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Hepatic Enzymes of Glutamine and Ureagenesis in Metabolic Acidosis (42358)

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Abstract. The activities of key glutamine and urea cycle enzymes were assayed in liver homogenates from control and chronically acidotic rats and compared with citrulline and urea productions by isolated mitochondria and intact liver slices, respectively. Glutamine-dependent urea and citrulline synthesis were increased significantly in isolated mitochondria and in liver slices; the activities of carbamoyl phosphate synthetase and arginase were unchanged and increased, respectively. Glutamine was not a precursor in the carbamoyl phosphate synthetase system, suggesting that the glutamine effect is an indirect one and that glutamine requires prior hydrolysis. Increased mitochondrial citrulline synthesis was associated with enhanced oxygen consumption, suggesting glutamine acts both as a nitrogen and fuel source. Hepatic phosphate-dependent glutaminase was elevated by chronic acidosis. The results indicate that the acidosis-induced reduction in ureagenesis and reversal from glutamine uptake to release observed *in vivo* are not reflections of corresponding changes in the hepatic enzyme content. Rather, when available, glutamine readily supports ureagenesis, suggesting a close coupling of hepatic glutaminase flux with citrulline synthesis.

The liver normally removes delivered ammonia (1) and glutamine (2, 3) and releases urea (3) and glutamate (4). However, during chronic metabolic acidosis urea release (3) and subsequent urinary excretion is reduced (3, 5, 6) while hepatic glutamine extraction decreases (4) and reverts to net release (2, 3). Since the delivered ammonia load increases (3, 7), the alteration in urea and glutamine metabolism suggests a redistribution of ammonia uptake between carbamoyl phosphate synthetase (EC 2.7.2.5) and glutamine synthetase (EC 6.3.1.2). Such a redistribution could account for the shift in urinary nitrogen partitioning from urea to ammonia that is observed in metabolic acidosis (3, 5, 6). We therefore measured the activity of both enzymes as well as that of arginase (EC 3.5.3.1) and phosphate-dependent glutaminase (EC 3.5.1.2) in control and chronically acidotic rat livers; in addition, the role of glutamine as a direct precursor of urea was investigated. The results indicate that glutamine plays a unique role in supporting ureagenesis, an effect which depends upon its prior hydrolysis by the hepatic glutaminase.

Materials and Methods. *Materials.* All biochemicals were obtained from Sigma Chemical Company (St. Louis, Mo.) with the exception of antipyrine and 2,3-butanedione monoxime which were purchased from Aldrich Chemical Company (Milwaukee, Wisc.).

Methods. Rats were housed in metabolic cages and maintained on Purina laboratory chow *ad libitum*. Metabolic acidosis was induced by feeding rats 1.2% NH_4Cl in 5% glucose; controls were pair-fed equimolar NH_4HCO_3 in 5% glucose. After 4 days urinary ammonium excretion plateaued at 1692 ± 170 μmole/day/100 g, some 10-fold greater than pair-fed controls (139 ± 38 μmole/day/100 g); urea excretion had decreased from 3506 ± 404 to 1661 ± 228 μmole/day/100 g. Arterial blood pH was slightly but significantly decreased to 7.32 ± 0.02 compared to 7.40 ± 0.01 in controls. Therefore all assays were performed on the fourth day of acid administration. At that time the animals were anesthetized with Inactin, 120 mg/kg, and 1 g of the middle liver lobe was removed and homogenized in 9 vol of 0.3 *M* sucrose containing 50 m*M* Tris–HCl buffer and 1 m*M* EGTA, pH 7.2. Aliquots of the homogenate were then used in the arginase and glutaminase assays; carbamoyl phosphate synthetase and glutamine synthetase were assayed on mitochondrial and postmitochondrial supernatant fractions obtained by differential centrifugation (8). For urea formation by liver tissue, liver slices of 30 to 40 mg wet wt were cut using a Stadie Riggs microtome;

