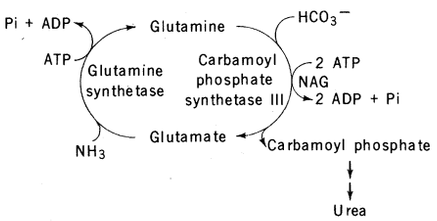
from glutamine. The studies of Webb and Brown (*12*) are consistent with this view; their studies indicate that liver extracts of all urea-retaining species of fish also have very high levels of glutamine synthetase activity (one to two orders of magnitude higher than the levels found in any other species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

PAUL M. ANDERSON
Department of Biochemistry, University of Minnesota, Duluth 55812, and *Laboratory of Biochemical Ecology, College of Fisheries, University of Washington, Seattle 98195*

References and Notes

1. S. Ratner, *Adv. Enzymol.* **39**, 1 (1973).
2. M. Marshall, in *The Urea Cycle*, S. Grisolia, R. Baguena, F. Mayor, Eds. (Wiley, New York, 1976), p. 133; R. Raijman, in *ibid.*, p. 243; M. Tatibana and K. Shigesada, in *ibid.*, p. 301.
3. A. J. Makoff and A. Radford, *Microbiol. Rev.* **42**, 307 (1978).
4. P. R. Trammel and J. W. Campbell, *Comp. Biochem. Physiol. B* **40**, 395 (1971).
5. P. M. Anderson, *ibid.* **54**, 261 (1976).
6. J. D. Wood, *Can. J. Biochem. Physiol.* **36**, 1237 (1958); M. R. Urist and K. A. Van de Putte, in *Sharks, Skates, and Rays*, P. W. Gilbert, R. F. Mathewson, D. P. Rall, Eds. (Johns Hopkins Univ. Press, Baltimore, 1967), p. 271.
7. G. W. Brown, Jr., in *Biochemical and Biophysical Perspectives in Marine Biology*, D. C. Malins and J. R. Sargent, Eds. (Academic Press, New York, 1976), vol. 3, p. 319; L. Goldstein and R. P. Forster, in *Comparative Biochemistry of Nitrogen Metabolism*, J. W. Campbell, Ed. (Academic Press, New York, 1970), vol. 2, p. 492.
8. A. K. Huggins, G. Skutsch, E. Baldwin, *Comp. Biochem. Physiol.* **28**, 587 (1969).
9. V. A. Cvancara, *Comp. Biochem. Physiol. B* **49**, 785 (1974); R. P. Wilson, *ibid.* **46**, 625 (1973); L. J. Read, *ibid.* **39**, 409 (1971).
10. L. Goldstein, in *Sharks, Skates, and Rays*, P. W. Gilbert, R. F. Mathewson, D. P. Rall, Eds. (Johns Hopkins Univ. Press, Baltimore, 1967), p. 207; H. W. Smith, *Biol. Rev.* **11**, 49 (1936); V. L. de Vlaming and M. Sage, *Comp. Biochem. Physiol. A* **45**, 31 (1973); L. Goldstein and R. P. Forster, *Am. J. Physiol.* **220**, 742 (1971); R. T. Jones and K. S. Price, Jr., *Comp. Biochem. Physiol. A* **47**, 971 (1974).
11. D. C. Watts and R. C. Watts, *Comp. Biochem. Physiol.* **17**, 785 (1966).
12. J. T. Webb and G. W. Brown, Jr., *Science* **208**, 293 (1980); see also *Comp. Biochem. Physiol. B* **54**, 171 (1976).
13. P. Lund and M. Watford, in *The Urea Cycle*, S. Grisolia, R. Baguena, F. Mayor, Eds. (Wiley, New York, 1976), p. 479.
14. O. H. Lowry, N. J. Rosebrough, A. L. Farr, R. J. Randall, *J. Biol. Chem.* **193**, 265 (1951).
15. Most of this study was done while I was on leave from the University of Minnesota as a visiting professor at the University of Washington. I thank Dr. G. W. Brown, Jr., for the use of his laboratory and facilities and both Drs. G. W. Brown, Jr., and J. T. Webb for their advice and comments. This work was supported by PHS grant GM 22434 and a grant from the Minnesota Medical Foundation.

