HEMOGLOBIN HETEROGENEITY IN TWO SPECIES OF COMMERCIAL FISH, THE CROAKER AND SPOT

A Thesis

Presented to

the Faculty of the Department of Biology

University of Houston

in Partial Fulfillment

of the Requirements for the Degree

Master of Science

by

Richard Michael Gersberg

August 1968

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ABSTRACT

The croaker (Micropogon undulatus) and spot (Leiostomus xanthurus) are closely related to each other and commercially utilized as industrial bottom fish. This study was begun to determine whether these species exist as genetically different and reproductively isolated subpopulations or as one freely interbreeding population. Polyacrylamide gel electrophoresis of CO-hemoglobin (CO-Hb) from fifty individaul croakers reveal three phenotypes occur in Galveston Bay. Patterns of croaker CO-Hb show multiple bands, whereas those of spot CO-Hb show no individual variation and only two components. Sedimentation analysis of croaker CO-Hb shows one component ($s_{20,w}$=4.3S) and indication of dissociation in presence of acid, base, and strong salts. Globin prepared by acid-acetone precipitation and fractionated on CM-cellulose with urea-mercaptoethanol-phosphate buffer gave three or four fractions. Resolution is poor and a better fractionation procedure is required to separate globins of croaker. These and other results indicate that in croaker genetic polymorphism exists, and at least three sub-populations occur.

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INTRODUCTION

In trying to understand the abundance, distribution, or migration of a species of fish, it is essential that breeding units within the species be identified, since subpopulations form the basic genetic units with which one must deal. A subpopulation is defined as that fraction of a population that is itself genetically self-sustaining. Hereditable differences between subpopulations show that barriers to gene flow exist between them (Marr, 1957).

The Atlantic croaker (Micropogon undulatus Linnaeus) and spot (Leiostomus xanthurus Lacépède) of the family Sciaenidae, are marine bottom fishes living in the shallow waters of the Gulf of Mexico. These fishes are relatively abundant, closely related to each other, and commercially utilized as industrial bottom fish. However, their commercial utilization, especially as a source of food, is only slightly realized today. Approximately 4/5 of a trawler's catch is discarded as scrap fish, because a suitable market is not available. This amounts to about 28,000 tons a year of discarded resource. This study was begun to determine whether these species exist as genetically different and reproductively isolated subpopulations, or whether they exist as one freely interbreeding population.

Morphological characteristics have frequently been used in an effort to distinguish subpopulations. An important limitation to this type of study is that these characters are influenced by both environmental and biological factors, and difficult to quantitate. They exhibit variation depend-

ing upon age, season, and hydrographical conditions of habitat. If morphological characteristics are used, the risk exists of studying the effects of environment rather than the effects of genetic isolation, and it is impossible to tell whether we are dealing with genetically different subpopulations or rather with environmentally determined modifications of one freely interbreeding population.

One of the best ways to determine the population structure of a species is through the investigation of biochemical characters such as blood groups or hemoglobin types, that are genetically determined, easily quantitated, and unaffected by environmental factors. Genetically isolated subpopulations where allelic genes are in Hardy-Weinberg equilibria are shown to have frequencies that differ among the subpopulations concerned (Cushing, 1964).

The initial work with biochemical characters and their relation to subpopulation studies of fishes were based upon blood types (Cushing, 1964). Blood types are under genetic control, and gene frequency data may be used to determine whether a single species of a marine organism is subdivided into reproductively isolated subpopulations. The disadvantages of this approach are the difficulty of keeping blood cells, and the lack of good reagents to distinguish among the various fish blood groups. The value of this work, however, was to show the great promise of this approach in studying population structure.

Although most homologous proteins of individuals of a given species have very similar electrophoretic properties, others may show enough individual variation to allow individuals to be identified. With current high resolution techniques (starch gel, acrylamide gel electrophoresis) variation between blood proteins of individuals has been demonstrated in many vertebrate species. Electrophoretically distinct haptoglobins, lipoproteins, phosphatases, esterases, transferrins, and albumins have been observed in single populations of vertebrate species (Leone,. 1962).

Polymorphism has been defined as the occurrence together in the same environment of two or more discontinuous forms of a species in proportions such that the rarest of them cannot be maintained by recurrent mutation (Ford, 1965). Genetic polymorphism is a type of variation in which individuals with sharply distinct qualities co-exist as normal members of a population, and at the molecular level is expressed as protein polymorphism. Using such polymorphic characters, subpopulations can be distinguished. The control of polymorphic phases is almost always genetic in nature, polymorphic characters will occur with different frequencies in separate subpopulations.

