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Proline Metabolism in Isolated Rat Liver Cells

By HUBERTUS E. S. J. HENSGENS,*† ALFRED J. MEIJER,† JOHN R. WILLIAMSON,‡ JOKE A. GIMPEL‡ and JOSEPH M. TAGER†

†*Laboratory of Biochemistry, B.C.P. Jansen Institute, University of Amsterdam, Plantage Muidergracht* 12, *Amsterdam, The Netherlands, and* ‡*Department of Biochemistry and Biophysics, University of Pennsylvania,* 37*th and Hamilton Walk, Philadelphia, PA* 19174, *U.S.A.*

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The metabolism of proline was studied in liver cells isolated from starved rats. The following observations were made. 1. Consumption of proline could be largely accounted for by production of glucose, urea, glutamate and glutamine. 2. At least 50% of the total consumption of oxygen was used for proline catabolism. 3. Ureogenesis and gluconeogenesis from proline could be stimulated by partial uncoupling of oxidative phosphorylation. 4. Addition of ethanol had little effect on either proline uptake or oxygen consumption, but strongly inhibited the production of both urea and glucose and caused further accumulation of glutamate and lactate. Accumulation of glutamine was not affected by ethanol. 5. The effects of ethanol could be overcome by partial uncoupling of oxidative phosphorylation. 6. The apparent K_m values of argininosuccinate synthetase (EC 6.3.4.5) for aspartate and citrulline in the intact hepatocyte are higher than those reported for the isolated enzyme. 7. 3-Mercaptopicolinate, an inhibitor of phosphoenolpyruvate carboxykinase (EC 4.1.1.32), greatly enhanced cytosolic aspartate accumulation during proline metabolism, but inhibited urea synthesis. 8. It is concluded that when proline is provided as a source of nitrogen to liver cells, production of ammonia by oxidative deamination of glutamate is inhibited by the highly reduced state of the nicotinamide nucleotides within the mitochondria. 9. Conversion of proline into glucose and urea is a net-energy-yielding process, and the high state of reduction of the nicotinamide nucleotides is presumably maintained by a high phosphorylation potential. Thus when proline is present as sole substrate, the further oxidation of glutamate by glutamate dehydrogenase (EC 1.4.1.3) is limited by the rate of energy expenditure of the cell.

When urea is synthesized by the liver, one of the two nitrogen atoms of the molecule is derived from aspartate and the other from ammonia. Aspartate is formed by transamination between glutamate and oxaloacetate, a process that can occur both in the mitochondria and in the cytosol. Ammonia is delivered to the liver via the portal-venous blood and in addition it is produced directly in the cell during amino acid breakdown. The generally held view is that ammonia is mainly formed via glutamate dehydrogenase (EC 1.4.1.3). McGivan & Chappell (1975), however, have proposed that ammonia is formed from aspartate via the purine nucleotide cycle, first described by Lowenstein (1972) in skeletal muscle.

To obtain information about the factors that control the production of aspartate and ammonia for urea synthesis we have investigated the metabolism of ammonia and of a number of amino acids, including proline, in isolated rat liver cells (Hensgens *et al.*, 1976; Meijer *et al.*, 1975; Williamson *et al.*, 1976). Proline is particularly suitable for such studies because it is readily converted into glutamate in the liver. Glutamate itself cannot be used because, as Hems *et al.* (1968) have shown, it does not permeate readily through the liver plasma membrane.

The conversion of proline into glutamate occurs via two oxidation steps, the first being oxidation of proline to 1-pyrroline-5-carboxylate by mitochondrial proline oxidase (Johnson & Strecker, 1962), and then oxidation of the latter compound to glutamate by 1-pyrroline-5-carboxylate dehydrogenase (EC 1.5.1.12) (Strecker, 1960), an NAD-linked enzyme that has been found both in the mitochondria and in the cytosol (Brunner & Neupert, 1969).

We have observed that the further metabolism of glutamate formed from proline is limited by the rate of reoxidation of mitochondrial NADH by the respiratory chain. Partial uncoupling of oxidative phosphorylation stimulates ureogenesis and gluco-

* To whom requests for reprints should be addressed.

neogenesis from proline (Hensgens *et al.*, 1976). Similarly, the inhibitory effect of ethanol on the metabolism of proline to glucose and urea, first observed by Krebs (1971) and Fellenius *et al.* (1973), can be overcome by partial uncoupling of oxidative phosphorylation (Hensgens *et al.*, 1976).