12 September 1979; revised 26 December 1979

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

0036-8075/80/0418-0293$00.50/0

Table 1. Liver, kidney, and brain glutamine synthetase of urea-retaining and non-urea-retaining fish species. The species and enzyme activities are compiled into physiological groups on the basis of osmoregulatory adaptation. Standard assay conditions were used. The mean of specific activities are expressed as units per milligram of protein ± standard deviation. The number of specimens is indicated by parentheses.

Fish	Specific activity			
	Liver	Kidney	Brain	Liver/brain
Urea-retaining	0.41 ± 0.16 (20)*	0.47 ± 0.18 (12)*	0.27 ± 0.07 (20)*	1.55 ± 0.61 (19)*
Non-urea-retaining	0.01 ± 0.01 (48)*	0.02 ± 0.02 (43)	0.65 ± 0.29 (51)*	0.02 ± 0.02 (48)*

*These values also include results from a previous study (*10*).

assay based on Eq. 3 (*10, 15*). Activity of glutamine synthetase was linear with time and enzyme concentration. The apparent K_m (Michaelis constant) for hydroxylamine was 3.3 ± 0.2 m*M* with the enzyme extracted from liver acetone powder of the shark *Squalus acanthias*. Glutamine synthetase activity of the extract was fully dependent upon L-glutamine, hydroxylamine, Mn^{2+}, and ADP and arsenate. Maximal activity in coelacanth liver homogenate was also dependent on the same components. Complete inhibition of glutamine synthetase activity in *S. acanthias* liver acetone powder extract by 3 m*M* methionine sulfoximine was dependent upon presence of ATP and Mg^{2+}. An alternative assay (Eq. 2) for glutamine synthetase activity was also carried out with an acetone powder extract of *S. acanthias* liver as the enzyme source. L-Glutamate, hydroxylamine, and MgATP were substrates and γ-glutamylhydroxamate was the measured product (Eq. 2) under otherwise standard conditions (*10*). The glutamate-dependent assay produced only 6.3 percent as much activity as the more sensitive glutamine-dependent transferase assay (Eq. 3) used throughout our studies (*15*). The dependencies for activity, apparent K_m for hydroxylamine, inhibition properties by methionine sulfoximine, and ratio of activity yielded by the two assays were consistent with earlier findings on glutamine synthetase derived from various animal, plant, and bacterial sources (*11, 12*).

Four urea-retaining species (marine Chondrichthyes) were studied: ratfish, *Hydrolagus colliei*; big skate, *Raja binoculata*; spiny dogfish, *Squalus acanthias*; and the blue-spotted stingray, *Taeniura lymma* (*15*). The other 14 species surveyed (Fig. 1 and Table 1) do not retain urea (*5, 6*).

The average specific activities of glutamine synthetase in liver and kidney of all urea-retaining species surveyed are about 40 and 28 times higher, respectively, than the analogous specific activities of all species surveyed that do not retain urea (Table 1). The specific activity of glutamine synthetase in brain was high in all species, a result that is consistent with observations of high activity of this enzyme in fish brain (*10*). The activity in brain is apparently related to factors other than urea levels and osmoregulation. The specific activities of glutamine synthetase in liver of all species surveyed are shown in Fig. 1 as a ratio of the specific activity in liver relative to that in brain for each species. Each urea-retaining species, without exception, had significantly higher ratios than any of the 14 species that do not retain urea. The average ratios of all species in each of the two physiologically different groups differed by almost two orders of magnitude (Table 1).

