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$$Mb^{+}H_2O + e^{-} \rightarrow Mb + H_2O$$
$$V68D^{+}H_2O + H^{+} + e^{-} \rightarrow V68DH^{+} + H_2O$$
$$V68E^{+} + H^{+} + e^{-} \rightarrow V68EH^{+}$$

where e^{-} is an electronic charge. The half-cell reactions in C110A and V68N′ are identical with the one shown for Mb. If we neglect changes in protein structure and charge distribution that occur upon decreasing the *p*H from 7.0 to 6.0, then for the half-cell reactions shown above for V68D and V68E, $E^{\circ\prime} = E^{\circ}(6.0) - 2.303RT/F$. Differentiating with respect to T we obtain $\Delta S^{\circ\prime}_{rc} = \Delta S^{\circ}_{rc}(6.0) - 2.303R$, where R is the gas constant and F is the Faraday constant.
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Evolution of Urea Synthesis in Vertebrates: The Piscine Connection

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Elasmobranch fishes, the coelacanth, estivating lungfish, amphibians, and mammals synthesize urea by the ornithine-urea cycle; by comparison, urea synthetic activity is generally insignificant in teleostean fishes. It is reported here that isolated liver cells of two teleost toadfishes, *Opsanus beta* and *Opsanus tau*, synthesize urea by the ornithine-urea cycle at substantial rates. Because toadfish excrete ammonia, do not use urea as an osmolyte, and have substantial levels of urease in their digestive systems, urea may serve as a transient nitrogen store, forming the basis of a nitrogen conservation shuttle system between liver and gut as in ruminants and hibernators. Toadfish synthesize urea using enzymes and subcellular distributions similar to those of elasmobranchs: glutamine-dependent carbamoyl phosphate synthethase (CPS III) and mitochondrial arginase. In contrast, mammals have CPS I (ammonia-dependent) and cytosolic arginase. Data on CPS and arginases in other fishes, including lungfishes and the coelacanth, support the hypothesis that the ornithine-urea cycle, a monophyletic trait in the vertebrates, underwent two key changes before the evolution of the extant lungfishes: a switch from CPS III to CPS I and replacement of mitochondrial arginase by a cytosolic equivalent.

THE SYNTHESIS OF UREA BY THE ORnithine-urea cycle is widespread among the vetebrates. Marine elasmobranchs (sharks, skates, and rays), the coelacanth, and holocephalan fishes use urea as an important osmolyte (*1*), whereas estivating lungfishes (*2*) and amphibians (*3*) synthesize urea to detoxify ammonia during periods of water stress. Mammals synthesize urea to detoxify ammonia (*4*), a probable prerequisite for living on land, whereas ruminants and some hibernating mammals recycle nitrogen between liver and gut through urea (*5*). Urea cycle enzymes have been detected in only minute activities in some teleostean fishes (*6*) and are absent in others (*7*). Significant urea synthesis has not been reported in teleosts (*8*), and uricolysis or hydrolysis of dietary arginine through arginase are the suggested sources for urea found in teleostean urine (*9*). Components of the cycle (*10*) are identical in all vertebrates, with the exclusion of mitochondrial CPS, which in elasmobranchs is glutamine-dependent (CPS III), and arginase, which is mitochondrial (*11*). In amphibians and mammals, CPS depends on ammonia (CPS I) and a cytosolic arginase liberates urea (*12*).

With few exceptions, teleostean fishes spend most of their lives in water, disposing of waste nitrogen as ammonia with little, if any, metabolic expenditure (*13*). Therefore, Read's early description (*14*) of significant levels of all urea cycle enzymes in the liver of oyster toadfish (*Opsanus tau*), a marine teleost, has remained an anomaly in the literature. Also, the physiological and evolutionary significance of this observation was neglected and the actual occurrence of urea synthesis in *O. tau* was not assessed.

We report that the liver of the related Gulf toadfish, *O. beta*, contains a high titer of all urea cycle enzymes (Table 1) (*15*). Isolated liver cells of *O. beta* and *O. tau* (*16*) rapidly synthesize and release urea (*17*) (Table 2). Rates of urea synthesis in these toadfishes are higher than in elasmobranch hepatocytes and for *O. beta* approach one-third of the mammalian rate (*18*). Otherwise, toadfish liver cells reveal no metabolic peculiarities, with enzyme activities and metabolic flux rates similar to those of other teleosts (Tables 1 and 2) (*19*).