The metabolism of proline has now been examined in more detail. The results in the present paper suggest that the synthesis of urea from proline is primarily limited by the rate of formation of ammonia from glutamate via glutamate dehydrogenase.

Materials and Methods

Liver cells

Liver cells from 18–24h-starved rats were prepared by the procedure of Berry & Friend (1969), with the modifications described previously (Meijer *et al.*, 1975).

Incubation conditions

The incubations were performed in sealed plastic 25-ml Erlenmeyer flasks in a final volume of 2.5–4ml of Krebs Ringer medium (Krebs & Henseleit, 1932), pH7.4, containing 2.5–4% (w/v) dialysed serum albumin (fraction V; Sigma Chemical Co., St. Louis, MO, U.S.A.). The gas atmosphere was O_2/CO_2 (19:1). The cell concentration was 5–10mg dry wt./ml. Sampling of the cells, deproteinization with $HClO_4$ and neutralization of the extracts were carried out as described previously (Meijer *et al.*, 1975). The metabolic reactions were linear with time, unless stated otherwise. For determination of the intracellular contents of metabolites, the cells were centrifuged through a layer of silicone oil (ρ 1.015g/ml) into a layer of 14% (w/v) $HClO_4$ as described previously (Meijer *et al.*, 1975). In some experiments the mitochondrial–cytosolic distribution of metabolites was determined by using the digitonin technique of Zuurendonk & Tager (1974) with the modifications described by Zuurendonk *et al.* (1976). The cells were exposed to digitonin (2mg/ml) at about 5°C for 15s to rupture the plasma membrane before the cellular fragments (including the mitochondria) were centrifuged through silicone oil (ρ 1.027g/ml) into $HClO_4$. Lactate dehydrogenase (EC 1.1.1.27), as the cytosolic marker, and adenylate kinase (EC 2.7.4.3), as the mitochondrial marker, were assayed in the supernatant by conventional methods (Bergmeyer *et al.*, 1970).

Oxygen was determined either by the manometric method described by Krebs *et al.* (1974) or polarographically by using a Clark-type oxygen electrode (Oxygraph). In the latter case, 1.5ml samples of the cell suspension were withdrawn from the incubation vessel at regular intervals and the rate of oxygen consumption was monitored in the Oxygraph for 5min before quenching of the cells with $HClO_4$. The two methods yielded values for the rate of oxygen consumption that agreed within 10%. Measurements of oxygen consumption were always done in duplicate.

Determination of metabolites

Glucose was determined by the method of Bergmeyer *et al.* (1970). Ammonia and urea were determined consecutively by using glutamate dehydrogenase followed by addition of urease (EC 3.5.1.5) (Bernt & Bergmeyer, 1970*b*). Glutamate was determined with NAD^+ and glutamate dehydrogenase (Bernt & Bergmeyer, 1970*a*). Glutamine was determined with glutaminase (EC 3.5.1.2) and glutamate dehydrogenase (Lund, 1970). β-Hydroxybutyrate and acetoacetate were determined by the methods of Williamson & Mellanby (1970) and Mellanby & Williamson (1970) respectively. For metabolites present at low concentrations (ATP and aspartate), fluorimetric assay techniques were used as described by Williamson & Corkey (1969). Citrulline was determined by the method of Hunninghake & Grisolia (1966) after treatment of the neutralized samples with urease. Proline was determined with a Beckman Multichrome M amino acid analyser.

Materials and enzymes

Enzymes, coenzymes and intermediary metabolites were obtained from Sigma Chemical Co., St. Louis, MO, U.S.A., or from Boehringer Mannheim G.m.b.H., Mannheim, Germany. Collagenase (EC 3.4.24.3) was obtained from Worthington Biochemical Corp., Freehold, NJ, U.S.A. 3-Mercaptopicolinic acid was generously given by Dr. H. L. Saunders of Smith, Kline and French Laboratories, Philadelphia, PA, U.S.A. Carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone was a gift from Dr. Peter Heytler of E. I. DuPont de Nemours and Co., Wilmington, DE, U.S.A.

Results

Proline metabolism and its inhibition by ethanol

Although gluconeogenesis and ureogenesis with proline as the substrate are slow processes, large amounts of glutamate accumulate in the cell suspension (Hensgens *et al.*, 1976). This suggests that the rate-limiting step of proline metabolism is distal to the production of glutamate. Fig. 1 shows that gluconeogenesis and ureogenesis from proline were already maximal at 5mM-proline. On the other hand, glutamate accumulation increased still further when the proline concentration was increased to above 5mM.