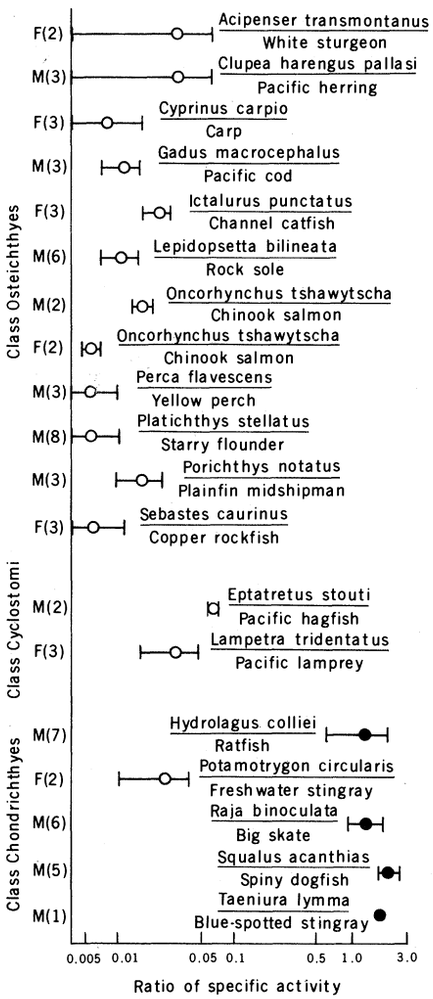


Fig. 1. Ratio of specific activities of glutamine synthetase in liver and brain of various fishes. The ratio was calculated separately for each specimen. Specific activity was expressed as units per milligram of protein. Means for non-urea-retaining species (○). Means for urea-retaining species (●). Line segments indicate one standard deviation. The number of specimens examined is given in parentheses after the habitat listing for each species. *F*, freshwater; *M*, marine.

The freshwater stingray, *Potamotrygon circularis*, which does not retain urea has very low specific activity of glutamine synthetase in both liver and kidney. The activity is comparable to that in species which do not retain urea and is in contrast to activity in closely related members of the Chondrichthyes (Fig. 1). A single, long-frozen sample of liver from the only known urea-retaining species (*Latimeria chalumnae*) of Osteichthyes was assayed for glutamine synthetase activity. This coelacanth liver (*16*) showed a fourfold greater specific activity than liver of non-urea-retaining species (Table 1).

These results and observations provide evidence that the incidence of high glutamine synthetase activities in liver of fish is limited to those species which retain urea as an osmoregulatory solute. This direct correlation between glutamine synthetase activity and production of osmoregulatory urea suggests that there may be a metabolic relationship between production of glutamine and urea in these species.

We have calculated the rate of glutamine synthesis in the liver of *Squalus acanthias* on the basis of tissue activity (Eq. 3) corrected for the lower activity of the forward assay (Eq. 2) (*15*). The approximate rate, 124 mmole of glutamine per kilogram of body weight of *S. acanthias* per day, is about 20 times the previously reported rates of urea synthesis (*17*) or urea loss (*18*) from *S. acanthias*.

The first step in the biosynthesis of urea is formation of carbamoyl phosphate (*19*). Brown (*20*) demonstrated the presence of carbamoyl phosphate synthetase activity in shark liver with ammonia as the nitrogen donor. Watts and Watts (*21*) subsequently found that shark liver extracts can also use the amide amino group of glutamine as the nitrogen source for biosynthesis of citrulline via carbamoyl phosphate formation. Anderson (*22*), has shown that the livers of urea-retaining species of fish have relatively high levels of glutamine-dependent carbamoyl phosphate synthetase III.

This supports the theory that the capability for a high rate of glutamine synthesis in livers of ureosmoregulating fish may be directly related to production of urea.

Leech *et al.* (*23*) reported that glutamine was not detectable in the blood of the shark *Squalus acanthias* during various starvation periods. If ureosmoregulatory fish in general do exhibit a very low blood glutamine level, the utility of their having a high tissue glutamine synthetase activity becomes at once obvious.

The ornithine-urea cycle has been considered to be a route for detoxification of ammonia in typically ureotelic vertebrates (*8*). Our findings are consistent with this view. But, for the group of animals that retain urea for osmoregulation, we emphasize that urea synthesis serves not only to detoxify ammonia, but also represents an economical physiological adaptation that makes osmoregulatory use of the glutamine synthetase and the ornithine-urea cycle pathway. The assimilation of ammonia by glutamine synthetase and subsequent glutamine-dependent synthesis of urea would support the osmoregulation of these species and would provide a route for excretion of nitrogen (ammonia) through loss of urea via the gills and kidney of ureosmoregulators.

Our study suggests that glutamine synthesis may be the initial step in assimilating ammonia for subsequent synthesis of urea in marine, ureosmoregulating species of fish. Our findings and those of Anderson (*22*) in the accompanying report are mutually supporting.

