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Ornithine-Urea Cycle Enzymes in the African Lungfish, Protopterus aethiopicus

Abstract. *The presence of all five enzymes of the ornithine-urea cycle has been demonstrated in the liver of the African lungfish* Protopterus aethiopicus. *Levels of activity of the rate-limiting enzymes, carbamoyl phosphate synthetase and argininosuccinate synthetase, are similar to those in the premetamorphic tadpole of* Rana catesbeiana *and considerably lower than the levels reported for other ureotelic animals. They are thus consistent with the predominantly ammonotelic metabolism of the lungfish in an aquatic environment.*

In a recent report, Brown (*1*) demonstrated the presence of ornithine carbamoyltransferase in the liver of African lungfish, but he was unable to detect carbamoyl phosphate synthetase activity. Evidence is given in this report for the occurrence of all five enzymes of the ornithine-urea cycle of Krebs and Henseleit (*2*) in the liver of the free-living lungfish.

Lungfish were collected by one author (P.A.J.) during a visit to Makerere College, Uganda, from the University of Sheffield, England, in the spring of 1964, and were identified as *Protopterus aethiopicus*. Identification was based on the following criteria: (i) The fish were collected from Lake Victoria in which *P. aethiopicus* is the sole representative of the genus (*3*). (ii) After the livers were removed for enzyme analysis the fish were examined and found to have between 38 and 40 pairs of ribs, which is characteristic of the species (*4*). The fish were flown to Sheffield in 1964 and then to Madison in October 1965, where they were kept in individual plastic tanks in still tap-water at a temperature of 23°C. They were fed twice weekly on minced lamb heart or chopped calf liver, and the containers were cleaned out on the day after each feeding.

Activities of carbamoyl phosphate synthetase (*5*), ornithine carbamoyltransferase, argininosuccinate synthetase, and argininosuccinate lyase (*6*) were measured by the method of Brown and Cohen (*7*); arginase (*6*) activity was assayed by the method of Balinsky and Baldwin (*8*). The tissue was prepared by the following procedure. Lungfish were weighed and then killed by decapitation. The liver was removed, weighed, and immediately homogenized in four volumes of 0.1 percent CTB (cetyltrimethylammonium bromide) in a glass homogenizer with a Teflon pestle. Homogenization was effected with a top-drive motor while the homogenizer was immersed in a mixture of ice and water. The homogenate was centrifuged at 10,000*g* for 10 minutes at 3°C, and the supernatant fraction (S_1) was decanted. The pellet was rehomogenized in three volumes of 0.1 percent CTB, and the homogenate was centrifuged at 10,000*g* for 10 minutes at 3°C. Supernatant fraction (S_2) was decanted. The following enzyme assays were carried out: argininosuccinate synthetase activity on fraction S_1; carbamoyl phosphate synthetase on fraction S_3 (four volumes of fraction S_1+ three volumes of fraction S_2); ornithine carbamoyltransferase and argininosuccinate lyase on fraction S_3 diluted with four volumes of water; and arginase on fraction S_3 diluted with 24 volumes of water and then mixed with an equal volume of 28 m*M* manganous sulfate.

Assays indicated presence of all five enzymes of the ornithine-urea cycle in the liver of *Protopterus* (Table 1). In each case, enzyme activity was linear with respect to both enzyme concentration and time of incubation under the experimental conditions employed. The presence of carbamoyl phosphate synthetase, which Brown (*1*) was unable to detect (for reasons which we cannot comment on since experimental conditions were not revealed), was established by the demonstration that enzyme activity was dependent on the presence of acetylglutamate in the incubation medium (Table 1).

Values for enzyme activities reported here are similar to those previously given for ornithine carbamoyltransferase (*1*) and arginase (*9*) in the lungfish. Argininosuccinate synthetase appears to be the rate-limiting enzyme in the ornithine-urea cycle (*10*); the level of activity of this enzyme in lungfish is similar to that in the premetamorphic tadpole of *Rana catesbeiana* but considerably lower than in adult *R. catesbeiana* (*10*) and the great majority of ureotelic animals that were examined (*11*). The same pattern is evident for levels of activity of both carbamoyl phosphate synthetase and ornithine carbamoyltransferase. The level of arginase activity is extremely high in lungfish but this is also the case in *Necturus maculosus maculosus* Rafinesque (*11*), an amphibian which is almost exclusively ammonotelic (*12*). Fed lungfish of a group from which the experimental animals were taken excreted both ammonia and urea in the ratio 8.5 : 1.5 (*13*), which is in agreement with previous work by Smith (*14*). The only data to the contrary (*15*) was obtained with a large fish (450 g) in a small volume of water (500 ml) in which, on the first day of the experiment, the ammonia concen-

Table 1. Levels of enzymes of the ornithine-urea cycle in the liver of *Protopterus*.

Animal	Body wt. (g)	Liver wt. (g)	Carbamoyl phosphate synthetase	Ornithine carbamoyl-transferase	Arginino-succinate synthetase	Arginino-succinate lyase	Arginase
			Micromoles of product per milligram of protein per hour				
1	92	1.52	0.90	36.8	0.20	2.12	790
2	60	0.95	.50	38.6	.12	0.55	800
3	37	.65	.50				
3	37	.65	.08*				
			Micromoles of product per gram of wet tissue per hour				
1	92	1.52	37.2	1530	7.7	88.3	32,000
2	60	0.95	23.8	1820	5.5	25.4	37,600
3	37	.65	32.7				
3	37	.65	4.8*				

* Acetylglutamate omitted from incubation medium.

tration rose to more than 2.5 mM. This may well have affected the relative rates of ammonia and urea excretion. Enzyme activities reported are thus consistent with the predominantly ammonotelic metabolism of lungfish in an aquatic environment.