The existence of a polymorphic character in a population, means that is must have possessed some selective advantage to the organism in order to have reached a frequency greater than that attainable merely by mutation pressure. A gene

which controls a polymorphic phase therefore must preserve an overall advantage, and through selection, it will spread through the population until the former "normal" allele is reduced to the status of a rare mutant. Ford (1965) defines this process as a transient polymorphism which ends when the original allele no longer exists in the population.

A permanent diversity cannot arise as a result of a transient polymorphism. It can only occur when there exists a dynamic balance such that a gene which controls a polymorphic character possesses a selective advantage when rare; however this same gene possesses a selective disadvantage when it becomes more frequent. In this manner a balanced polymorphism is maintained and diversity remains in the population. The most general mechanism for maintaining such a condition is when the heterozygote possesses more advantage than both homozygotes, and thus both polymorphic characters in the population are maintained.

The hemoglobin system is a classical example of polymorphism at the molecular level. Hemoglobin has been found to exhibit extensive intraspecific variation in vertebrates. In humans, in addition to normal adult hemoglobin, Hb A, certain individuals possess Hb S, or sickle cell hemoglobin. Hb S is composed of normal α chains, with β^S chains having one amino acid difference from the normal β^A. An additional polymorphic hemoglobin, Hb C, has been detected which also contains variant β chains. It has been found that β^A, β^S

and β^C are multiple alleles at the β locus and therefore constitute genetically polymorphic characters (Ingram, 1963).

Barnicot and Jolly (1966) have shown four different hemoglobin phenotypes in the orangutan. In addition to the presumed "normal" major hemoglobin A ($\alpha_2^A \beta_2^A$), the orangutan hemoglobin B contains variant alpha chains, α^B, and hemoglobin C contains variant beta chains, β^C. Doubly heterozygous individuals demonstrate hemoglobin D, composed of the two "variant" polypeptide chains. Hemoglobin of the chimpanzee, monkey, and gibbon has also been found to evidence polymorphism (Hoffman, 1967; Crawford, 1966).

The hemoglobins A and B of domestic sheep described by Harris and Warren (1955) form an electrophoretically distinguishable genetic polymorphism. The occurrence of two types of bovine hemoglobins A and B was observed by Cabannes and Serain (1955a). As in sheep, these hemoglobins are probably the products of identical α genes and allelic β genes (Cabannes and Serain, 1955b).

In the deer, polymorphic hemoglobins I, II, III and V can be demonstrated by electrophoresis. Multiple differences in the β chains that are the products of a single pair of alleles account for the structural differences in the polymorphic hemoglobins III and V of the deer, and preliminary studies indicate that the polymorphic deer hemoglobins other than III and V indicate differences in the β chain structure (Kitchen et al., 1967).

Hemoglobin polymorphism has also been demonstrated in reptiles (Manwell, 1966a), and in amphibians (Manwell, 1966b); however, the genetic mechanism underlying this polymorphism is not as yet known.

Electrophoresis of hemolysates from the primative hagfish reveal five hemoglobin phenotypes. It is postulated that the monomeric hemoglobin chain of the hag fish is controlled by genes at four loci. A four band pattern is then interpreted to show homozygosity at each of the four loci for monomeric hemoglobin. Variants at two of these loci have been detected in some of these individuals revealing a genetic polymorphism (Ohno and Morrison, 1966).

Complex heterogeneity of hemoglobin in many species of fish has been demonstrated by electrophoresis. However if these multiple hemoglobins appear in all individuals of a particular species, these multiple components are most probably the expression of independent, non-allelic gene loci, and not the products of polymorphic genes. We are interested in polymorphic hemoglobins, because these are controlled by allelic genes and as such will differ between genetically isolated subpopulations.

Zone electrophoresis of fish hemoglobin has been reported by Buhler and Shanks (1959), Chandrasekhar (1959), Manwell *et al.* (1963), and Tsuyuki (1965). An exceedingly complex heterogeneity has been demonstrated in most species, with as many as sixteen hemoglobin components in an indivi-

dual. In these studies no intraspecific variation of hemoglobin patterns was reported. In view of the very complex electrophoretic pattern of fish hemoglobin, a genetic polymorphism within a species may not easily be interpreted, and an investigation of the structure of each component is needed in order to resolve the genetic mechanism of the polymorphism. Indeed, in the few cases of hemoglobin polymorphism reported for fish no complex heterogeneity was found, and, as a result, the genetics of the polymorphism could be more easily elucidated.

