# Avian and Mammalian Mitochondrial Ammonia-Detoxifying Systems in Tortoise Liver

Abstract. *Liver mitochondria from the desert tortoise* Gopherus agassizii *and the Texas tortoise* G. berlandieri *contain glutamine synthetase, the enzyme used by birds and higher reptiles to detoxify ammonia generated intramitochondrially during amino acid catabolism. Tortoise mitochondria also contain carbamyl phosphate synthetase-I and ornithine transcarbamylase, the enzymes used by mammals for this purpose. Since chelonid reptiles have changed little since their divergence from the stem reptiles, this finding suggests that both systems were present in the latter before the emergence of the ruling reptile, avian, and mammalian lines of descent.*

In mammals and ureotelic amphibians, ammonia generated intramitochondrially during amino acid gluconeogenesis in liver cells is converted to citrulline for exit to the cytosol and subsequent conversion to urea for excretion. In birds and higher reptiles that are uricotelic, ammonia generated in this manner is converted to glutamine for exit to the cytosol and subsequent conversion to uric acid for excretion (*1*). Neither the amide nitrogen of glutamine nor the carbamoyl nitrogen of citrulline are protonated at physiological *p*H's, and we have suggested that these two mechanisms convert intramitochondrially generated ammonia to a form that does not interfere during its exit with the normal proton gradient across the inner mitochondrial membrane (*2*). Glutamine synthetase, the enzyme reponsible for intramitochondrial glutamine synthesis in the uricotelic system, is in the extramitochondrial compartment in mammalian liver (*3*). There was thus a fundamental change in the compartmentation of this enzyme in liver cells during the evolution of birds and mammals. Glutamine synthetase is also in the extramitochondrial compartment in brain tissue of birds (*4*). This suggests that a similar change may occur during tissue differentiation in these species.

Of the extant vertebrates, the chelonid reptiles (turtles and tortoises) are the closest relatives of the cotylosaurians or "stem reptiles" that gave rise to the avian and mammalian lines of descent and are generally believed to have changed little since their divergence (*5*). Aquatic turtles excrete mainly urea (*6*) and have the full complement of hepatic urea cycle enzymes including carbamyl phosphate synthetase and ornithine transcarbamylase (*7*), the enzymes responsible for intramitochondrial citrulline formation. Tortoises also have the full complement of urea cycle enzymes in liver but excrete more uric acid than urea (*8*). We now report that glutamine synthetase is a mitochondrial enzyme in liver of the tortoises *Gopherus agassizii* and *G. berlandieri* and that liver mitochondria from these two species form and release citrulline and glutamine during glutamate degradation.

As shown in Table 1, 70 to 80 percent of the glutamine synthetase activity in liver tissue of the tortoises was present in the mitochondrial fraction after fractionation of this tissue by differential centrifugation. The distribution of glutamine synthetase activity among the different cellular fractions generally followed that of glutamate dehydrogenase, a mitochondrial matrix enzyme, and cytochrome oxidase, an enzyme of the inner mitochondrial membrane. For example, 10 to 20 percent of the recovered glutamine synthetase activity was present in the nuclear fraction. That this is most likely due to mitochondrial contamination of this fraction is indicated by the presence of a somewhat similar percentage of glutamate dehydrogenase and cytochrome oxidase activities. While there was some contamination of the mitochondrial fraction by microsomes, as assessed with the microsomal marker glucose 6-phosphatase (*9*), there was essentially no glutamine synthetase activity in the microsomal fraction. This indicates that the glutamine synthetase activity in the mitochondrial fraction was not due to contaminating microsomes. Glutamine synthetase is a matrix enzyme in chicken mitochondria (*10*), so the 5 percent or so present in the soluble fraction is presumably due, in part, to mitochondrial breakage during fractionation. Again, a similar percentage of the glutamate dehydrogenase activity was also present in the soluble fraction in support of this interpretation. When fractions of *G. berlandieri* liver, prepared in another experiment, were probed with antibody to chicken mitochondrial glutamine synthetase (*11*), immunoreactive protein was detected only in the nuclear and mitochondrial fractions (Fig. 1). The nuclear fraction contained 19 percent of the recovered glutamine synthetase activity and 21 and 23 percent, respectively, of the glutamate dehydrogenase and cytochrome oxidase activities. As shown in Fig. 1, ornithine transcarbamylase, also a mitochondrial matrix enzyme (*12*), gave results similar to those with glutamine synthetase. The results shown in Fig. 1 also demonstrate that the subunit molecular weight of the tortoise glutamine synthetase (45,000 daltons) is similar to that of other vertebrate glutamine synthetases (*13*) and that it is immunochemically cross-reactive with antibody