In lungfish, which are capable of alternating between periods of ammonotelism and ureotelism, it is quite probable that levels of activity of the enzymes ornithine carbamoyltransferase and arginase would not undergo such striking changes as would the rate-limiting enzymes, carbamoyl phosphate synthetase and argininosuccinate synthetase. Thus a comparison of the levels of activity of ornithine carbamoyltransferase in lungfish with that in elasmobranchs may not be appropriate in the context of ascribing an ammonotelic or ureotelic status. Validation of this position will have to await the outcome of experiments recently initiated to determine levels of activity of the enzymes of the ornithine-urea cycle in aestivating lungfish when the animal is in the ureotelic phase.

In previous experiments, Janssens (*9*) demonstrated synthesis of urea from ammonia and bicarbonate by liver slices of *Protopterus* at a rate of approximately 2 μmole per gram of tissue per hour. This is consistent with the activity of the rate-limiting enzyme, argininosuccinate synthetase, which is reported here, and it is therefore not surprising that addition of ornithine to the incubation medium did not produce an increase in the rate of synthesis of urea (*9*).

Goldstein and Forster (*16*) recently suggested that, in lungfish, urea might be formed from uric acid and not by way of the ornithine-urea cycle. While this remains a possibility, our demonstration of the presence of all enzymes of the ornithine-urea cycle in *Protopterus* establishes this pathway for urea biosynthesis. Further investigation will be necessary to determine whether it is the major pathway.

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$$ATP + NH_3 + CO_2 \rightleftharpoons ADP + \text{carbamoyl phosphate} \quad (1)$$

This reaction is not dependent on acetylglutamate. Carbamoyl phosphate synthetase catalyzes the sequential reaction:

$$ATP + CO_2 \xrightarrow{\text{acetyl-glutamate}} ADP + P_i + \text{"active } CO_2\text{"} \quad (2)$$

$$ATP + \text{"active } CO_2 + NH_3 \xrightarrow[\text{acetyl-glutamate}]{} ADP + \text{carbamoyl phosphate} \quad (3)$$

If reactions 2 and 3 are summed, then

$$2ATP + CO_2 + NH_3 \xrightarrow{\text{acetyl-glutamate}} 2ADP + P_i + \text{carbamoyl phosphate} \quad (4)$$

where reaction 2 is irreversible and reactions 2 and 3 are dependent on the presence of acetylglutamate [see review by P. P. Cohen in *The Enzymes*, P. D. Boyer, H. Lardy, K. Myrbäck, Eds. (Academic Press, ed. 2, New York, 1962), vol. 6, p. 477].
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Sedimentation of an Initially Skewed Boundary

Abstract. *An asymmetric initial boundary is often formed when a synthetic boundary cell is used in the analytical ultracentrifuge. Model calculations show that, when such a boundary diffuses, the peak of the corresponding gradient pattern moves. The movement of the peak resulting from diffusion, when superimposed on the sedimentation of the boundary, may produce errors in the measurement of the sedimentation coefficient.*

The sedimentation velocity of a solute in the ultracentrifuge is correctly evaluated by measuring the rate of movement of the square root of the second moment ($\bar{r}$) of the gradient of solute concentration. The identification of the weight-average sedimentation coefficient (S) in the plateau region with $1/\omega^2 \cdot d \ln \bar{r}/dt$, where ω is the angular velocity and t is time, is correct for any boundary shape (*1*). If the gradient curve is symmetrical, then, unless the boundary is unusually broad, the position of the maximum gradient (r_{max}) lies within a few microns of $\bar{r}$ (*2*). Conditions giving rise to symmetrical boundaries are quite often met; it is, therefore, a common practice to derive sedimentation coefficients from measurements of r_{max} rather than $\bar{r}$, since the peak position is more easily located than is the square root of the second moment.

In order to produce a symmetrical gradient curve, a solute must be homogeneous with respect to sedimentation coefficient. Its diffusion coefficient (D) must be independent of concentration (*3*), and its sedimentation coefficient must either be independent of concentration or else be a linear function of concentration (*4*). In addition, the shape of the boundary must not be perturbed by restricted diffusion against the top of the solution column (*5*). This last condition generally requires the use of a synthetic boundary cell, which is designed to form, at the beginning of the experiment, a sharp boundary between solvent and solution some distance from the meniscus.

Synthetic boundary cells do not always function properly. There may be some mixing when the solvent compartment empties; moreover, convection may occur during acceleration of the centrifuge rotor after the boundary has been formed. Whatever the cause, it is not unusual to find that the boundary is somewhat skewed by the time the rotor reaches the speed at which sedimentation is to be observed. I now report that, in such a case, the use of successive peak positions to calculate the sedimentation coefficient may lead to serious errors.

The behavior of asymmetric boundaries has been studied theoretically by a simulation of the diffusion of such boundaries with a simple numerical model and a digital computer. The model, resembling one suggested by Vink (*6*), has been somewhat modified to take account of the sector shape of