Although toadfish accumulate urea in their plasma (*20*), they are not ureotelic. Like other aquatic teleosts, both toadfish species are ammoniotelic (*21*), whereas excretion of urea is below the limit of detection in *O. beta* and in trace amounts in *O. tau* (*14*). Because the concentrations of urea achieved in the plasma of the toadfish are

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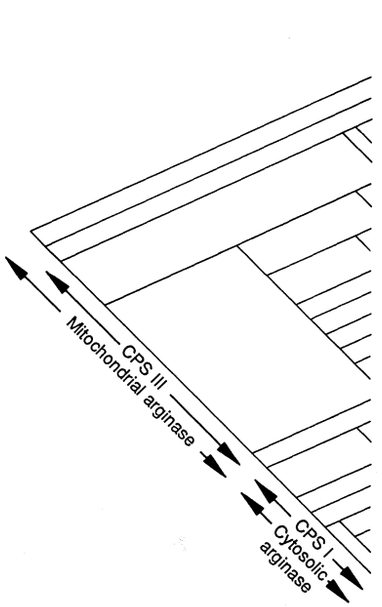
small compared with other plasma components (*22*), urea is unlikely to serve as a relevant osmolyte. Ammoniotelism, low levels of plasma urea compared to substantial synthetic activity in the liver (*23*), and considerable activity of urease in toadfish gut contents (*24*) suggest that urea is used as a transport form for nitrogen and that the synthesis of urea in the toadfish is a nitrogen salvage mechanism. Therefore, we propose an intertissue nitrogen cycle in these toadfish involving hepatic urea synthesis and gut urease, analogous to urea-based nitrogen cycling in ruminants and some hibernating mammals (*5, 25*). Because toadfish lose little or no urea to the environment, permeability for urea, which is high in teleosts, is likely to be extremely low in toadfish gills (and kidneys).

Within the Batrachoididae, the capability for urea synthesis may be restricted to the genus *Opsanus* [*Porichthys notatus*, another member of the same family, displays comparatively low levels of CPS (*11*)]. The recent observation of high activities of all urea cycle enzymes in *Heteropneustes fossilis*, an air-breathing catfish (*26*), suggests that species with semi-aquatic life-styles will be appended to the list of ureogenic teleosts, with urea assuming the familiar detoxification function. The selective pressures peculiar to toadfish leading to the expression of genes that appear silent in most other extant teleotsts are not obvious: present-day toadfish are not routinely exposed to air, and their dietary physiology has yet to be examined.

Once we had established the activity and function of the urea cycle in the toadfishes, we became interested in the evolutionary implications. Toadfish synthesize urea with enzymes and enzyme compartmentation (*27*) identical to those in elasmobranchs (*11*), including CPS III and mitochondrial arginase (Table 2). Subsequently, we analyzed CPS and arginase characteristics in a variety of fishes: the coelacanth, two species of African lungfish, hagfish, ratfish, bichir, bowfin, trout, and carp. The resulting cladogram (Fig. 1) strongly suggests that urea synthesis within the vetebrate line is a monophyletic trait that has undergone biochemical and functional changes in the course of vertebrate evolution. Only minor biochemical changes need be postulated to explain this development: (i) the switch



Vertebrate	Urea synthesis (function)	CPS type	Arginase	Glutamine synthetase
Hagfish	No		M	C
Lampreys	No			
Sharks	Yes (1)	III	M	M
Rays, skates	Yes (1)	III	M	M
Chimaera	Yes (1)	III	M	M
Sturgeon	No			
Paddlefish	No			
Bichir	No	III	M	
Bowfin	No	III	M	C
Trout	No		M	C
Bass	No	III		
Carp	No	III	M	M
Midshipman	No	III		
Oyster toadfish	Yes (3)	III	M	C
Gulf toadfish	Yes (3)	III	M	M
Coelacanth	Yes (1)	III	M	
Lungfish*	Yes (2,1)	I	C	C
Lungfish+	Yes (2,1)		C	C
Amphibians	Yes (2)	I	C	C
Turtles, tortoises	Yes (2)	I		M
Snakes, lizards	No	I		C
Crocodiles	No			M
Birds	No			M
Mammals	Yes (2)	I	C	C
Hibernators Ruminants	Yes (2,3)	I	C	C

