Interrelationships between Gluconeogenesis and Ureogenesis in Isolated Hepatocytes*

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DL-Cycloserine (5 to 10 mM), when added to isolated hepatocytes, is shown to inhibit aspartate aminotransferase predominantly in the cytosol. At low concentrations (0.1 to 0.2 mM) it selectively inhibits alanine aminotransferase. Aminooyacetate (0.5 mM) irreversibly inhibits all transaminase activity provided the inhibitor is added prior to substrate addition. Neither cycloserine nor aminooyacetate caused appreciable inhibition of gluconeogenesis from pyruvate, but both inhibitors greatly decreased ureogenesis from lactate. These observations confirm earlier findings that malate efflux from mitochondria predominates with pyruvate as glucose precursor while aspartate efflux predominates with lactate as substrate.

Urea synthesis from ammonia by isolated hepatocytes was limited by endogenous ornithine. Half-maximal stimulation of urea synthesis in the presence of pyruvate was obtained with 0.5 mM exogenous ornithine. Measurements of citrulline accumulation showed (a) that carbamyl phosphate formation was not rate-limiting and (b) that the apparent $K_\text{m}$ of argininosuccinate synthetase for citrulline was about 1.5 mM. Urea formation in the presence of ornithine and either lactate or pyruvate was limited by the activity of argininosuccinate synthetase. Urea synthesis was completely inhibited by aminooyacetate, but was largely unaffected by cycloserine. These data show that aspartate required for urea synthesis must be generated in the mitochondria. Accumulation of aspartate showed that its rate of formation was not limiting for urea synthesis. Cycloserine addition in the absence of ornithine stimulated urea formation from ammonia. This effect was caused by an increase of intracellular citrulline levels because of inhibition of ornithine transaminase.

Ammonia addition to hepatocytes incubated with oleate and either lactate or pyruvate caused an inhibition of gluconeogenesis and accumulation of amino acids (primarily alanine, glutamate, and aspartate). Decreased gluconeogenesis correlated with lowered malate levels, suggesting limitation of flux through P-enolpyruvate carboxykinase by a fall of the oxalacetate concentration. Pyruvate carboxylase flux was also inhibited, possibly because of elevated mitochondrial glutamate levels. On the other hand, pyruvate dehydrogenase was stimulated by ammonia addition as a result of the decreased mitochondrial NADH/NAD ratio. Ammonia addition with ornithine increased malate production in the cytosol as a consequence of increased urea formation, and released inhibition of flux through pyruvate carboxylase and P-enolpyruvate carboxykinase when pyruvate was added as substrate. With lactate as substrate, high rates of urea synthesis induced by ornithine did not stimulate gluconeogenesis, and malate levels remained low because of constraints imposed by maintenance of near-equilibrium of cytosolic aspartate aminotransferase. No evidence was obtained in the present experiments that transport of anions across the mitochondrial membrane provided a rate-limiting step in the pathways of either gluconeogenesis or urea synthesis.

Urea synthesis from ammonia or amino acids requires a stoichiometric production of carbamyl phosphate and aspartate. Carbamyl phosphate is formed in the mitochondria from ammonia, CO$_2$, and ATP, and reacts with ornithine to produce citrulline. Citrulline is transported to the cytosol, where its carbamyl group condenses with the amino group of aspartate in the presence of ATP to form argininosuccinate, AMP, and pyrophosphate. Cleavage of argininosuccinate yields arginine and fumarate. Fumarate is converted to malate by cytosolic fumarase, where arginine is split to produce urea and ornithine. The overall pathway of urea formation involves enzyme reactions in both the cytosol and mitochondria, which are subject to control by the concentrations of their substrates, activators, and inhibitors. In addition, compartmentation of urea cycle enzymes necessitates transport of intermediates across the mitochondrial membrane. Hence many factors may contribute to the regulation of urea synthesis under different metabolic conditions.
In previous studies using isolated liver cells from fasted rats, various rate-limiting steps in the pathway of urea synthesis from externally added ammonia in the absence of gluconeogenic precursors were described (1, 2). It was concluded that one of the factors limiting the rate of urea synthesis under these conditions was the supply of oxalacetate for mitochondrial aspartate production, this being determined by the rate of transport of malate from the cytosol to the mitochondria. However, it may be expected that in the presence of carbon substrates capable of generating pyruvate, and hence oxalacetate via pyruvate carboxylase, aspartate generation no longer becomes rate-limiting for ureogenesis. The question then arises whether aspartate needed for condensation with citrulline in the cytosol is formed directly in the cytosol by transamination between oxalacetate and glutamate or is generated in the mitochondria and thereafter transported to the cytosol. Whichever process predominates will determine which metabolic anions are transported across the mitochondrial membrane and their flux. Thus the overall rate of gluconeogenesis or ureogenesis, under particular substrate availability conditions, may be regulated by kinetic constraints imposed upon the translocator steps (2-4). Our current work focuses on an elucidation of the role which the anion translocators play in determining the concentration of metabolic anions in the mitochondrial and cytosolic spaces, and their quantitative interactions with enzymes of the gluconeogenic and ureogenic pathways.

Previous studies by Lardy et al. (5) and Krebs et al. (6) suggested that, in the liver and kidney of the rat, where P-enolpyruvate carboxykinase is located predominately in the cytosol (7), gluconeogenesis is associated with transfer of oxalacetate from the mitochondria to the cytosol in the form of malate or aspartate. Oxalacetate as such is transported very slowly across the mitochondrial membrane at physiological concentrations because of its low affinity for the dicarboxylate (8) and a-ketoglutarate (9) translocators. From stoichiometric considerations of carbon and hydrogen balances (6, 10), it appeared likely that with pyruvate as a gluconeogenic precursor, malate efflux from the mitochondria predominates, while with lactate as substrate aspartate is the principal anion transported. Subsequent studies using perfused rat liver (11, 12) or rat kidney cortex slices (13, 14) confirmed these predictions by showing that gluconeogenesis from lactate is largely inhibited by aminooxyacetate, a powerful transaminase inhibitor (15), while gluconeogenesis from pyruvate is little affected. When gluconeogenesis and ureogenesis occur simultaneously, the patterns of anion transport are more complex (2, 16). In particular, cytosolic malate formed as a consequence of the operation of the urea cycle becomes potentially available for conversion to glucose (5, 10), and thus may alter the availability conditions, may be regulated by kinetic considerations of carbon and hydrogen balances (6, 10), it may be expected that in the presence of carbon substrates capable of generating pyruvate, and hence oxalacetate via pyruvate carboxylase, aspartate generation no longer becomes rate-limiting for ureogenesis. The question then arises whether aspartate needed for condensation with citrulline in the cytosol is formed directly in the cytosol by transamination between oxalacetate and glutamate or is generated in the mitochondria and thereafter transported to the cytosol. Whichever process predominates will determine which metabolic anions are transported across the mitochondrial membrane and their flux. Thus the overall rate of gluconeogenesis or ureogenesis, under particular substrate availability conditions, may be regulated by kinetic constraints imposed upon the translocator steps (2-4). Our current work focuses on an elucidation of the role which the anion translocators play in determining the concentration of metabolic anions in the mitochondrial and cytosolic spaces, and their quantitative interactions with enzymes of the gluconeogenic and ureogenic pathways.

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The interrelationships between gluconeogenesis and urea synthesis can be explored in greater detail by use of different transaminase inhibitors. Aminooxyacetate inhibits transaminases located in both the cytosol and mitochondria and inhibits urea synthesis completely (1). m-Cycloserine, also a transaminase inhibitor (17, 18), is a more powerful inhibitor of alanine aminotransferase than aspartate aminotransferase and acts predominantly in the cytosolic space because of its relative impermeability across the mitochondrial membrane (1, 16). In this paper we compare the effects of aminooxyacetate and m-cycloserine on isolated liver cells incubated with ammonia and either lactate or pyruvate as carbon substrate. Studies with butyramalate, an inhibitor of malate-phosphate exchange on the mitochondrial dicarboxylate carrier (19, 20), are also presented. Supplementation with ornithine to stimulate urea synthesis and with oleate to stimulate gluconeogenesis has allowed the interactions between gluconeogenesis and urea synthesis to be investigated under a wide variety of intracellular substrate conditions. Preliminary accounts of parts of this study have been published (16, 21, 22).

