COMPARATIVE STUDIES ON THE HEMOGLOBINS OF REPRESENTATIVE SALAMANDERS OF THE FAMILIES CRYPTOBRANCHIDAE, PROTEIDAE AND HYNOBIIDAE

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Abstract—1. Properties of hemoglobins in the blood of the salamanders Cryptobranchus alleganiensis, Necturus maculosus and Hynobius tsuensis were examined by starch gel electrophoresis and peptide mapping.

2. Multiple hemoglobin components were found in individual specimens of N. maculosus and H. tsuensis, but a single component was observed in C. alleganiensis.

3. Sedimentation coefficients of 4.8 S were found for the N. maculosus and C. alleganiensis hemoglobins.

4. Cryptobranchus hemolysates exhibited a relatively low oxygen affinity, a Hill constant of 2.7 and an absence of a Bohr effect at pH near neutrality whereas N. maculosus hemolysates showed a higher oxygen affinity, a Hill constant of 1.7 and a Bohr effect.

INTRODUCTION

Comparative biochemical studies on amphibian hemoglobins are of interest in understanding molecular evolution and respiratory adaptations. Amphibians occupy an intermediate position between bony fishes and terrestrial vertebrates and utilize a variety of modes of respiration including gills, skin, buccopharyngeal adaptations and lungs (Noble, 1931). Some detailed structural and functional studies on frog and tadpole hemoglobins have been made (Prosser & Brown, 1961; Baglioni & Sparks, 1964; Riggs, 1964; Brunori et al., 1968), but little is known of other amphibian hemoglobins. Among urodeles the paper electrophoretic mobilities of hemoglobins from several members of various families have been described (Dessauer et al., 1957). The hemoglobins from Necturus maculosus (Proteidae) seem to have gained the most biochemical attention (Lenfant & Johansen, 1967).

We have recently examined the hemoglobins from members of different geographic populations of the large aquatic salamanders, Cryptobranchus, including two populations of C. alleganiensis bishopi, and one of C. alleganiensis alleganiensis (Nickerson, Wortham & Taketa, 1973). In addition, limited samples from N. maculosus (Proteidae) and Hynobius tsuensis (Hynobiidae) were studied.
Apparently there is no other work that characterizes the hemoglobins of the cryptobranchid salamanders, and although there are earlier reports on the electrophoretic mobility of the protein of the proteid (*N. maculosus*), details on its structural and functional characteristics are lacking.

*Cryptobranchus* loses its gills before reaching adulthood (Bishop, 1941) and although it has lungs, adult respiration is mainly cutaneous (Guimond, 1970). It is characteristically found in highly spring-fed and/or well-oxygenated streams. In contrast, *N. maculosus* has external gills, is a gill breather and may be found in sluggish, less oxygenated waters. Members of the Hynobiidae are purportedly one of the most primitive urodeles. Some are semi-aquatic, and adult respiration is presumably primarily pulmonary and cutaneous.

These considerations made it of interest to examine and compare the properties of their respective hemoglobins. This work describes their starch gel electrophoretic mobilities as well as their tryptic peptide fingerprints, and compares the oxygen equilibrium characteristics of *C. alleganiensis* and *N. maculosus* hemolysates. Sufficient material was not available for studies on oxygen binding with *H. tsuensis* hemoglobin. It is shown that blood of members from the three families of salamanders exhibit distinct differences in hemoglobin electrophoretic patterns and that the characteristics of the oxygen equilibria of *C. alleganiensis* and *N. maculosus* hemolysates are also quite different.