Sick (1961) has found hemoglobin polymorphism in two species of fish, namely whiting and cod. In both of these species the situation is similar to the hemoglobin polymorphism described in man, cattle, and sheep. Sick presents a genetic hypothesis involving two alleles to explain the genetics of the hemoglobin variation in these two species. According to this hypothesis, two co-dominant allelic genes Hb I^1 and Hb I^2 determine the hemoglobin components Hb I-1, and Hb I-2 respectively. These allelic genes probably control one pair of polypeptide chains in the hemoglobin tetramer, while another pair of allelic genes control the other pair of chains, in analogy with the α and β chain loci of human hemoglobin. The Hb I^1 and Hb I^2 genes are polymorphic as are the β^A and β^S in humans. Type Hb I-1 and Hb I-2 are considered as homozygous for the corresponding allele, and type Hb I 1-2 would represent the heterozygote. Pheno-

types corresponding to these three cases were identified by agar gel electrophoresis. In addition to cod and whiting distinct intraspecific variation in hemoglobin has been observed in sole and eel pout (Sick, 1961).

Further hemoglobin studies on cod populations by Sick (1965) have shown that the cod in the Belt Sea and Western Baltic is genetically different from the cod that lives in the eastern part of the Baltic. Both populations are polymorphic for the hemoglobin alleles Hb I^1 and Hb I^2, but with radically different gene frequencies.

These results indicate that hemoglobin polymorphism is a rather common occurrence in fish, and that the zone electrophoresis of hemoglobins is a valuable tool to analyze intraspecific variation and population structure. These results found for other fish suggested that it would be worthwhile to analyze the hemoglobins of croaker and spot to differentiate genetic isolates within a species. This approach requires first and most fundamentally, determining whether or not intraspecific hemoglobin variation does exist. Secondly, determining whether a genetic hypothesis can be adopted to explain such variation. This entails knowledge of the genetic mechanism that controls the formation of the hemoglobin tetramer. Thirdly, once such mechanisms are elucidated, can subpopulations be distinguished on the basis of an analysis of gene frequencies.

The present study on these two species of fish will show

that intraspecific hemoglobin variation does exist in the croaker, and will attempt to determine the genetic mechanism controlling such variation.

METHODS AND MATERIALS

Fish were bled from the caudal artery by severing the tail, and in the case of larger fish, by cardiac puncture. All blood was kept cold (4° C) from time of collection and no anti-coagulant was used.

The erythrocytes were washed three times with 1% saline, with little or no noticeable hemolysis, and after each washing the cells were packed by low speed centrifugation for thirty minutes. Erythrocytes were hemolyzed by adding approximately six volumes of cold de-ionized water to one volume of packed red cells. After fifteen minutes in the cold, hemolysis was complete. Cell stroma and nuclei were removed by centrifugation. The clear supernatant hemoglobin solution thus obtained was converted to carbon-monoxy hemoglobin (CO-Hb) by saturation of the hemolysate with pure carbon monoxide gas (Matheson, Coleman & Bell). The carbon monoxide derivative of fish hemoglobin is much more stable than the oxyhemoglobin form. The carbon-monoxy hemoglobin was dialyzed overnight either against CO-saturated phosphate buffer (0.81M K_2HPO_4, 1.18M KH_2PO_4, 0.05 ionic strength, 0.1M KCl, pH 6.85) for electrophoretic analysis, or Tris-HCl buffer (0.05M Tris-HCl, pH 8.6) for chromatographic analysis. All solutions were clarified by low speed centrifugation and saturated with CO gas once again. Other than bleeding, all preparative steps were carried out at 4° C. All electrophoretic, chromatographic, and sedimentation analyses of the hemoglobins were carried out within three days after collec-

tion of the blood samples. Sick (1965) points out that unlike mammalian hemoglobins, fish hemoglobins cannot be stored in the freezer even after converting to CO-hemoglobin. However, it was found that the hemoglobin of the croaker and the spot could be kept at 4° C for up to 14 days without detectable changes.

