylation of pyruvate many-fold (Davis-van Thienen & Davis, 1981). This would tend to pull the pyruvate cycling process.

For gluconeogenesis from malate, pyruvate recycling is not necessary for cytoplasmic NADH production. This is documented by the lack of effect of α -CHC on the $[\text{lactate}]/[\text{pyruvate}]$ ratio (Table 1). Since the energy requirements to sustain the kidney must now be provided by malate-derived pyruvate, it is not surprising that inhibition of pyruvate entry into mitochondria would suppress gluconeogenesis from malate.

Glutamate was also used as sole substrate to evaluate the effects of α -CHC on its metabolism, since there is no need for pyruvate cycling to generate cytoplasmic reducing equivalents from this substrate for gluconeogenesis, nor is pyruvate entry into mitochondria required for energy (ATP) production. Glucose synthesis was only marginally suppressed by α -CHC, the $[\text{lactate}]/[\text{pyruvate}]$ ratio was sustained in the physiological range, but there was a net increase in accumulation of lactate plus pyruvate. Therefore gluconeogenesis from glutamate and maintenance of cytoplasmic redox state are principally independent of pyruvate cycling (Table 1).

More direct evidence for the operation of pyruvate cycling during the metabolism of glycerate was obtained through an investigation of the effects caused by inhibition of PEPCK by 3-MPA. As shown in Table 2, the presence of 0.5 mM-3-MPA inhibited the rate of

Table 1. Effects of α -cyanohydroxycinnamate on utilization of substrates and release of metabolites by kidneys perfused under various conditions

Values are expressed as $\mu\text{mol}/\text{min}$ per kidney (means $\pm$ S.E.M. for the numbers of each type of perfusion, shown in parentheses). Perfusions were for 60 min; [lactate]/[pyruvate] ratios reported are those found at 60 min. + and - refer to perfusions done in the presence or absence of 0.5 mM- α -cyano-*p*-hydroxycinnamate respectively. * Different from control without inhibitor ($P < 0.05$ in all cases).

Substrate(s)	Metabolite...	Substrate	NH ₃	Glucose	Serine	Glutamate	Glutamine	Fumarate	Lactate	Pyruvate	Lactate + pyruvate	[Lactate]/[pyruvate]
3 mM-Aspartate	(6)	-	1.18 $\pm$ 0.16	0.30 $\pm$ 0.03	0.32 $\pm$ 0.03	0.37 $\pm$ 0.03	0.11 $\pm$ 0.12	-	0.05 $\pm$ 0.01	0.02 $\pm$ 0.00	0.07 $\pm$ 0.02	2.50 $\pm$ 0.18
	(3)	+	1.22 $\pm$ 0.06	0.08 $\pm$ 0.01*	0.25 $\pm$ 0.02	0.37 $\pm$ 0.02	0.11 $\pm$ 0.01	-	0.15 $\pm$ 0.01*	0.24 $\pm$ 0.01*	0.39 $\pm$ 0.01*	0.64 $\pm$ 0.09*
5 mM-Glutamate	(3)	-	1.04 $\pm$ 0.07	0.59 $\pm$ 0.02	0.32 $\pm$ 0.01	-	-	-	0.10 $\pm$ 0.02	<0.02	0.10 $\pm$ 0.12	>5.0
	(3)	+	0.73 $\pm$ 0.09	0.42 $\pm$ 0.02*	0.25 $\pm$ 0.01*	-	-	-	0.22 $\pm$ 0.02*	0.04 $\pm$ 0.00*	0.26 $\pm$ 0.02*	4.6 $\pm$ 0.4
3 mM-Glycerate	(3)	-	0.17 $\pm$ 0.02	0.21 $\pm$ 0.01	-	-	-	-	0.08 $\pm$ 0.00	0.02 $\pm$ 0.00	0.10 $\pm$ 0.00	3.87 $\pm$ 0.34
	(3)	+	0.21 $\pm$ 0.03	0.02 $\pm$ 0.00*	-	-	-	-	0.15 $\pm$ 0.02*	0.28 $\pm$ 0.04*	0.43 $\pm$ 0.07*	0.56 $\pm$ 0.07*
3 mM-Glycerate + 1 mM-octanoate	(5)	-	0.15 $\pm$ 0.01	0.25 $\pm$ 0.01	-	-	-	-	0.06 $\pm$ 0.00	0.04 $\pm$ 0.00	0.10 $\pm$ 0.00	1.57 $\pm$ 0.12
	(3)	+	0.12 $\pm$ 0.02	0.23 $\pm$ 0.02	-	-	-	-	0.21 $\pm$ 0.01*	0.48 $\pm$ 0.02*	0.69 $\pm$ 0.03*	0.44 $\pm$ 0.01*
5 mM-Malate	(3)	-	0.11 $\pm$ 0.01	0.44 $\pm$ 0.04	-	-	-	0.54 $\pm$ 0.03	0.15 $\pm$ 0.01	0.03 $\pm$ 0.00	0.18 $\pm$ 0.01	5.59 $\pm$ 0.25
	(3)	+	0.15 $\pm$ 0.02	0.06 $\pm$ 0.01*	-	-	-	0.70 $\pm$ 0.08	0.30 $\pm$ 0.00*	0.07 $\pm$ 0.00*	0.37 $\pm$ 0.01*	4.32 $\pm$ 0.20