**MATERIALS AND METHODS**

*Cryptobranchus a. bishopi* were collected in the North Fork of White River, Ozark County, Missouri and in the Spring River, Fulton County, Arkansas and *C. a. alleganiensis* in the Niangua River, Dallas County, Missouri. *Necturus maculosus* were obtained from the Mukwonago River, Waukesha County, Wisconsin. The *Hynobius tsuensis* came from Kechi, Tsushima, Japan. Blood samples were obtained via caudal incision or cardiac puncture and collected in 0.9% saline containing 2 µg/ml heparin. The erythrocytes were collected by centrifugation at 2500 rev/min in a clinical centrifuge and washed by resuspension in 0.9% NaCl. This procedure was repeated three times. The packed cells were lysed with about 5 vol. of distilled water and then centrifuged for 20 min at 1500 rev/min in a SS-1 rotor of a Sorvall Refrigerated Centrifuge to obtain clear hemoglobin solutions. Hemoglobin concentration was estimated spectrophotometrically by the cyanmethemoglobin method (Hainline, 1958).

Horizontal starch gel electrophoresis using Tris–EDTA–borate buffer pH 8.4 was carried out as described earlier (Taketa & Morell, 1966), and the separated bands of hemoglobin were visualized using the benzidine stain (Sunderman, 1964). Sedimentation analysis was carried out using a Beckman Model E analytical centrifuge. Hemoglobin —SH groups were measured spectrophotometrically using the reagent 4, 4'-dipyridinedisulfide (PDS) as described before (Taketa & Morell, 1969). Typically, 2.0 ml of a 1.5 x 10^{-6} M solution of hemoglobin was reacted with 0.2 ml of 1 x 10^{-3} M solution of PDS to measure the “reactive” —SH groups. One ml of a 1% sodium dodecyl sulfate solution was then added to the reaction mixture to denature the protein and to measure “total” —SH.

The peptide mixtures obtained by tryptic digestion of the hemoglobins were analyzed by a fingerprinting technique similar to that described by Baglioni (1961). Oxygen saturation measurements were made by the spectrophotometric technique of Rossi-Fanelli & Antonini (1958).
RESULTS

Electrophoresis

The hemoglobins from fifteen adult specimens of C. a. bishopi, nine of C. a. alleganiensis, three of N. maculosus and six H. tsuensis, were examined in this work. Starch gel electrophoretic analysis showed that the blood of C. a. bishopi and C. a. alleganiensis contained a single hemoglobin component of identical electrophoretic mobility, whereas samples from all three N. maculosus and all six H. tsuensis contained two major components.

Figure 1 shows the relative electrophoretic mobilities of these hemoglobins compared with adult human HbA at pH 8.4. The Cryptobranchus hemoglobin and one of the N. maculosus hemoglobins (fast component) exhibited a decidedly greater anodic mobility than human HbA. The second N. maculosus hemoglobin (slow component) showed only a slightly greater anodic mobility than human HbA. The mobility of the more anodic component of H. tsuensis (fast component) was similar to that of the slow component of N. maculosus. The slow component of H. tsuensis migrated at nearly the same rate as adult human HbA. The relative proportions of the fast and slow components in both N. maculosus and H. tsuensis were approximately 40/60. The results on the N. maculosus hemoglobins are in contrast with the report of Dessauer et al. (Baglioni & Sparks, 1964), which indicated the presence of a single component of slightly slower anodic migration than human HbA at pH 8.5. It was possible that one of the two components observed was a polymer of the other as polymerization by disulfide bond formation is apparently of common occurrence among amphibian and reptile hemoglobins. Svedberg & Hedenius (1934) reported the presence of two hemoglobin components with sedimentation coefficients of about 4 S and 7 S respectively in Salamandra
maculosa (Salamandridae) blood and postulated the formation of a \( \sim 7 \) S polymer from a \( \sim 4 \) S tetrameric hemoglobin. Such a process seems to occur by disulfide bond formation in frog (*Rana catesbeiana*) and turtle hemoglobins following hemolysis and can be prevented by alkylation of hemoglobin —SH groups prior to hemolysis of the erythrocyte (Riggs *et al.*, 1964). It can also be reversed by reaction of the 7 S polymer with mercaptoethanol (Riggs *et al.*, 1964). To test the possibility of polymerization, *N. maculosus* red cells were reacted with N-ethylmaleimide prior to hemolysis. Analysis indicated that under the conditions of reaction (pH 6.8 Hendry’s phosphate buffer, 23°C, NEM/Hb = 20, 1 hr) all of the reactive —SH groups were modified. Nevertheless, the same two electrophoretic components were present in about the same proportions as in the untreated control sample. In addition, reaction of the fresh hemolysate with an excess of mercaptoethanol did not cause a change in the electrophoretic pattern.

