Hemoglobins of the Killifish Fundulus heteroclitus

SEPARATION, CHARACTERIZATION, AND A MODEL FOR THE SUBUNIT COMPOSITIONS*

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Polyacrylamide and starch gel electrophoresis of the hemoglobin of the killifish Fundulus heteroclitus reveal the presence of four clearly distinguishable components. These isohemoglobins, each tetramers consisting of $\alpha$ and $\beta$ chains, can be preparatively separated by ion exchange chromatography on DEAE-cellulose and are homogeneous according to isoelectric focusing in polyacrylamide gels. Oxygen equilibria of the isolated hemoglobin components (Hb I, Hb II, Hb III, and Hb IV) show only minor differences in the magnitude of the Bohr effect and in the effect of ATP on the binding of oxygen.

Four different globin chains, $\alpha^a$, $\alpha^b$, $\beta^a$, and $\beta^b$, can be separated by ion exchange on CM-cellulose. Hb I is a homotetramer of $\alpha^a$ and $\beta^a$ chains, Hb IV consists of $\alpha^b$ and $\beta^b$ subunits, and components II and III are heterotetramers consisting of all four chains. The $\alpha$ and $\beta$ chains differ significantly in amino acid composition. A model suggesting the existence of 10 different isohemoglobins, 6 of which have stable intersubunit contacts, has been proposed to account for the qualitative and quantitative aspects of the electrophoretic behavior of the components. Separations of the isohemoglobins on DEAE-cellulose under slightly modified conditions provide additional support for the model.

The presence of multiple hemoglobins in fish and other lower vertebrates is a well established phenomenon. However, there have been discrepancies regarding the actual number, proportions, and structural and functional properties of species-specific hemoglobin components (1-14). While these differences can arise from such factors as thermoacclimatory variation (7, 8), developmental changes (9, 10), polymerization (11, 12), dissociation (13, 14), and susceptibility to autooxidation (1, 14), they are often the result of variations in the procedures and techniques employed to analyze and preparatively separate the components of a hemolysate. Due to such qualitative and quantitative uncertainties, an analysis of the subunit structure of the multiple hemoglobins in a particular species has only rarely been attempted (3, 6, 15). We have analyzed the isohemoglobins of the killifish Fundulus heteroclitus; as a result of our study, we propose a model to explain the molecular basis for the hemoglobin multiplicity. An analysis of this type should improve our perception of the structural properties of multiple hemoglobins in lower vertebrates and provide a basis for the understanding of even more complex systems.

EXPERIMENTAL PROCEDURES

Purification of Hemolysate—Fish were obtained from shallow inlets of the Chesapeake Bay. Blood samples were taken with a syringe by cardiac puncture and pooled with excess 9.5% NaCl. The cells were washed, lysed, and centrifuged for 60 min at 12,000 x g.

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The membrane-free hemolysate was dialyzed at 4°C against 10 mM Tris-HCl, pH 8.5, and subjected to pressure filtration (Amicon Corp.). The concentrated hemoglobin (500 mg in 2 ml) was purified by gel filtration in a Sephadex G-100 column (2.5 x 100 cm). The concentration of hemoglobin was calculated by assuming millimolar extinction coefficients (equivalent $M_x = 16,000$/hemel of 13.8 at 541 nm for oxyhemoglobin and 13.4 at 540 nm for carboxyhemoglobin (16).

Separation of Hemoglobin Components—The four electrophoretically distinguishable hemoglobin components were separated by ion exchange chromatography on a DEAE-cellulose (Whatman DE52) column (0.9 x 27 cm) at 4°C. The hemoglobin sample was dialyzed against 30 mM Tris-HCl, pH 8.6, and concentrated by pressure filtration. The small volume (approximately 1 ml) was critical to obtain resolution of the components since hemoglobins I and II separate without fully binding to the resin. The flow rate was maintained at 20 ml/h and 1-ml fractions were collected. After the elution of the first two components, hemoglobins III and IV were eluted with a linear gradient between 50 ml of the starting buffer and 50 ml of 30 mM Tris-HCl, 0.1 M NaCl, pH 8.5. Subsequent separations of the components were performed at higher pH (8.6) with 30 mM Tris-HCl, and with a lower salt concentration (20 mM Tris-HCl) at pH 8.4, utilizing a stepwise salt gradient to elute component III at 40 mM Tris-HCl and component IV at 0.1 M NaCl, 40 mM Tris-HCl.

