brain regions (*3*). It is therefore likely that the physiological and behavioral effects of these drugs may be a function of both their ability to elevate GABA in nerve terminals and their ability to exert this effect in specific brain regions.

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24. The percentages by which GABA increased in the various compartments were calculated as follows:

$$\text{Total} = 100\,\frac{(x' - x)}{x}$$

$$\text{Non-nerve terminal} = 100\,\frac{(y' - y)}{y}$$

$$\text{Nerve terminal} = 100\,\frac{[(x' - x) - (y' - y)]}{(x - y)}$$

where x is GABA concentration in the intact SN; y is GABA concentration in the denervated SN; x' is GABA concentration in the intact SN after drug treatment; and y' is GABA concentration in the denervated SN after drug treatment.
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Glutamine- and *N*-Acetylglutamate–Dependent Carbamoyl Phosphate Synthetase in Elasmobranchs

Abstract. *High levels of glutamine- and* N*-acetyl-*L*-glutamate-dependent carbamoyl phosphate synthetase activity are present in liver extracts of marine species of fish that retain high levels of urea in their tissues for the purpose of osmoregulation. The function of the synthetase in these species appears to be related to urea synthesis.*

Carbamoyl phosphate is utilized in the first step of two major metabolic pathways, one leading to biosynthesis of uridine monophosphate and the pyrimidine nucleotides and the other leading to biosynthesis of arginine or urea (or both) (*1*). At least three different kinds of enzymes catalyzing carbamoyl phosphate formation have been identified on the basis of substrate specificity (that is, the nitrogen donating substrate) and cofactor requirements. Carbamoyl phosphate synthetase I (CPSase I) is located specifically in the liver mitochondria of ureogenic animals, requires *N*-acetyl-L-glutamate (NAG) as a cofactor, and utilizes only ammonia as the nitrogen donating substrate (*1, 2*). The properties of CPSase I are related to its function in ammonia detoxification via the urea cycle (*2*). The K_m (Michaelis constant) for ammonia is relatively low (about 2 m*M*), and the requirement of NAG for CPSase I activity is considered a mechanism for regulating urea cycle activity in response to the level of the amino acid pool.

CPSase II utilizes glutamine rather than ammonia as the physiologically significant nitrogen donating substrate, and NAG is not required for catalytic activity (*1, 3*). Like other amidotransferases, CPSase II from most sources will utilize ammonia in place of glutamine as the nitrogen-donating substrate, but concentrations of at least 0.1*M* are required for full activity. The function of CPSase II is related to pyrimidine nucleotide biosynthesis in higher animals and to both arginine and pyrimidine nucleotide biosynthesis in other animal species and probably in plants.

Trammel and Campbell have reported the presence in several invertebrate species of a third type of CPSase, CPSase III (*4*). This enzyme, like CPSase II, utilizes glutamine as the nitrogen-donating substrate, but, like CPSase I, it requires NAG as a cofactor. We have found CPSase III activity in liver of largemouth bass (*Micropterus salmoides*), a freshwater teleost fish, thus establishing that CPSase III is also present in vertebrates (*5*).

The requirement for NAG suggests that the function of CPSase III may be related to the urea cycle. In the case of fish, most species show various levels of urea in their tissues and do excrete urea (*6, 7*). The level of urea in the tissues of most bony fish such as the teleosts in Osteichthyes is usually very low (0.01 to 0.03 percent) (*6, 8*). Although all five of the enzymes required for the urea cycle, including CPSase, are present in several species of teleosts, the activities are generally low (*8, 9*). In contrast, marine cartilaginous fish in the class Chon-

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drichthyes (elasmobranchs, such as sharks, skates, and rays, and holocephalans) synthesize and retain urea in their tissues (about 2 percent), primarily as a mechanism for osmoregulation in seawater (*7, 8, 10*). In these species, urea is apparently produced via the well-known urea cycle; all five of the urea cycle enzymes are present at relatively high levels in livers of these fish. The few CPSase activities that have been reported to be present in fish extracts have not been well characterized. They have apparently been assumed to be analogous to the CPSase I activity in mitochondria of ureotelic, vertebrate species. Watts and Watts, however, reported that liver extracts from several elasmobranch species would catalyze carbamoyl phosphate formation with glutamine as a nitrogen-donating substrate in place of ammonia, but a dependence of this reaction on NAG was not established (*11*).