JAMES T. WEBB*
G. W. BROWN, JR.
Laboratory of Biochemical Ecology, College of Fisheries, University of Washington WH-10, Seattle 98195

References and Notes

1. H. W. Smith, *Biol. Rev. Biol. Proc. Cambridge Philos. Soc.* **11**, 49 (1936).
2. G. W. Brown, Jr., and S. G. Brown, *Science* **155**, 570 (1967).
3. G. E. Pickford and F. B. Grant, *ibid.*, p. 568.
4. M. S. Gordon, K. Schmidt-Nielsen, H. M. Kelly, *J. Exp. Biol.* **38**, 659 (1961).
5. T. B. Thorson, C. M. Cowan, D. E. Watson, *Science* **158**, 375 (1967); T. B. Thorson, *Life Sci.* **9**, 893 (1970).
6. W. N. Holmes and E. M. Donaldson, in *Fish Physiology*, W. E. Hoar and D. G. Randall, Eds. (Academic Press, New York, 1969), vol. 1, pp. 1-89.
7. H. A. Krebs and K. Henseleit, *Z. Physiol. Chem.* **210**, 33 (1932).
8. G. W. Brown, Jr., and P. P. Cohen, *Biochem. J.* **75**, 82 (1960); P. P. Cohen and G. W. Brown, Jr., in *Comparative Biochemistry*, M. Florkin and H. S. Mason, Eds. (Academic Press, New York, 1960), vol. 2, pp. 161-244.
9. L. Goldstein, S. Harley-DeWitt, R. P. Forster, *Comp. Biochem. Physiol. B* **44**, 357 (1973).
10. J. T. Webb and G. W. Brown, Jr., *ibid.* **54**, 171 (1976). Standard assay conditions were: 60 m*M* L-glutamine; 15 m*M* hydroxylamine-HCl; 0.4 m*M* Na_2ADP; 20 m*M* KH_2AsO_4; 3 m*M* $MnCl_2$; 40 m*M* imidazole; 10-minute incubation period at 25°C. Tissues from freshly killed fish were homogenized (the concentration was dependent on tissue activity and quantity), cooled to 0° to 4°C, and assayed within 1 hour. Tissues from urea-retaining species were assayed at *p*H 6.7 (optimal), and tissues from non-urea-retaining species were assayed at *p*H 6.4 (optimal). The alternative assay (Eq. 2) was conducted at *p*H 7.5. A unit of glutamine synthetase activity is defined as the production of 1 μmole of γ-glutamylhydroxamate per minute at 25°C, as determined by spectrophotometrically measuring the complex with $FeCl_3$ (in HCl) at 500 nm. Protein was determined by the biuret method adapted from S. Zamenhof [*Methods Enzymol.* **3**, 702 (1957)].
11. A. Meister, in *The Enzymes*, P. Boyer, Ed. (Academic Press, New York, ed. 3, 1974), vol. 10, pp. 699-754.
12. E. R. Stadtman and A. Ginsburg, in *ibid.*, pp. 755-807; D. Rhodes, G. A. Rendon, G. R. Stewart, *Planta* **125**, 201 (1975).
13. A. Meister, *Adv. Enzymol.* **31**, 183 (1968); S. Prusiner and E. R. Stadtman, Eds., *The Enzymes of Glutamine Metabolism* (Academic Press, New York, 1973); D. A. Bender, *Amino Acid Metabolism* (Wiley, New York, 1975).
14. S. D. Dunn and R. V. Klucas, *Can. J. Microbiol.* **19**, 1493 (1973); C. M. Brown and M. J. Dilworth, *J. Gen. Microbiol.* **86**, 39 (1975); C. V. Givan, *Planta* **122**, 281 (1975); U. Grafe, H. Bocker, H. Thrumm, *Z. Allg. Mikrobiol.* **17**, 201 (1977).
15. J. T. Webb, thesis, University of Washington, Seattle (1979). The urea content of *Taeniura lymma* liver [2.4 percent (by weight), 0.40*M*] was determined with the use of urease and Nessler's reagent.
16. The coelacanth liver studied here was from *Latimeria chalumnae* No. 78 which was made available, in part, through the cooperation of the California Academy of Sciences, J. McCosker (director, Steinhart Aquarium), and the Society for the Protection Of Old Fishes (G. W. Brown, Jr., president).
17. L. Goldstein, in *Sharks, Skates and Rays*, P. W. Gilbert, R. F. Mathewson, D. P. Rall, Eds. (Johns Hopkins Press, Baltimore, 1967), pp. 207-214.
18. Calculated from the data of R. P. Forster and F. Berglund, *J. Cell. Comp. Physiol.* **49**, 281 (1957); J. W. Boylan, in *Sharks, Skates and Rays*, P. W. Gilbert, R. F. Mathewson, D. P. Rall, Eds. (Johns Hopkins Press, Baltimore, 1967), pp. 197-206; J. W. Burger, in *ibid.*, pp. 177-185.
19. M. E. Jones, L. Spector, F. Lipmann, *J. Am. Chem. Soc.* **77**, 819 (1955).
20. G. W. Brown, Jr., in *Taxonomic Biochemistry and Serology*, C. A. Leone, Ed. (Ronald, New York, 1964), pp. 407-416.
21. D. C. Watts and R. L. Watts, *Comp. Biochem. Physiol.* **17**, 785 (1966).
22. P. M. Anderson, *Comp. Biochem. Physiol. B* **54**, 261 (1976); P. M. Anderson, *Science* **208**, 291 (1980).
23. A. R. Leech, L. Goldstein, C. Chung-Ja, J. M. Goldstein, *J. Exp. Zool.* **207**, 73 (1979).
24. Supported in part by a fellowship from the National Wildlife Federation and a Pacific Fisheries Biologists' scholarship (J.T.W.). Publication No. 13 from the Laboratory of Biochemical Ecology; contribution No. 517, College of Fisheries, University of Washington.
* Present address DFCBS/Air Force Academy, Colorado Springs, Colo. 80840.

