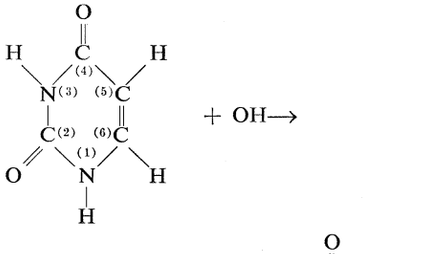
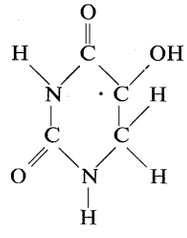
produce similarly detectable signals from O—H addition radicals for thymine, cytosine, adenine, and guanine.

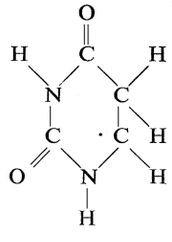
The upper curve of Fig. 1 represents the observed ESR of uracil in powdered form at 300°K, previously evacuated and subjected to gaseous O—H radicals produced by dissociation of H_2O_2 in an electric discharge. Production of O—H radicals from H_2O vapor gave similar results, but with some evidence for H-addition radicals also. The bars of the upper diagram of Fig. 1 represent the theoretical pattern expected for radical (I) below, which would be formed by OH addition to C(5) (the numbers in parentheses indicate the ring position in the diagram below) with a subsequent transfer of the H from C(5) to C(6).





(I)

The corresponding radical formed by H bombardment of uracil is structure (II):



(II)

It gives rise to the lower pattern of Fig. 1 which was obtained earlier (1) and is repeated here for comparison what that of radical (I). That the primary addition occurs on C(5) rather than C(6) is in agreement with the theory of Pullman and Mantione (*2*).

At room temperature the couplings on the CH_2 hydrogens are equivalent for both (I) and (II). This indicates that the two C—H bonds have symmetrical orientation relative to the molecular plane. For symmetrical orientation, the dihedral angle θ between the plane determined by C(5) — C(6) — H and the plane of the π-orbital lobes between C(5) and C(6) must be 30°. From the relation (*3*)

$$A_\beta = \rho_\alpha Q \cos^2 \theta$$

with θ equal to 30° and Q equal to 58 gauss (*4*), and with the observed coupling A_β equal to 28 gauss for radical (I), the spin density on C(5) is estimated to be 0.64. A similar calculation with $A_\beta = 33$ gauss yields $\rho_\alpha = 0.76$ for radical (II). From the α H coupling, a value of $\rho_\alpha = 0.71$ is obtained for radical (II). Although there is no α H for calculation of ρ_α for radical (I), this comparison indicates that it may be slightly lower than the 0.64 estimated from the β H's, possibly 0.60 (Table 1).

Table 1. Coupling constants and spin densities.

Radical species	Proton coupling (gauss)		π Spin density on C_α
	$C_\alpha H$	$C\beta H$	ρ_α
Uracil + OH		28	0.64
Uracil + H	18.5	33	.71

Unless special care is taken, a sharp singlet is produced in the center of the pattern for samples bombarded with H, D, or OH. In all instances the singlet increases in strength with the increase in energy delivered to the sample per unit time. Elimination of these singlets is more difficult in D or OH bombardment than in H bombardment. The singlet occurs for other nucleic acid bases as well as for uracil and for the nucleosides and nucleotides. This nonspecific signal results from a localized degradation or a surface charring of the sample by the bombardment.

We have bombarded the other nucleic acid bases with OH, but so far have been unable to produce detectable signals from OH addition radicals. These failures may be due to an inability of the OH radicals to penetrate these solid samples rather than their inability to add to the carbons of these rings. By other methods Ekert (*5*) has shown that the stable molecular compounds thymine glycol and dihydrothymine are formed in γ-irradiated solutions of thymine in de-aerated water.

JANKO N. HERAK*
WALTER GORDY
Department of Physics, Duke University, Durham, North Carolina

References and Notes

1. J. N. Herak and W. Gordy, *Proc. Nat. Acad. Sci. U.S.* **54**, 1287 (1965).
2. B. Pullman and M J. Mantione, *Compt. Rend.* **261**, 5679 (1965).
3. C. Heller and H. M. McConnell, *J. Chem. Phys.* **32**, 1535 (1960).
4. R. W. Fessenden and R. H. Schuler, *ibid.* **39**, 2147 (1963).
5. B. Ekert, *Nature* **194**, 278 (1962).
6. Supported by the Army Research Office (Durham), grant DA-ARO(D)-31-124-G731 and by the U.S. Air Force Office of Scientific Research, grant AF-AFOSR-493-66.
* Permanent address: Institute Ruder Boskovic, Zagreb, Yugoslavia.