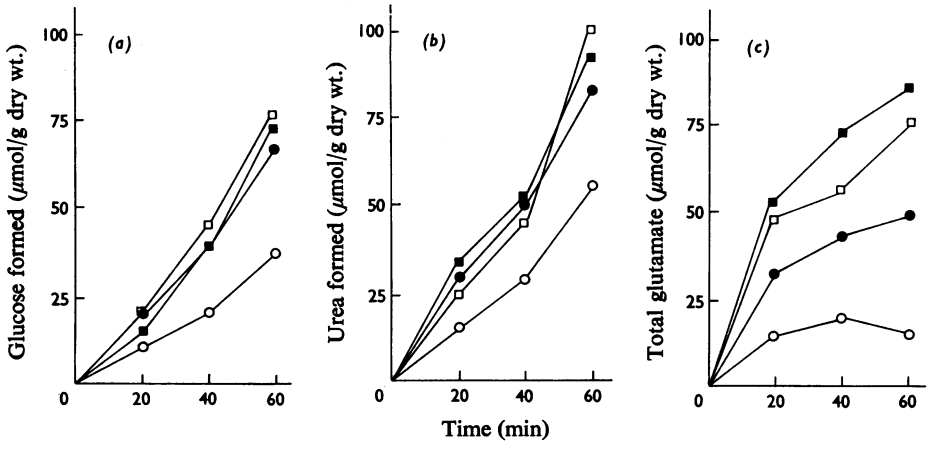

Fig. 1. *Effect of proline concentration on (a) glucose formation, (b) urea synthesis and (c) glutamate formation from proline in isolated rat liver cells*

For experimental details see the Materials and Methods section. Ornithine (3mM) was present in all incubations. ○, No proline; ●, 5mM-proline; □, 10mM-proline; ■, 20mM-proline.

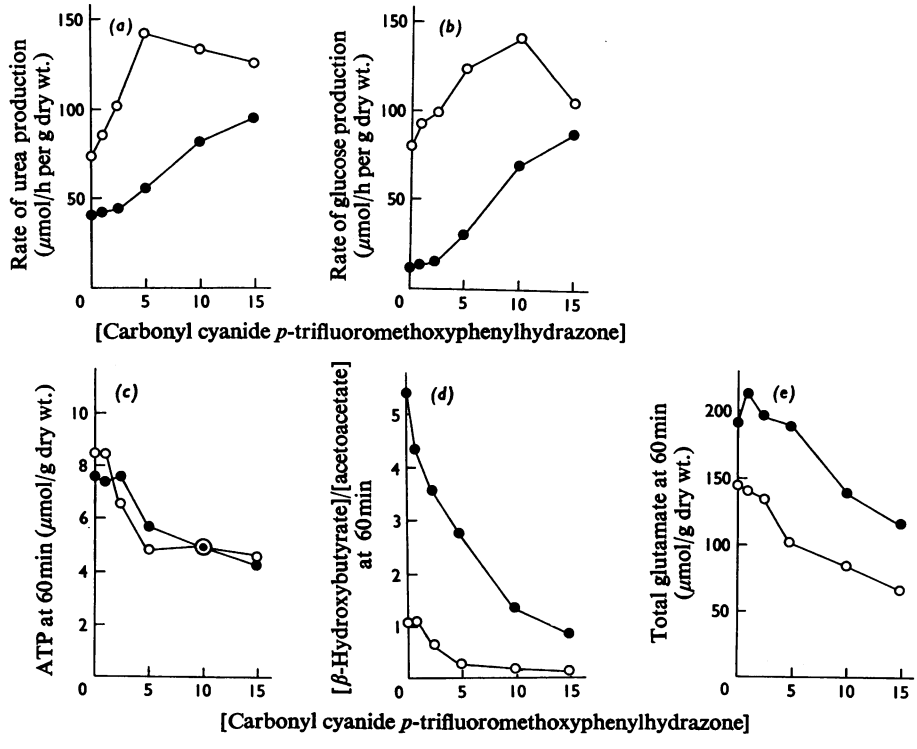

Fig. 2. *Effect of carbonyl cyanide p-trifluoromethoxyphenylhydrazone on proline metabolism in the absence and presence of ethanol*

For experimental details see the text and the Materials and Methods section. Proline (10mM) and ornithine (3mM) were present in all incubations. Carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone was added at the concentrations indicated. ○, Ethanol absent; ●, 10mM-ethanol present.