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slices were then incubated in a modified Krebs–Henseleit solution containing 1.7 m*M* L-glutamine and 25 m*M* HCO_3^- equilibrated with 95% O_2–5% CO_2, pH 7.4. For citrulline formation by isolated mitochondria, freshly isolated mitochondria were incubated at 37°C in a modification of the media described by Charles *et al.* (9); the media contained (m*M*) 15 KCl, 5 $MgCl_2$, 50 Tris–HCl buffer, 25 sucrose, 16 $KHCO_3^-$, 10 ornithine, 2 ATP, and either 10 NH_4Cl or 10 L-glutamine with equilibration and pH adjustment to 7.2 just prior to use. Citrulline formation was detected colorimetrically (10); supernatant glutamate was determined enzymatically (11). Oxygen consumption by isolated mitochondria was determined manometrically (12) using 2 m*M* L-glutamine or 4 m*M* succinate as the sole exogenous substrate; oxygen uptake was linear over a 45-min time course.

Enzyme assays. Phosphate-dependent glutaminase activity was assayed using liver homogenates and derived mitochondrial and postmitochondrial fractions (8) employing 10 m*M* L-glutamine in the medium described by Curthoys and Lowry (13); glutamate formed was measured enzymatically (11). Arginase activity was determined in homogenates after first heating the homogenate for 10 min at 55°C (14); urea formed was measured as previously described (10). Carbamoyl phosphate synthetase was assayed in the citrulline assay system above using freeze-thawed mitochondria in the presence of 0.5 m*M* *N*-acetylglutamate (15). Glutamine synthetase was assayed in the homogenate and mitochondrial and postmitochondrial supernatant, using the previously described media (16) containing tracer amounts of L-[U-^{14}C]glutamate; formed [^{14}C]glutamine was separated from [^{14}C]glutamate on a Dowex ion-exchange column (16). All reactions, with the exception of citrulline synthesis, were linear with time and expressed as nanomoles per minute per milligram of protein with the protein concentration determined using a dye-binding assay (Bio-Rad, Richmond, Calif.) with bovine serum albumin as the standard. Citrulline synthesis from glutamine by the intact mitochondria exhibited a 5-min lag after which the reaction assumed linearity. Results are expressed as means ± SE; differences between nonacidotic and acidotic rat liver activities are significant at the 0.05 percentile level using the Student *t* test.

Results. Table I presents the activity of carbamoyl phosphate synthetase, CPS, and arginase in freeze–thawed liver mitochondria and liver homogenates, respectively, from NH_4HCO_3- and NH_4Cl-fed rats. CPS activity in the absence of *N*-acetylglutamate was 13% of the activity expressed in its presence; metabolic acidosis had no effect on the expression of either activity. Substitution of L-glutamine for NH_4Cl gave rates only 21–23% of those with NH_4Cl; in addition, supplementing the complete system, NH_4Cl + *N*-acetylglutamate, with L-glutamine did not further enhance its activity, suggesting the low rate observed with glutamine may be due to NH_4 present. Although there was no effect upon CPS, arginase activity exhibits a slight but significant increase in acidosis. Consequently, alterations in ureagenesis do not appear to be reflections of decreased CPS activity. Table II shows that citrulline formation from glutamine by intact mitochondria was increased in chronic acidosis; in contrast, citrulline formation from equimolar NH_4Cl was unchanged. In the presence of both NH_4Cl and L-glutamine, the rate of citrulline formation was greatly increased although the rate of formation by acidotic preparations still exceeds that of the control preparation; ammonium therefore potentiated citrulline synthesis since the rate far exceeded the sum of either substrate alone. The ammonia-induced stimulation of citrulline formation may be due to activation of the