Table 1. Subcellular localization of enzymes in tortoise liver. Tissue from individual animals was fractionated in Hepes-buffered 0.25*M* sucrose and the individual fractions assayed for enzyme activity as described (*19*). The activities, as micromoles per hour per gram of tissue, recovered in the fractions for the Texas and desert tortoises, respectively, were: glutamine synthetase, 222 and 95; glutamate dehydrogenase (GDH), 3588 and 2923; cytochrome oxidase, 194 and 192; carbamyl phosphate synthetase-I (CPS-I), 24 and 30 (homogenate value); ornithine transcarbamylase (OTC), 1453 and 3857; lactate dehydrogenase (LDH), 2027 and 3580; and glucose 6-phosphatase, 345 and 363. Values in upright print are for the Texas tortoise *G. berlandieri*; those in italics are for the desert tortoise *G. agassizii*.

| Fraction | Protein (mg/g) | Percentage of total units recovered in fractions | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | | Glutamine synthetase | GDH | Cytochrome oxidase | CPS-I | OTC | LDH | Glucose 6-phosphatase |
| Nuclear | 2.4, *7.6* | 10.2, *19.1* | 8.0, *28.5* | 3.0, *27.5* | 9.4, *23.9* | 8.0, *30.3* | 0, *0* | 0, *6.6* |
| Mitochondrial | 27.6, *8.8* | 80.1, *72.0* | 85.4, *63.5* | 97.0, *66.3* | 82.2, *55.0* | 83.1, *69.7* | 0, *0* | 29.4, *7.4* |
| Microsomal | 15.7, *8.8* | 1.2, *3.2* | 1.3, *2.8* | 0, *6.1* | 1.3, *21.1* | 0.5, *0* | 3.5, *2.3* | 65.0, *75.8* |
| Soluble | 59.1, *33.0* | 8.4, *5.8* | 5.2, *5.1* | 0, *0* | 7.2, *0* | 8.4, *0* | 96.5, *97.7* | 5.3, *10.2* |
| Percentage of homogenate value recovered | 95.3, *93.1* | 87.5, *75.0* | 99.0, *83.9* | 111.6, *78.0* | 117.2, *29.1* | 106.8, *107.6* | 97.9, *78.7* | 75.7, *63.5* |

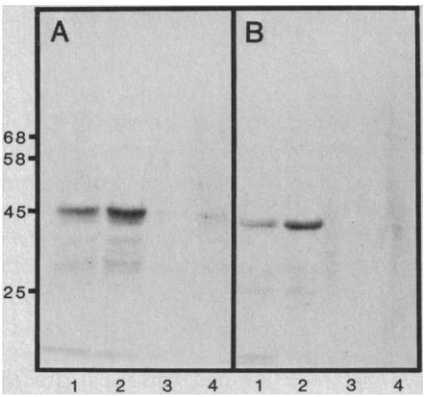


Fig. 1. Western blot of glutamine synthetase and ornithine transcarbamylase in subcellular fractions of *Gopherus berlandieri* liver. Fractions were prepared as in Table 1. The proteins in each fraction were separated by 10 percent sodium dodecyl sulfate–polyacrylamide gel electrophoresis and then electrophoretically transferred to a nitrocellulose filter (*20*). Half of the filter (A) was probed with antiserum to chicken glutamine synthetase (dilution, 1:50) (*4*) and the other half (B) with antiserum to beef liver ornithine transcarbamylase (dilution, 1:50). This was followed by $^{125}$I-labeled protein A ($10^6$ count/min; 17 μCi per microgram). The filters were exposed to Kodak XAR-15 film at −80°C for 12 hours with an intensifying screen and then developed. Each lane contained 100 μg of protein of the respective subcellular fraction: (lane 1) nuclear, (lane 2) mitochondrial, (lane 3) microsomal, and (lane 4) soluble. The migration of the molecular weight markers (in kilodaltons) is indicated on the left of (A).