Nitrogen content of hemoglobin in solution was determined with a Coleman Model 29 nitrogen analyzer. This is an automated instrument for the rapid determination of the nitrogen content of materials. Samples of hemoglobin of unknown concentration were placed in aluminum boats and dried to a constant weight in a vacuum oven at 70° C. These samples were then placed in a combustion tube, and following combustion, total nitrogen evolved was measured. Concentrations of the samples were then estimated using the standard value of 6.25 as the conversion factor from nitrogen to protein.

The absorbance of samples of hemoglobin of known nitrogen concentration was then determined using a Beckman DB-G spectrophotometer, standard 1cm cuvettes, at 574 mu. This wavelength corresponds to the maximum absorbance of the band in the visible range for fish CO-hemoglobin. While fish CO-hemoglobin shows a maximum at 574 mu, oxyhemoglobin shows a maximum absorbance at 578 mu. Using the following equation:

$$O.D. = \varepsilon_{sp} Cl, \text{ where } \varepsilon_{sp} = \text{specific extinction coeffient}$$

1 = path length of cuvette
C = concentration in gms/liter
O.D. = absorbance at 574 mu

The extinction coefficient of carbon-monoxy hemoglobin at 574 mu was found to be .068 at a concentration of 10gm/liter. Concentrations of CO-hemoglobin solutions thereafter were determined by measuring absorbance at 574 mu.

Polyacrylamide gel electrophoresis was performed on individual CO-hemoglobin samples using the method of Davis (1963). The only modifications of the procedure of Davis are explained below. A sample gel was not used. Instead the sample was carefully layered, utilizing a micro capillary tube, above the spacer gel, and Tris-glycine buffer was carefully layered above the sample. The current was adjusted to 2 milliamps per tube during the initial 30 minutes of electrophoresis. After the sample entered the spacer gel the current was increased to 4 milliamps per tube. Electrophoresis was continued until the Bromphenol Blue marker dye migrated to approximately 5 mm from the end of the small pore gel. Destaining was carried out overnight at a current of 5 milliamps per tube.

The volume of sample added to each tube depended upon the concentration of the particular sample and was adjusted so that the total amount of protein applied was approximately 200 ugms for all samples.

In the Tris-glycine system previously described, in which the running pH is 9.5, most hemoglobin components

migrate as anions. Any cationic components either will not migrate or will migrate backwards in a large zone and be lost in the resevoir. Therefore to determine whether any such cationic components exist in the fish hemoglobins, an acidic system was tried using the procedure of Reisfeld et al. (1962), in which cationic proteins migrate at a running pH of 4.0. However it was found that at this low pH denaturation of fish hemoglobin occurs, as indicated by a change in color of the hemoglobin solution from red to reddish brown and decrease in the $s_{20,w}$ value. Such denaturation results in the formation of a diffuse staining band of hemoglobin near the origin, rather than the distinct hemoglobin bands observed at a higher value of pH.

As the pH 4.0 buffer system is not suitable for the electrophoretic analysis of hemoglobin, a single system allowing the simultaneous separation of oppositely charged proteins was used according to the procedure of Racusen (1967). In this procedure two discontiuous buffer systems (pH 7.5 & pH 8.3) provide concentrating and running conditions for both anionic and cationic proteins in the same sample. No cationic proteins were observed in this system. However, a cationic component was detected in cellulose acetate electrophoresis in Barbital buffer, 0.1 ionic strength, pH 8.6 (See discussion).

After chromatographic analysis, the various hemoglobin components that were separated and eluted were relatively

dilute (.02-.1% protein). Therefore the Tris-glycine electrophoretic procedure was modified as suggested by Davis (1963). A more concentrated large pore solution was used, and a sample gel was prepared by dilution with a protein sample. The volume of the sample gel was increased so that the total amount of protein was approximately 200 ugms.