**Experimental Procedures**

Isolation and Incubation of Liver Cells—Male Sprague-Dawley rats weighing 180 to 240 g were fasted for 18 to 24 h prior to use. The liver was isolated and mounted in a recirculation perfusion system as described by Williamson et al. (23). The procedure used for the cell isolation was similar in principle to that described by Berry and Friend (24) with modifications as described in the previous paper (1). Liver cells (usually 4 to 6 mg dry weight/ml) were incubated with gentle shaking at 37°C in Krebs' bicarbonate medium (pH 7.4) containing 2 to 4% (w/v) defatted bovine serum albumin which had been exhaustively dialyzed against Krebs' bicarbonate medium. The incubation medium was equilibrated with 95% O2 plus 5% CO2, and aliquots were removed for metabolic analyses usually after 20, 40, and 60 min. Analysis of intracellular metabolite contents was achieved by centrifuging the cells through a layer of silicone oil into 0.1 ml of buffer containing 200 mM sucrose, 50 mM KCl and 10 mM 4-morpholinepropanesulfonate, pH 7.4. The cells were washed three times with the same buffer, resuspended in 0.5 ml of buffer, and sonicated in the cold to release enzyme activity from both the mitochondrial and cytosolic spaces. Enzyme activity in the mitochondrial fraction was measured after rapid disruption of the cells (25) followed by centrifugation of the particulate fraction through silicone oil into sucrose/KCl buffer. The pellet was washed and sonicated to release mitochondrial enzyme activity. Alanine and aspartate transaminase activities were assayed as described by Bergmeyer (26). Ornithine transaminase activity was assayed as described by Strackar (27).

Metabolites in the cell supernatant were generally determined by standard spectrophotometric enzymic procedures (26). Ammonia and urea were measured consecutively using glutamate dehydrogenase followed by the addition of urease (26). Metabolites present in the cellular or mitochondrial neutralized perchloric acid extracts were assayed by fluorometric enzyme techniques (28). Ornithine, citrulline, and glutamine, together with other amino acids in some of the experiments, were assayed using a Beckman 121 amino acid autoanalyzer with a lithium carbonate buffer system (29).

**Materials—Enzymes, coenzymes, and metabolites were obtained from Sigma Chemical Co. or Boehringer Mannheim Corp. Bovine serum albumin (fraction V, low in fatty acids) was purchased from Sigma Chemical Co. or Miles Laboratories, Inc., m-cycloserine from Regis Chemical Co., and aminooxyacetate from Eastman Kodak Co. n-Butyramalate was prepared from the diethyl ester (Columbia Organic Chemical Co.) as described by Vogel (30). m-Cycloserine is currently unavailable from Regis Chemical Co., but can be obtained from Dr. W. E. Scott, Hoffman La Roche, Nutley, N. J.

**Results and Discussion**

Subcellular Location of D.L-Cycloserine Inhibition of Aspartate Aminotransferase—In previous communications (1, 16, 31) we have suggested that 10 mM D.L-cycloserine, when added to liver cells, inhibits only cytosolic transaminases because of the poor permeability of the mitochondrial membrane for this compound. The development of rapid cell disruption (25) or lysis techniques (32) allows measurement of the activities of aspartate aminotransferase directly in the cytosolic and mito-
pyruvate kinase (39, 40).

of lactate formation presumably depending on the activity of glucose and lactate formation from pyruvate require flux enolpyruvate and thence to pyruvate (37, 38). Thus, both through P-enolpyruvate carboxykinase, with the relative rate metric amounts when malate is the hydrogen donor, it has been proposed that the major mechanism by which the cell accomplishes this conversion to glucose and lactate in the approximate ratio of 1:2 (35). The large lactate production represents an attempt of the liver cells as sole substrate, its metabolism is characterized by the presence of cycloserine, half the total aspartate aminotransferase activity was found in the mitochondrial fraction (cf. Ref. 33). About a two-thirds loss of activity was obtained with 5 mM cycloserine in the whole cells, while the mitochondrial fraction was little affected, indicating that cytosolic aspartate aminotransferase was almost completely inhibited. The results confirm that cycloserine inhibits aspartate aminotransferase more readily in the cytosol than in the mitochondria and show that an apparent half-maximal effect was obtained with about 2 mM DL-cycloserine in the cell preincubation medium. Partial inhibition of the mitochondrial enzyme develops with time, as shown directly by incubating isolated liver mitochondria with cycloserine.

Alanine aminotransferase activity is predominantly cytosolic (33) and was completely inhibited by incubating the cells with 0.2 mM cycloserine. Preincubation of the cells with 0.5 or 1 mM aminooxyacetate caused an almost complete loss of both alanine and aspartate aminotransferase activities whether lactate or pyruvate was subsequently added as substrate. The inability of pyruvate to reverse the inhibition of aspartate aminotransferase in liver cells preincubated with 1 mM aminooxyacetate confirms our earlier findings (22), and is in contrast with the observations of Smith et al. (34) using 0.2 mM aminooxyacetate, who found a partial release of inhibition after addition of pyruvate.

Effects of Cycloserine and Aminooxyacetate with Pyruvate and Ammonia as Substrates—When pyruvate is added to liver cells as sole substrate, its metabolism is characterized by conversion to glucose and lactate in the approximate ratio of 1:2 (35). The large lactate production represents an attempt of the cell to achieve equilibration of the lactate/pyruvate ratio with the cytosolic NADH/NAD ratio via lactate dehydrogenase. Addition of aminooxyacetate or cycloserine with pyruvate alone as substrate produced negligible effects on the rates of glucose and lactate production (Table I), in confirmation of earlier studies (11, 12, 36). These data indicate that gluconeogenesis from pyruvate does not involve aspartate formation, and they furnish experimental support for the suggestion (6) that malate efflux from the mitochondria provides the carbon and reducing equivalents necessary for glucose and lactate formation. Since generation of cytosolic NADH needed for reduction of pyruvate to lactate forms oxaloacetate in stoichiometric amounts when malate is the hydrogen donor, it has been proposed that the major mechanism by which the cell disposes of cytosolic oxaloacetate is by conversion first to P-enolpyruvate and thence to pyruvate (37, 38). Thus, both glucose and lactate formation from pyruvate require flux through P-enolpyruvate carboxykinase, with the relative rate of lactate formation presumably depending on the activity of pyruvate kinase (39, 40).

Addition of ammonia together with pyruvate provides the cell with a readily available nitrogen source by reductive amination of α-ketoglutarate to glutamate via glutamate dehydrogenase in the mitochondria. Amino acids accumulate, principally in the form of alanine, with smaller amounts of glutamate (Table I). Their accumulation in the medium and the cells is roughly equal to the difference between the rates of ammonia uptake and twice the rate of urea synthesis (Table I). Citrulline formation was appreciable (100 to 120 μmol/g dry weight/h) only when ornithine was added. Urea formation was only marginally stimulated by ammonia alone compared with rates obtained when ornithine was also added, indicating that the low endogenous ornithine content of the cells made citrulline rate-limiting for urea synthesis. As seen from Table I, ammonia addition caused 45% inhibition of the sum of twice the glucose plus the lactate formation, this inhibition being completely reversed by ornithine addition (16).

In the absence of ornithine (ammonia present), the inhibited rate of glucose and lactate formation was unaffected by aminooxyacetate and was slightly stimulated by cycloserine. However, in the presence of ornithine and ammonia, the sum of twice the glucose and the lactate formation was 50% inhibited by aminooxyacetate but was much less affected by cycloserine. Both cycloserine and aminooxyacetate inhibited the accumulation of alanine but not of glutamate. Aminooxyacetate suppressed urea synthesis in the presence of ammonia and ornithine by 80% and stimulated glutamate accumulation as a consequence of suppressing its utilization by mitochondrial aspartate aminotransferase (Table I). In contrast, cycloserine stimulated urea formation 4-fold in the absence of ornithine and had a small inhibitory effect in its presence. The mechanism of this effect is discussed in detail later in this paper. The important conclusion which may be drawn from these data is that the aspartate requirements for urea synthesis are met by aspartate generation in the mitochondria, followed by transport to the cytosol, and not by the formation of aspartate directly in the cytosol.