Characterization of Hemoglobins by Gel Electrophoresis—The separated hemoglobin components were compared with the unfractionated mixture by electrophoresis in starch gel (17) using the Tris/borate/EDTA system of Boyer et al. (18) and a modification of the discontinuous buffer system developed by Poulik (19). The two procedures gave similar results. Electrophoresis was carried out at 25 mA (140 V) for 6 h at 4°C.

Polyacrylamide gel electrophoresis using the standard analytical system of Davis (20) was employed to determine the apparent molecular weights of the isolated hemoglobin components. This system of Davis (20) was employed to determine the apparent molecular weights of the isolated hemoglobin components. This system of Davis (20) was employed to determine the apparent molecular weights of the isolated hemoglobin components.
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relative proportions of the four isohemoglobin. The erythrocytes from 16 fish were washed and lysed as described and the separate hemolysates, after saturation with carbon monoxide to minimize autooxidation, were subjected to gel chromatography at 30°C (5 m
ma/gel) for 4 hr at 4°C. The running pH for the gels was 8.9. The gels were scanned at 540 nm using a Gilford spectrophotometer.

Isoelectric Focusing of Hemoglobin Components — Electrofocusing of the separated hemoglobin components was performed in poly-
acrylamide gel after the method of Mott et al. (22). Electrofocusing was carried out at 4°C, initially at 130 V (1.5 mA/gel). The current was maintained at 1.5 mA/gel until 200 V was reached. Focusing was complete (about 3 h) when the current dropped to zero. The pH of each hemoglobin component was determined from the pH gradient produced from eluates of gel sections or from a direct measurement of the pH of the gel slices containing the hemoglobin (23, 24). The pH values reported are the averages of six determinations in each case.

Gel Filtration — Apparent molecular weights of the hemoglobin components were determined by gel filtration in a Sephadex G-100 column (50 × 0.8 cm) equilibrated with 0.1 M Tris HCl, pH 7.2, with 0.1 m NaCl and 1 mM EDTA. The concentration of hemoglobin applied to the column varied from 3 μM to 200 μM (M, = 64,000). The column was standardized with the following proteins from a calibration kit (Pharmacia Fine Chemicals Inc.): aldolase, bovine serum albumin, ovalbumin, chymotrypsinogen, and ribonuclease A.

Ultracentrifugation — Sedimentation velocity measurements were made on the isolated carboxyhemoglobin components of F. heterocli-
tus using a Beckman model E ultracentrifuge. These studies were performed at initial protein concentrations between 0.001 g/ml and 0.01 g/ml in 0.1 m phosphate, pH 7.5, with 0.1 M NaCl, and the S20,w values were determined by extrapolation to zero protein concentration.