Table 2. Citrulline and glutamine synthesis by *G. agassizii* liver mitochondria. The basic reaction mixture and reaction conditions were as described in Fig. 2, with 4.2 mg of mitochondrial protein. The additions to the basic reaction mixture are indicated. Citrulline and glutamine, separated as described in Fig. 2, were eluted from the chromatograms into 200 μl of water and counted. The amount of citrulline formed was calculated on the basis of the specific radioactivity of [$^{14}$C]bicarbonate and the amount of glutamine on that of [$^{14}$C]glutamate. Recovery of added radioactivity averaged 84.2 percent.

| Compounds added | Product formed (nmol) | |
|---|---|---|
| | Citrulline | Glutamine |
| [$^{14}$]Bicarbonate (10 μmol; 2 μCi per micromole)<br>L-Ornithine (5 μmol)<br>Ammonium chloride (10 μmol)<br>Succinate (5 μmol) | 203.3 | 0 |
| [U-$^{14}$C]L-Glutamate (10 μmol; 1 μCi per micromole)<br>Ammonium chloride<br>Succinate<br>Rotenone (7.5 nmol) | 0 | 61.3 |
| [$^{14}$C]Bicarbonate plus [$^{14}$C]glutamate<br>L-Ornithine<br>Ammonium chloride<br>Succinate<br>Rotenone | 72.9 | 87.9 |
| [$^{14}$C]Bicarbonate plus [$^{14}$C]glutamate<br>L-Ornithine | 95.5 | 174.2 |

to chicken liver mitochondrial glutamine synthetase.

In addition to mitochondrial glutamine synthetase, tortoise liver contains both carbamyl phosphate synthetase-I (*14*) and ornithine transcarbamylase, and these enzymes are mitochondrial (Table 1) as they are in mammals and ureotelic amphibians (*12*). For three desert tortoises, glutamine synthetase activity ranged from 95 to 126 units (micromoles per hour at 30°C) per gram of tissue. The values for two Texas tortoises were 178 and 222. Carbamyl phosphate synthetase-I ranged from 24 to 30 units per gram for both species. Ornithine transcarbamylase activity in the desert tortoise ranged from 3584 to 3857 units per gram, and the values for the Texas tortoise were 1383 and 1453. In general, the values for both the glutamine and citrulline systems are lower than those in exclusively uricotelic (*1*) or exclusively ureotelic vertebrates (*15*). When liver mitochondria were incubated with [$^{14}$C]bicarbonate plus [$^{14}$C]glutamate and with glutamate as the sole source of ammonia, both [$^{14}$C]citrulline and [$^{14}$C]glutamine were synthesized (Fig. 2 and Table 2). This shows that both systems operate in intact mitochondria. For the Texas tortoise, it was also shown that both compounds are released to the medium (Fig. 2A) as is glutamine in uricotelic species (*2*) and citrulline in ureotelic species (*12*). The synthesis of citrulline and glutamine by *G. agassizii* liver mitochondria under different experimental conditions is shown in Table 2. Because tortoises do not feed well in captivity, the general physiological state of the animal used was unknown. We therefore attribute little quantitative significance to the relative amounts of citrulline and glutamine synthesized under