Basically similar effects of cycloserine in the absence of ornithine were observed using 0.3 mM DL-cycloserine (Fig. 2). These data show an almost complete inhibition of alanine accumulation combined with increased accumulation of glutamate and aspartate, indicating that the metabolic changes must be a direct consequence of inhibition of alanine transamination rather than of aspartate transamination in the cytosol.

1 P. F. Zuurendijk, H. de Bakker, and J. M. Tager, unpublished observations.
switch is particularly evident in the presence of ornithine. Interestingly, inhibition of aspartate generation (and hence aspartate efflux) by aminooxyacetate failed to cause a compensatory increase of malate efflux. This indicates that malate efflux was suppressed by the presence of ammonia and that this inhibition was not a consequence of a stimulated rate of urea formation.

Effects of Ammonia and Ornithine on Pyruvate Metabolism—The data in Table I showed that addition of ammonia to liver cells incubated with pyruvate and oleate as substrates had the interesting effect of inhibiting gluconeogenesis. Further addition of ornithine partially relieved this inhibition and caused a 2-fold increase of lactate formation. Additional information pertaining to these effects is shown in Table II. Pyruvate carboxylase flux was assumed equal to the sum of twice the glucose formation plus the lactate formation, so that the difference of flux between these two enzymes is equal to the accumulation of the dicarboxylic amino acids. As seen from Table II, pyruvate carboxylase flux in the absence of ammonia completely accounted for the measured rate of pyruvate uptake, indicating negligible flux through pyruvate dehydrogenase. Pyruvate dehydrogenase was presumably inactivated under control conditions by the presence of oleate, which promotes conversion of the enzyme to the inactive phosphorlyated form (41). Addition of ammonia, however, stimulated pyruvate uptake and inhibited flux through both pyruvate carboxylase and P-enolpyruvate carboxykinase, indicating that the presence of ammonia induced a greatly enhanced rate of pyruvate decarboxylation. This effect has also been demonstrated recently in perfused rat liver (42). Inhibition of P-enolpyruvate carboxykinase flux by ammonia addition can be accounted for by the 3-fold decrease of the malate content of the cells (Table II). Assuming near equilibrium of cytosolic malate dehydrogenase together with relatively small changes of the cytosolic NADH/NAD ratio after ammonia addition (43), decreased malate levels will be associated with a fall of the cytosolic oxalacetate concentration.

From the data presented in Table I and the conclusion that aspartate generation in the cytosol is negligible, it is possible to calculate the rate of net malate efflux from the mitochondria. Since urea synthesis is accompanied by a stoichiometric formation of malate in the cytosol, it follows that malate efflux = Δ lactate + 2 Δ glucose − Δ urea. Values for malate efflux calculated for the different experimental conditions are shown in the last column of Table I. Aspartate efflux from the mitochondria may also be calculated from the sum of the rates of urea synthesis and aspartate accumulation (Table I). With pyruvate as substrate, the accumulation of aspartate is rather small so that the calculated rates of aspartate efflux are essentially equal to the rates of urea production. From a comparison of the rates of malate and aspartate efflux it is evident that with pyruvate as substrate, ammonia addition causes a switch from malate efflux to aspartate efflux. This switch is particularly evident in the presence of ornithine. Interestingly, inhibition of aspartate generation (and hence aspartate efflux) by aminooxyacetate failed to cause a compensatory increase of malate efflux. This indicates that malate efflux was suppressed by the presence of ammonia and that this inhibition was not a consequence of a stimulated rate of urea formation.

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Gluconoegenesis and Ureogenesis in Rat Liver Cells

Rat liver cells were incubated as described in Table I. Metabolic rates were calculated for the time interval from 0 to 40 min of incubation. The cell contents of glutamate, aspartate, and malate were averaged for the 20- and 40-min time points. Pyruvate carboxylase (PC) flux and P-enolpyruvate carboxykinase (PEPCK) flux were calculated as follows: PC flux = 2 Δglucose + Δlactate + Δglutamate + Δaspartate. PEPCK flux = 2 Δglucose + Δlactate.

The fall of cellular malate levels is best interpreted as a direct consequence of stimulation of flux through glutamate dehydrogenase by the increased ammonia concentration. This results in a 4-fold increase of cell glutamate content, which in turn promotes flux through mitochondrial aspartate aminotransferase causing depletion of the cell oxalacetate plus malate pool and an increased aspartate content (Table II). Stimulation of flux through pyruvate dehydrogenase can be accounted for by the fall of the mitochondrial NADH/NAD ratio after ammonia addition (42, 43) which decreases pyruvate dehydrogenase kinase activity and, therefore, increases the proportion of pyruvate dehydrogenase in the active non-phosphorylated form (44, 45). Decreased flux through pyruvate carboxylase after ammonia addition is less readily explained. At first sight it would appear reasonable that flux is inhibited by the accumulation of glutamate since the estimated mitochondrial glutamate concentration is at least 4-fold higher than the reported Kₘ of 6 mM for glutamate with isolated rat liver pyruvate carboxylase (46).

Ornithine addition in the presence of ammonia increased flux through both P-enolpyruvate carboxykinase and pyruvate carboxylase (Table II). The increased malate level is consistent with the increased flux through P-enolpyruvate carboxykinase, but since the glutamate content did not change, some other factor must be responsible for the activation of pyruvate carboxylase. Ornithine addition in the presence of substrate and ammonia stimulates the rate of oxygen uptake by the hepatocytes (47). Hence increased β oxidation required to support the increased respiration could result in an elevation of the acetyl-CoA level in the mitochondria with consequent stimulation of pyruvate carboxylase.

The kinetics of changes of intracellular metabolites following ornithine addition in the presence of ammonia and pyruvate are shown in Fig. 3. In this experiment, ornithine was added after 20 min and caused an increase of malate, decrease of aspartate, and increase of α-ketoglutarate levels, together with a prompt stimulation if urea synthesis (latter not shown). These changes result directly from an enhanced rate of citrulline synthesis and an increased intracellular citrulline concentration (see below), which permits stimulation of flux through argininosuccinate synthetase and thereby an enhanced rate of aspartate utilization in the cytosol. Malate levels increased as a result of the increased rate of urea synthesis, and hence an acceleration of malate generation in the cytosol. The lowered aspartate concentration in the absence of an appreciable change of glutamate concentration caused α-ketoglutarate levels to rise as a consequence of maintenance of the aspartate aminotransferase mass action ratio. Thus, it is seen that when citrulline is no longer a rate-limiting factor in the urea cycle, the intracellular metabolite profile is perturbed in such a way as to secondarily affect the rates of glucose and lactate formation from pyruvate.

Effects of Butylmalonate with Pyruvate and Ammonia as Substrates—It is known from studies with isolated liver mitochondria that malate efflux from the mitochondria can occur in exchange either with phosphate on the dicarboxylate carrier, with α-ketoglutarate on the α-ketoglutarate carrier, or with citrate on the tricarboxylate carrier (48). Transport of malate on the dicarboxylate carrier is by far the most sensitive to inhibition by butylmalonate (9, 49). Thus, it may be predicted from the data in Table I that addition of ammonia to liver cells incubated with pyruvate should cause decreased sensitivity of glucose and lactate formation to inhibition by butylmalonate because of decreased flux through the malate-phosphate carrier under these conditions.