Oxygen Equilibria — Studies of oxygen equilibria were performed at several pH values by the method of Riggs and Wolbach (25) and the micro method described by Powers et al. (26) employing the Hem-O-Scan oxygen dissociation analyzer (American Instrument Co.). Phosphate and other ions were removed from the individual component samples by passage through an ion exchange column according to the method of Dintzis (27). The hemoglobin was concentr-
ed by pressure filtration, and each stripped hemoglobin was frozen in liquid nitrogen until oxygen equilibria studies were performed. During use, samples were kept on ice and appropriate buffers were added to give final concentrations of 0.1 to 4 mm hemoglobin (M, = 64,000). The pH of a 20-μl aliquot of each sample was then measured with a microcombination glass pH electrode (Microelectrodes, Inc.). The F. heteroclitus hemoglobin solutions were buffered with 0.1 M Tris or bis(2-hydroxyethyl)iminom-
itrile(hydroxymethyl)ethane in the pH range from 5.5 to 9.0 and were analyzed at 20°C. The human Hb A “standard” was analyzed at 21.5°C in 0.1 M Tris, 0.1 M NaCl, 1 mM EDTA, pH 7.4. A 5-μl aliquot of the human Hb A was placed on a radial immunodiffusion plate (18 mm number 2 thickness), and covered with an 18-mm circular piece of a silicone co-polymer membrane (General Electric Co.). The sample was tamped with the Aminco sample assembly and placed in the sample holder of the oxygen dissociation analyzer. After the sample had been equilibrated to the temperature and partial pres-
sure of the machine, the 100% oxygenation was set, assuming that the hemoglobin was fully saturated at the current barometric pressure. This assumption appears to be valid for these hemoglobins because the solutions were checked for full saturation spectrophoto-
metrically by the method of Riggs and Wolbach (25) at various pH
tual values, and full saturation was achieved for all hemoglobin
deviation of organic phosphates. However, at pH values less than pH 6.7 in the presence of ATP, full saturation of the hemoglobins was not achieved. Therefore, analyses of hemoglobins at lower pH values in the presence of ATP were not performed by the automatic method.

Once the 100% saturation level was determined, the sample was deoxygenated with high purity nitrogen (99.999%) and the 0%
ABSORPTION AT 540 nm

Fig. 1. Densitometer trace of a polyacrylamide gel following electrophoresis of a fresh hemolysate. The carboxyhemoglobin components are designated I, II, III, and IV in order of increasing mobility from the top of the gel (cathode) to the bottom (anode).
F. heteroclitus hemoglobin can be fractionated into as many as 4 components by ion exchange on DEAE-cellulose. Components II and III focused as wider bands than Hb I and Hb IV. The pI values in four isolated hemoglobin components gave single bands in starch gel electrophoresis, and that Hb consists of three different hemoglobins, II A, II B, and II C, as shown in Table I. Variations in the competing effects of increased buffer ionic strength and higher pH show that component II is separable into two fractions, II A and II B, which are also electrophoretically indistinguishable. Peak III B appears to contain still another component which is not separable under these conditions.

Characterization of Hemoglobin Components - Each of the 4 isolated hemoglobin components gave single bands in polyacrylamide gel isoelectric focusing. Components II and III focused as wider bands than Hb I and Hb IV. The pI values in the hemolysate as described under "Experimental Procedure."  

Table I reflect relatively large net charge differences between hemoglobins I and IV in comparison with those of the four hemoglobins of trout (38) and of the eight components of plaice (39). One of the hemolysates was stored at 0°C for 5 days, and the small amount of brown precipitate which had formed upon thawing was removed by centrifugation. When this sample was thus subjected to electrophoresis, it became apparent that, following storage, components I and II comprised less of the total hemoglobin (see Table I) than in the fresh hemolysate, suggesting that Hb I and Hb II are more susceptible to autooxidation and denaturation than components III and IV. 

Separation of Hemoglobin Components - Virtually all of the hemoglobin purified by gel filtration on Sephadex G-100 was recovered in a single peak which emerged at an elution volume characteristic of the tetramer. The fractionation of this hemoglobin into four components by ion exchange on DEAE-cellulose is illustrated in Fig. 2. Of 500 mg applied to the column, 379 mg were recovered, a yield of 76%. The average preparative yield of each of the components is given in Table I. The losses due to autooxidation and denaturation are greatest for Hb I and Hb II which together comprise only 44% of the total recovered. This result is in agreement with the observation of differential losses of components I and II following storage of the hemolysate. Preparative yields of component I as low as 5% (of the total Hb) have been obtained. Some loss of Hb III has also taken place as indicated by the increase in the proportion of Hb IV relative to Hb III.