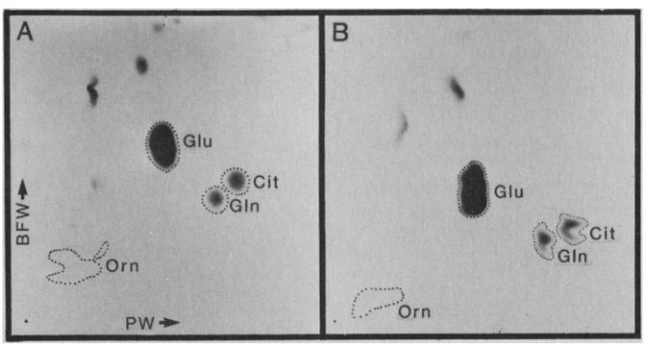


Fig. 2. Identification of citrulline and glutamine as products of glutamate metabolism by tortoise liver mitochondria. Twice-washed liver mitochondria (4.2 to 7 mg of protein; respiratory control ratio with succinate, 2.2 to 2.6) were incubated for 30 minutes at 30°C in a basic reaction mixture containing (in micromoles per milliliter): magnesium chloride, 5; potassium phosphate, 2.5; Hepes, 50; potassium chloride, 10; and aminooxyacetate, 2; plus 50 μg of atractyloside and 0.75 mg of bovine serum albumin (fatty acid free). To this were added 5 μmol of L-ornithine, 10 μmol of [$^{14}$C]bicarbonate (2.2 μmol of [$^{14}$C]sodium bicarbonate plus 7.8 μmol of potassium bicarbonate; 2 μCi per micromole); and 10 μmol of [U-$^{14}$C]L-glutamate (1 μCi per micromole). Reagents were adjusted to *p*H 7.4 with potassium hydroxide as required. Sucrose was added to approximately 300 mos*M*. The reaction was stopped with 50 μl of 70 percent perchloric acid. This was removed as the potassium salt after removal of precipitated protein. The compounds in 10 μl of the neutralized extract were separated on 20 by 20 cm cellulose plates with a mixture of *sec*-butyl alcohol, formic acid, and water (BFW) (75:15:10 by volume) in the first dimension and 80 percent (by volume) phenol (PW) in the second. The dried plates were sprayed with EN$^3$HANCE (New England Nuclear), exposed to Kodak XAR-15 film at −80°C for 60 hours, and then developed. The dotted lines indicate ninhydrin-positive—or, in the case of citrulline—Erhlich reagent-positive reactions on the chromatograms; Glu, glutamate; Gln, glutamine; Cit, citrulline; and Orn, ornithine. (A) *Gopherus berlandieri* and (B) *G. agassizii* mitochondria. For (A) the mitochondria were removed from the reaction mixture by rapid centrifugation before the addition of perchloric acid.

the conditions used for the data in Table 2 since it seems likely that the ratio of formation of these two products would be influenced by the physiological state. The data do, however, indicate that ammonia is a better substrate than glutamate for citrulline synthesis, whereas the reverse is true for glutamine synthesis.

The evolutionary precedent for a mitochondrial localization of glutamine synthetase in vertebrate liver was set in the elasmobranchs (*14*), where the enzyme functions in conjunction with the glutamine-utilizing carbamyl phosphate synthetase-III in the synthesis of urea for osmotic purposes. In amphibians, hepatic mitochondrial ammonia detoxication is via carbamyl phosphate synthetase-I. Immunochemical and other properties of carbamyl phosphate synthetases-I and -III suggest that the two are evolutionarily related (*16*). The utilization of glutamine synthetase for the intramitochondrial detoxication of ammonia appears not to occur in amphibians, even in those species that excrete a large percentage of their excretory nitrogen as uric acid (*17*). However, our data indicate that both systems may have been present in the stem reptiles that subsequently gave rise to the ruling reptile, avian, and mammalian lines of descent. Both carbamyl phosphate synthetase-I and cytosolic glutamine synthetase in mammalian liver show a heterogeneous distribution within this organ (*18*), so whether the same or different populations of tortoise hepatocytes contain both detoxification systems remains a question.