The metabolism of proline can be inhibited by adding ethanol (Krebs, 1971; Fellenius *et al.*, 1973; Hensgens *et al.*, 1976). Studies with isolated mitochondria (see Hoek *et al.*, 1974), the perfused rat liver (Sies *et al.*, 1974) and isolated rat liver cells (Williamson *et al.*, 1976) have demonstrated that flux through glutamate dehydrogenase is strongly controlled by the redox state of the mitochondrial nicotinamide nucleotides, the production of ammonia being greater when they become more oxidized. Since ethanol is known to be a powerful reductant of NAD in both cytosol and mitochondria (Forsander *et al.*, 1965; Williamson *et al.*, 1969), it is possible that the inhibitory effect of ethanol on proline metabolism is due to diminished flux through glutamate dehydrogenase in the direction of ammonia production, caused by increased reduction of the mitochondria. Indeed, the inhibition by ethanol of glucose formation and urea synthesis from proline is accompanied by an increased accumulation of glutamate in the cell suspension (Hensgens *et al.*, 1976).

Of particular importance is the fact that the effect of ethanol on the synthesis of urea and glucose from proline can be reversed by the addition of the uncoupler of oxidative phosphorylation carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone (hereafter termed 'the uncoupler'), as is shown in Fig. 2. It must be stressed that rather large amounts of the uncoupler were required because it was bound in part to the albumin present in the incubation medium.

Figs. 2(*a*) and 2(*b*) show that concentrations of uncoupler up to 10μM almost doubled the rate of synthesis of both urea and glucose with proline as the substrate. This effect was paralleled by decreases in cellular ATP and in the [β-hydroxybutyrate]/[acetoacetate] ratio (Figs. 2*c* and 2*d*). In the absence of ethanol, beyond a concentration of 5–10μM-uncoupler, the rates of urea and glucose production decreased again, presumably because of too extensive uncoupling. Ethanol inhibited synthesis of both urea and glucose (Figs. 2*a* and 2*b*). This inhibition was accompanied by an increased accumulation of glutamate (Fig. 2*e*). Addition of the uncoupler released the inhibition by ethanol (Figs. 2*a* and 2*b*). Fig. 2(*e*) shows that the acceleration of ureogenesis from proline by the uncoupler was accompanied by a decreased accumulation of glutamate in the cell suspension, in both the absence and the presence of ethanol. This illustrates that the oxidation of glutamate is the rate-controlling step in proline degradation.

To obtain more information about the inhibitory effect of ethanol on proline metabolism, not only the products of proline catabolism, but also the uptake of proline and the rate of oxygen consumption, were measured. Table 1 gives the results of several such experiments.

In the presence of proline alone, the amounts of nitrogen-containing products glutamate, glutamine and urea were about 35% higher than could be accounted for by proline uptake. The difference was partly accounted for by ornithine consumption, which was 20–30μmol/h per g dry wt. of cells (results not shown), and possibly by urea production from endogenous sources, since urea synthesis in the absence of added substrates was 38μmol/h per g dry wt. of cells. At least 50% of the total oxygen consumption could be accounted for by proline oxidation, since oxygen consumption increased from 437 to 926μmol/h per g dry wt. on proline addition. This is consistent with the stoicheiometry of oxygen consumption during conversion of proline into urea (5mol of

Table 1. *Effect of ethanol and the uncoupler carbonyl cyanide p-trifluoromethoxyphenylhydrazone on proline metabolism in isolated rat liver cells*

For details see the Materials and Methods section. The concentrations of the substrates were: proline, 7mM; ornithine, 3mM; ethanol, 10mM. Carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone was added at 10μM. The values represent the means ± S.E.M. for five experiments. The first four incubations were carried out with each of the five separate cell preparations. The incubation with no substrate was performed only in two of the five preparations. Nitrogen products are the sum of glutamate plus twice the amounts of urea and glutamine formed. In each of the individual experiments the percentage stimulation of urea synthesis by the uncoupler in the absence of ethanol was 25 ± 3 and that of glucose production 16 ± 4. In other experiments the stimulation by the uncoupler was higher (cf. Fig. 2).