In confirmation of earlier findings with perfused rat liver and isolated liver cells (21, 50), the data presented in Table III show that with pyruvate alone as substrate about 50% inhibition of glucose and lactate formation was obtained after addition of 5 mM butylmalonate. However, with ammonia and ornithine also present, glucose and lactate production were only 20% inhibited by butylmalonate. Urea formation was similarly affected. With oleate also added to optimize flux through pyruvate carboxylase, butylmalonate caused even smaller effects. A comparison of these results with the calculated rates of malate efflux shown in Table I indicates that in the presence of oleate, ammonia, and ornithine only a small part of the apparent total malate efflux is affected by butylmalonate, and thus occurs via the dicarboxylate carrier. The butylmalonate-insensitive part of malate efflux presumably reflects malate efflux in exchange with α-ketoglutarate influx. Entry of α-ketoglutarate into mitochondria (together with glutamate efflux) is necessary for alanine formation in the cytosol via alanine aminotransferase. Residual malate efflux is thus more sensitive to further inhibition by cycloserine than to inhibition by butylmalonate (cf. Table I).

Taken together, the data emphasize the minor role of malate transport across the mitochondrial membrane compared with aspartate transport under optimal conditions for

Table II

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Pyruvate</th>
<th>Pyruvate carboxykinase flux</th>
<th>P-enolpyruvate carboxykinase flux</th>
<th>Cell contents of:</th>
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<th>Aspartate</th>
<th>Malate</th>
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<tbody>
<tr>
<td></td>
<td>μmol/g dry wt</td>
<td></td>
<td></td>
<td>μmol/g dry wt</td>
<td></td>
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<td>845</td>
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<td>2.0</td>
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<td></td>
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<tr>
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<td>547</td>
<td>449</td>
<td>44.6</td>
<td>8.7</td>
<td>0.9</td>
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<td>887</td>
<td>789</td>
<td>43.1</td>
<td>2.4</td>
<td>1.5</td>
<td></td>
</tr>
<tr>
<td>NH₄⁺ + ornithine</td>
<td>1402</td>
<td>887</td>
<td>789</td>
<td>43.1</td>
<td>2.4</td>
<td>1.5</td>
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</tbody>
</table>

FIG. 3. Effect of ornithine addition on intracellular metabolite contents. Rat liver cells (7.7 mg dry weight/ml) were incubated with 10 mM pyruvate and 10 mM ammonia. After 20 min, 3 mM ornithine was added to the cell suspension. Samples were taken every 5 min for separation of the cells from the suspension medium by rapid centrifugation through silicone oil. Values shown represent intracellular contents.

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Taken together, the data emphasize the minor role of malate transport across the mitochondrial membrane compared with aspartate transport under optimal conditions for
urea synthesis from ammonia when pyruvate is supplied as carbon substrate. Measurements of the ATP content of cells (control values, about 10 µmol/g dry weight) showed that addition of the different inhibitors used in these experiments had a negligible effect, indicating that they did not interfere with the generation of energy in the cells.

Effects of Aminoxyacetate and L-D-Cycloserine in Presence of Lactate and Ammonia: When lactate is supplied to the liver as a gluconeogenic precursor instead of pyruvate, reducing equivalents for glucose synthesis are generated in the cytosol by lactate dehydrogenase, and previous work has indicated that carbon precursors are exported from the mitochondria largely in the form of aspartate (5, 11, 12, 30). Support for this conclusion is illustrated by the data in the first three lines of Table IV, which show that both lactate uptake and glucose production were largely prevented by addition of either cycloserine or aminoxyacetate. Thus with lactate as substrate, transamination in the cytosol is essential in order to regenerate oxalacetate from the aspartate translocated out of the mitochondria (5, 10). Inhibition of gluconeogenic flux by the transaminase inhibitors also results in inhibition of flux through pyruvate carboxylase since malate and aspartate accumulate only to a limited extent (not shown). Because of inhibition of the transfer of reducing equivalents into the mitochondria by the malate-aspartate cycle, the cytosolic NADH/NAD ratio is increased and the intracellular pyruvate concentration falls (from 0.32 to 0.01 mM), which may account for the decreased rate of oxalacetate generation via pyruvate carboxylase. Addition of ammonia in the presence of lactate, especially with ornithine also present, decreased the sensitivity of gluconeogenesis to inhibition by L-cycloserine (Table IV and Fig. 4). Urea synthesis from ammonia in the absence of ornithine was increased by addition of L-cycloserine, as also observed with pyruvate as substrate (see Table I). Fig. 4 shows that this effect was maximal with 1 mM cycloserine. Ornithine produced a large stimulation of urea production, which was increased further by low concentrations of cycloserine but was slightly inhibited at high cycloserine concentrations. In contrast to L-cycloserine, aminoxyacetate totally abolished both glucose and urea synthesis under all conditions (Table IV) since it prevented the intramitochondrial generation of aspartate.

These differential effects of L-cycloserine and aminoxyacetate on gluconeogenesis and ureogenesis in the presence of lactate and ammonia show that aspartate generated in the mitochondria can still be used for gluconeogenesis in the cytosol by lactate dehydrogenase, and previous work has indicated that carbon precursors are exported from the mitochondria largely in the form of aspartate (5, 11, 12, 30).

### Table III

**Effect of butylmalonate on pyruvate metabolism with and without ammonia in isolated rat liver cells**

<table>
<thead>
<tr>
<th>Additions</th>
<th>ΔGlucose</th>
<th>ΔLactate</th>
<th>2 × ΔGlucose + ΔLactate</th>
<th>ΔUrea</th>
<th>ΔNH₃</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pyruvate</td>
<td>− 133 ± 12</td>
<td>381 ± 49</td>
<td>647 ± 75</td>
<td></td>
<td></td>
</tr>
<tr>
<td>+ 65 ± 3</td>
<td>200 ± 19</td>
<td>326 ± 26</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pyruvate + NH₃ + ornithine</td>
<td>− 123 ± 21</td>
<td>394 ± 45</td>
<td>640 ± 33</td>
<td>531 ± 59</td>
<td>1230 ± 100</td>
</tr>
<tr>
<td>+ 96 ± 13</td>
<td>320 ± 26</td>
<td>515 ± 33</td>
<td></td>
<td>417 ± 31</td>
<td>1020 ± 40</td>
</tr>
<tr>
<td>Pyruvate + NH₃ + ornithine + oleate*</td>
<td>126</td>
<td>467</td>
<td>717</td>
<td>602</td>
<td>1380</td>
</tr>
<tr>
<td>+ 98</td>
<td>430</td>
<td>626</td>
<td></td>
<td>505</td>
<td>1130</td>
</tr>
</tbody>
</table>

* Mean of two experiments.