JAMES W. CAMPBELL
DARWIN D. SMITH, JR.
JEAN E. VORHABEN

*Department of Biology,*
*Rice University,*
*Houston, Texas 77251*

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14. Tortoises are predominantly uricotelic in their nitrogen excretion (*8*). Hence their synthesis of urea could be to prevent water loss by raising the osmolarity of body fluids, such as occurs in elasmobranchs. In the latter species, glutamine synthetase is also a mitochondrial enzyme in liver and provides the substrate for carbamyl phosphate synthetase-III, an *N*-acetyl-L-glutamate–dependent, glutamine-utilizing mitochondrial enzyme [C. A. Casey and P. M. Anderson, *J. Biol. Chem.* **257**, 8449 (1982)]. It was therefore necessary to establish that the carbamyl phosphate synthetase in tortoise liver was enzyme I and not enzyme III. When glutamine was substituted for ammonia in the colorimetric assay (*19*), only 6.8 percent of the ammonia-dependent activity was obtained with the Texas tortoise. With a similar assay that contained [$^{14}$C]bicarbonate and one in which the acid-stable counts were used as criterion for citrulline formation, 10.9 percent of the ammonia-dependent activity was obtained when glutamine was substituted for ammonia with the desert tortoise. In the latter case, the reaction mixture was determined to have trace amounts of ammonia (0.4 to 0.6 m*M*). The activity was adenosine triphosphate (ATP)– and *N*-acetyl-L-glutamate–dependent in both species. The tortoise enzyme is thus carbamyl phosphate synthetase-I.
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19. Tissue fractionation and the assay of glutamine synthetase and the marker enzymes, glutamate dehydrogenase, cytochrome oxidase, lactate dehydrogenase, and glucose 6-phosphatase were essentially as previously described (*1, 10*). The 30*g* residue was not assayed for enzyme activity. Ornithine transcarbamylase was determined as described by M. Marshall and P. P. Cohen [*J. Biol. Chem.* **247**, 1641 (1972)]. Carbamyl phosphate synthetase was assayed for in a mixture containing (in micromoles per milliliter): potassium bicarbonate, 10; ammonium chloride, 10; magnesium sulfate, 10; ATP, 5; *N*-acetyl-L-glutamate, 5; L-ornithine, 5; Hepes, 40; and phosphoenolpyruvate, 2.5. Substrates were adjusted to *p*H 7.4 to 7.5 with potassium hydroxide as required. Beef liver ornithine transcarbamylase prepared as described by Marshall and Cohen (15 units), pyruvate kinase (6 units), and oligomycin (2 μg) were also added. Citrulline formed was measured colorimetrically by the method of R. M. Archibald [*J. Biol. Chem.* **156**, 121 (1944)]. For the assay of carbamyl phosphate synthetase in the desert tortoise shown in Table 1, the ATP-generating system (phosphoenolpyruvate + pyruvate kinase) was omitted. The low recovery of activity was thus most likely due to high mitochondrial adenosinetriphosphatase that was not completely inhibited by added oligomycin. Freeze-thawing was shown to have no effect on the activities of glutamine synthetase, carbamyl phosphate synthetase, or ornithine transcarbamylase.
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21. Liver tissue from three male desert tortoises was fractionated at the University of New Mexico-Albuquerque and the fractions frozen for transport on solid carbon dioxide to Houston for assay. We thank Tom Fritts and Randy Jennings for providing this tissue as well as the necessary laboratory facilities. A single female desert tortoise was shipped from the Sonoran Desert Museum in Tucson to Houston with permission from the state of Arizona. We thank Howard Lawler of the Desert Museum for providing this specimen. Male Texas tortoises were collected under permit 501 from the state of Texas near Laredo. We thank Kent Campbell for helping with this collection. This work was supported by grant PCM82-14901 from the National Science Foundation, Metabolic Biology Program.

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## Hypertension and Sodium Salts

Whitescarver *et al.* (*1*) suggest that "hypertension development in the Dahl S [Dahl salt-sensitive] rat may be more closely related to dietary chloride consumption than to sodium consumption." In drawing this conclusion, the authors overlooked several animal and human studies assessing the effects of potassium chloride on blood pressure.

In the Dahl S rat (*2*), spontaneously hypertensive rats (*3*), and albino rats subjected to ligature of the contralateral kidney (Grollman's operation) (*4*), the addition of potassium chloride to diets containing high levels of sodium chloride mitigates the expected rise in blood pressure. Similarly, the addition of potassium chloride to diets containing "usual" (*5*) or low sodium (*6*) levels causes a decrease in blood pressure in mild hypertensives (*5*) and in normotensives with a family history of hypertension (*6*).

If chloride were the only electrolyte to affect blood pressure, the addition of potassium chloride to these animal or human diets would, by Whitescarver's theory, raise, not lower, blood pressure. Alternatively, it is possible that chloride does raise blood pressure, but to a far lesser extent than potassium lowers blood pressure, so that the net effect of administering potassium chloride would be to lower blood pressure. MacGregor observed an average decrease of 7 mmHg in systolic blood pressure when he added 60 mmol of KCl to the usual diets of 23 mild hypertensives for 4 weeks (*5*) and an average increase of 10 mmHg in systolic blood pressure after adding 100 mmol of NaCl to low sodium diets consumed by 23 mild hypertensives for 4 weeks (*7*). In these experiments, each 100 mmol of KCl lowered blood pressure an average 12 mmHg, and each 100 mmol of NaCl raised systolic blood pressure an average 13 mmHg. If chloride, not sodium, were the pressor component of salt, potassium would have to be roughly twice as potent in lowering blood pressure as chloride is in raising it.

Whitescarver *et al.* conclude that "the development of hypertension in the Dahl S rat is dependent on the provision of sodium as sodium chloride." This conclusion could only be reached if sodium chloride had been compared with a num-