Our study was initiated to establish whether CPSase III activity exists in other species of fish. Liver extracts of a number of available and representative species of Chondrichthyes and marine teleosts were assayed for CPSase activity (Table 1). Of the teleosts surveyed in our study, only one species (plainfin midshipman, *Porichthys notatus*) had CPSase III activity, and the level of activity was low, comparable to that observed with largemouth bass (*5*). All other species of teleosts appeared to have significant CPSase II activity, comparable to that of CPSase III observed for largemouth bass and plainfin midshipman. These activities are tentatively attributed to a CPSase II because the ammonia-dependent activity was about the same as the glutamine-dependent activity, and NAG was not required for either.

Our significant observation was that all four representative species of marine fish in the class Chondrichthyes (three different species of elasmobranchs and one holocephalan), all of which retain urea for osmoregulation, had very high CPSase III activity (Table 2). In all cases, activity with ammonia was less than that with glutamine, and the presence of NAG stimulated the activity, with either glutamine or ammonia as the nitrogen-donating substrate. The results of the single experiment with the freshwater stingray indicate that CPSase III activity is also present in this freshwater elasmobranch, but that the activity may be significantly lower than that in the marine elasmobranchs. The freshwater stingray does not retain high levels of urea in its tissues.

Our studies have resulted in a 40-fold purification of the CPSase III from the spiny dogfish (*Squalus acanthias*) by a two-step procedure involving ammonium sulfate fractionation and affinity chromatography. The enzyme at this stage of purification retains the same requirements for activity as observed in the crude extract (Table 2). Partial purification of CPSase III has indicated that, except for the fact that the enzyme can utilize glutamine, the general kinetic properties are similar to those of CPSase I from frog (*2*).

These observations support the view that the function of CPSase III, at least in the urea-retaining marine elasmobranchs (and holocephalans), is related to urea synthesis and that, in these species, carbamoyl phosphate utilized for urea synthesis is derived directly

Table 1. Carbamoyl phosphate synthetase activity in liver of several species of teleosts. Extracts were prepared as follows. Fresh liver tissue was cut into small sections and homogenized in a blender for 20 seconds at high speed with three volumes of a solution containing glutamine (50 m*M*), dithiothreitol (1 m*M*), KCl (100 m*M*), and Hepes buffer (50 m*M*, *p*H 7.6) at 4°C. The homogenate was centrifuged at 25,000*g* for 15 minutes, and the resulting precipitate was discarded. Glutamine and the components of lower molecular weight in the supernatant solution were removed by passing 15 to 20 ml of the solution through a Sephadex G-25 column (2 by 25 cm) equilibrated with a solution containing dithiothreitol (1 m*M*), KCl (100 m*M*), and Hepes buffer (50 m*M*, *p*H 7.6) at 4°C. About 5 ml of the center of the protein peak was collected and used for measurement of CPSase activity. CPSase activity was measured essentially as described (*5*). The reaction mixtures contained enzyme extract (0.4 ml), adenosine triphosphate (20 m*M*), $MgSO_4$ (20 m*M*), [^{14}C]$NaHCO_3$ (5 m*M*, 8×10^6 count/min), KCl (100 m*M*), Hepes buffer (50 m*M*, *p*H 7.6), NAG (2 m*M*) where indicated, and glutamine (2 m*M*) or NH_4Cl (100 m*M*) as indicated in a final volume of 1.0 ml. The [^{14}C]carbamoyl phosphate formed after 30 minutes at 26°C was measured as described (*5*). All assays were carried out in duplicate. The variation in duplicates was less than 10 percent. Protein was measured by the method of Lowry *et al.* (*14*). Unless indicated otherwise, the specimens were captured in Puget Sound and maintained in recycled seawater aquariums at 9° to 12°C.