TABLE I. EFFECT OF CHRONIC ACIDOSIS ON CPS AND ARGINASE ACTIVITY

	nmole/min/mg		
	Control	Acidotic	*P*
CPS[a]			
−Ac-Glu + NH_4Cl	9.2 ± 1.4[b]	9.4 ±1.4	NS
+Ac-Glu + NH_4Cl	69.3 ± 4.5	70.0 ± 5.8	NS
+Ac-Glu + L-Gln	15.9 ± 2.7	16.1 ± 3.8	NS
+Ac-Glu + L-Gln + NH_4Cl	74.9 ± 9.4	65.6 ± 9.4	NS
Arginase	654 ± 40	810 ± 23	<0.05

[a] Carbamyl phosphate synthetase assayed with 0.5 m*M* *N*-acetylglutamate and 10 m*M* NH_4Cl and/or 10 m*M* L-Gln.

[b] Results are means ± SE from four to six rats.

TABLE II. EFFECT OF CHRONIC ACIDOSIS ON CITRULLINE AND UREA SYNTHESIS

	nmole/min/mg		
	Control	Acidotic	*P*
Citrulline[a]			
NH_4Cl	0.70 ± 0.24	1.05 ± 1.10	NS
L-Gln	0.49 ± 0.15	1.14 ± 0.25	<0.05
NH_4Cl + L-Gln	3.49 ± 1.94	12.2 ± 1.8	<0.01
NH_4Cl + succinate	2.23 ± 0.58	6.91 ± 1.20	<0.05
Urea[b]			
NH_4Cl	148 ± 22	143 ± 38	NS
L-Gln	97 ± 19	267 ± 51	<0.025
NH_4Cl + L-Gln	168 ± 52	359 ± 61	<0.05

[a] Studied in isolated mitochondria with 10 mM substrate concentrations except for succinate which was 4 m*M*.

[b] Studied in liver slices with 1 m*M* NH_4Cl and 1.7 m*M* L-glutamine. Results are means ± SE. Respiratory control ratio measured manometrically was 4.85 ± 0.27 in isolated mitochondria respiring 4 mM succinate, $N = 5$.

intramitochondrial glutaminase (17); consistent with this, glutamate production from glutamine by mitochondria from control rats rose from 4 ± 1 to 32 ± 6 nmole/min/mg consistent with ammonia enhancement of phosphate-dependent glutaminase. An accelerated glutamine deamidation would provide ammonia for citrulline synthesis and as well an oxidizable fuel in the form of glutamate. That citrulline formation from NH_4Cl was limited under the present conditions by an energy source was shown by the sevenfold increase with NH_4Cl and succinate as substrates (Table II). However, citrulline synthesis from NH_4Cl plus glutamine even exceeded the rate with succinate as the fuel source, 12.2 ± 1.8 vs 6.91 ± 1.20 nmole/min/mg. Oxygen consumption by mitochondria utilizing 10 m*M* glutamine was increased from 3.6 ± 1.2 to 12.2 ± 3.7 nmole/min/mg, $P < 0.05$, in acidosis; with 4 m*M* succinate oxygen consumption tended to be increased 34 ± 5 to 43 ± 4 nmole/min/mg which, however, did not achieve statistical significance, $0.10 < P < 0.05$. These findings are therefore consistent with increased citrulline synthesis resulting from glutamine's role both as a nitrogen donor and as an oxidizable substrate. Similarly, urea formation by liver slices from 1.7 m*M* L-glutamine was markedly increased by chronic acidosis, 97 ± 19 to 267 ± 51 nmole/min/g, while urea formation rates from NH_4Cl were not different. Adding ammonium chloride to liver slices, metabolizing glutamine increased urea production far less than it did in mitochondrial preparations; nevertheless the difference between control and acidotic preparations was maintained. The effect of chronic acidosis on two key enzymes of hepatic glutamine metabolism, glutamine synthetase and phosphate dependent glutaminase activity, is shown in Table III. Glutamine synthetase activity measured in the postmitochondrial supernatant was unchanged, 46 ± 4 vs 54 ± 6 nmole/min/mg; similarly, when assayed in the homogenate the specific activity was less, but again without any effect of acidosis, 32 ± 4 vs 36 ± 5 nmole/min/mg. On the other hand, homogenate glutaminase specific activity was increased in acidosis, 84 ± 7 to 105 ± 4 nmole/min/mg.