Electrophoresis in Starch Gel - A comparison of the four isohemoglobins separated on DEAE-cellulose with those in the unfractionated mixture is shown in Fig. 3. In starch gel, component I corresponds to the cathodal band in the hemolysate, while the remaining components migrate in the anodal direction.

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The gel filtration studies in 0.1 M NaCl at low initial protein concentrations (e.g. 10^{-5} mol (heme)/liter) indicated that, while human Hb A significantly dissociated (apparent Mr = 52,000), the F. heteroclitus components remained essentially undissociated (apparent Mr = 64,000 for each component).

Sedimentation velocity measurements in 0.1 M NaCl were consistent with the gel filtration results. All of the carboxyhemoglobin components of F. heteroclitus had sedimentation coefficients characteristic of the tetrameric species (i.e. s_{20,w} = 4.4 S).
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I. EFFLUENT VOLUME, ml

FIG. 4. Separation of F. heteroclitus hemoglobin components by ion exchange chromatography on DEAE-cellulose. Components I and II were separated by the starting buffer, 20 mM Tris-HCl, pH 8.4. The increase in buffer salt concentration to 40 mM Tris-HCl, pH 8.4, fractionated component III into Hb III A and Hb III B. Component IV was eluted with 40 mM Tris-HCl, 0.1 M NaCl, pH 8.4. The isolated components were identified by starch gel electrophoresis as in Fig. 3.

II. EFFLUENT VOLUME, ml

FIG. 5. Separation of F. heteroclitus hemoglobin components by ion exchange chromatography on DEAE-cellulose. The starting buffer, 30 mM Tris-HCl, pH 6.6, fractionated Hb II into three components: II A, II B, and II C (see "Discussion"). Hemoglobins III and IV were separated using a linear NaCl gradient as in the initial separation (see Fig. 2). The isolated components were identified by starch gel electrophoresis as in Fig. 3.

Oxygen Equilibria—Fig. 6A shows the results of oxygen binding studies of unfraccionated Fundulus hemoglobin, stripped of organic phosphates, at pH 7.2 and pH 6.4. These data, which were obtained by manual gasometric methods (25), show that the unfraccionated hemoglobin has a Bohr effect similar in magnitude to that of other fish hemoglobins. In Fig. 6B, the $p_{50}$ of the hemoglobin devoid of ATP at pH 6.7 is essentially identical with that obtained by the automatic method (see Fig. 7). In addition, $p_{50}$ values for human Hb A, determined by the Hem-O-Scan, were consistently the same as those obtained under identical conditions by Mills et al. (38) according to the procedure of Imai et al. (39). These consistencies between the results obtained by different methods are especially important since there is no published comparative analysis to document the accuracy of the oxygen dissociation analyzer for hemoglobin solutions. A recent study has shown that the automatic method yields consistently reproducible data for solutions of hemoglobin from several fish species (40).

Fig. 6B also shows that ATP shifts the oxygen equilibrium curve. Increases in $p_{50}$ values in the presence of ATP were also observed using the Hem-O-Scan (see Fig. 7). An analysis of the effect of ATP on the oxygen equilibria at pH 7 indicates that all of the components (as well as the unfraccionated hemoglobin) are similarly affected by ATP. Although Hb I sometimes showed a reduced sensitivity to modulation by ATP, this result is apparently related to the relative structural instability of this component, and its tendency toward autooxidation.

The effect of pH on the $p_{50}$ of the unfraccionated hemoglobin and of the isolated components I, II, III, and IV is illustrated in Fig. 7. All of the components are similarly affected by pH. The data in Fig. 7 suggest that components III and IV have slightly higher oxygen affinity at pH values greater than 7.5. Although subtle differences may exist among the components, these differences in general appear to lie within the range of our experimental variation. A more detailed oxygen equilibrium analysis of the components at high pH and various salt conditions is required to further characterize these differences.