14 June 1966

Urea Synthesis in the Lungfish: Relative Importance of Purine and Ornithine Cycle Pathways

Abstract. *The relative importance of the purine pathway (uricolysis) and the ornithine cycle as routes for urea synthesis was assessed in isolated liver preparations from the African lungfish* Protopterus dolloi. *Incorporation of C^{14}-labeled precursors into urea was used for comparison. Both pathways are present in the lungfish, but the ornithine cycle is quantitatively more important.*

During its aquatic phase, the African lungfish obtains about 50 percent of its total metabolic energy from protein, and, as in some aquatic amphibians, the nitrogenous endproducts are excreted mainly as ammonia and urea (*1, 2*). During aestivation the fish is entirely enveloped by a leathery cocoon, urine formation is completely suspended, and the metabolic rate markedly decreases; the animal may survive for several years entirely without food and water. Endogenous protein provides most of the fish's metabolic energy during this period; ammonia does not accumulate, and the nitrogen from catabolized protein is almost entirely quantitatively incorporated into urea, which accumulates in the animal's body fluids and comes to constitute 0.5 to 1 percent of its total weight within a year (*1, 3*).

The need to detoxify ammonia during aestivation is evident, but the mechanism whereby the accumulation of ammonia is suppressed in favor of ureogenesis is not known. Brown (*4*) and Janssens and Cohen (*5*) have provided evidence for the presence of all of the enzymes of the ornithine cycle in the liver of the lungfish. These studies indicate that in the lungfish urea may be synthesized via the classical ornithine

cycle. Urea may also be formed as an endproduct of purine synthesis and degradation (uricolysis). This pathway appears to be solely responsible for the relatively small amounts of urea synthesized by teleost fishes, in which the ornithine cycle is completely absent (*6*). All three of the uricolytic enzymes (urate oxidase, allantoinase, and allantoicase) are present in the liver of *Protopterus annectens* (*7*). Dietary arginine could be a potential third source of urea in Dipnoans and high activities of arginase have been reported in *Protopterus* (*5*). However, it is improbable that degradation of this single amino acid could make a significant contribution to the total amount of urea produced.

Our study was undertaken to determine relative rates of urea synthesis by the ornithine cycle and purine pathway in livers taken from lungfish both in their aquatic phase and during aestivation. The ornithine cycle is the only known route for the synthesis of urea from ammonia and carbon dioxide in vertebrates. As pointed out by Brown (*8*), urea synthesized via the ornithine cycle can be distinguished from that arising from purine and uric acid synthesis by use of $C^{14}O_2$. We determined the rates of urea synthesis via the ornithine cycle by incubating liver slices in vitro in a balanced isotonic medium containing ammonium ion, C^{14}-bicarbonate, ornithine, and an energy source; we then measured the radioactivity (count/min per gram per hour) in urea recovered after incubation. We also studied the damping effect that unlabeled citrulline, one of the intermediates in the synthetic cycle, had on the rate of production of C^{14}-labeled urea. To evaluate rates of ureogenesis via the purine pathway we used a similar in vitro preparation with L-serine labeled with C^{14} in the third position as the source of the "formate" carbon atoms 2 and 8 in uric acid (*9*), which were detected eventually as the C^{14} constituents in recovered urea. We added uric acid in some experiments to observe the possible damping effect that introduction of this "cold" intermediate had on the incorporation of C^{14} into urea derived from C^{14}-serine.

Thin liver slices (100 to 150 mg) were suspended in 3.0 ml of isotonic Krebs-Ringer bicarbonate solution or tissue culture medium (*10*) in stoppered 25-ml flasks. Depending upon the specific experiment, the isotopically labeled substrates, or the unlabeled amino acids or salts, were then dissolved in the incubation medium; the flasks were gassed with pure oxygen and incubated for 1 hour at 30°C in a Dubnoff metabolic shaker at 120 oscillations per minute. After the tissues were incubated 0.15 ml of 60 percent perchloric acid was added to the flasks to stop the reaction, and the slices were homogenized in the incubation medium with an all-glass homogenizer. Unlabeled CO_2 was bubbled through the homogenate for 2 hours to remove C^{14}-labeled carbon dioxide. The homogenate was then neutralized with KOH and centrifuged and the supernatant solution was removed.