*Sedimentation analysis*

Sedimentation analysis of the *N. maculosus* hemolysate also indicated the presence of only a single 4-8 S component (Fig. 2). It thus appears that the two *N. maculosus* hemoglobin components do not bear a monomer—polymer relationship and are indeed separate hemoglobins with different primary structures. Further analyses of these components was not possible in this work due to lack of sufficient material and their further characterization must await their isolation from one another. A sedimentation coefficient of 4-8 S was also observed with the *C. alleganiensis* hemoglobin. The molecular weights of these hemoglobins are therefore about 68,000, a value that is similar to that found in a number of other amphibian hemoglobins (Tentori *et al.*, 1965).

*Sulfhydryl analysis*

Table 1 shows the results of —SH titration of hemoglobins in freshly appeared hemolysates of *C. a. bishopi, C. a. alleganiensis* and *N. maculosus*. An average of about six —SH per hemoglobin molecule, all of which are readily reactive, was found in the three hemolysates. Since the *N. maculosus* hemolysate contains two

<table>
<thead>
<tr>
<th>Sample</th>
<th>—SH/Hb</th>
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<tbody>
<tr>
<td><em>C. alleganiensis</em></td>
<td>6.1</td>
</tr>
<tr>
<td><em>C. alleganiensis</em> bishopi</td>
<td>6.2</td>
</tr>
<tr>
<td><em>N. maculosus</em></td>
<td>6.0–8.0</td>
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* Measured with 4,4′dipyridyldisulfide. Number of —SH groups is calculated for a molecular weight of 68,000.
major components, the exact distribution of —SH groups on the individual components is not known at this time. However, the occurrence of significant numbers of —SH groups distinguishes these hemoglobins from that of the tadpole in which there are none. The adult frog (*R. catesbeiana*) hemoglobin contains four reactive —SH groups (Brunori et al., 1968) and has a tendency to polymerize by disulfide bond formation. In spite of the presence of six reactive —SH groups, no evidence for polymerization was found in any of the salamander hemoglobins.

**Fingerprinting analysis**

Figure 3 shows the tryptic peptide maps prepared from the hemolysates from *C. a. bishopi*, *C. a. alleganiensis* and *N. maculosus*. Peptide maps of *H. tsuensis* hemolysates were also prepared but are not shown because of poor photographic reproduction. In each case, the number and distribution of peptides are those expected of tetrameric hemoglobins with two kinds of chains. As with the electrophoretic mobility of the intact hemoglobins, the peptide maps of *C. a. bishopi* and *C. a. alleganiensis* appear to be identical. They are, however, clearly different from that of *N. maculosus* and *H. tsuensis* even though extensive homologies seem to exist. In general, a greater number of neutral and acidic peptides is found in salamander hemoglobin fingerprints when compared with those from mammalian hemoglobins.

**Oxygen equilibrium**

Figure 4 shows the oxygen equilibrium curves obtained from hemolysates of *C. a. bishopi* and *N. maculosus* in 0·14 M phosphate buffer pH 6·5 and 7·4 at 20°C. The partial pressure of oxygen required to half saturate (*P*<sub>50</sub>) the hemoglobins in *N. maculosus* hemolysate at pH 7·4 was 5 mm Hg, whereas it was 20 mm Hg for the *Cryptobranchus* hemoglobin. Clearly, the high oxygen affinity (1/*P*<sub>50</sub>) of

![Graph](image)