	Metabolite changes (μmol/h per g dry wt. of cells)						
Additions	O_2	−Proline	Glucose	Urea	Glutamate	Glutamine	Nitrogen products
Proline+ornithine	926 ± 63	254 ± 19	88 ± 8	96 ± 9	57 ± 9	48 ± 4	345
Proline+ornithine+ethanol	928 ± 91	253 ± 25	22 ± 6	49 ± 6	126 ± 13	45 ± 3	314
Proline+ornithine+uncoupler	1287 ± 97	272 ± 14	101 ± 7	119 ± 10	45 ± 7	30 ± 8	343
Proline+ornithine+ethanol +uncoupler	1408 ± 95	260 ± 17	62 ± 11	75 ± 12	85 ± 13	41 ± 2	317
None	352, 522	—	12, 22	25, 51	8, 20	16, 20	126

O_2/mol of urea), glutamate (1mol of O_2/mol of glutamate) and glutamine (3.5mol of O_2/mol of glutamine, assuming that the 2-oxoglutarate formed by deamination of glutamate is quantitatively converted into glucose).

Addition of ethanol had hardly any effect on the rate of oxygen consumption, which demonstrates that oxidation of ethanol competes with that of proline for transport of reducing equivalents via the respiratory chain to oxygen. Ethanol had little effect on proline uptake. The inhibition of synthesis of urea and glucose was accompanied by a considerable accumulation of glutamate in the cell suspension (cf. Fig. 2). Glutamine production was not affected by ethanol. The results show that the inhibitory effect of ethanol on proline catabolism is located at the level of glutamate oxidation and not at the level of proline oxidation to glutamate.

The uncoupler reversed the inhibitory effect of ethanol on proline catabolism (Table 1; cf. Fig. 2). Although oxygen consumption increased on addition of the uncoupler, the uptake of proline was little influenced. By contrast, the uncoupler greatly stimulated ethanol oxidation under these conditions (Hensgens *et al.*, 1976).

Distribution of glutamate and aspartate across the mitochondrial membrane during proline metabolism in isolated rat liver cells

Since during proline metabolism very large amounts of glutamate accumulate in the cell suspension, it was decided to find out where this glutamate is located in the cell. Cells were treated with digitonin after a 30-min incubation with proline in the absence or presence of ethanol, as described by Zuurendonk & Tager (1974). Table 2 shows the results of such an experiment. To ensure that the digitonin treatment resulted in rupture of the plasma membrane but did not affect the integrity of the mitochondrial membranes, both lactate dehydrogenase and adenylate kinase were measured in the extraparticulate fluid. The treatment with digitonin caused an increase in the leakage of lactate dehydrogenase from the cells

Table 2. *Distribution of glutamate and aspartate across the mitochondrial membrane during proline metabolism in isolated rat liver cells*

For details see the text and the Materials and Methods section. The concentrations of the substrates were: proline, 10mM; ethanol, 10mM. The incubation time was 30min. The values in the Table are the results of duplicate incubations. The mitochondrial and cytosolic concentrations of glutamate and aspartate were calculated by assuming a mitochondrial water space of 0.2ml/g dry wt. and a cytosolic water space of 2.0ml/g dry wt. (see the text). To rule out the possibility that the glutamate associated with the mitochondrial fraction was formed from proline during the transfer of mitochondria through the silicone oil, control experiments (results not shown) were carried out in which proline was omitted during the 30min incubation but was added during treatment of the cells with digitonin. No glutamate was formed under those conditions. Results similar to those shown in the Table were obtained in several experiments.

	Cells incubated with	
	Proline	Proline+ethanol
Lactate dehydrogenase in supernatant (% of total)		
In control cells	13, 15	15, 17
In digitonin-treated cells	79, 85	87, 97
Adenylate kinase in supernatant (% of total)		
In control cells	3, 4	3, 4
In digitonin-treated cells	11, 12	11, 11
Glutamate (μmol/g dry wt. of cells)		
Mitochondrial	12, 14	17, 18
Cytosolic	17, 18	21, 21
Extracellular	23, 23	24, 26
Aspartate (μmol/g dry wt. of cells)		
Mitochondrial	0.1, 0.1	0.1, 0.1
Cytosolic	2.0, 2.1	0.8, 1.0
Extracellular	1.4, 1.5	0.5, 0.6
Glutamate concentration (mM)		
Mitochondrial	65	87
Cytosolic	8.7	10.5
Aspartate concentration (mM)		
Mitochondrial	0.5	0.5
Cytosolic	1.0	0.5

from control values of 15% to values of greater than 80%. Leakage of adenylate kinase, on the other hand, increased only slightly, thus demonstrating adequate separation of mitochondria and cytosol (see Zuurendonk & Tager, 1974). It is important to stress that at a leakage of lactate dehydrogenase of as low as 50% the plasma membrane is already fully permeable to small molecules such as sucrose (Zuurendonk *et al.*, 1976).