The method used to measure C^{14}-urea was similar to that used by Brown (*8*). One milliliter of the supernatant solution was transferred to the main compartment of a Warburg flask containing 1.0 ml of urease solution so that urea might be converted to carbon dioxide (10 mg of urease per milliliter of 0.2*M* citrate buffer, *p*H 5.0). The center well contained a solution for trapping the carbon dioxide [0.2 ml of hydroxide of Hyamine-10-X (*11*) plus 0.2 ml of toluene], and the sidearm contained 0.4 ml of 5.5*N* sulfuric acid. The flask was stoppered and incubated for 60 minutes at 30°C. The sulfuric acid was then tipped into the main compartment to ensure the complete liberation of carbon dioxide, and the incubation was continued for 30 minutes. The contents of the center well were transferred to a glass counting vial containing 15 ml of counting medium, and measurements were made in a liquid scintillation spectrometer (*12*).

The lungfish (*Protopterus dolloi*), 50 to 150 g, were maintained in the aquatic state in unaerated glass aquaria for 1 to 3 weeks without feeding (*13*). We successfully induced two animals to aestivate during the summer in mud obtained locally at the Mount Desert Island Biological Laboratory in Salisbury Cove, Maine, using a procedure similar to one described by Janssens (*3*). Aestivation was continued for 6 weeks during July and August when the temperature in the laboratory varied between 16° and 20°C. The aestivating fish were then broken out of the completely dried mud cake and found to be dormant within a tough brown "co-

Table 1. Incorporation of L-serine-3-C^{14} and C^{14} labeled bicarbonate into urea by liver slices. Incubation medium for urea synthesis in vitro in *Protopterus* specimens 1, 2, and 3 contained Krebs-Ringer solution with either (i) 0.5 μc C^{14}-labeled bicarbonate, 30 μmole of NH_4Cl, 3 μmole of L-ornithine, 27 μmole of sodium lactate, and 16 μmole of sodium bicarbonate; or (ii) 0.9 μc of L-serine-3-C^{14}, 25 μmole of L-glutamine, 30 μmole of glycine, and 10 μmole of L-serine. The incubation medium for *Protopterus* specimens 4, 5, and 6, and for *Necturus* 1 and 2 was tissue culture medium containing 30 μmole of $NaHCO_3$, 0.72 μmole of L-serine, and either 0.5 μc of C^{14}-$NaHCO_3$ for evaluation of ornithine cycle participation or 0.5 μc of L-serine-3-C^{14} for assessing purine pathway activity.

C^{14}-labeled substrate	Incorp. C^{14} into urea* (%)	Substrate activity (count min^{-1} $\mu mole^{-1}$)†	C^{14}-urea (count min^{-1} g^{-1} hr^{-1})	Urea synthesis‡ ($\mu mole$ g^{-1} hr^{-1})
		Protopterus No. 1 (aestivating)		
$NaHCO_3$	0.041§	9.45×10^3	2,890	3.1×10^{-1}
Serine	.001	1.82×10^5	162	8.9×10^{-4}
		Protopterus No. 2 (aestivating)		
$NaHCO_3$	.005	9.45×10^3	368	3.9×10^{-2}
Serine	not detect.	1.82×10^5		
		Protopterus No. 3 (aquatic)		
$NaHCO_3$	.003	8.51×10^3	202	2.4×10^{-2}
		Protopterus No. 4 (aquatic)		
$NaHCO_3$	.378	2.1×10^4	26,600	1.3
Serine	.089	1.54×10^6	6,200	4.1×10^{-3}
		Protopterus No. 5 (aquatic)		
$NaHCO_3$	.034	2.56×10^4	2,360	9.2×10^{-2}
Serine	.024	1.54×10^6	1,710	1.1×10^{-3}
		Protopterus No. 6 (aquatic)		
$NaHCO_3$	.727	2.1×10^4	51,172	2.4
Serine	.101	1.54×10^6	7,134	4.6×10^{-3}
		Necturus No. 1		
$NaHCO_3$	.266	2.15×10^4	18,750	8.7×10^{-1}
		Necturus No. 2		
$NaHCO_3$	.160	2.50×10^4	11,200	4.5×10^{-1}

* The percentage is [count min^{-1} in urea/count min^{-1} in flask] × 100. † Specific activity of substrate in incubation medium. ‡ Calculated by dividing (count min^{-1} g^{-1} hr^{-1}) by specific activity of substrate in medium. § Average of duplicate assays.

