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Arginase activity in *Bufo arenarum* embryos

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The evolution of arginase activity during development of ***Bufo arenarum*** *embryos was studied. The enzyme activity was detected after the neural groove stage, being low at the following early stages and increasing progressively until the end of the embryonic development. Arginase activity was first found in the neurula stage, when an outline of basic structures for renal and hepatic function is present. The activity increased two- or three-fold at the beginning of the larval stages.*

Key terms: *anuran embryos,* ***Bufo arenarum****, embryonic arginase.*

Free-living aquatic larvae of most terrestrial anurans excrete nitrogen wastes mainly as ammonia, showing a shift to urea during the metamorphosis when the animal emerges from water. In this larval-juvenile transition, there is a correspondence between the excreted main nitrogen products and the activity of enzymes associated with protein catabolism, linked to a parallel transition in their digestive model from herbivorous to carnivorous regime (10).

It is well known that arginase (L-arginine ureohydrolase; EC 3.5.3.1.), the terminal enzyme of the urea cycle which catalyzes the hydrolysis of L-arginine into urea and L-ornithine, is a prominent enzyme in the liver and kidney of ureogenic adult anurans. Changes in the activity of this enzyme during the larval-juvenile transition period have already been determined (2, 15). Since the pronephros excretory function and the presence of hepatic diverticulum can be shown as early as at the tail bud-neurula stages (3), we have extended those observations to the very early amphibian developmental stages. We measured the arginase specific activity in different embryonic stages of *Bufo arenarum*.

Embryos were obtained inducing ovulation by means of ip injection of a suspension of homologous hypophysis and subsequent fertilization of the eggs of toads, whose body mass interval was 159-170 g. At the beginning of the oviposition, animals were spinal pithed and the oocytes carefully removed from the ovisacs. The oocytes were fertilized *in vitro* with a sperm suspension made by disrupting testes in 10% Holtfreter's solution. The composition of the solution was (in $g \cdot l^{-1}$) as follows: NaCl 0.35; KCl 0.005; $CaCl_2$ 0.01; and $NaHCO_3$ 0.002. Eleven different ovulations were made. In some cases, jelly coated oocytes from 2-3 females were pooled and fertilized with sperm from 2-3 males. In each ovulation 1-3 samples of each embryonic stage were taken. Embryos were allowed to develop in glass Petri dishes containing 10% Holtfreter's solution. The solutions were replaced daily. Dishes were kept in a Ghilon TC 120 incubation chamber with the temperature set constant at $20 \pm 1°C$ and 12 h light-dark cycle. The experiments were carried out irrespective of the season.

Developmental stages of embryos were determined according to the table of Del Conte and Sirlin (7). Wet and dry weights of all embryos used in this study were 4.62 ± 0.27 and 0.71 ± 0.01 mg/embryo (means ± SEM's; n = 80), respectively. When necessary, embryos were freed from jelly coat by

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a short treatment with neutralized 2% thioglycolic acid, and rinsed thoroughly with 10% Holtfreter'solution.

Groups of 150-300 non-fertilized oocytes and morphologically normal embryos of each stage were homogenized on ice with 0.5 - 1.0 ml of 19 mM buffer Tris-HCl, pH 9, using a motor-driven glass-teflon homogenizer at 5000 rpm (20 strokes). Arginase activity was measured at 15 different developmental stages, from oocyte up to complete operculum, and at the first larval stage characterized by the presence of limb buds.

Arginase was estimated by the method of Shoemaker and McClanahan (15). The assay mixture was activated incubating with 0.05 M manganese maleate (pH 7) for 15 minutes at 25°C in a water bath. The reaction was started by the addition of 0.1 ml 0.7 M L-arginine, pH 9.5 to 0.2 ml of homogenate, and it was incubated for 30 min at 25°C. The reaction was stopped by adding 0.2 ml 0.4 M NaH_2PO_4 and by heating in boiling water for 5 min. Two blanks were run: one was a reagent blank and the other was a tissue blank (reaction stopped at zero time of incubation). In some cases, incubation mixtures were kept at -18°C for 4-6 days; we found that this procedure did not affect the enzyme activity. Total homogenates were centrifuged at 1600 g for 10 min at 5°C in a Sorvall RC 5B centrifuge. The amount of urea produced was determined in the supernatants by Fawcett and Scott method (9). Absorbances were read at 540 nm in a Shimadzu UV-240 spectrophotometer. Urea assays were carried out using kits of Wiener Labs. Enzymatic activity was expressed as nmoles urea/min/mg protein.

Protein was estimated by the procedure of Lowry *et al* (13) with bovine serum albumin (BDH) as the standard, and total content was expressed as mg protein/embryo.

All measurements were conducted in duplicate or triplicate. All chemicals used were of analytical grade. Normality in the distribution of the values was checked by the Kolmogorov-Smirnov goodness of fit procedure. Standard least-square exponential regression analysis between the specific activity of the enzyme, and the time (h) post *in vitro* fertilization was done. Linear regression was made for total protein (8). The regression parameters can be seen on Table I. For all tests the fiducial significance level was $p < 0.05$.