Table 2 shows that a considerable portion of the intracellular glutamate was associated with the mitochondrial fraction. Aspartate, on the other hand, was mainly in the cytosol. Table 2 also shows the actual (calculated) cytosolic and mitochondrial concentrations, assuming that the cytosolic water space is 2.0ml/g dry wt. of cells and the mitochondrial water space 0.2ml/g dry wt. (Williamson, 1969; see also Tischler *et al.*, 1977). The extremely high intramitochondrial concentration of glutamate is particularly striking. Ethanol caused a small increase in both mitochondrial and cytosolic concentrations of glutamate, so that the concentration gradient of glutamate across the mitochondrial membrane was not significantly altered. The aspartate concentration was very low in the mitochondrial fraction, in both the absence and the presence of ethanol. The accuracy of the measurements did not allow us to draw a conclusion about the precise mitochondrial/cytosolic concentration ratio for aspartate. The calculated cytosolic aspartate concentration in the present experiment was about 1mM. This value is much higher than the K_m value of rat liver argininosuccinate synthetase for aspartate, which is about 20μM (Saheki *et al.*, 1975). This suggests that, with proline as the substrate, the flux through argininosuccinate synthetase is controlled by the supply of citrulline and not by the supply of aspartate (but see the Discussion section).

Urea synthesis from proline: limitation by ammonia?

The experiments described above show that the high mitochondrial reduction state prevents the further oxidation of glutamate formed from proline. Glutamate oxidation proceeds via two pathways, namely deamination to 2-oxoglutarate and ammonia and transamination to aspartate (see, for example, de Haan *et al.*, 1967). If, during ureogenesis, neither ammonia nor aspartate accumulates, fluxes through both pathways must be equal. The strong correlation between urea synthesis from proline and the mitochondrial redox state (Hensgens *et al.*, 1976) can be explained on the basis of redox control of glutamate dehydrogenase, but oxidation of the mitochondrial nicotinamide nucleotides will also lead to increased flux through the transamination pathway of glutamate oxidation. To test whether aspartate production also controls urea synthesis from proline, we have studied the effect of 3-mercaptopicolinate on proline metabolism. This compound is a powerful inhibitor of phosphoenolpyruvate carboxykinase (DiTullio *et al.*, 1974). Goodman (1975) has shown that addition of 3-mercaptopicolinate to livers perfused with lactate or alanine leads to accumulation of oxaloacetate, malate and aspartate, as does quinolinate, another inhibitor of phosphoenolpyruvate carboxykinase (Veneziale *et al.*, 1967).

During proline metabolism, glutamate accumulates in large amounts within the cells (Table 2). Therefore inhibition of phosphoenolpyruvate synthesis by 3-mercaptopicolinate can be expected to lead to an accumulation of large amounts of aspartate. Table 3 shows the result of such an experi-

Table 3. *Effect of 3-mercaptopicolinate on proline metabolism in isolated rat liver cells*

For details see the Materials and Methods section. The concentrations of the substrates were: proline, 10mM; ethanol, 7mM; ammonia, 8mM. Ornithine (3mM) was present in all incubations. 3-Mercaptopicolinate was added at the concentrations indicated. Results similar to those shown in the Table were obtained in several experiments.

Additions	3-Mercaptopicolinate (mM)	Metabolite changes (μmol/h per g dry wt. of cells)			Intracellular metabolite concentrations (μmol/g dry wt. of cells after 30 min)		
		Glucose	Urea	Lactate	Aspartate	Glutamate	ATP
Proline	0	76	112	7	2.1	50	9.0
	0.1	30	69	6	12.2	51	9.2
	0.5	9	58	16	16.4	60	10.1
	3.0	2	58	30	16.2	75	9.1
Proline+ethanol	0	12	60	34	1.6	63	8.0
	0.1	2	57	58	1.5	74	9.2
	0.5	2	56	56	1.6	72	8.1
	3.0	1	64	60	1.7	80	7.5
Proline+ammonia	0	72	574	—	—	—	—
	0.1	33	598	—	—	—	—
	0.5	8	588	—	—	—	—
	3.0	1	574	—	—	—	—

Table 4. *Distribution of aspartate across the mitochondrial membrane during proline metabolism in the presence of 3-mercaptopicolinate*

For details see the Materials and Methods section. The concentrations used were: proline, 10mM; 3-mercaptopicolinate, 1mM. Ornithine (3mM) was also present. The incubation time was 40min. The values in the Table are the results of duplicate incubations.