Species	Experiment	Tissue activity* (μmole/hour per gram of liver)	Specific activity (nmole/hour per milligram of protein)			
			Glutamine		Ammonia	
			+NAG	−NAG	+NAG	−NAG
Lepidopsetta bilineata (rock sole)	1	0.10	0.9	0.9	1.1	1.1
	2	0.21	2.2	2.0	2.2	2.1
Platichthys stellatus (starry flounder)	1	0.23	2.2	2.0	2.0	1.9
	2	0.17	1.6	1.5	1.7	1.7
Gadus macrocephalus (Pacific cod)	1	0.05	0.6	0.6	0.6	0.6
	2	0.02	0.3	0.3	0.3	0.3
	3	0.10	1.2	1.2	1.0	1.0
Sebastes caurinus (copper rockfish)	1	0.20	1.4	1.4	1.2	1.1
	2	0.10	0.7	0.8	0.6	0.6
Porichthys notatus (plainfin midshipman)	1	0.13	1.2	0.1	0.7	0.1
	2		1.7	0.4	1.1	0.3

*Glutamine and NAG present.

Table 2. Carbamoyl phosphate synthetase activity in liver of several species of elasmobranchs and a holocephalan. The CPSase activity was determined as described in Table 1.

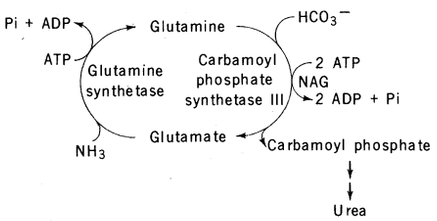
Species	Experiment	Tissue activity* (μmole/hour per gram of liver)	Specific activity (nmole/hour per milligram of protein)			
			Glutamine		Ammonia	
			+NAG	−NAG	+NAG	−NAG
Squalus acanthias (spiny dogfish)	1	5.39	96.1	13.4	9.6	0.1
	2	2.77	53.2	3.2	2.3	0.2
	3	6.97	120.1	18.5	12.0	0.1
Raja binoculata (big skate)	1	2.38	29.5	0.2	1.4	<0.1
	2	5.24	72.7	4.3	7.2	0.1
	3	12.6	120.1	7.2	21.2	0.1
Taeniura lymma (blue-spotted stingray)†	1	8.26	45.3	6.6	6.5	0.2
Potamotrygon circularis (freshwater stingray)‡	1		6.4	<0.1	0.2	<0.1
Hydrolagus colliei (ratfish)§	1	1.75	43.5	0.7	8.5	0.1
	2	1.09	38.9	1.6	7.8	0.1
	3	9.12	198.0	2.2	56.0	0.6

*Glutamine and NAG present. †Purchased from Pacwest Aquatics, Seattle, Washington. ‡Purchased from C and C Fisheries, Seattle, Washington; the liver sample had been stored at −20°C for 60 days before analysis. §A holocephalan.

from glutamine. The studies of Webb and Brown (*12*) are consistent with this view; their studies indicate that liver extracts of all urea-retaining species of fish also have very high levels of glutamine synthetase activity (one to two orders of magnitude higher than the levels found in any other species of fish included in their survey) and that there is a direct correlation between high levels of glutamine synthetase and retention of urea for osmoregulation.

The mean rate of carbamoyl phosphate synthesis for spiny dogfish calculated from the data in Table 2 is about 12 mmole per kilogram of body weight per day. The rate of daily urea production in this species has been estimated to be 6.5 mmole per kilogram of body weight (*10, 12*). Thus, the CPSase III activity measured in vitro is in the same range as the rates of urea production in vivo.

