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A COMPARISON OF THE HEMOGLOBINS OF LARVAL AND
TRANSFORMED Ambystoma tigrinum

by

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INTRODUCTION

Amphibians which undergo metamorphosis from a larval aquatic to a terrestrial adult stage exhibit accompanying physiological adjustments to cope with the different environment. When the two life history stages are compared, hemoglobins of larvae generally have different polypeptide chains and are present in lower concentrations than in the terrestrial stage. Although Hahn (1962) did not measure hemoglobin in his study of metamorphosing Ambystoma tigrinum, he did report an increase in the hematocrit which would suggest an increase in hemoglobin. McCutcheon (1936) found larger and more numerous erythrocytes in tadpoles of Rana catesbeiana than in adults and suggested that this difference might be due to a change in the locus of erythropoiesis, to the type of hemoglobin present, or to the respiratory demands of the hemoglobin associated with the nature of the environment and the metabolic needs of the organism. Riggs (1951) confirmed the findings of McCutcheon and added that the molecular weight of adult bullfrog and tadpole hemoglobin is about the same (68,000) as is the heme-heme interaction. However, the oxygen affinity of the tadpole hemoglobin, in contrast to the ontogenetic changes exhibited by amphibians, is about 7 times as great as

that of the adult at pH 6 and twice as great as pH 9. Brown and DeWitt (1970) in an electrophoretic study of the hemoglobins of the newt, Notophthalmus viridescens, found that adult and eft stages had identically migrating hemoglobins; however, the larvae had one hemoglobin similar to the minor hemoglobin of the eft and one different. They explained their results recalling the similarities in respiration of the adult and the eft, both of which are terrestrial. Moss and Ingram (1968a) suggested that all polypeptide hemoglobin chains were different between tadpoles and adult Rana catesbeiana. They (1968b) later reported that cytological and radiographic studies suggested a new population of red blood cells to be responsible for synthesis of adult hemoglobin in Rana catesbeiana. Frieden (1961) found the heme portion the same in tadpoles and adults with identical absorption peaks at 541 m μ and 578 m μ . He confirmed earlier findings that there were variations in the amino acid composition. Edwards and Justus (1969) reported that the hemoglobin pattern of Ambystoma maculatum changed during natural metamorphosis but that the pattern of A. mexicanum did not change upon thyroxine-induced metamorphosis. They suggested that the genetic information required for metamorphic changes in the hemoglobin pattern and for spontaneous metamorphosis may have become lost or inactive in laboratory strains of A. mexicanum.

The ability of A. tigrinum marvortium to metamorphose

is variable. Normally animals in West Texas metamorphose at about 100 mm snout to vent; however, others remain larval for longer periods and reach snout to vent lengths of up to 170 mm. Many of these larger larvae lack the ability to metamorphose even when injected with thyroxine (Hahn, 1962). In view of the complexity of metamorphosis in this species, and its similarity to A. mexicanum, a study was initiated to analyze hemoglobin for possible changes during natural metamorphosis.

METHODS

Larval and transformed Ambystoma tigrinum used in this study were collected at Reese Air Force Base, Hurlwood, Texas. Each animal was measured from snout to vent, sexed, and sacrificed by decapitation. Approximately three ml. of blood were drained into a 50 ml. beaker which was coated with 4% sodium flouride - 4% potassium oxalate to retard clotting. One ml. of the blood was pipetted into a vial filled with 10 ml. of a 0.9% NaCl solution and centrifuged. The cells were washed twice in the saline, the fluid decanted and the cells lysed by the addition of 2 ml. of distilled water, followed by freezing. On thawing, the cell debris was packed by centrifugation and the hemoglobin solution was pipetted into a vial and stored at -10°C. Hemoglobin stored over two days gave poor resolution;

therefore, only fresh hemoglobin samples were used.

For hematocrit determinations blood was collected in heparinized tubes and spun in an International Micro-Capillary Centrifuge for 5 minutes at 11,500 rpm. Hemoglobin concentrations were determined with an American Optical Hemoglobinometer.

A spectral analysis was made using 0.02 ml. of whole blood and 5 ml. of Drabkin's solution (Hawk, 1923). In this analysis 5 larvae and 5 transformed animals were used. An absorbance scan was conducted for blood samples from each animal at 10 m μ increments from 340 m μ to 680 m μ .

The electrophoretic mobility of the hemoglobins was determined on cellulose acetate strips (Beckman Microzone) and horizontal starch (Smithies, 1959). Starch gel electrophoresis was carried out on a 14% gel using a borate buffer (pH 8.6). Thirty μ l. of hemolysate were placed in each slot and electrophoresed at 160 volts for 15-16 hours. To dissociate the hemoglobins into their component polypeptide chains, the samples were spotted in a formate gel (pH 1.8) 2 mm. in thickness and electrophoresed at 250 volts for 2 hours.