Fig. 1. Urea synthesis by the ornithine-urea cycle in vertebrates. Function of urea: 1, osmolyte; 2, ammonia detoxification; 3, nitrogen cycling between liver and gut; CPS III is glutamine-dependent; CPS I is ammonia-dependent; M, mitochondrial; C, cytosolic. Hagfish (*Eptatretus stouti*, Myxiniformes); sharks (Elasmobranchii) (*11, 36*); rays and skates (*Raja erinacea*) (Elasmobranchii) (*11, 37*); chimaera (*Hydrolagus colliei*, Holocephail) (*11, 34, 38*); sturgeons and paddlefishes (Chondrostei) (*39*); bowfin (*Amia calva*, Holostei); bichir (*Polypterus* spp., Holostei); trout (*Salmo gairdneri*, Teleostei); bass (*Micropterus salmoides*) (*40*); carp *Cyprinus carpio*) (*41*); midshipman (*Porichthys notatus*, Batrachoididae) (*11*); oyster toadfish (*Opsanus tau*, Batrachoididae); Gulf toadfish (*O. beta*); coelacanth (*Latimeria chalumnae*, Coelacanthiformes); lungfish (*) (*Protopterus aethiopicus*, Dipnoi); lungfish (+) (*Protopterus annectens*); amphibians (*3*); reptiles, crocodiles, birds (*42*); and mammals (*12, 43*).

Table 1. Enzyme activities in toadfish liver. Enzyme activities were measured at 24°C (*O. beta*) or 20°C (*O. tau*) under saturation conditions.

Enzymes	Activities (micromoles of product per gram of liver per minute ± SEM)	
	Opsanus beta	*Opsanus tau*
	Urea cycle	
Ornithine carbamoyl transferase	52.11 ± 5.09 (18)*	40.3 ± 4.00 (9)
Argininosuccinate synthetase	0.08 ± 0.01 (7)	
Argininosuccinate lyase	0.33 ± 0.03 (7)	
Arginase†	31.49 ± 4.22 (9)	417.1 ± 54.2 (10)
	Carbamoyl phosphate synthetase‡	
Nitrogen donor: glutamine	7.80 ± 1.2 (7)	3.66 ± 1.0 (10)
Nitrogen donor: ammonia	0.84 ± 0.06 (2)	0.91 ± 0.23 (3)
	Intermediary metabolism	
Glutamine synthetase§	5.39 ± 0.67 (18)	1.50 ± 0.26 (10)
Glutamate dehydrogenase	103.4 ± 17.5 (5)	
Citrate synthase	1.57 ± 0.29 (13)	2.12 ± 0.26 (10)
Lactate dehydrogenase	25.1 ± 1.61 (12)	6.65 ± 0.64 (10)

*Number of independent duplicate determinations. †Arginase fractionates with citrate synthase, ornithine carbamoyl transferase, and glutamate dehydrogenase, that is, as a mitochondrial matrix enzyme. ‡CPS was measured in isolated mitochondria, and activity was calculated on the basis of the yield in mitochondria; activities are given in micromoles per gram per hour; toadfish CPS shows the characteristics of CPS III, higher activity with glutamine than with ammonia. CPS I does not accept glutamine as a nitrogen donor. §Glutamine synthetase is mitochondrial in *O. beta* (n = 5). The enzyme is cytosolic in *O. tau*, that is, behaves like lactate dehydrogenase during cell fractionation (n = 10).

Table 2. Metabolic activities of isolated toadfish hepatocytes. Experiments were conducted at 24°C (*O. beta*) or 20°C (*O. tau*).

Metabolic pathway	Activity (micromoles of product per gram of hepatocytes per hour ± SEM
Opsanus beta	
Urea synthesis	
With glutamine (5 m*M*)	17.26 ± 3.16 (10)*
With ammonia (5 m*M*)	3.38 ± 0.50 (6)
L-Lactate oxidation	3.22 ± 0.46 (7)
L-Lactate gluconeogenesis	0.96 ± 0.19 (7)
L-Alanine oxidation	8.14 ± 1.22 (6)
L-Alanine gluconeogenesis	1.40 ± 0.34 (6)
Opsanus tau	
Urea synthesis	
With glutamine (5 m*M*)	1.48 ± 0.27 (10)

*Number of independent duplicate determinations.