FIG. 6. Oxygen equilibria curves determined by the manual gasometric method of Riggs and Wolbach (25). A, stripped unfraccionated Hb. $\circ$, pH 7.2; $\bigcirc$, pH 6.4. B, both curves are at pH 6.7. $\bigcirc$, stripped Hb; $\blacksquare$, 60 $\mu$M ATP added.

FIG. 7. A plot of the variation of log $p_{50}$ with pH for F. heteroclitus hemoglobins. Data were obtained using the Amino-o Hem-O-Scan oxygen dissociation analyzer. Open symbols represent stripped Hb; solid symbols represent Hb in the presence of 10 ATP/Hb. $\bigcirc$ and $\blacksquare$, unfraccionated Hb; $\times$, Hb I; $\bigtriangleup$, Hb II; $\bigcirc$, Hb III; $\triangle$, Hb IV.
concentrations and temperatures is currently in progress. Unless such analyses conclusively demonstrate functional variation between components, we must tentatively conclude that there are no significant differences in oxygen equilibria among F. heteroclitus hemoglobins when stripped of organic phosphates.

Separation of Globin Chains — The separation of the globin chains of isohemoglobins I, II, III, and IV by ion exchange on CM-cellulose is shown in Fig. 8. The elution positions of the chains for the four hemoglobins are directly comparable since the conditions were identical for the four separations. In comparison to Hb II, component III appears to contain a greater proportion of chains 1 and 3 relative to 2 and 4. However, since the total yield of the globin from the column is low (approximately 50% by lyophilized weight), any conclusions drawn from the relative proportions of the four polypeptide chains must remain tentative. It was possible to separate only freshly prepared globin which had not been previously chromatographed.

Characterization of Globin Chains — The four polypeptide chains separated by ion exchange were each shown to migrate as single bands by isoelectric focusing in 8 M urea in polyacrylamide gels. While all four chains had pI values between 7.8 and 8.0, it was clear that there is a slight increase in the isoelectric points going from chain 1 to 4, as anticipated from their relative elution positions on CM-cellulose. These pI values are similar to that obtained for human Hb A globin (41).

The COOH-terminal sequence obtained by carboxypeptidase digestion and the NH₂-terminal amino acid determined by dansylation for each of the four chains are given in Table II. Quantitative results for the determination of the COOH-terminal sequences are presented in Table III. The same results were obtained for a particular chain regardless of which hemoglobin component was used for the chain separation. For example, the p⁺ globin has an NH₂-terminal valine and a COOH-terminal sequence of -tyrosylhistidine whether isolated from Hb II, Hb III, or Hb IV. By analogy with trout

![Image](https://example.com/image.png)

**Fig. 8.** Separation of the globin chains of the isolated hemoglobin components by ion exchange chromatography on CM-cellulose. After sample application, 100 ml of 0.2 N formic acid, 0.02 M pyridine were passed through the column prior to elution with a formic acid gradient as described. The four different polypeptide chains, p⁺, β⁺, α⁺, and α⁺⁻ were identified in each case by end group analyses (see Table II).
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... (36), carp (42), and catostomid (43, 44), chains 1 and 2 appear to be of the \( \beta \) variety and are designated \( \beta^a \) and \( \beta^b \), respectively, while chains 3 and 4 are \( \alpha^a \) and \( \alpha^b \). Hb I thus appears to be a homotetramer (i.e. a tetramer which yields identical dimers upon dissociation) consisting of \( \alpha^a \) and \( \beta^a \) chains, while Hb IV can be described as a homotetramer of \( \alpha^b \) and \( \beta^b \) chains. Components II and III each consist of both types of \( \alpha \) and \( \beta \) chains. The finding that both \( \beta \) chains have a COOH-terminal histidine residue is consistent with the presence of a Bohr effect for each of the components (45). The amino acid compositions of the globin chains are given in Table IV. The order of chain elution, \( \beta \)'s before \( \alpha \)'s, may be partially accounted for by the noticeably higher content of glutamic acid in the \( \beta \) chains and lysine in the \( \alpha \) chains. In carp (42, 46) and Rio Grande cichlid (47) the \( \beta \) chains elute from CM-cellulose before the \( \alpha \)'s in a similar system, and in both cases the \( \beta \)-globins contain more glutamic acid and the \( \alpha \)'s have a higher lysine content.