### Table IV

**Effects of cycloserine and aminoxyacetate on lactate metabolism in isolated rat liver cells**

<table>
<thead>
<tr>
<th>Substrate</th>
<th>CS</th>
<th>AOA</th>
<th>ΔGlucose</th>
<th>ΔLactate</th>
<th>ΔNH₃</th>
<th>ΔUrea</th>
<th>Transport of reducing equivalents to mitochondria</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lactate</td>
<td>− 300 ± 58</td>
<td>775 ± 95</td>
<td>25 ± 9</td>
<td>200</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>+ 34 + 3</td>
<td>122 ± 30</td>
<td>17 ± 8</td>
<td>63</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>− + 47 ± 6</td>
<td>131*</td>
<td>27*</td>
<td>64</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lactate + NH₃</td>
<td>− 235 ± 15</td>
<td>811 ± 126</td>
<td>712 ± 118</td>
<td>193 ± 47</td>
<td>534</td>
<td></td>
<td></td>
</tr>
<tr>
<td>+ 126 ± 42</td>
<td>999 ± 12</td>
<td>902 ± 19</td>
<td>349 ± 60</td>
<td>696</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>− + 2 ± 1</td>
<td>142*</td>
<td>184 ± 6</td>
<td>8 ± 1</td>
<td>146</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lactate + NH₃ + ornithine</td>
<td>− 195 ± 10</td>
<td>731 ± 102</td>
<td>2100 ± 190</td>
<td>816 ± 63</td>
<td>1157</td>
<td></td>
<td></td>
</tr>
<tr>
<td>+ 160 ± 33</td>
<td>623 ± 96</td>
<td>1710 ± 210</td>
<td>711 ± 79</td>
<td>1014</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>− + 20 ± 9</td>
<td>153 ± 35</td>
<td>323 ± 47</td>
<td>27 ± 9</td>
<td>140</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* Mean of two experiments.
cytosolic aspartate into malate by the urea cycle. Glutamate necessary for aspartate generation in the mitochondria is continuously supplied by recycling the α-ketoglutarate formed in the mitochondrial aspartate aminotransferase reaction via glutamate dehydrogenase. However, unlike the situation with pyruvate as substrate, when lactate is present as carbon precursor urea synthesis contributes to the production of reducing equivalents in the cytosol in excess of the requirements for gluconeogenesis. These must be transported to the mitochondria for oxidation by the mitochondrial respiratory chain. Estimated rates, calculated by subtracting twice the rate of glucose production from the sum of the rates of lactate uptake and urea formation, are shown in the last column of Table IV. In the absence of transaminase inhibitors these values represent the maximum flux through a hydrogen transport shuttle since theoretically cytosolic malate produced by the urea cycle can enter the mitochondria to produce oxaloacetate required for intramitochondrial aspartate amino transferase (see Ref. 1). Minimum flux is provided by subtracting twice the glucose production from the lactate uptake. Malate influx to the mitochondria is probably fairly minimal, however, since 5 mM butylmalonate caused only a 23% inhibition of glucose formation and a 19% inhibition of urea synthesis with lactate, oleate, ornithine, and ammonia as substrate (not shown). From these data it is evident that a pathway other than the malate-aspartate cycle is used for transfer of reducing equivalents from the cytosol to mitochondria, certainly in the presence of cycloserine and probably also in its absence. The identity of this alternative shuttle has not been ascertained, but possible candidates are the α-glycerophosphate (51) or fatty acid shuttles (52, 53). The former is certainly active in liver (16, 54-56), but its capacity appears to be too small in normal rats to account for the rates of hydrogen transport shown in Table IV. According to Berry et al. (55) maximal flux through the α-glycerophosphate cycle with glycerol as substrate in order to provide saturating levels of α-glycerophosphate was about 160 μmol/g dry weight/h, which is approximately equal to the activity of mitochondrial α-glycerophosphate oxidase in our liver cell preparations (177 μmol/g dry weight/h). The α-glycerophosphate content of cells with lactate as substrate was not appreciably affected by ammonia or cycloserine additions.

Three observations of interest presented in Table IV require further clarification. The first is the mechanism for inhibition of gluconeogenesis from lactate by ammonia, the second is the mechanism for stimulation of urea synthesis from ammonia by addition of cycloserine, and the third is the mechanism for stimulation of flux through glutamate dehydrogenase by ornithine in the presence of ammonia. This latter effect follows as a direct consequence of the stimulation of urea synthesis by ornithine since ammonia fixation via glutamate dehydrogenase provides the amino nitrogen for intramitochondrial aspartate generation.

**Inhibitory Effect of Ammonia on Gluconeogenesis** — As already shown in Table IV, with lactate as substrate (oat present), ammonia addition either in the absence or presence of ornithine caused inhibition of gluconeogenesis. Analogous results were obtained in other experiments using a low ammonia concentration (1 mM) and dilute cell suspensions. Furthermore, the inhibitory effect of ammonia plus ornithine on gluconeogenesis from lactate is also observed in the absence of oleate (cf. Ref. 47). Table V shows the results of a cell separation experiment with the substrates and incubation conditions similar to those of Table IV. Cells were incubated for 20 min with 10 mM lactate and 1 mM oleate either in the presence or absence of 3 mM ornithine, followed by addition of 5 mM ammonia and further incubation for 30 min. The mitochondria/cytosol gradients for malate, glutamate, and α-ketoglutarate varied from 1.7 to 7.1 under the different conditions, while the aspartate gradient was closer to unity (range, 0.7 to 1.8). It is of interest that the α-ketoglutarate gradient was always greater than the malate gradient, which is consistent with net transport of malate into mitochondria coupled with α-ketoglutarate efflux. Ammonia addition decreased the concentration of malate in the cytosol whether ornithine was

![FIG. 4. Effects of m-cycloserine on glucose and urea synthesis by rat liver cells.Liver cells (3.5 mg dry weight/ml) were preincubated with 1 mM oleate and different concentrations of cycloserine for 15 min, followed by addition of 10 mM lactate, 10 mM ammonia and, when present, 3 mM ornithine (Orn), and further incubation for 30 min. O, □, changes in the absence of ornithine; ●, ■, changes in its presence.](http://www.jbc.org/)

**TABLE V**

<table>
<thead>
<tr>
<th>Metabolite</th>
<th>M</th>
<th>C</th>
<th>NH₃</th>
<th>Ornithine</th>
<th>Ornithine + NH₃</th>
</tr>
</thead>
<tbody>
<tr>
<td>Malate</td>
<td>4.15 ± 0.70</td>
<td>2.07 ± 0.11</td>
<td>1.21 ± 0.26</td>
<td>0.29 ± 0.04</td>
<td>1.80 ± 0.33</td>
</tr>
<tr>
<td>Glutamate</td>
<td>7.5 ± 2.0</td>
<td>2.2 ± 0.3</td>
<td>54.1 ± 15.5</td>
<td>17.4 ± 2.2</td>
<td>22.8 ± 10.1</td>
</tr>
<tr>
<td>α-Ketoglutarate</td>
<td>1.26 ± 0.39</td>
<td>0.39 ± 0.10</td>
<td>0.42 ± 0.08</td>
<td>0.07 ± 0.01</td>
<td>1.01 ± 0.31</td>
</tr>
<tr>
<td>Aspartate</td>
<td>3.2 ± 0.2</td>
<td>4.4 ± 0.1</td>
<td>31.2 ± 3.6</td>
<td>24.9 ± 1.8</td>
<td>4.7 ± 1.4</td>
</tr>
</tbody>
</table>

Rat liver cells were incubated for 20 min with 10 mM lactate and 1 mM oleate either in the absence or presence of 3 mM ornithine. Ammonia (5 mM) was then added and incubation continued for another 30 min. Immediately prior to NH₃ addition and 10 and 30 min thereafter samples were removed for cell fractionation by the cell disruption technique (25). The values shown are averages for 4 separate experiments. In the presence of NH₃, averages were taken of 10- and 30-min values. Concentrations of metabolites in the cytosolic (C) and mitochondrial (M) spaces are expressed as μmol per mg of mitochondrial matrix protein per g dry weight of cells (25).
absent or present. These data demonstrate that the decreased rate of gluconeogenesis, i.e. the decreased flux through P-enolpyruvate carboxykinase, as well as accounted for by a fall in the cytosolic malate concentration, which in turn will cause a fall of the oxalacetate concentration. With high ammonia concentrations, mitochondrial and cytosolic aspartate and glutamate concentrations increased dramatically upon ammonia addition in the absence of ornithine, indicating that flux through pyruvate carboxylase was less affected than flux through P-enolpyruvate carboxykinase. However, a similar inhibition of gluconeogenesis and fall of malate content in the absence of ammonia accumulation has been observed using perifused rat liver cells where the lactate concentration was maintained at 0.8 mM and the ammonia concentration at 0.3 mM (57), indicating that pyruvate carboxylase was inhibited under these conditions. The fall of the malate concentration in the mitochondria after ammonia addition can be explained by a decrease of the free mitochondrial NADH/NAD ratio (43) and by increased flux through aspartate aminotransferase as a consequence of the increased mitochondrial glutamate concentration. The glutamate concentration increased and the α-ketoglutarate concentration decreased in the mitochondrial compartment because of the stimulation of flux through glutamate dehydrogenase by ammonia addition.