19 September 1979; revised 21 December 1979

Devonian Gametophytes with Anatomically Preserved Gametangia

Abstract. *The oldest anatomically preserved and physiologically apparently independent gametophytes are described from the Lower Devonian of Scotland. These gametophytes have upright, leafless axes with terminally borne, bowl-shaped gametangiophores. The antheridia are stalked and their walls are multicellular. The archegonia are clustered in groups on common bases.*

In the course of phylogeny of the Tracheophyta the gametophytes were reduced to effect only sexual reproduction. Therefore today in the Tracheophyta the sporophyte is dominant in size as well as in life-span and is the typical assimilating "green" plant. The gametophytes are very reduced in size and simplified in shape and structure. In the Bryophyta the gametophytes are the longer living and more differentiated generation; the sporophytes are physiologically more or less dependent on the gametophytes, to which they remain attached throughout their life-span. It was hoped that Devonian gametophytes would provide clues to the phylogenetic development and early ancestors of the land plants. Prior to the present discovery, however, all that had been found with any certainty among the ancient land plants was the sporophyte generation. It seemed that the gametophyte generation either did not exist as independent plants or failed to be fossilized.

Evidence of gametophytes was to be expected in the silicified fossil peat near Rhynie in Scotland. The Rhynie Chert has been placed in the Lower Devonian, at the Siegenian-Emsian boundary (*1*), for the last 15 years. The plants in the Rhynie Chert are preserved with cellular structure. It was from this material that Merker (*2*), Lemoigne (*3*), and Pant (*4*) claimed to find evidence of gametophytes. They interpreted parts of *Rhynia gwynne-vaughanii* as gametophytes. Previously, these parts had commonly been regarded as sporophytes, and they are taken as such even today by many paleobotanists.

In 1977 we collected samples of the Rhynie Chert; on the surface of one sample we noticed two fractured bowl-shaped plant structures, which bore blackish, globular bodies. Such plant remains had not previously been described from the Rhynie Chert. They seemed to lie among axes, which we first referred to the Rhyniaceae on the basis of their recognizable features. Ensuing investigations of approximately longitudinal sections revealed that two of these axes were stalks of the bowl-shaped plant remains. The stalks are in organic connection with them and are up to 2 cm

 0036-8075/80/0418-0295$00.50/0