Figure 1 shows the specific activity of arginase and protein content in homogenates of embryos along several stages of their development and the exponential regression for arginase. In non-fertilized oocytes and in the early stages, from two blastomeres up to gastrula, total arginase activity was below the sensitivity of the technique. The activity was detected from the stage of neural groove on, increasing gradually and being initially very low and more pronounced at the end of the embryonic development.

Total protein content of embryos did not show significant variations along their development. At the onset of the larval development when animals start exogenous feeding having completed the development of the gut, there was a considerable increase in the enzyme activity which was too- or three-fold the mean found at the complete operculum stage.

Since our results are referred to whole embryos, our preparations do not allow the precise location of the detected enzyme activity. However, the observed gradual increase of arginase activity in embryos could be related to the morphogenetic and functional development of the renal excretory system and of the liver. In anuran embryos, the pronephros excretory function and the presence of a hepatic diverticulum was described at the stage of tail bud (3).

The presence of arginase activity in embryos of *Bufo arenarum* might be interpreted considering that in early stages of their development it may play other physiological roles different from urea biosynthesis. It is

TABLE I

Regression analysis parameters for arginase activity and total protein content

	Arginase	Protein
Slope	1.10×10^{-2}	-5.12×10^{-4}
Intercept	0.65	0.47
Standard error of slope	9.74×10^{-4}	1.53×10^{-4}
Correlation coefficient	0.804	0.337
R-squared (%)	64.7	11.3
Standard error of estimation	0.599	0.124

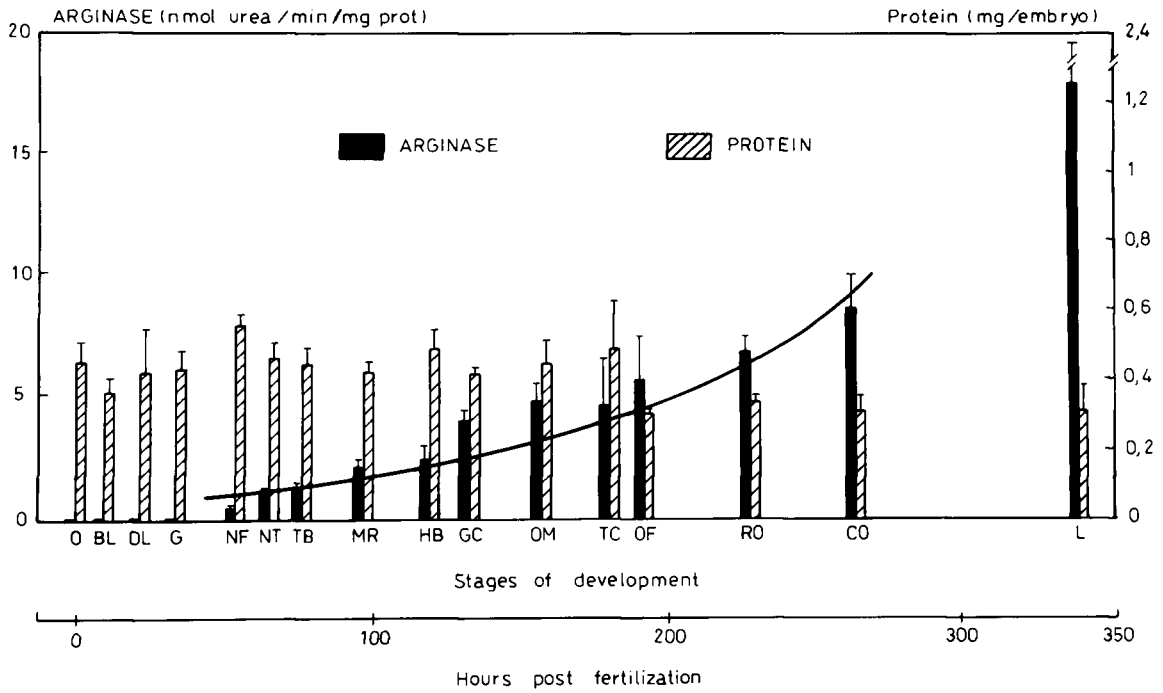

Fig 1. Specific activity of arginase (nmol urea/min/mg protein) and protein content (mg/embryo) of homogenates of *Bufo arenarum* embryos developed in Holtfreter solution along the stages of development (bars). Exponential regression indicated by solid line. Data expressed as means ± SEM. Stages (number of homogenates in parentheses): O, oocyte (6); BL, blastulas (3); DL, dorsal limb (2); G, gastrulae (7); NF, neural folds (2); NT, neural tube (7); TB, tail bud (6); MR, muscular response (10); HB, heart beat (4); GC, gill circulation (6); OM, open mouth (7); TC, tail fin circulation (3); OF, operculum fold (3); RO, right operculum (9); CO, complete operculum (12); L, first larval stage (11).

worth mentioning that, in both embryos and in prometamorphic tadpoles, arginase might be in considerable excess relative to the low amount of urea formed, yet it cannot function cyclically because the other reactions of the urea cycle are very limited. In this respect, it is important to note that arginase shows a universal distribution and, in a variety of tissues, it is associated with growth, protein synthesis, formation of aminoacids or transamination reactions for creatine synthesis (12).

This type of early ontogenetic anticipation of particular biochemical pathways has already been reported in anurans (1, 4, 6, 11) and suggests biochemical-physiological coupling along differentiation processes (5, 14).

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