from CPS III to CPS I; (ii) the replacement of mitochondrial arginase by a cytosolic counterpart; and (iii) adjustment of mitochondrial transporter specificity from arginine to ornithine. Further, these switches appear linked and occurred before the evolution of the extant lungfishes. Our finding of mitochondrial arginase and CPS III in the coelacanth liver leads to the positioning of the coelacanth with the teleost-elasmobranch group and thus away from the amphibians and the lungfishes, a suggestion that is likely to fuel the current controversy over *Latimeria*'s "true" position (*28*). Few minor biochemical changes are involved in the switch from CPS III to CPS I (*29*), two enzymes with strong immunological cross-reactivity (*30*). If the mitochondrial and cytosolic arginases are products of the same gene, only a loss of the mitochondrial leader sequence needs to be invoked to explain the subcellular distribution of vertebrate arginases.

Two distinct paths of urea synthesis in the vertebrates can thus be distinguished, leading to the liberation of urea in different compartments. In the presence of mitochondrial arginase—as in all fishes, including the coelacanth—arginine must enter the mitochondrion, and, after urea production, ornithine is directly available for citrulline synthesis by ornithine carbamoyl transferase, an enzyme of the mitochondrial matrix. If arginase is localized in the cytosol, as it is in lungfish, amphibians, and mammals, ornithine has to enter the mitochondrion for citrulline synthesis. Because glutamine plays a central role in urea synthesis, either directly as a substrate for CPS III, indirectly as a nitrogen transporter (*31*), or as a temporary nitrogen sink for the detoxification of ammonia, we have included glutamine synthetase (GNS) in Fig. 1. Before the switch to CPS I, ammonia was probably trapped or detoxified in liver (if required) initially by GNS and then became available to CPS. The subcellular localization of GNS appears to be plastic (Fig. 1), and ammonia could enter the mitochondrion either directly (as in *O. beta* with mitochondrial GNS) or be carried on glutamine (as in *O. tau*). In other ("higher") vertebrates, glutamine eventually assumes a pivotal role as an intertissue transporter for "nitrogen." Because of its localization around the hepatic venule (*32*), GNS in mammals fulfills a detoxification function subordinate to the urea cycle (*33*). The (cytosolic) enzyme is positioned to prevent hepatic ammonia not utilized in urea synthesis from spilling over into the circulation. Although our results shed new light on the distribution, function, and evolution of the urea cycle in vertebrates, they also suggest a reevaluation of the central role of glutamine, which is intricately linked to the route of urea synthesis.

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16. Gulf toadfish (*O. beta*, 9 to 130 g) were obtained locally and maintained at ambient temperature (29°C) without feeding for up to 7 days. Adult oyster toadfish (*O. tau*, 200 to 300 g) were supplied to the University of British Columbia, Vancouver, from Woods Hole Biological Laboratory and used within 3 days; animals had been starved for an unknown period. Fish were killed, and livers removed. One liver portion was used for isolation of mitochondria (*27*). Hepatocytes were isolated by liver perfusion with collagenase [P. J. Walsh, *J. Exp. Biol.* **130**, 295 (1987)]. Final cell concentration was 10 to 20 mg/ml. The gas phase consisted of 0.25% CO_2, and the balance air.
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22. Plasma osmolarity, 330 ± 3.8 mosm/liter ($n = 3$); plasma glucose, 1.27 ± 0.15 m*M* ($n = 6$); amino acid concentration, measured by high-pressure liquid chromatography [K. Mopper and R. G. Zika, *Nature* **325**, 246 (1987)]; liver, 19.55 mmol/kg; plasma, 2.49 m*M*.
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24. Urease activity, measured with a radiotracer (*35*) and presumably of microbial origin, was 0.88 ± 0.13 μmol ($n = 3$) of urea hydrolyzed per milliliter of gut contents in 1 hour at 24°C. Urease was determined in 0.5 ml of contents from 4-cm-long pieces of Gulf toadfish gut. Activity was not detectable in liver, gill, gut, kidney, or skeletal muscle.
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Phylogenetic Meaning of the Kingdom Concept: An Unusual Ribosomal RNA from *Giardia lamblia*

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An analysis of the small subunit ribosomal RNA (16*S*-like rRNA) from the protozoan *Giardia lamblia* provided a new perspective on the evolution of nucleated cells. Evolutionary distances estimated from sequence comparisons between the 16*S*-like rRNAs of *Giardia lamblia* and other eukaryotes exceed similar estimates of evolutionary diversity between archaebacteria and eubacteria and challenge the phylogenetic significance of multiple eukaryotic kingdoms. The *Giardia lamblia* 16*S*-like rRNA has retained many of the features that may have been present in the common ancestor of eukaryotes and prokaryotes.