**Fig. 4.** Oxygen equilibrium curves of *N. maculosus* and *C. a. alleganiensis* hemoglobins. Left panel *N. maculosus*; right panel, *C. a. alleganiensis* hemolysate. Data were obtained on hemolysates in 0·14 M phosphate buffer at 20°C; hemoglobin concentration was 0·7%.
FIG. 2. Schlieren pattern of *N. maculosus* hemolysate obtained at 60,000 rev/min. Hemoglobin concentration was 0·6%.
FIG. 3. Tryptic peptide maps of hemoglobins in hemolysates of (a) *C. alleganiensis* and (b) *N. maculosus*. 
*N. maculosus* hemoglobins resembles that of the tadpole (Riggs, 1951; Brunori *et al.*, 1968) and is apparently an adaptation for life in a sluggish aquatic habitat. In contrast, the oxygen affinity of *Cryptobranchus* hemoglobin is relatively low, and can be compared with values of $P_{50}$ for terrestrial mammals that range from about 10–30 mm under comparable conditions. The aquatic environment of *Cryptobranchus* is relatively well oxygenated and its cutaneous mode of respiration appears adequately efficient to enable its hemoglobin to resemble that of terrestrial mammals with respect to oxygen affinity. Furthermore, its oxygen equilibrium curve is distinctly sigmoidal, and the Hill interaction coefficient calculated from the slope of the plot of logarithm of the fractional saturation, log $Y/(1-Y)$, vs. the logarithm of the partial pressure of oxygen, log $pO_2$, was about 2·7, a value that is similar to Hill coefficients in mammalian hemoglobins. The Hill coefficient for the *N. maculosus* hemolysate was about 1·7, indicating a lower degree of co-operativity among the hemoglobin oxygen binding sites.

*Cryptobranchus* hemoglobin, like that of the tadpole, does not exhibit a Bohr effect, whereas the components of the *N. maculosus* hemolysate resemble the hemoglobin of the adult frog in that a Bohr effect is present and is quantitatively about one-half of that found in terrestrial mammals (Brunori *et al.*, 1968).

**DISCUSSION**

Multiple hemoglobin components are commonly found in the blood of vertebrates, and the occurrence of two major hemoglobins in *H. tsuensis* and *N. maculosus* fits this pattern. In contrast, the blood of *Cryptobranchus* apparently contains a single homogeneous hemoglobin and no evidence was found for the occurrence of polymorphic components among twenty-six individual specimens. The earlier report (Dessauer *et al.*, 1957) describing the presence of only a single electrophoretic component in *N. maculosus* blood may be due to a lack of resolution of the hemoglobins in the paper electrophoretic system used, or due to actual differences in the proteins of two forms tested, i.e. *N. m. maculosus* and *N. m. louisianensis* (assumed).

The oxygenation properties of *N. maculosus* hemoglobin, including the high oxygen affinity, the presence of a Bohr effect and a Hill constant of 1·7 at pH near neutrality are in sharp contrast with the properties of *Cryptobranchus* hemoglobin which include a relatively low oxygen affinity, an absence of a Bohr effect and a Hill constant of 2·7. Thus, although heterotropic (Bohr effect) and homotropic (Hill coefficient) effects presumably involve conformational changes that alter subunit interactions, they are reflections of changes that are distinct and independent of one another. One of the hemoglobins found in trout blood also exhibits a sigmoid oxygen equilibrium but no Bohr effect (Binotti *et al.*, 1971). Anuran tadpole hemoglobins resemble *N. maculosus* hemoglobins with respect to high oxygen affinity but are similar to *Cryptobranchus* hemoglobin in lacking a Bohr effect. These and other considerations make it of interest to obtain additional information on the structure and properties of these hemoglobins.
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REFERENCES


*Key Word Index*—Salamander; *Cryptobranchus*; *Necturus*; *Hynobius*; hemoglobin; electrophoresis; fingerprints; sedimentation; oxygen equilibrium; Bohr effect; Hill constant.