	Cells incubated with	
	Proline	Proline+3-mercaptopicolinate
Aspartate (μmol/g dry wt. of cells)		
Mitochondrial	0.3, 0.4	1.1, 1.3
Cytosolic	3.1, 4.3	27, 29
Extracellular	3.8, 4.4	14, 18
ATP (μmol/g dry wt. of cells)		
Mitochondrial	2.1, 2.2	1.9, 2.2
Cytosolic	7.5, 7.9	7.7, 7.9
Extracellular	None	None
Change in concentration of urea (μmol/g dry wt. of cells)	56, 57	24, 27
Change in concentration of glucose (μmol/g dry wt. of cells)	52, 53	4, 4

ment. 3-Mercaptopicolinate at 0.5mM or higher almost totally blocked gluconeogenesis from proline. The intracellular aspartate concentration increased 8-fold, whereas glutamate increased by up to 50%. Despite this large increase in aspartate, urea synthesis decreased by 50%. Cellular ATP was not significantly affected, even at 3mM-3-mercaptopicolinate, indicating that ATP deficiency was not the cause of the decreased urea production.

In the experiment of Table 4, the cellular distribution of aspartate in the presence of 3-mercaptopicolinate was examined. In the presence of the inhibitor, more than 90% of the cellular aspartate was located in the cytosol. The cytosolic aspartate concentration was as high as 13–15mM (assuming that 1g dry wt. is equivalent to 2ml of water volume). The ATP concentration was measured and the usual distribution between mitochondria and cytosol, also found by others (cf. Zuurendonk & Tager, 1974; Siess & Wieland, 1976; Katz & Jungerman, 1976) was shown, thus ruling out the possibility that damage to the mitochondria by the digitonin treatment might have led to a loss of aspartate.

In view of the high cytosolic concentrations of aspartate in the presence of 3-mercaptopicolinate, it must be concluded that it is the production of the other substrate of the urea cycle, i.e. ammonia, that is the rate-controlling step in urea synthesis from proline under these conditions.

In another experiment (results not shown) we found that 0.5mM-3-mercaptopicolinate in the presence of proline caused an increase in the [β-hydroxybutyrate]/[acetoacetate] ratio from 1.2 to 3.4 after 60min. This observation is in agreement with similar results obtained with 3-mercaptopicolinate in the intact rat (Blackshear *et al.*, 1975) or in rat liver perfused with lactate or alanine (Goodman, 1975). This increase in the mitochondrial reduction state must have been caused by the decreased utilization of reducing equivalents in the cell because of the inhibition of glucose production. Thus the inhibition by 3-mercaptopicolinate of urea synthesis from proline, like that caused by ethanol, can be explained by the fact that accumulation of reducing equivalents in the mitochondria leads to a diminished flux through glutamate dehydrogenase in the direction of ammonia production.

When 3-mercaptopicolinate was added to cells incubated with proline plus ethanol, no effect on cellular aspartate was seen, and only a slight increase in glutamate occurred (Table 3). This result is consistent with the fact that, in the presence of ethanol, glucose synthesis was already very low. Therefore flux through phosphoenolpyruvate carboxykinase must have been slow, and inhibition of this enzyme by 3-mercaptopicolinate would not have led to the large accumulation of aspartate seen in the absence of ethanol.

When ammonia was added in the presence of proline, a large increase in urea synthesis was observed. No effect of 3-mercaptopicolinate on this rate was observed, although glucose synthesis was again almost completely abolished. This result therefore rules out the possibility that 3-mercaptopicolinate has a direct inhibitory effect on any of the enzymes involved in the operation of the urea cycle.

Production of lactate during proline metabolism

Hensgens *et al.* (1976) have shown that during the metabolism of proline, especially in the presence of ethanol, considerable amounts of lactate are formed. The observation that 3-mercaptopicolinate increased lactate production even further (Table 3) indicates that lactate was not produced by back-flow of carbon via pyruvate kinase (EC 2.7.1.40) as is the case in lactate production during pyruvate metabolism (Friedmann *et al.*, 1971; Rognstad & Katz, 1972; Meijer & Williamson, 1974). Instead, we propose that the carbon for lactate production is provided for by the action of 'malic' enzyme (EC 1.1.1.40), since cellular malate content increased from 0.9 to 7.4 and from 1.9 to 4.9 μmol/g dry wt. of cells when 3 mM-mercaptopicolinate was added in the presence of proline or in the presence of proline plus ethanol respectively. The NADPH produced in the 'malic' enzyme reaction may then be oxidized via the isocitrate-2-oxoglutarate shuttle or may be used for fatty acid synthesis.