**Discussion**

Five years ago we showed that ATP, which decreases hemoglobin-oxygen affinity, is the major intraerythrocyte organic phosphate in *F. heteroclitus* (48). Moreover, we have shown that the regulation of hemoglobin function by ATP is tuned to the environmental needs of *F. heteroclitus* (48-50). Our oxygen equilibria studies indicate that the hemoglobin components are affected by pH and ATP. Moreover, Powers et al. (40) have shown that the oxygen equilibria of the unfracti- onated hemoglobins are affected by temperature. The killi- fish hemoglobins resemble those of the carp (42) in that there appear to be essentially no functional differences among the multiple hemoglobins. *F. heteroclitus* therefore falls into the Class I category of species (52) whose hemoglobins are sensitive to temperature and pH and are similar in function.

Contrary to what has been observed in many other fish, *F. heteroclitus* does not possess an isohemoglobin which repre- sents a "main component" (i.e. one comprising 50% or more of the total hemoglobin). The four electrophoretically distinguishable components are present (apparently in equilibrium) in symmetrical proportions (see Fig. 1 and Table I), suggesting that the constituent globin chains occur in comparable quantities and that there are no minor \( \alpha \) or \( \beta \) chains present in only a single, proportionally small component.

The globin chain separations reveal that among the hemo- globins there are four different polypeptides, two of the \( \alpha \) type (\( \alpha^a \) and \( \alpha^b \)) and two of the \( \beta \) type (\( \beta^a \) and \( \beta^b \)). Hb I consists of \( \alpha^a \) and \( \beta^a \) only, and Hb IV is composed of \( \alpha^b \) and \( \beta^b \) chains. Since components I and IV are tetrameric as shown by gel filtration, they can be represented as \( \alpha^a \beta^a \alpha^b \beta^b \) and \( \alpha^b \beta^b \alpha^a \beta^a \), respectively. In using this notation the assumption is made (54) that cleavage into dimers and reassociation into tetramers occurs along the interface between the \( \alpha \) and \( \beta \) subunits (and between \( \alpha \alpha \) and \( \beta \beta \)). That is, the equilibrium is of the following type:

\[
\alpha \beta + \alpha \beta \rightleftharpoons \alpha \beta \alpha \beta
\]

Dimers Tetramer

There are four different \( \alpha \beta \) (or \( \alpha \beta \)) dimers which can be formed from the four globin chains: \( \alpha^a \beta^a \), \( \alpha^a \beta^b \), \( \alpha^b \beta^a \), and \( \alpha^b \beta^b \), and these can associate to form ten different tetramers as shown in Fig. 9. Since the upper portion of the matrix contained hemoglobins identical with those in the lower section, the duplicate tetramers have been omitted. Therefore, with the exception of those at the top of each column, there are two ways to form each isohemoglobin.

In both polyacrylamide and starch gel electrophoresis there is no central band representing a hemoglobin with a charge intermediate between components II and III. This phenomenon, which is similar to that observed in the electrophoresis of sickle cell hemolysates (55, 56), suggests that there are limitations in the association of the hemoglobin subunits to form stable hybrid tetramers consisting of mixtures of the different globin chains. The model in Fig. 9 represents an attempt to reconcile, both qualitatively and quantitatively, the existence of 10 possible hemoglobins with the data presented in this study. If we assign relative charges of +1, +2, +3, and +4 to the subunits \( \beta^a \), \( \beta^b \), \( \alpha^a \), and \( \alpha^b \), respectively, according to their positions of elution from CM-cellulose, Hb I (\( \alpha^a \beta^a \alpha^b \beta^b \)) has a charge of +12 and Hb IV (\( \alpha^b \beta^b \alpha^a \beta^a \)) +8. This is consistent with their order of elution from DEAE-cellulose and their relative isoelectric points. Each of the four tetramers shown with **dotted lines** between the subunits in Fig. 9 would then have a charge of +10, i.e. intermediate between hemoglo-
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...if the absence of an electrophoretically intermediate hemoglobin band suggests that its theoretical constituents are relatively unstable, *i.e.* they are not detectable during the isolation of stable components.