Diethylaminoethyl (DEAE) cellulose, first used to separate hemoglobins by Huisman and Dozy (1962), was used for column chromatographic fractionation of individual hemoglobins. DEAE-cellulose, Bio-Rad Cellex-D, control no. 4727, capacity 0.65 meq/gm, (Calbiochem) was prepared according to the procedure of Huisman and Dozy (1965), and equilibrated with .05 M Tris-HCl buffer, pH 8.6. Columns 60cm x 0.9cm were used for analytical purposes. The anion exchanger was packed under 15 lbs air pressure until a final height of 38cm was reached. Prior to use the column was equilibrated at room temperature with the Tris-HCl buffer for 12 hours at 10 mls/hr. Carbon-monoxy hemoglobin solutions of individual specimens were dialyzed overnight at 4°C against the starting buffer (0.05M Tris-HCl, pH 8.6). The total amount of hemoglobin applied to the column varied, but approximately 10 mg were applied in a volume of 1-2 mls. The hemoglobin was pumped carefully into the column with the use of a Buchler polystaltic pump. Elution of hemoglobin fractions was obtained with an increasing linear Cl^- ion gradient, made by mixing 125 mls of starting buffer with 125 mls of a buffer

consisting of 2M NaCl, 0.05M Tris-HCl, pH 8.6, in a gradient device described by Bock and Ling (1954). All buffers were saturated with carbon monoxide before use. The flow rate of the column was maintained at 18 mls/hr by adjusting the speed of the pump. The effluent was monitored continuously at 415 mu, using a Beckman DB-G spectrophotometer. Percent transmission was recorded on a Beckman 10" recorder. The chromatographic experiments were carried out at room temperature (20-22° C). Fractions of 2 mls were collected and further analyzed by polyacrylamide gel electrophoresis.

Globin chains were prepared by acid-acetone precipitation according to the method of Anson and Mirsky (1930). Separation of the chains by CM-cellulose column chromatography with urea-mercaptoethanol-phosphate buffer was performed as described by Clegg et al. (1965). Globin was prepared only from pooled samples of croaker CO-hemoglobin.

Sedimentation analysis was made with Spinco Model E analytical ultracentrifuge equipped with Schlieren optics and temperature control. Samples of CO-hemoglobin were dialyzed overnight against the appropriate buffer prior to analysis. Samples were dialyzed against phosphate buffer (KH_2PO_4, K_2HPO_4, 0.05 ionic strength, o.1M KCl, pH 6.85) for determination of sedimentation coefficients of CO-hemoglobin. Prior examination of alkaline denaturation of fish hemoglobin as a function of pH indicated that complete denaturation was accomplished at pH 11.4. Therefore, before sedimentation

analysis of alkaline denaturation of hemoglobin, samples were dialyzed overnight against Glycine-NaOH buffer (0.1 ionic strength, pH 11.4). In the study of the sedimentation behavior hemoglobin at acid pH, samples were dialyzed overnight against Acetate buffer (0.1 ionic strength, pH 4.8). In the sedimentation study of the effects of high salt concentration on croaker hemoglobin, the hemoglobin samples were dialyzed overnight against a 2M NaCl-phosphate buffer at pH 6.85.

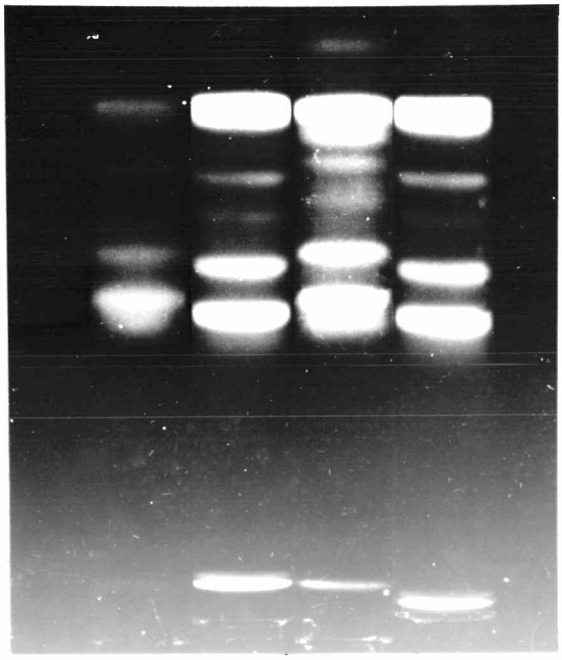
Spectrophotometric analyses of alkaline denaturation of croaker hemoglobin solutions were performed on individual CO-hemoglobin samples which were diluted 1:30 with Glycine-NaOH buffer, pH 11.4, giving a final pH of 11.2 for the solutions. The denaturation process was accompanied by marked changes in the visible absorption spectra as a function of time.