The globin portion of the molecule was precipitated using 10 ml. of acetone: HCl (100:1 v/v) to 1 ml. of hemoglobin solution. The precipitate was redissolved in 1 ml. of formate buffer (pH 1.8) and electrophoresed on a 2 mm. formate gel at 250 volts for 2 hours.

In order to examine the hemoglobin substructure, fresh samples were electrophoresed on a thick borate gel. Portions of the Hb S and Hb F hemoglobins for each larval and transformed animal were cut from the borate gel and placed in a formate gel (Dessauer and Nevo, 1969). Formate buffer was poured over each slot to further dissociate the hemoglobins into their polypeptide chains. The gel was run packed in ice at 160 volts for 2 hours. All gel electrophoresis was conducted at 5°C and upon completion the starch slabs were stained with Buffalo Black.

To determine the relative concentrations of the Hb F and Hb S hemoglobins on cellulose acetate, the strips were scanned in a Beckman Analytrol Densitometer (slit width 1.5 mm.) fitted with a Microzone attachment drive. The percent composition was ascertained by measuring the area under each peak. Statistical analyses include one standard deviation and two standard errors of the mean.

RESULTS

Spectral analysis revealed no qualitative difference in hemoglobin from larval and metamorphosed A. tigrinum. Both groups had identical spectral peaks at 540 mμ. Hemoglobin from transformed animals had an absorbance twice as great as that from larvae. This was confirmed (Figure 1) by quantitative analysis using an A and O optical

Fig. 1--Comparative hemoglobin levels of transformed and larval *A. tigrinum*. The mean, range, standard deviation, and two standard errors of the mean are represented.

hemoglobinometer. Values indicated include the mean $\pm$ one standard deviation and the range. The mean value for larvae was 8.0 ± 1.7 (5.8-12.0), however; the value for metamorphosed animals was 11.1 ± 1.7 mg HHb (8.9-14.6)/100 ml. of blood. Hematocrit values increased from 32.1 ± 7.0 (22-49) in the larvae to 40.4 ± 5.8 (32-52) in the adults (Figure 2). The increase in hemoglobin is a function of the increased hematocrit (Figure 3); the coefficient of correlation is .995.

Two hemoglobins were resolved on cellulose acetate strips. There was no difference in the migration pattern of the two hemoglobins between larval and metamorphosed animals (Figure 4). However, the fast migrating hemoglobin (Hb F) of the metamorphosed animals comprised 63% of the

Fig. 2--Comparative hematocrits of transformed and larval A. tigrinum.

total hemoglobin while the same hemoglobin of the larvae comprised only 57% (Figure 5). Thus, not only is there an increase in the total amount of hemoglobin but a relative increase in the Hb F hemoglobin during metamorphosis.

When electrophoresed for 15 hours on a borate gel, the hemoglobins resolved into the typical Hb F and Hb S hemoglobins. However, if electrophoresis continued for 24 hours the Hb F hemoglobin separated into two hemoglobins (Figure 6). The separation of the Hb F hemoglobin was subsequently verified using thin layer borate gels. There was no difference in the migration rate of the hemoglobins of larval and transformed animals on borate gels. A lightly staining band preceeding the Hb S hemoglobin showed no red color and is thought to be protein residue from incomplete washing of the cells, which emerged on staining with Buffalo Black.

Fig. 3--Hematocrit vs. mg. HHb/100 ml. blood of transformed and larval A. tigrinum.

Fig. 4--Comparison of Hb S and Hb F hemoglobins of transformed and larval *A. tigrinum* as determined on cellulose acetate electrophoresis.

Fig. 6--Hemoglobins of transformed and larval *A. tigrinum* on a borate gel (pH 8.6) electrophoresed for 24 hours at 160 volts. Larval and transformed animals alternate.

Hb S and Hb F hemoglobins isolated from a borate gel and electrophoresed in a formate gel showed strikingly similar migration mobilities (Figure 7). The Hb S hemoglobin dissociated into a darkly staining, slow migrating polypeptide preceded by a lightly staining one. The Hb F hemoglobin dissociated into two identically migrating

Fig. 7--Substructure of Hb S and Hb F hemoglobin isolated from borate gel (pH 8.6) and electrophoresed in a formate gel (pH 1.8). Hb S and Hb F alternate.

polypeptides as those seen in the Hb S hemoglobin plus a slower, lightly staining polypeptide. Again there were no differences between larval and transformed animals.