Discussion

In principle, proline is an ideal substrate for the study of the mechanisms that control the production of ammonia and aspartate for urea synthesis. After two oxidation steps, glutamate, the direct precursor of ammonia and aspartate, is formed in the mitochondria; 1 mol of ammonia and 1 mol of aspartate are required for each mol of urea synthesized.

When 2 mol of proline are completely metabolized to glucose and urea, 28 mol of ATP are synthesized by oxidative phosphorylation, whereas only 8 mol of ATP are required for the synthesis of glucose and urea (see Hensgens *et al.*, 1976). Thus with proline as the substrate, ATP production is in large excess of the energy demands for gluconeogenesis and ureogenesis.

The results described in the present paper demonstrate that, during metabolism of proline, the oxidation of the glutamate formed is inhibited by the high state of mitochondrial reduction. This situation is even more pronounced when ethanol is also present. The fact that synthesis of both glucose and urea was accelerated by low concentrations of an uncoupler of oxidative phosphorylation strongly suggests that ATP utilization in the cells was not fast enough to allow re-oxidation of mitochondrial NAD(P)H by oxygen to occur at a sufficiently high rate.

It is likely that, of the two pathways of glutamate oxidation, glutamate deamination rather than transamination is most affected by the high state of mitochondrial reduction, for two reasons. Firstly, the rate of urea synthesis with glutamine as the substrate is about three times that observed with proline, despite the fact that with both substrates equal amounts of glutamate accumulate in the cell suspension, as shown previously (Hensgens *et al.*, 1976). Secondly, in the presence of 3-mercaptopicolinate large amounts of aspartate were formed from proline, but urea synthesis was still slow (Tables 3 and 4). Since the cytosolic aspartate concentration under these conditions was very high (13–15 mM; see Table 4) it can also be concluded that ammonia could not be produced from aspartate via the AMP cycle, as proposed by McGivan & Chappell (1975).

Krebs *et al.* (1976) postulated that stoicheiometry between carbamoyl phosphate and aspartate formation for urea synthesis is achieved by the fact that the aspartate aminotransferase system in the normal liver is close to equilibrium. They proposed that, whenever aspartate is removed for urea synthesis by the formation of argininosuccinate from citrulline and aspartate, aspartate aminotransferase re-establishes equilibrium, thus maintaining aspartate at a constant concentration. In agreement with this proposal we have found, using the digitonin technique to fractionate hepatocytes (Zuurendonk & Tager, 1974), that mitochondrial aspartate aminotransferase in the intact hepatocyte incubated with proline is indeed very close to equilibrium (M. E. Tischler & A. J. Meijer, unpublished work).

It is noteworthy that in the presence of proline both the cytosolic aspartate concentration (Tables 2 and 4) and the cellular citrulline content (see Hensgens *et al.*, 1976) are in the millimolar range, which is well above the K_m values of either aspartate (20 μM) or citrulline (44 μM) for the isolated rat liver argininosuccinate synthetase (Saheki *et al.*, 1975). To account for the weak flux through this enzyme, with proline as the only added substrate, we can only assume that the apparent K_m values of argininosuccinate synthetase for both aspartate and citrulline in the intact hepatocyte are higher than those found for the isolated enzyme.

Finally, we wish to comment on the production of glutamine in our experiments. In contrast with the observations of Lund & Watford (1976), we found appreciable production of glutamine from proline. This difference in result may be explained by a difference in experimental conditions; we used 7 mM-proline and hepatocytes prepared from 24 h-starved rats, whereas Lund & Watford (1976) used 5 mM-proline and hepatocytes from 48 h-starved rats. It is noteworthy that ethanol had little effect on glutamine production, despite the fact that glutamate oxidation and urea synthesis were inhibited (see Table 1). Apparently, in the hepatocyte, glutamine synthesis has a greater affinity for ammonia than has synthesis of carbamoyl phosphate, although *in vitro* the K_m values of ammonia for glutamine synthetase (EC 6.3.1.2) (see Pamiljans *et al.*, 1962; Tate & Meister, 1971) and for carbamoyl phosphate synthase (ammonia) (EC 2.7.2.5) (see Elliott & Tipton, 1974) are of the same order of magnitude. The uncoupler carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone decreased net glutamine synthesis in the absence

but not in the presence of ethanol (Table 1). At present we do not have a satisfactory explanation for this phenomenon.

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