Polyacrylamide gel electrophoresis of hemoglobin of the croaker (Micropogon undulatus) revealed three phenotypes. Examples of these three phenotypes are shown by actual photographs of the gels in Figure 1, and labelled A, B, and C. Shown in Figure 2 is the electrophoretic pattern of hemoglobin of the spot (Leiostomus xanthurus), and the pattern of normal adult human hemoglobin, along with three representative patterns of phenotypes A, B, and C of the croaker.

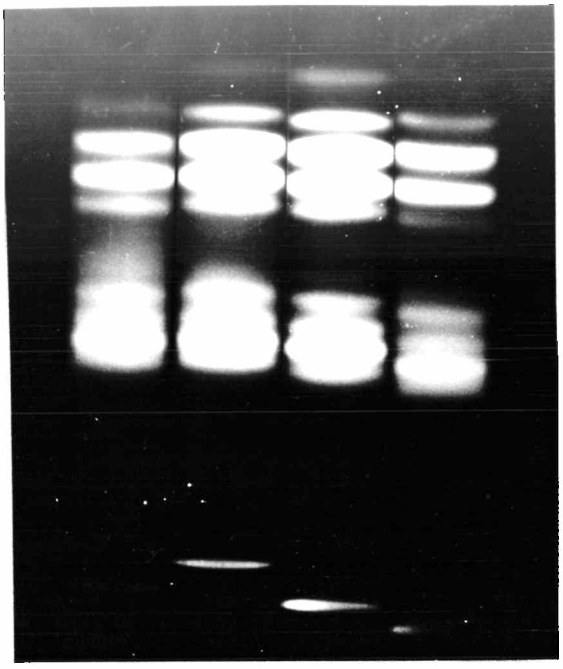
Of the 50 individual croakers analyzed (all from Galveston Bay, Texas), 5 individuals demonstrated phenotype A, 14 demonstrated phenotype B, and 31 individuals demonstrated phenotype C. No other patterns besides these were evidenced. Hemoglobin from the spot demonstrated only one major electrophoretic component, and one minor band close enough to the major component so as to be masked by the dense staining of the major band. Normal adult human hemoglobin, shows the predominance of Hb A and a few minor components in agreement with the results of Huisman (1960) using starch gel electrophoresis.

The croaker shows extreme complexity in its hemoglobin pattern, a property which has been found in other fishes. In order to be assured that all components stained with the non-specific amido-black were indeed hemoglobin components, a sample of hemoglobin was applied to three gels. After electrophoresis the natural red color of the hemoglobin components were compared to gels stained with non-specific amido black

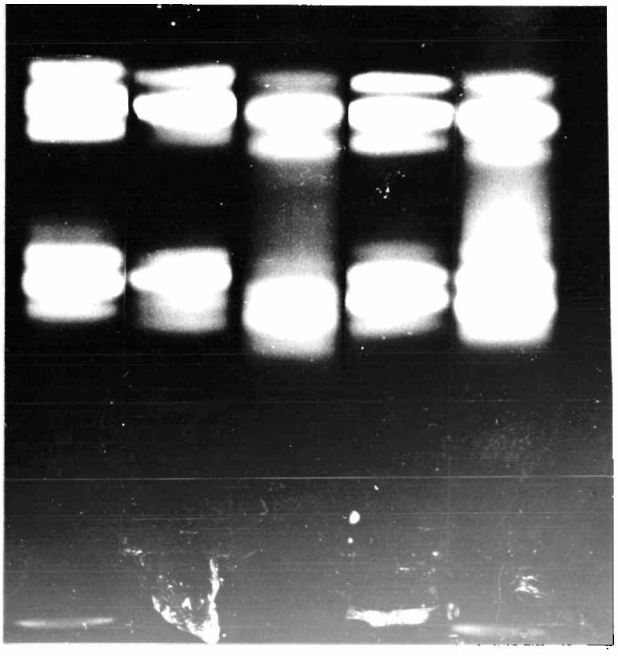
Figure 1. Representative polyacrylamide gel electrophoretic patterns of phenotypes A, B, and C of croaker hemoglobin.