From the above data, it appears that the mechanism for inhibition of gluconeogenesis by ammonia addition is similar when either pyruvate or lactate is the primary substrate, namely decreased availability of cytosolic oxalacetate to P-enolpyruvate carboxykinase. A major difference in behavior of the cell to the two substrates is that subsequent ornithine addition reversed this inhibition only with pyruvate as substrate even though rates of urea formation were high in both cases. With pyruvate present, the cell malate content increased (Table II) while with lactate as substrate the malate content remained low after ornithine addition (Table V). This difference may be related to the fact that with pyruvate and ammonia as substrates, addition of ammonia increased the cell α-ketoglutarate content 2-fold (from 0.9 to 1.8 μmol/g dry weight, Fig. 3) while with lactate, oleate, and ammonia as substrates, ornithine only increased the cell α-ketoglutarate content from 0.22 to 0.27 μmol/g dry weight (Table V). When the α-ketoglutarate concentration is elevated, maintenance of near-equilibrium of cytosolic aspartate aminotransferase permits a higher oxalacetate concentration, which directly affects the malate concentration via near equilibrium of malate dehydrogenase. Flux through P-enolpyruvate carboxykinase is in fact much greater with pyruvate than lactate as substrate since lactate production from pyruvate involves flux through P-enolpyruvate carboxykinase, as already described, in addition to that involved in glucose production.

Stimulatory Effect of Cycloserine on Urea Synthesis in Absence of Ornithine—The time course of changes of the contents of the major amino acids in liver cells incubated with 10 mM lactate, 1 mM oleate, and 10 mM ammonia in the absence and presence of a 15-min preincubation with 10 mM DL-cycloserine are shown in Fig. 5. This figure also illustrates that cycloserine in the absence of added ornithine caused an immediate increase in the rate of urea formation (cf. Table IV). Another difference between presence of lactate or pyruvate as carbon substrate is that with lactate there was a large accumulation of aspartate (cf. Table II) of which 80 to 90% was intracellular. Cycloserine greatly inhibited the accumulation of aspartate during the first 30 min of incubation and completely abolished the accumulation of alanine. Under control conditions, 50% of the alanine in the cell incubation was intracellular after 30 min, falling to 35% after 60 min. The cell glutamate content increased rapidly during the first 15 min of incubation with 90% being intracellular, and thereafter declined gradually as glutamate leaked from the cells to the medium. Cycloserine increased the glutamate accumulation, presumably as a consequence of the diminished glutamate utilization for alanine and aspartate accumulation. Under control conditions the cell glutamine content increased linearly at a slow rate, with this synthesis being abolished by the presence of cycloserine. Of particular interest is the finding that the cellular citrulline content was increased up to 3-fold by cycloserine. The increased rate of urea synthesis, therefore, may be ascribed to the increased citrulline content, which apparently is rate-limiting for argininosuccinate synthetase. With an increased availability of citrulline, flux through argininosuccinate synthetase is stimulated, thereby causing less of an accumulation of aspartate.

Regulation of Tissue Ornithine Content—The reason for the increased citrulline content of cells incubated with cycloserine remains to be established. The most probable explanation is that cycloserine slows the transamination between endogenous ornithine and α-ketoglutarate by inhibition of ornithine transaminase in the cytosol, thereby allowing increased conversion of ornithine to citrulline when ammonia is also present. The mitochondrial ornithine transaminase, however, would remain active. Measurement of the activity of ornithine transaminase in whole cells and in the mitochondrial fraction after cell disruption showed that its activity in the cytosol, which was about 50% of the total (cf. Ref. 27), was completely inhibited by 5 mM DL-cycloserine. After 30 min of incubation of liver cells in the presence of 10 mM lactate, 1 mM oleate, and 5 mM ammonia, the ornithine content was 0.6 μmol/g dry weight and the sum of ornithine plus citrulline was 3.2 μmol/g dry weight. With 5 mM cycloserine also present, the ornithine content was 0.95 μmol/g dry weight and...
the sum of ornithine plus citrulline increased to 5.75 μmol/g dry weight, in accordance with the above suggestion.

In the absence of added ammonia, the ornithine content of liver cells incubated for 30 min with 10 mM lactate and 1 mM ornithine was 3.5 μmol/g dry weight compared with a citrulline content of 0.25 μmol/g dry weight. As noted above, addition of ammonia decreased the ornithine content to 0.6 μmol/g dry weight and increased the citrulline content to 2.6 μmol/g dry weight. During 30 min of incubation in the absence of ammonia, the sum of ornithine and citrulline contents decreased from 6.0 to 3.7 μmol/g dry weight, while the arginine content also decreased from 1.4 to 0.7 μmol/g dry weight. The total activity of ornithine transaminase in the liver is sufficient to metabolize ornithine at the rate of 300 to 400 μmol/g dry weight/h (27), but is presumably highly inhibited in the tissue, e.g. by branched chain amino acids (58). The purified enzyme from rat liver (27) has a K_m for ornithine of 2.8 mM and a K_m for α-ketoglutarate of 0.23 mM, both of which are in the region of the tissue concentrations. With 3 mM ornithine present, ornithine transamination was increased in the absence of ammonia, as shown by the increased glutamate concentration in both the mitochondria and cytosol (Table V). Thus, glutamate accumulated at a rate of 77 μmol/g dry weight/h as a result of addition of 3 mM ornithine. This may be compared with a maximum rate of ornithine catabolism of 84 μmol/g dry weight of liver/h observed with isolated liver mitochondria at 30°C (59), calculated on the basis of a mitochondrial content of 226 mg of protein/g dry weight. Possibly ornithine metabolism by mitochondrial ornithine transaminase is limited by ornithine transport to the mitochondria at low ornithine concentrations (59). Furthermore, ammonia protects against ornithine metabolism (a) by converting most of the ornithine to citrulline as a result of increased carbamyl-phosphate formation, and (b) by decreasing the concentration of α-ketoglutarate needed as acceptor for the ε-amino group of ornithine (Table V). Ornithine transaminase has a K_m for ornithine of about 1.4 mM (60), but its very high activity in liver (61) ensures a very rapid turnover of ornithine at elevated ammonia levels.

Regulation of Arginosuccinate Synthetase—Since arginosuccinate synthetase reacts with aspartate and citrulline in the cytosolic compartment of the cell, the distribution of these metabolites between the mitochondrial and cytosolic compartments was measured using the cell disruption technique of Tischler et al. (25). Table VI summarizes the effects of 10 mM cycloserine on the distributions of aspartate and citrulline in liver cells incubated with lactate, oleate, and ammonia in the absence and presence of ornithine. The effects of cycloserine on urea synthesis were similar to those shown in Table IV, indicating the reproducibility of different batches of liver cells. Citrulline was found to be concentrated in the mitochondrial compartment with a gradient of 3 to 6 in different experiments. In the absence of ornithine, incubation of cells with substrate and cycloserine produced an increase of the cytosolic citrulline concentration from 0.8 to 2.5 mM in conjunction with an approximately 4-fold stimulation of urea synthesis. The cytosolic aspartate concentration under control conditions was very high at 25 mM, falling to 16 mM in the presence of cycloserine. In the presence of ornithine with urea synthesis maximally stimulated, the cytosolic citrulline concentration was in the range of 14 to 22 mM while the cytosolic aspartate concentration was 4 to 5 mM and essentially unaffected by the presence of cycloserine. Since this aspartate concentration is approximately 4-fold higher than that observed with pyruvate, ammonia, and ornithine (cf. Table II) for similar rates of urea production, arginosuccinate synthetase activity would appear not to be limited by the aspartate concentration under all conditions with lactate as substrate. The apparent K_m values of isolated arginosuccinate synthetase (62) for aspartate (20 μM) and citrulline (44 μM) are both very low compared with their concentrations in the cytosol, suggesting that there is at least 1 order of magnitude discrepancy between the in vitro and in vivo apparent K_m values. The present data suggest that the in vivo apparent K_m for citrulline is in the range of 1 mM.

This conclusion is supported by a separate series of experiments with 10 mM pyruvate and 10 mM ammonia as substrates to which different concentrations of ornithine were added. Fig. 6A shows the concentration dependency of the stimulation of urea production from ammonia by ornithine, indicating that maximum effects were obtained with 3 mM ornithine and a half maximal stimulation with 0.5 mM ornithine. In Fig. 6B the rate of urea synthesis is plotted against the intracellular citrulline content at 30 min found with the different concentrations of ornithine added. The capacity of carbamyl phosphate synthetase was clearly sufficient to allow a large accumulation of citrulline (cf. Ref. 63). From Fig. 6B an apparent K_m of citrulline for arginosuccinate synthetase of about 4 μmol/g dry weight can be calculated. This is equivalent to an intracellular citrulline concentration of 1.5 mM (cf. Ref. 64). It is evident that under optimal concentration the activity of arginosuccinate synthetase is rate-limiting for urea synthesis. This enzyme has the lowest activity of all the enzymes of the urea cycle, with a maximum capacity of about 1000 μmol/g dry weight of liver/h at 37°C for rats fed a diet containing 15% protein (61).