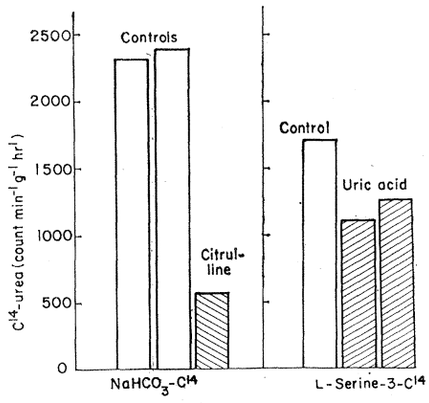


Fig. 1. Damping effects of nonradioactive intermediates of the ornithine cycle and of the purine pathway on the generation of C^{14}-urea from C^{14}-bicarbonate (left) and from L-serine-3-C^{14} respectively. L-Citrulline was added in $10^{-2}M$ concentration, and uric acid in $3 \times 10^{-3}M$. The specific activities of C^{14}-$NaHCO_3$ and L-serine-3-C^{14} in the medium were 2.56×10^4 and 1.54×10^5 count min^{-1} $\mu mole^{-1}$, respectively.

coon" with the tail curved over the head. Within a few minutes after exposure and removal of the "cocoon" they breathed heavily and snorted characteristically when handled. The animals were quickly pithed, and the livers were taken for study *in vitro*.

The radioisotopic data (Table 1) show that the C^{14} of $C^{14}O_2$ is incorporated into urea by slices of lungfish liver. Under the conditions of these experiments an average of 0.2 percent of the counts present as $C^{14}O_2$ in the incubation medium were converted to urea. The rate of urea synthesis from CO_2 was 2.4×10^{-2} to 2.4 μmole per gram of liver per hour (Table 1) with an average 6.4×10^{-1} $\mu mole\ g^{-1}\ hr^{-1}$. These values may be compared with the rates of urea synthesis observed in liver slices from *Necturus* (8.7×10^{-1} and 4.5×10^{-1} $\mu mole\ g^{-1}\ hr^{-1}$ Table 1) an amphibian in which the activities of the ornithine cycle enzymes (*14*) are similar to those found in *Protopterus* (*5*). To determine whether labeled CO_2 was being incorporated into urea via the ornithine cycle, "cold" citrulline was added to the incubation mixture. Unlabeled citrulline would be expected to substitute for the C^{14}-labeled form in the condensation reaction which forms argininosuccinate within the ornithine cycle, and to diminish, thereby, the fraction of urea labeled with isotopic CO_2. With nonradioactive citrulline in the medium the rate of C^{14} urea generation was reduced 75 percent below control values (Fig. 1). The rates of urea synthesis shown (Table 1) for livers of aestivating lungfish, 3.9×10^{-2} and 3.1×10^{-1} $\mu mole\ g^{-1}\ hr^{-1}$, were in the range found in aquatic lungfish (2.4×10^{-2} to 2.4). The urea content of livers taken from aestivating fish was seven times that of aquatic fish (14 μmole/g as opposed to 2 μmole) at death. The cause of the large variation in rates of urea synthesis from animal to animal is not clear but might be related to a difference in history of the animals before being brought to our laboratory.