A

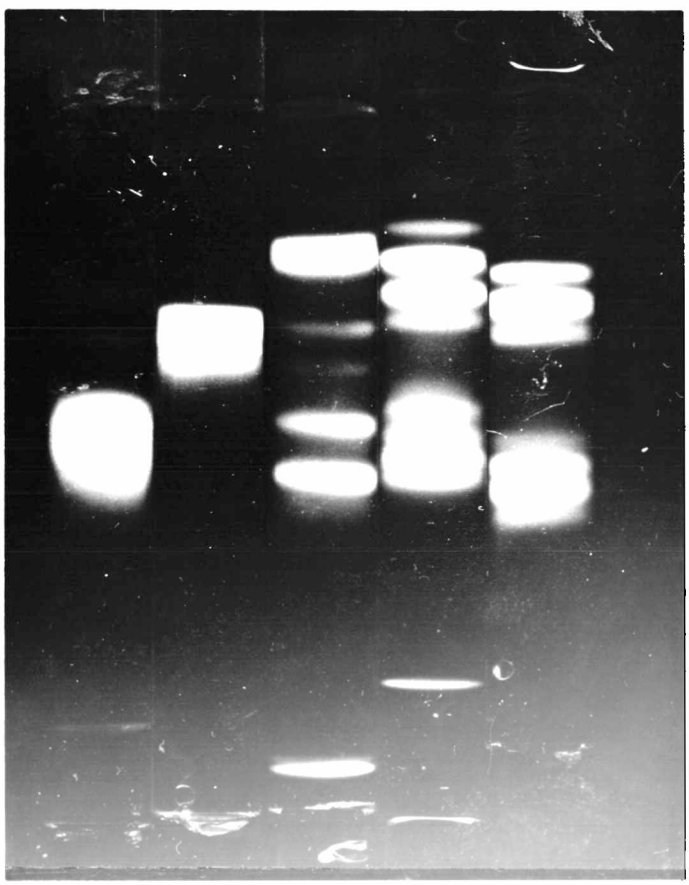


B



C

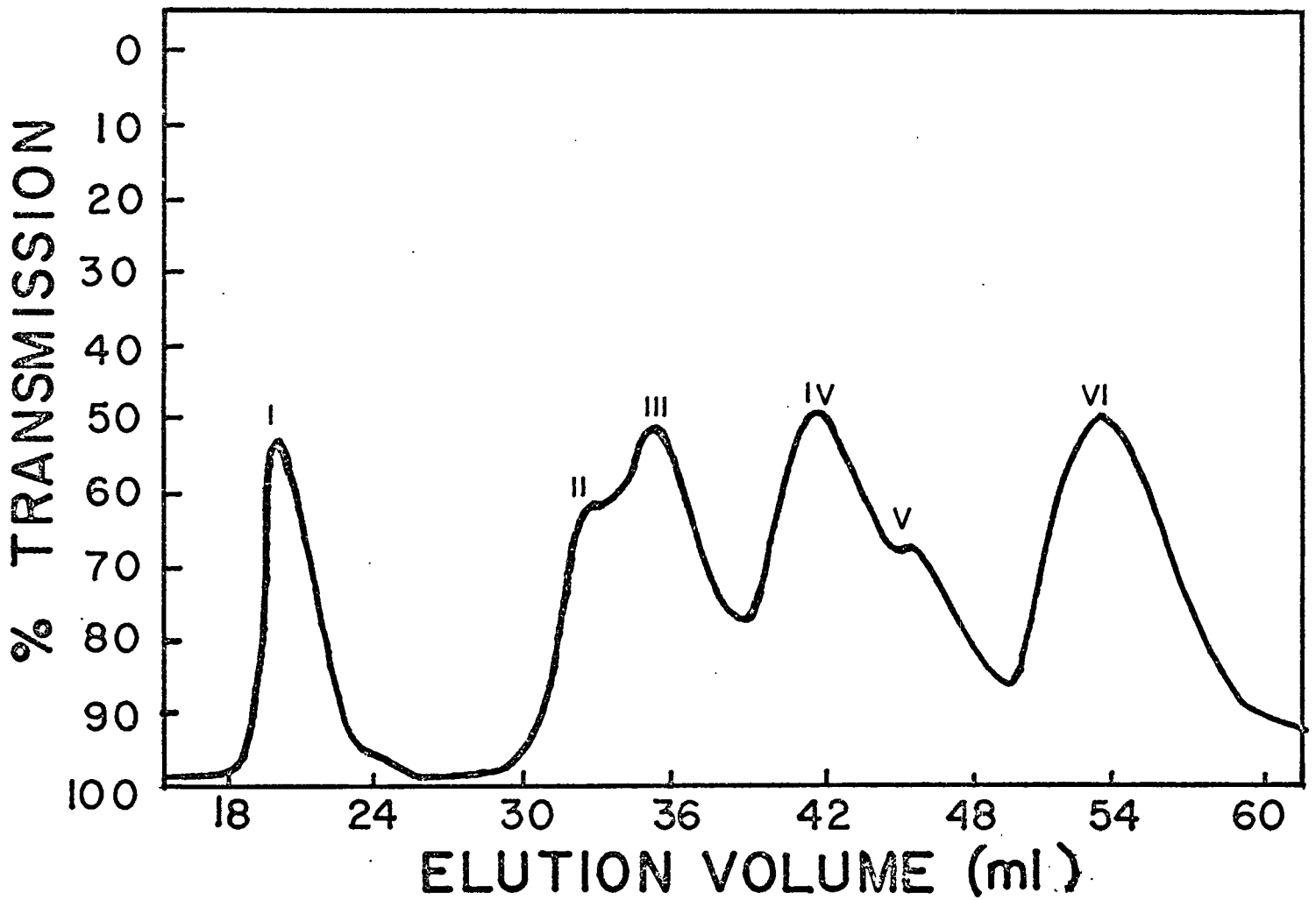
Figure 2. Polyacrylamide gel electrophoretic patterns shown left to right are normal human adult hemoglobin, spot hemoglobin, and phenotypes A, B, and C of croaker hemoglobin.