Discussion. The results clearly establish that the reduced urea production *in vivo* during chronic metabolic acidosis is not attributable to a decrease in the inherent capacity of several key urea cycle activities. Other factors influencing *in situ* cycle activity would therefore appear to play dominant roles *in vivo*. Amongst these could be prevailing pH (18) and bicarbonate concentration (18) as well as substrate availability (3). In the perfused liver, doubling the hydrogen ion concentration reduces the rate of urea production (18) and enhances net glutamine release (18–20). Both of these responses are indeed observed *in vivo* although in this situation the elevation in hydrogen ion concentration was far less, 40 to

TABLE III. EFFECT OF CHRONIC ACIDOSIS ON Gln METABOLIZING ENZYMES

	nmole/min/mg		
	Control	Acidotic	*P*
Gln synthetase			
Homogenate	32 ± 4	36 ± 5	NS
Mitochondria	11 ± 2	12 ± 3	NS
PMS[a]	46 ± 4	50 ± 6	NS
PD glutaminase			
Homogenate	84 ± 7	105 ± 4	<0.05
Mitochondria	126 ± 15	147 ± 17	NS
PMS	9 ± 2	14 ± 4	NS

[a] Postmitochondrial supernatant; results are means ± SE.

48 nmole/liter in contrast to the doubling, or more, employed *in vitro.* Under the conditions employed in the present study, citrulline synthesis (Table II) and ureagenesis (Table II) were shown to be highly dependent upon glutamine as a substrate; rates with ammonia as the nitrogen donor were similar in both control and acidosis. Part of this effect no doubt reflects glutamine contributing its amide nitrogen as a nitrogen source; consistent with this is the increased hepatic glutaminase activity. It may be that glutamine nitrogen, in contrast to extracellular ammonium nitrogen, is more readily accessible for carbamoyl phosphate synthetase. The importance of the glutaminase activity as the first step in making glutamine nitrogen available is underlined by the confirmation of previous studies showing that mammalian CPS (21), in contrast to microorganisms (22) and elasmobranchs (23), does not utilize glutamine. Another role of glutamine in acidosis may be to supply glutamate for intramitochondrial *N*-acetylglutamate synthesis; the presence of increased *N*-acetylglutamate could be a powerful stimulant for carbamoyl phosphate synthesis (Table I and (10)). Finally, glutamate derived from glutamine may provide an oxidizable substrate to fuel the heavily energy-dependent ureagenesis. That citrulline synthesis was limited by the available energy was suggested by the enhanced rate occurring with the addition of succinate. *In vivo,* of course, exogenous succinate is not available but must be derived from blood-borne fuels. Mitochondrial preparations from acidotic rat livers consumed more oxygen when incubated with glutamine, suggesting that this physiological fuel acts as both a nitrogen donor and oxidizable fuel under these *in vitro* conditions. *In vivo,* on the other hand, the kidneys syphon off tremendous amounts of glutamine in this condition, providing substrate for renal ammoniagenesis and perhaps, as well, depriving hepatic ureagenesis of substrate.

The shift in portal-delivered ammonium nitrogen from ureagenesis to glutamine release in acidosis is not dependent upon an increased amount of glutamine synthetase as previously suggested for liver (7) and which apparently plays a role in skeletal muscle (16). It is not clear why the increased ammonium load bypasses the upstream ureagenesis for incorporation into glutamine at the perivenous site (24). However, one possibility is that the upstream urea production is relatively deprived of oxidizable substrate and relinquishes ammonia for downstream glutamine synthesis, a process which requires much less energy for deposition of a mole of ammonium than does ureagenesis. Further studies are required to evaluate this as well as other possible mechanisms involved in nitrogen partitioning occurring during chronic metabolic acidosis.

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