THE TAXONOMIC SEGREGATION OF organisms into two or more kingdoms is a legacy from early systematic biologists who relied on morphological variation at the macroscopic level to differentiate plants from animals. With the discovery of the microbial world and the development of analytical tools for defining subcellular features, the number of proposed kingdoms has increased, and the debate about evolutionary relationships between the major groups of eukaryotic organisms has intensified (*1*). Controversies over conflicting taxonomic schemes are usually due to a lack of consensus about which characteristics are most useful for inferring phylogenies. As an alternative to traditional methods, comparisons of gene sequences that share a common ancestry can be used to infer objective phylogenetic frameworks (*2*). The 16*S*-like rRNAs have proved to be particularly well suited for estimating relationships between even the most divergent taxa (*3*). Here we describe extensive differences between the 16*S*-like rRNAs of *Giardia lamblia* and other eukaryotes, including diverse protozoans.

The protozoan parasite *G. lamblia* is a diplomonad, which can be propagated in vitro but normally lives attached to the intestinal mucosa of its host. *Giardia lamblia* has two nuclei and eight flagella but lacks mitochondria and normal endoplasmic reticulum (ER) or Golgi (*4*). Either these features were introduced into other eukaryotes after the divergence of diplomonads or they were lost in the *G. lamblia* evolutionary lineage. To identify its phylogenetic placement, we determined the sequence of the *G. lamblia* 16*S*-like rRNA coding region and compared it to the rRNAs of divergent taxa.

The *G. lamblia* 16*S*-like rRNA sequence (*5, 6*) is unusually rich in G+C content (75%) and has only 1453 nucleotide positions, a size more typical of prokaryotes than of eukaryotes. A collection of 41 eukaryotic (*7–9*), 6 archaebacterial (*10*), and 7 eubacterial 16*S*-like rRNAs (*11*) were aligned with the *G. lamblia* sequence by a computer-assisted procedure that considers the conservation of both primary and secondary structure features. Evolutionary distances (*6*) were used in the distance matrix methods (*12*) to infer the phylogenetic tree shown in Fig. 1. In this phylogenetic framework, *G. lamblia* represents the earliest diverging lineage in the eukaryotic line of descent. The *G. lamblia* branching is followed by the microsporidian *Vairimorpha necatrix* and then by euglenoids (*Euglena gracilis*) and kinetoplastids (*Trypanosoma brucei*). Late in the evolution of eukaryotes there was a nearly simultaneous splitting of animals, fungi, chlorophytes plus plants, chromophyte algae, and ciliates plus dinoflagellates. The precise branching order for these lineages is statistically uncertain, since it spans a distance of fewer than one nucleotide change per 100 positions. Yet the general branching pattern is nearly constant in similar phylogenetic trees that include different representatives of these major eukaryotic lineages. Similar tree topologies are observed by using the parsimony methods implemented by Swofford's computer program, "Phylogenetic analysis using parsimony" (PAUP) (*13*), with the significant difference that *V. necatrix* branches before *G. lamblia*. Unequal rates of change in one or more lineages (which are represented by long segments in distance matrix trees) sometimes produce anomalously deep branching patterns or generate different tree topologies when parsimony rather than distance methods are used (*14, 15*). The segment connecting *G. lamblia* to the eukaryotic subtree is not abnormally long and therefore its early divergence is not due to an unusually high mutation rate in its rRNA genes. In contrast, *V. necatrix* seems to have evolved more rapidly than the rRNAs of other eukaryotes, which may explain the alternate branching orders in distance and parsimony analyses.

The tree geometry in Fig. 1 could be biased by the high G/C content in the 16*S*-like rRNAs of *G. lamblia* (75% G/C) and *Sulfolobus solfataricus* (67% G/C). If G/C-rich 16*S*-like rRNAs are used to represent the eubacteria and archaebacteria, then convergence toward G or C at a number of sites might influence the phylogenetic position of

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