Stimulatory Effect of Ornithine on Glutamate Dehydrogenase—In the experiment shown in Table VI, with lactate, oleate, and ammonia as substrates, the calculated flux through glutamate dehydrogenase increased from 354 to 805 μmol/g dry weight/h after addition of ornithine. A similar qualitative effect was obtained in numerous other experiments and was not dependent on the presence of oleate or the concentration of ammonia above 1 mM. Glutamate dehydro-
which has a ferase, while the decreased cytosolic aspartate concentration will diminish product inhibition of aspartate aminotransferase. The decreased mitochondrial aspartate concentration will fall of aspartate concentrations in both the mitochondrial and extramitochondrial compartments as enhanced citrulline availability increases aspartate utilization by argininosuccinate synthase. An explanation previously advanced for the ornithine stimulation of flux through glutamate dehydrogenase is subject to mass action regulation via the NAD system.

CONCLUDING REMARKS

Gluconeogenesis and urea synthesis represent the major biosynthetic pathways of fasted liver. Both processes utilize enzymes located in the mitochondria and in the cytosol and require transport of metabolic anions across the mitochondrial membrane (see Fig. 7). Certain enzymes and transport systems are common to both pathways, notably aspartate aminotransferase, malate dehydrogenase and the transport systems for malate, α-ketoglutarate, glutamate, and aspartate. Interaction between the two pathways is thus to be expected, particularly if both pathways separately are operating under optimal conditions. Moreover, because urea synthesis is associated with an equimolar production of malate in the cytosol, different effects might be expected whether pyruvate or lactate is used as gluconeogenic precursor. Finally, we were concerned with evaluating the possibility that transport of specific anions across the mitochondrial membrane might provide a rate-limiting step for either gluconeogenesis or ureogenesis.

The present study using transaminase inhibitors confirms our earlier work (16) in demonstrating that aspartate needed in the cytosol for argininosuccinate formation must first be generated within the mitochondria and then transported to the cytosol. A comparison of maximum urea synthetic rates observed in the present study (600 to 800 μmol/g dry weight/h) with rates obtained in our previous work (1) using hepatocytes incubated in the absence of gluconeogenic substrates (200 to 250 μmol/g dry weight/h), clearly shows that an intramitochondrial generation of oxalacetate via pyruvate carboxylase is required for maximum rates of urea synthesis. This eliminates the necessity, during urea synthesis, for malate transport into the mitochondria, which was previously shown to be a rate-limiting step (1). Urea formation by isolated hepatocytes incubated with ammonia, ornithine, and different concentrations of lactate shows saturation kinetics (47, 64) and, although not proven, it may be inferred that the aspartate flux can be calculated in two ways, either as the sum of urea formation and accumulation of all amino acids except citrulline, or by subtraction of the sum of urea formation and accumulation of citrulline and glutamine from the measured ammonia uptake. Both estimates agreed well in particular experiments where analyses were available to make a direct comparison between the two methods of calculation. If flux through glutamate dehydrogenase is subject to mass action control, ornithine addition in the presence of ammonia would be expected to bring about an increase of substrate concentrations or a decrease of product concentrations, or both. Inspection of the appropriate data in Table V shows in fact that the mitochondrial glutamate concentration decreased after ornithine addition in the presence of ammonia, while the α-ketoglutarate concentration increased. The changes are relatively small, however, and the glutamate/α-ketoglutarate ratio only decreased from 129 to 89. A comparison of the mitochondrial concentrations of glutamate and α-ketoglutarate with the kinetic constants of isolated glutamate dehydrogenase shows that the α-ketoglutarate concentration is in the range of the reported Kₘ of 0.7 mM for bovine liver enzyme (65) and 0.5 mM for rat liver enzyme (66). The observed mitochondrial glutamate concentration (about 50 mM), however, is much higher than the reported Kₘ values of 1.8 and 4.7 mM for bovine (65) and rat (66) liver enzymes, respectively, indicating that it is likely to be highly inhibitory in the direction of glutamate synthesis. An explanation previously advanced for the ornithine stimulation of flux through glutamate dehydrogenase was based on an increased availability of mitochondrial α-ketoglutarate as a result of stimulation of flux through mitochondrial aspartate aminotransferase (2). Increased flux through this enzyme in turn is permitted by the fall of aspartate concentrations in both the mitochondrial and cytosolic compartments as enhanced citrulline availability increases aspartate utilization by argininosuccinate synthetase. The decreased mitochondrial aspartate concentration will diminish product inhibition of aspartate aminotransferase, while the decreased cytosolic aspartate concentration will aid removal of mitochondrial aspartate by release of aspartate inhibition of the glutamate-aspartate translocator, which has a Kₘ for extramitochondrial aspartate of 4 mM and a Kₘ for external glutamate of 7 mM (67). It may be noted from Table V that ornithine addition in the presence of ammonia decreased the cytosolic aspartate concentration from 25 to 7 mM while the cytosolic glutamate concentration decreased from 17 to 11 mM.

While the above explanation appears to account at least qualitatively for the observed coordination of flux through the glutamate-aspartate translocator and mitochondrial aspartate aminotransferase, the observed increase of mitochondrial α-ketoglutarate concentration appears to be too small to account for regulation of glutamate dehydrogenase flux by a purely mass action effect. An indirect alternative or additional stimulatory effect of ornithine on glutamate dehydrogenase may be provided by an increased state of reduction of the mitochondrial pyridine nucleotides. Thus, in an experiment similar to that of Table V, addition of ornithine increased the mitochondrial NADPH concentration from 1.4 to 1.8 mM, while the β-hydroxybutyrate/acetocetate ratio also increased slightly, from 0.07 to 0.11. These changes are also small and the NADPH concentrations is far in excess of the value of 25 μM reported for the Kₘ of bovine glutamate dehydrogenase for NADPH (65). Tischler et al. (68) have recently shown that while glutamate dehydrogenase in the intact liver cell remains in near-equilibrium with the mitochondrial NADPH system, it deviates from equilibrium with the mitochondrial NADP system by 3 to 4 orders of magnitude. It appears, therefore, that flux through glutamate dehydrogenase is more likely to be regulated by the NADPH/NADP ratio than by mass action regulation via the NAD system.
content of the cells increased along with urea synthesis. Our own studies show that the estimated cytosolic aspartate concentration of hepatocytes incubated with ammonia and ornithine (i.e. having low rates of urea synthesis) was 0.4 to 0.5 mM, while that with 10 mM pyruvate also present was 1.0 to 1.5 mM. With lactate as substrate, the aspartate concentration was considerably higher (cf. Table V). It is obviously not possible to reconcile these values with the apparent K_m of 44 μM observed for aspartate with purified argininosuccinate synthetase (62). Nevertheless, it is evident that aspartate availability in the cytosol can limit urea synthesis from ammonia under conditions of substrate deficiency. Probably this regulation is not very important under nonpathological conditions in vivo since the substrate supply to the liver would be expected to be sufficient to maintain hepatic aspartate levels. Furthermore, only a part of the ammonia requirements for urea synthesis is derived from the portal blood (the remainder being from deamination of glutamate).