Except in one *Protopterus*, small but significant amounts of C^{14} from serine were incorporated into urea. Approximately 0.04 percent of the radioactivity present as C^{14}-L-serine was incorporated into urea (Table 1). The rate of urea synthesis from serine ranged from being undetectable to being 4.6×10^{-3} μmole per gram of liver per hour (average 2.1×10^{-3}). Specific evidence for the operation of the purine pathway was found in the damping effect of unlabeled uric acid on the generation of C^{14}-urea from C^{14}-serine (Fig. 1). Addition of unlabeled uric acid to the incubation mixture reduced the incorporation of C^{14} from serine into urea by about 30 percent. The damping effect was not as marked as when citrulline was added. This may be due to one or both of the following: (i) The low solubility and highly ionized nature of uric acid at physiological *p*H's probably resulted in a much lower concentration of added urate entering the cells than that of citrulline added to slices in the previous experiment; (ii) Some of the added C^{14}-serine may have been converted to $C^{14}O_2$ by the liver slices and incorporated into urea via the ornithine cycle. Partial inhibition of incorporation of C^{14} from L-serine-3-C^{14} into urea by unlabeled uric acid does indicate, however, that at least a portion of the synthesis of urea from serine proceeds via the purine pathway. Thus, both pathways appear to be present in lungfish livers, but the rate of urea synthesis by the ornithine cycle is at least 100 times greater than that of the purine pathway under the conditions of these experiments. Similar results were obtained by Carlisky *et al.* (*15*) and Schooler *et al.* (*16*) in comparisons of the rates of urea synthesis via the ornithine cycle with those via the purine pathway in the frog kidney and dogfish liver, respectively.

It is not necessary to invoke a radical alteration in biochemical pathways in order to account for the shift from "ammoniotelism" to "ureotelism" when the lungfish undergoes aestivation. Activity of the ornithine cycle, which is present even in the aquatic phase, would enable them to continue synthesizing urea in the usual manner, and the accumulating ammonia would be captured by carbamoylphosphate synthetase to be incorporated into carbamoylphosphate along with carbon dioxide, and thence fed into the cycle. It can be calculated that at the rate of urea synthesis observed in nonaestivating lungfish (approximately 1 μmole per gram of liver per hour) a 100-gram lungfish whose liver weighs approximately 1 gram could synthesize approximately 0.5 gram of urea in a year. This figure is within the range of urea found in the tissues of lungfish aestivating for this period of time (*1*).

R. P. FORSTER
Department of Biological Sciences, Dartmouth College, Hanover, New Hampshire 03755
LEON GOLDSTEIN
Department of Physiology, Harvard Medical School, Boston, Massachusetts

References and Notes

1. H. W. Smith, *J. Biol. Chem.* **88**, 97 (1930).
2. W. H. Sawyer, *Amer. J. Physiol.* **210**, 191 (1966).
3. P. A. Janssens, *Comp. Biochem. Physiol.* **11**, 105 (1964).
4. G. W. Brown, Jr., *Science* **149**, 1515 (1965).
5. P. A. Janssens and P. P. Cohen, *ibid.* **152**, 358 (1966).
6. L. Goldstein and R. P. Forster, *Comp. Biochem. Physiol.* **14**, 567 (1965).
7. M. Florkin and G. Duchateau, *Arch. Inter. Physiol.* **53**, 267 (1943).
8. G. W. Brown, Jr., *Biochim. Biophys. Acta* **60**, 185 (1962).
9. J. M. Buchanan and S. C. Hartman, *Advance. Enzymol.* **21**, 199 (1959).
10. Grand Island Biological Co. medium diluted two to three times.
11. Packard Instrument Co., Inc.
12. Counting medium was 100 mg of 2,5-diphenyloxazole (Packard) and 3.0 g of 2,2′-*p*-phenylene-bis-5-phenyloxazole (Packard) in 1 liter of toluene; spectrometer was Packard Tri-Carb Scintillation counting machine. Counting efficiency was approximately 80 percent.
13. Lungfish were imported from Africa and supplied commercially by Paramount Aquarium, Ardsley, New York. They are shipped by air in polyethylene bags containing a little water, and they withstand this kind of travel very well.
14. G. W. Brown, Jr. and P. P. Cohen, *Biochem. J.* **75**, 82 (1960).
15. N. J. Carlisky, S. Jard, F. Morel, *Compt. Rend., Ser. D* **262**, 290 (1966).
16. J. M. Schooler, L. Goldstein, S. C. Hartman, R. P. Forster, *Comp. Biochem. Physiol.* **18**, 271 (1966).
17. Supported by PHS grant HE-04457 and NSF grant GB-285. We thank Dr. J. M. Schooler for assistance in analyses performed during the preliminary phase of this study. Most of the work was done at the Mount Desert Island Biological Laboratory, Salisbury Cove, Maine.

20 June 1966