glucose formation from 3 mM-D-glycerate by about 20%. However, this inhibition was associated with a marked lowering of the [lactate]/[pyruvate] ratio and an accumulation of the tissue content of malate plus fumarate (from 0.051 ± 0.002 to $0.140 \pm 0.018 \mu\text{mol}/\text{g}$), indicating a role for PEPCK in the shuttling of reducing potential from the mitochondria. Evidently, the reaction catalysed by PEPCK, a step that is not required for the direct conversion of glycerate carbon into glucose, normally functions as part of a pyruvate cycle. However, its activity does not appear to be obligatory for the shuttling process, since synthesis of glucose and lactate is not strongly suppressed by 3-MPA. This concentration of 3-MPA is sufficient to inhibit glucose production from 3 mM-aspartate by about 94% (Scaduto & Davis, 1985). Thus these data are of considerable importance, since they illustrate a PEPCK-independent mechanism for the shuttling of reducing potential.

The operation of a PEPCK-independent pathway for formation of pyruvate can be estimated by calculating the rate of aspartate oxidation from the difference in aspartate consumption and the synthesis of all products of its metabolism. In perfusions in which PEPCK flux is completely blocked (1.5 mM-3-MPA; glucose and serine synthesis blocked, without affecting NH₃ production), the calculated residual rate of PEPCK-independent oxidation of aspartate (and therefore pyruvate oxidation) was $1.07 \pm 0.10 \mu\text{mol}/\text{min}$ per kidney, and NH₃ production was the same as aspartate oxidation (1.01 ± 0.02) in three perfusions. Also, in terms of total nitrogen balance $1.35 \pm 0.08 \mu\text{equiv.}/\text{min}$ was lost from aspartate, and 1.29 ± 0.03 was recovered in products. Therefore both the carbon and nitrogen balances indicate a capacity of about $1 \mu\text{mol}/\text{min}$ for PEPCK-independent pathway(s) for pyruvate flux.

'Malic' enzyme activities in the rat renal cortex

Since the experiments performed with either aspartate or glycerate as substrate indicated that the kidney was capable of converting malate into pyruvate in the presence of 3-MPA, the activity of 'malic' enzyme in the kidney cortex was evaluated to assess its possible role in this regard. These results, after correction for the cross-contamination of mitochondrial and cytosolic activities, are shown in Table 3. Although we have identified a previously unrecognized NAD-linked 'malic' enzyme activity in rat kidney cortex, this activity is considerably less than the NADP-linked activity estimated to be present in both cellular compartments. These data show that the kidney cortex contains sufficient NADP-linked, but not NAD-linked, 'malic' enzyme activity to catalyse the estimated rate of pyruvate formation from aspartate during inhibition of PEPCK. This, however, does not provide any proof of its activity in the perfused kidney.

Scheme 2 summarizes how we perceive the potential pathways of generation of reducing equivalents for gluconeogenesis from aspartate or glycerate. Since both are more-oxidized molecules than is glucose, cytoplasmic NADH must be provided by mitochondrial processes. For glycerate, the carbon precursor is provided independently of mitochondrial processes, whereas the required NADH comes ultimately (indirectly) from mitochondria. Hence, some glycerate is anabolized to glucose and some is catabolized to pyruvate. The pyruvate can be carboxylated, the oxaloacetate thus formed reduced to

Table 2. Effects of 0.5 m-3-mercaptopycolinate on renal glycerate metabolism

Values are expressed as means $\pm$ S.E.M. for *n* perfusions; **P* $\leq$ 0.05 versus no inhibitor.

Metabolite(s)	Substrate... <i>n</i> ...	3 mM-Glycerate 5	3 mM-Glycerate + 0.5 mM-3-MPA 3
Glycerate (μ mol/min per kidney)		-1.42 ± 0.15	-1.39 ± 0.08
Glucose (μ mol/min per kidney)		0.22 ± 0.01	$0.16 \pm 0.01^*$
NH ₃ (μ mol/min per kidney)		0.17 ± 0.02	0.13 ± 0.01
Lactate (μ mol/kidney)		4.71 ± 0.03	$2.73 \pm 0.22^*$
Pyruvate (μ mol/kidney)		1.24 ± 0.10	$5.30 \pm 0.27^*$
Lactate + pyruvate (μ mol/kidney)		5.96 ± 0.13	$8.10 \pm 0.43^*$
[Lactate]/[pyruvate]		3.87 ± 0.34	$0.53 \pm 0.04^*$

Table 3. Enzyme activities in rat kidney cortex

Activities were determined at 30 °C, except for the cytosolic NAD-linked 'malic' enzyme activity, which was determined at 37 °C. For comparative purposes this activity, as shown, has been corrected to that expected at 30 °C, by assuming a Q_{10} of 2. All activities have been corrected for cross-contamination and represent the average activities of two (mitosol 'malic' enzymes) or three separate preparations; 1 unit of activity is 1 μ mol of substrate transformed to product/min.