Citrulline availability to argininosuccinate synthetase is probably a more important physiological modulator of urea synthesis. Whereas citrulline formation by ornithine transcarbamylase is dependent on the mitochondrial concentrations of both ornithine and carbamyl phosphate, it appears that ornithine is more likely to be regulatory for urea synthesis under most circumstances in the intact liver than carbamyl phosphate, which is present at concentrations (estimated at 0.5 mM) considerably in excess of the K_m value of 24 μM (69). However, further work is required to assess the possibility of regulation by the carbamyl phosphate concentration. Possible limitation of citrulline synthesis by the rate of carbamyl phosphate formation was intentionally avoided in the present investigation by use of relatively high ammonia concentrations and inclusion of oleate as a respiratory fuel to maintain both ATP and N-acetyl glutamate levels, the latter compound being the only known physiological activator of carbamyl phosphate synthetase (see Ref. 70 for kinetic constants). Citrulline formation (and accumulation) was increased not only by the obvious expedient of adding ornithine to the hepatocyte incubation (half-maximal effect with 0.5 mM), but also, more interestingly, by inhibition of ornithine degradation via cytosolic ornithine transaminase as a result of cycloserine addition. Although the relative roles of cytosolic and mitochondrial ornithine transaminase in ornithine catabolism need to be investigated in greater detail under a variety of more physiological conditions (cf. Ref. 59), it is evident from the data presented in this paper that ornithine transamination plays an important role in regulating the hepatic ornithine content, which in turn is the decisive factor in determining citrulline formation when ammonia is not rate-limiting.

In contrast to the fact that a gluconeogenic precursor is needed for maximum rates of urea formation from ammonia, it is apparent that the rate of gluconeogenesis itself is inhibited by ammonia addition. As pointed out by Krebs et al. (47), this effect is observed at both high and low rates of urea formation with lactate as substrate. Data presented in this paper contribute towards elucidating this phenomenon, which is of interest because of the principles of regulation it illustrates. In the absence of ammonia, flux through pyruvate carboxylase and P-enolpyruvate carboxykinase are equal since dicarboxylic keto and amino acids do not accumulate. The accumulation of these intermediates when P-enolpyruvate carboxylyase is inhibited (71) suggests that pyruvate carboxylase is normally rate-limiting for gluconeogenesis. The rate of conversion of P-enolpyruvate to glucose may, however, be less than flux through pyruvate carboxylase due to loss of P-enolpyruvate back to pyruvate via pyruvate kinase, which in liver is subject to metabolite and hormonal regulations. Active recycling of carbon between pyruvate and P-enolpyruvate in liver cells has, in fact, been quantitated and shown to be greater with pyruvate than with lactate as substrate (40). As pointed out earlier, this difference relates to the fact that transport of reducing equivalents from the mitochondria to the cytosol (see Refs. 37 and 38) exceeds the stoichiometric equivalent for gluconeogenesis with pyruvate as substrate because of net lactate production. Apart from a stimulation of carbamyl phosphate (and hence citrulline) formation, the primary effect of ammonia addition to hepatocytes is a stimulation of reductive amination of α-ketoglutarate via glutamate.
dehydrogenase. This causes a large oxidation of the mitochondrial NADP system, a smaller oxidation of the NAD system, and stimulation of flux through NADP-linked isocitrate dehydrogenase (68, 72). In the presence of suitable carbon precursors, pyruvate carboxylation to oxalacetate, via pyruvate carboxylase, replenishes the supply of oxalacetate and α-ketoglutarate, the latter secondarily through mediation of the citric acid cycle, and aspartate and glutamate accumulate. Thus, flux through pyruvate carboxylase becomes greater than flux through P-enolpyruvate carboxykinase. Under these conditions we suggest that gluconeogenesis is regulated (a) primarily by the cytosolic free oxalacetate concentration, which determines flux through P-enolpyruvate carboxykinase, (b) by the activity of pyruvate kinase, which determines the relative proportion of P-enolpyruvate formation available for gluconeogenesis, and (c) secondarily by pyruvate carboxylase activity, which becomes inhibited by accumulation of mitochondrial glutamate. Interestingly, the ratio of lactate production to glucose production increased from 2.0 to 2.5 to 4.1 upon addition of ammonia and ammonia plus ornithine to hepatocytes incubated with pyruvate (Table 1). These data suggest that pyruvate kinase flux (reflected by the rate of lactate production) is stimulated by ammonia plus ornithine, despite the high alanine concentration in the cell which should be inhibitory.

Although addition of ammonia, particularly with ornithine also present, causes a diversion of intramitochondrially formed oxalacetate from net malate to net aspartate production, the present data provide no evidence that efflux of either malate or aspartate is rate-limiting for glucose and lactate production from pyruvate. The driving force for increased intramitochondrial transamination is provided by the increased glutamate concentration. Because of increased α-ketoglutarate utilization for glutamate synthesis, flux through the span of the citric acid cycle from α-ketoglutarate to malate is lower than that in the span from oxalacetate to α-ketoglutarate, and malate levels fall. Increased malate levels, and hence increased P-enolpyruvate carboxykinase flux after ornithine addition, appear to be secondary to increased aspartate utilization for argininosuccinate synthesis, which produces an enhanced rate of malate generation in the cytosol as a result of increased urea synthesis.

In considering the reason for the observed inhibition of gluconeogenesis from lactate in the presence of ammonia, in combination with oleate, ornithine, or both, Krebs et al. (47) suggested that the critical factor was the fall of cytosolic α-ketoglutarate, which caused a shortage of cytosolic α-ketoglutarate for transamination with aspartate, and hence a decreased supply of oxalacetate in the cytosol for gluconeogenesis. This explanation implies a disequilibrium of cytosolic aspartate aminotransferase (see also Ref. 73). Our data under similar experimental conditions (this paper and Ref. 68), indicate that cytosolic aspartate aminotransferase remains in near-equilibrium after ammonia addition; hence, the above explanation cannot be valid. Furthermore, with ammonia and ornithine present, the rate of urea synthesis (and hence cytosolic malate generation) even with cyclonoxime present is greater than twice the rate of glucose formation (Table IV). Flux through cytosolic aspartate aminotransferase is thus probably minimal, and there is in fact a requirement for net transport of malate into the mitochondria, as illustrated in Fig. 7.

It is commonly accepted that control of flux from oxalacetate to glucose is likely to reside at a disequilibrium step. The present data indicate that gluconeogenesis from lactate, as with pyruvate, in the presence of ammonia is regulated by oxalacetate availability to P-enolpyruvate carboxykinase. Increased activity of pyruvate kinase will, however, also diminish flux from P-enolpyruvate to glucose, but at present this remains an unknown factor. As an alternative to the postulation of cytosolic aspartate aminotransferase being rate-controlling in the sense that it is forced out of equilibrium by the low α-ketoglutarate concentration, we propose that the transaminases have a passive, though important, role in regulation of the cytosolic oxalacetate concentration, being proportional not only to the α-ketoglutarate concentration but also to the aspartate/glutamate ratio. Alanine accumulation will tend to maintain α-ketoglutarate levels near equilibrium with alanine aminotransferase.

Direct proof that anion translocation steps are, or are not involved in the regulation of the gluconeogenic pathway is difficult to obtain with intact cell preparations because of the cyclic nature of the processes involved. Probably anion translocation flux is a passive reflection of regulation of specific nonequilibrium enzymic steps, although the translocator kinetic constants are likely to be important in determining the cell concentration of individual metal-oligates. Two examples from the present data illustrate this point. First, the fact that malate generation in the cytosol with lactate, ammonia, and ornithine as substrates is greater than conversion of malate to glucose requires explanation. From the data in Table V it is apparent that the gradient of malate across the mitochondrial membrane is less than the α-ketoglutarate gradient. Presumably the relative concentration gradients of these anions determines net flux through the α-ketoglutarate-malate translocator, so that malate influx to the mitochondria may follow passively because of the high flux through mitochondrial aspartate aminotransferase and the consequent drain on intramitochondrial oxalacetate. The second example relates to the glutamate aspartate translocator. Since exchange of these anions is an irreversible process under conditions when energization of the mitochondrial membrane is maintained (67), this translocator appears a likely candidate for a regulatory site. However, although flux is kinetically regulated by the cytosolic glutamate/aspartate ratio at high glutamate concentrations (74), its activity does not appear to be rate-limiting for either gluconeogenesis or ureogenesis since cytosolic aspartate concentrations remain relatively high. Further, more detailed evaluations of the interactions between anion translocator flux and key regulatory enzymes of the various metabolic pathways can probably only be achieved by careful titrations of metabolite concentrations in the appropriate cell compartment and comparison with flux through specific enzyme or translocator steps.

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