Such limitations in the formation of “stable” (detectable) hybrid tetramers have been observed in several other hemoglobin systems. For example, when human $\alpha_1\beta_1\beta_2\gamma_2$ and canine $\alpha_C\beta_C\gamma_C\delta_C$ hemoglobins are mixed, hybridization results in only two observable tetramers—those containing identical dimers, $\alpha_1\beta_1\beta_2\gamma_2$ and $\alpha_C\beta_C\gamma_C\delta_C$, in addition to the original hemoglobins (56–58). Guidotti has maintained that electrophoretically intermediate $\alpha_1\beta_1\alpha_C\beta_C\gamma_C\delta_C$ hybrids will be formed but will be impossible to detect due to the rapid attainment of equilibrium with the original homotetramers.

$$2(\alpha_1\beta_1\gamma_2\delta_2) \Leftrightarrow \alpha_1\beta_1\gamma_2\delta_2 + \alpha_1^*\gamma_2^*\delta_2^*$$

during the comparatively slow process of separation (56).

The unstable $\alpha_1\beta_1\alpha_C\beta_C\gamma_C\delta_C$ hybrid is analogous to the $\alpha_1\beta_1\alpha_2\beta_2$ tetramer of *F. heteroclitus* hemoglobins (see Fig. 9). We might therefore expect this tetramer to be unstable and, following dissociation, to re-form the homotetramers $Hb$ I and $Hb$ IV as follows:

$$2(\alpha_1\beta_1\gamma_2\delta_2) \Leftrightarrow \alpha_1\beta_1\gamma_2\delta_2 + \alpha_1^*\gamma_2^*\delta_2^*$$

The $\alpha_1\beta_1$ and $\alpha_2\beta_2$ contacts in the unstable tetramer are both of the $a-b$ variety (*i.e.*, $\alpha_1\beta_1\gamma_2\delta_2$ and $\alpha_1^*\gamma_2^*\delta_2^*$). If $a-b$ contacts are less stable than the $a-a$ ($\alpha_1\beta_1\gamma_2\delta_2$ and $\alpha_1^*\gamma_2^*\delta_2^*$) and $b-b$ ($\alpha_2\beta_2\gamma_2\delta_2$ and $\alpha_2^*\gamma_2^*\delta_2^*$) types, then the two hemoglobins at the top of the columns (see Fig. 9) which also contain double $a-b$ contacts (*e.g.*, $\alpha_1\beta_1\gamma_2\delta_2$ and $\alpha_1^*\gamma_2^*\delta_2^*$) are likewise unstable. It is likely that the other hybrid hemoglobin (in the center of the matrix in Fig. 9) which contains four different polypeptide chains, $\alpha_1^\delta_1^\gamma_2^2\delta_2^2$, is also unstable. We suggest that these four hemoglobins, which would be expected to have intermediate charge (59) and migrate electrophoretically midway between components II and III are unstable, and are therefore depicted with dotted lines between the subunits in Fig. 9.