Ammonia assimilation for carbamoyl phosphate and urea synthesis may, therefore, occur as follows in these species:



Formation of carbamoyl phosphate utilized for urea synthesis directly from glutamine has not been previously demonstrated for any species, although this possibility has been considered, at least indirectly (*13*).

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Glutamine Synthetase: Assimilatory Role in Liver as Related to Urea Retention in Marine Chondrichthyes

Abstract. *The levels of glutamine synthetase specific activity in hepatic and renal tissue are higher in fish that are ureosmoregulators than in those that are not. Enzyme activities in the liver and kidney of 18 species of fish correlated directly with the ureosmoregulatory adaptation of each species.*

The occurrence and function of high levels of urea in biological tissues were reviewed by Smith (*1*), who concluded that urea serves as an osmolyte in the tissues and fluids of marine Chondrichthyes, such as sharks, skates, rays, and chimeras. Additional evidence to support such ureosmoregulation has been found in other marine vertebrates. The coelacanth, *Latimeria chalumnae*, a marine fish of the class Osteichthyes, retains high levels of urea (about 1.7 to 2.3 percent by weight or weight to volume) in its tissues and fluids (*2, 3*). Even the unusual amphibian *Rana cancrivora*, the crab-eating frog of saltwater habitat, retains as much as 2.9 percent urea in its plasma (*4*). Not all members of the class Chondrichthyes are ureosmotic. The freshwater stingrays of the family Potamotrygonidae that have been studied do not retain urea. The level of urea in the fluids and tissues of members of this family (*5*) is approximately the same (about 0.01 percent) as found in other species of aquatic vertebrates that do not retain urea (*6*).

In fish a direct correlation exists between retention of urea for osmoregulation and high activity of enzymes of the ornithine-urea cycle (*7*). Those species of Chondrichthyes that retain high levels of urea, 1 percent (0.17*M*) or more, also have high activities of the enzymes of the ornithine-urea cycle in liver tissue (*8*). The urea-retaining coelacanth also has high levels of these enzymes in its liver (*2, 9*). Liver tissue from species that do not retain urea has low or undetectable activities of most of these enzymes (*8*).

Our study of the properties and occurrence of glutamine synthetase activity in six species of fish showed that the activity of this enzyme in liver is high in three urea-retaining marine species of Chondrichthyes and is not detectable, if present, in three marine and freshwater teleosts that do not retain urea (*10*). These results suggested that, as with the urea cycle enzymes, there might also be a direct correlation between retention of urea for osmoregulation in fish and high glutamine synthetase activity in liver. A more extensive survey of glutamine synthetase activity in fish was therefore initiated in order to verify this relationship.

Glutamine synthetase from microorganisms, plants, and animals (*11, 12*) catalyzes the following reactions, all of which have been used to assay enzyme activity (ATP, adenosine triphosphate; ADP, adenosine diphosphate):

$$\text{L-glutamate} + \text{ATP} + \text{NH}_3 \xrightarrow{\text{Me}^{2+}} \text{L-glutamine} + \text{ADP} + \text{P}_i \quad (1)$$

$$\text{L-glutamate} + \text{ATP} + \text{NH}_2\text{OH} \xrightarrow{\text{Me}^{2+}} \gamma\text{-glutamylhydroxamate} + \text{ADP} + \text{P}_i \quad (2)$$

$$\text{L-glutamine} + \text{NH}_2\text{OH} \xrightarrow[\text{AsO}_4^{3-}]{\text{ADP Mn}^{2+}} \gamma\text{-glutamylhydroxamate} + \text{NH}_3 \quad (3)$$

The biosynthetic activity of glutamine synthetase (Eq. 1) is the only known physiologically significant route to glutamine synthesis in all species studied (*11–13*). Although an ammonia assimilatory role of glutamine synthetase has been proposed in microorganisms and plants (*14*), this function has not been seriously considered in vertebrates.

We determined glutamine synthetase activity in liver, brain, and kidney tissues from 18 species of fish (Fig. 1) by an

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