Three polypeptides were resolved from the globin precipitate electrophoresed in the formate gel (Figure 8). The fast migrating polypeptide is the major component and is followed immediately by a lightly staining polypeptide. The third substructure has a RF value of about 40%. There was no difference in the migration pattern on larval and transformed animals.

Fig. 8--Electrophoresis of globin precipitate of hemoglobin from transformed and larval A. tigrinum electrophoresed on formate gel (pH 1.8). Larval and transformed animal alternate.

DISCUSSION

There were no observed differences in the electrophoretic mobility patterns of the hemoglobins of larval and transformed Ambystoma tigrinum. If there are difference at the amino acid level, they were undetected by our techniques. Similarities of the Hb S and Hb F hemoglobins between the two groups were confirmed in alkaline buffers on cellulose acetate and starch gel. When these two hemoglobins were isolated and electrophoresed in formate buffers, the globins dissociated into five substructures. Two of these polypeptides were common to each hemoglobin and a third was unique to the Hb F hemoglobin. In addition, the

globins precipitated in acetone: HCl from larval and transformed animals showed identical migration patterns.

There was a significant increase in the amount of hemoglobin from larval to transformed animals. This was confirmed by spectral analysis and on cellulose acetate. Apparently the increase in hemoglobin of transformed animals is due to a relative increase in the number of circulating erythrocytes. However, besides an increase in the total amount of hemoglobin per unit of blood there is a relative increase in the Hb F hemoglobin of transformed animals.

Edwards and Justus (1969) reported no change in the hemoglobin pattern of A. mexicanum during transformation. They suggested that this might be due to a loss of the genetic capability to change the hemoglobins. Although the taxonomic status of A. mexicanum is not clear, it undoubtedly is closely allied to A. tigrinum. Quite possibly the inability to change the hemoglobins at transformation is a characteristic of this species group.

Although water has certain disadvantages when compared to air as a respiratory medium, apparently the gills and lower metabolism of larvae (Whitford and Hutchison, 1967 and Whitford and Sherman, 1968) more than compensate for these disadvantages. The loss of the filamentous gills and higher metabolism of transformed animals probably triggers the reported polycythemia. Rather than changing the

substructure of the hemoglobin molecules to increase the oxygen binding capacity after transformation, A. tigrinum apparently increases the relative number of erythrocytes and amount of hemoglobin in the circulating blood.

LIST OF REFERENCES

- Brown, Eleanor R., and DeWitt, William. 1970. Hemoglobin changes during metamorphosis in Triturus viridescens. Comp. Biochem. and Physiol. 35:495-7.
- Beckman Model R-101 Microzone Electrophoresis Cell Instruction Manual. Fullerton, California: Beckman Instruments, Inc. (1965).
- Dessauer, Herbert C., and Nevo, Eviatar. 1969. Geographic variation of blood and liver proteins in cricket frogs. Biochem. Genetics. 3:171-88.
- Edwards, J. A., and Justus, J. T. 1969. Hemoglobins of two urodeles: changes with metamorphosis. Proc. Soc. Exptl. Biol. and Med. 132:524-6.
- Frieden, Earl. 1961. Biochemical adaptation and anuran metamorphosis. Am. Zool. 1:115-49.
- Hahn, William E. 1962. Serum protein and erythrocyte changes during metamorphosis in paedogenic Ambystoma tigrinum mavortium. Comp. Biochem. and Physiol. 7:55-61.
- Hawk, Philip B. Practical Physiological Chemistry. Philadelphia: P. Blakiston's Son and Co., 1923.
- McCutcheon, F. H. 1936. Hemoglobin function during the life history of the bullfrog. J. of Cell. and Comp. Physiol. 8:63-81.
- Moss, Bernard, and Ingram, Vernon M. 1968a. Hemoglobin synthesis during amphibian metamorphosis - I. chemical studies on the hemoglobins from the larval and adult stages of Rana catesbeiana. J. Molecular Biol. 32:481-92.
- _____. 1968b. Hemoglobin synthesis during amphibian metamorphosis - II. synthesis of adult hemoglobin following thyroxine administration. J. Molecular Biol. 32:493-504.

- Riggs, Austen. 1951. The metamorphosis of hemoglobin in the bullfrog. J. Gen. Physiol. 35:23-40.
- Smithies, O. 1959. An improved procedure for starch gel electrophoresis: further variations in the serum proteins of normal individuals. Biochem. J. 71: 585-7.
- Whitford, Walter G., and Hutchison, Victor H. 1967. Body size and metabolic rate in salamanders. Physiol. Zool. 40:127-33.
- _____, and Sherman, Robert E. 1968. Aerial and aquatic respiration in axolotl and transformed Ambystoma tigrinum. Herpetologica. 24:233-7.