If the assumption of the instability of tetramers with double $a-b$ contacts between dimers is true, the proposed model predicts that there are six stable isohemoglobins and that components II and III each consist of two hemoglobins of nearly identical relative net charge. This aspect of the model is supported by the following observations. 1) Isoelectric focusing of components II and III results in wider bands than those obtained for hemoglobins I and IV. 2) Fig. 4 shows the separation of $Hb$ III into two components, $Hb$ III A and $Hb$ III B, which are electrophoretically indistinguishable. Sedimentation velocity analysis has shown that they are tetrameric species, and therefore must be of different subunit compositions. 3) In Fig. 5 we see that component II is also not a single isohemoglobin, but is separable into hemoglobins II A, II B, and II C. While it is possible that $Hb$ II C is a stable component, it is more likely a mixture of the dimers $\alpha_1^*\beta_2^*$, $\alpha_2^*\beta_1^*$, and $\alpha_1^*\beta_2^*$ generated through interaction of the tetramers II A and II B with the ion exchange resin, and in equilibrium with one or more of the unstable tetramers (and II A and II B). Accordingly, $Hb$ II C has been shown to electrophoretically co-migrate with II A and II B in starch gel. By this reasoning, hemoglobins III A and III B should be capable of generating a similar mixture of dimers (or unstable tetramers); these may be present in the shoulder of peak III B in Fig. 4. 4) Since the electrophoresis and ion exchange chromatography separations were performed at hemoglobin concentrations which are sufficiently high to preclude any significant dissociation, the proportions of the six components (I, II A, II B, III A, III B, and IV) would theoretically be 1:2:2:2:2:1, which corresponds to 1:4:4:1 for the four electrophoretically distinguishable hemoglobins. These proportions are indeed observed for the electrophoresis of a fresh hemolysate in polyacrylamide gel (see Fig. 1 and Table I).

In the model in Fig. 9 these components, which are both mixtures of isohemoglobins, have simply been designated “$Hb$ II” and “$Hb$ III” due to the uncertainty of the actual identities of hemoglobins II A, II B, III A, and III B. That hemoglobins II and III are most likely the tetramers shown is also supported by a recent series of experiments in which Bernstein and Bowman, using electrophoresis under anaerobic conditions at very low ionic strength, demonstrated the existence of the asymmetric hybrid tetramers $\alpha_1^*\beta_2^*$ and $\alpha_2^*\beta_1^*$ when blood from individuals with both $\alpha$ and $\beta$ chain variants were examined (61). Wilkins (15) has shown that several of the anodal hemoglobins of the Atlantic salmon are hybrids of the $\alpha_1^*\beta_2^*$ variety. *Fundulus* hemoglobins II and III appear to be mixtures of asymmetric hybrids of this type.

While the model in Fig. 9 is presented as one that is consistent with the data, we recognize that others may be proposed to account for some or all of the phenomena observed in these studies. Additional experiments designed to test the accuracy of this model are presently underway. For example, the use of a cross-linking agent such as p,p'-difluoro-m- m'-dinitrodiphenylsulfone, as employed to demonstrate the sickle cell hybrid hemoglobin (62), we expect to obtain an electrophoretically intermediate band representing the unstable tetramers which are not detectable using current separation techniques.

In comparison with mammalian hemoglobins, unusually stable tetramers, as indicated by tetramer-dimer dissociation constants, may be a widespread characteristic of fish hemoglobins (63). However, there are strong indications that the hemoglobins of lower animals, including fish, autooxidize more rapidly than the corresponding mammalian proteins (64). This is consistent with our observations of *F. heteroclitus* hemoglobin. It is interesting to note that with storage or during separation on DEAE-cellulose, hemoglobins I and II in particular undergo autooxidation and/or denaturation at a greater rate, a phenomenon which may be reflected in higher dissociation constants for these components. Hemoglobins I, II A, and II B each contain the $\alpha_1^*\beta_2^*$ dimer which may be unusually susceptible to autoxidation.

When the isolated components are kept at 4°C for a week or longer, starch gel electrophoresis has shown that hemoglobins II (A + B) and III (A + B) each generate all four components (65). This is due to the fact that all four different globin chains are present in both $Hb$ II and $Hb$ III. As expected, components I and IV do not form any of the other hemoglobins. These hybridizations are similar to those observed for isolated cichlid hemoglobins (47). Additional studies of the tetramer-dimer-monomer equilibria are currently being conducted.

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