	Activity		
	(munits/mg of cytosol)	(munits/mg of mitosol)	(units/g of cortex)
'Malic' enzyme			
NAD-linked	1.62	6.44	0.24
NADP-linked	25.1	20.4	1.38
Lactate dehydrogenase	3040	—	135
Glutamate dehydrogenase	—	2680	54

Scheme 2. Possible pathways for recycling pyruvate carbon derived from glycerate and aspartate for provision of cytoplasmic NADH for use in gluconeogenesis from these two substrates

The parallel curved lines represent the mitochondrial inner membrane.

malate, and the latter transported into the cytosol. As discussed above, the cytoplasmic malate can, in principle, be recycled back to pyruvate by at least three mechanisms, with the coupled generation of NAD(P)H. The PEPCK-dependent pathway is clearly functional, but, in addition, there is a PEPCK-independent pathway. This leaves the 'malic' enzymes and oxaloacetate decarboxylase as the remaining known possibilities for the latter. A weak oxaloacetate decarboxylase has been shown to occur in both rat kidney and liver mitochondria (Wojtczak & Walajtys, 1974), but this location is of no direct function in generating cytoplasmic NAD⁺. Furthermore, the high K_m for oxaloacetate (>0.5 mM) probably negates its function in this pathway. Finally, we have demonstrated the presence of both NAD- and NADP-linked 'malic' enzyme activities. For the NAD-linked enzyme, the total activity is too low to account for the calculated PEPCK-independent cycling of pyruvate. Furthermore, most of this activity was found to be of mitochondrial origin. Although this activity could function in recycling pyruvate, it would not be functional in generating cytoplasmic NADH. NADP-linked 'malic' enzyme activity in kidney cortex has been previously reported (Alleyne *et al.*, 1978; Watford *et al.*, 1980). Our own estimate of this activity was calculated to be sufficient to account for aspartate oxidation in the presence of 3-MPA. However, if this activity is also involved in pyruvate cycling linked to glucogenesis, this implies a substrate-mediated transhydrogenation from NADPH to NAD⁺.

In contrast with glucogenesis from glycerate, glucose synthesis from aspartate requires carbon flux through PEPCK (Scheme 2). We have also shown a PEPCK-independent pathway of pyruvate cycling from aspartate. A bifurcated pathway of aspartate carbon is therefore inferred: (a) recycling of aspartate-derived pyruvate to provide cytoplasmic NADH, coupled to (b) direct conversion of aspartate carbon into glucose precursors, which is not necessarily linked to pyruvate formation via pyruvate kinase.

In the present context it is important to cite the earlier work on renal gluconeogenesis from pyruvate (Saggerson, 1978) or from 2-oxoglutarate or glutamine (Watford *et al.*, 1980). Saggerson (1978) reached the guarded conclusion that some of the supply of reducing equivalents for glucose and lactate formation from pyruvate is provided by a pathway not involving cycling through pyruvate carboxylase, PEPCK and pyruvate kinase ('malic' enzyme?). In like manner, Watford *et al.* (1980) noted that inhibition of renal gluconeogenesis from glutamine or 2-oxoglutarate was without effect on complete oxidation of these substrates to CO₂ (in some species). On the basis of this and other evidence, these workers implicated 'malic' enzyme in this cycling process.

Finally, it has been reported that liver cells form lactate from citrate-cycle intermediates under certain conditions, and that this process is strongly stimulated by ethanol (see, e.g., Hensgens *et al.*, 1978; Davis & Baugh, 1985). 3-MPA blocked gluconeogenesis, but further increased lactate accumulation. Once again, these studies implicate, but do not prove, involvement of 'malic' enzymes in this cycling process.

The accumulated evidence from our and other laboratories has clearly established that citrate-cycle intermediates can be converted by gluconeogenic tissues

(liver and kidney cortex) into pyruvate by pathway(s) independent of the PEPCK- and pyruvate kinase-linked process. Although the exact identity of this reaction(s) remains to be established, this flux would be expected to play an important role in the homeostasis and substrate disposition in these multi-functional tissues. We propose that this pathway becomes an important by-pass, allowing for the complete oxidation of citrate-cycle intermediates and their precursors under conditions in which gluconeogenesis is not required. In a similar manner, we have proposed previously (Davis *et al.*, 1972; Davis & Bremer, 1973; Lin & Davis, 1974) the mitochondrial NAD-linked 'malic' enzyme as a mechanism for removal of excessively generated cycle intermediates in muscle.

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