and gels stained with benzidine specific for hemoglobin. The hemoglobin zones from all three gels corresponded well to each other, so that it appears that minor components, such as serum constituents which remain in spite of repeated washing of the red cells, or erythrocytic components do not account for the zones which are stained by amido black. Comparison of patterns of CO-hemoglobin with those of oxyhemoglobin does not indicate any differences in the electrophoetic patterns. The possibility that some of these zones might be aggregates may be ruled out, because ultracentrifugal studies to be discussed later on, indicate a uniformity in molecular weight. In addition, spot hemoglobin, under the same conditions as croaker hemoglobin during preparation and electrophoresis, shows a consistent homogenous pattern. In fact, spot hemoglobin serves as a good control when examining croaker hemoglobin by various techniques. Also normal adult human hemoglobin showed the normal components indicated in previous electrophoretic separations in other laboratories.

In order to further examine the heterogeneity of the individual hemoglobins and to separate the various hemoglobin components of an individual sample of croaker hemoglobin, column chromatography on DEAE-cellulose was carried out. A relatively close relationship between heterogeneity demonstratable by acrylamide gel electrophoresis and by column chromatographic procedures was evidenced. Figure 3 shows

Figure 3. Elution profile on DEAE-cellulose of croaker hemoglobin demonstrating phenotype B.


% TRANSMISSION
0
10
20
30
40
50
60
70
80
90
100
I
II
III
IV
V
VI
18
24
30
36
42
48
54
60
ELUTION VOLUME (ml)

the resulting elution profile for croaker hemoglobin demonstrating phenotype B, and Figure 4, for croaker hemoglobin demonstrating phenotype C. Because of the lack of sufficient samples of phenotype A, chromatographic analysis of this phenotype was not carried out. Figure 5 shows the elution profile of the hemoglobin of the spot, demonstrating chromatographic homogeneity. In the case of hemoglobin phenotype B, six peaks were resolved. These peaks are labelled I-VI, and the electrophoretic properties of the hemoglobin fractions contained in these peaks are shown schematically in Figure 6a. The elution profile of hemoglobin of phenotype C, demonstrates 5 peaks (I-V), and the electrophoretic patterns of fractions collected from these peaks are diagrammed schematically in Figure 6b. These figures show that the hemoglobins were eluted as predicted by their electrophoretic properties. However, the first major fraction eluted in both the chromatography of phenotype A and phenotype B could not be detected on acrylamide gel electrophoresis. Previous analyses of these phenotypes by both acrylamide gel electrophoresis (Tris-glycine system) and the double gel technique were unable to detect the presence of cationic components. However, analysis of individual hemoglobins by cellulose acetate electrophoresis using Barbital buffer (pH 8.6, 0.1 ionic strength) demonstrated a single component migrating as a cation while all other components migrated as anions. This single cationic component might be the same component which is eluted at the

Figure 4. Elution profile on DEAE-cellulose of croaker hemoglobin demonstrating phenotype C.

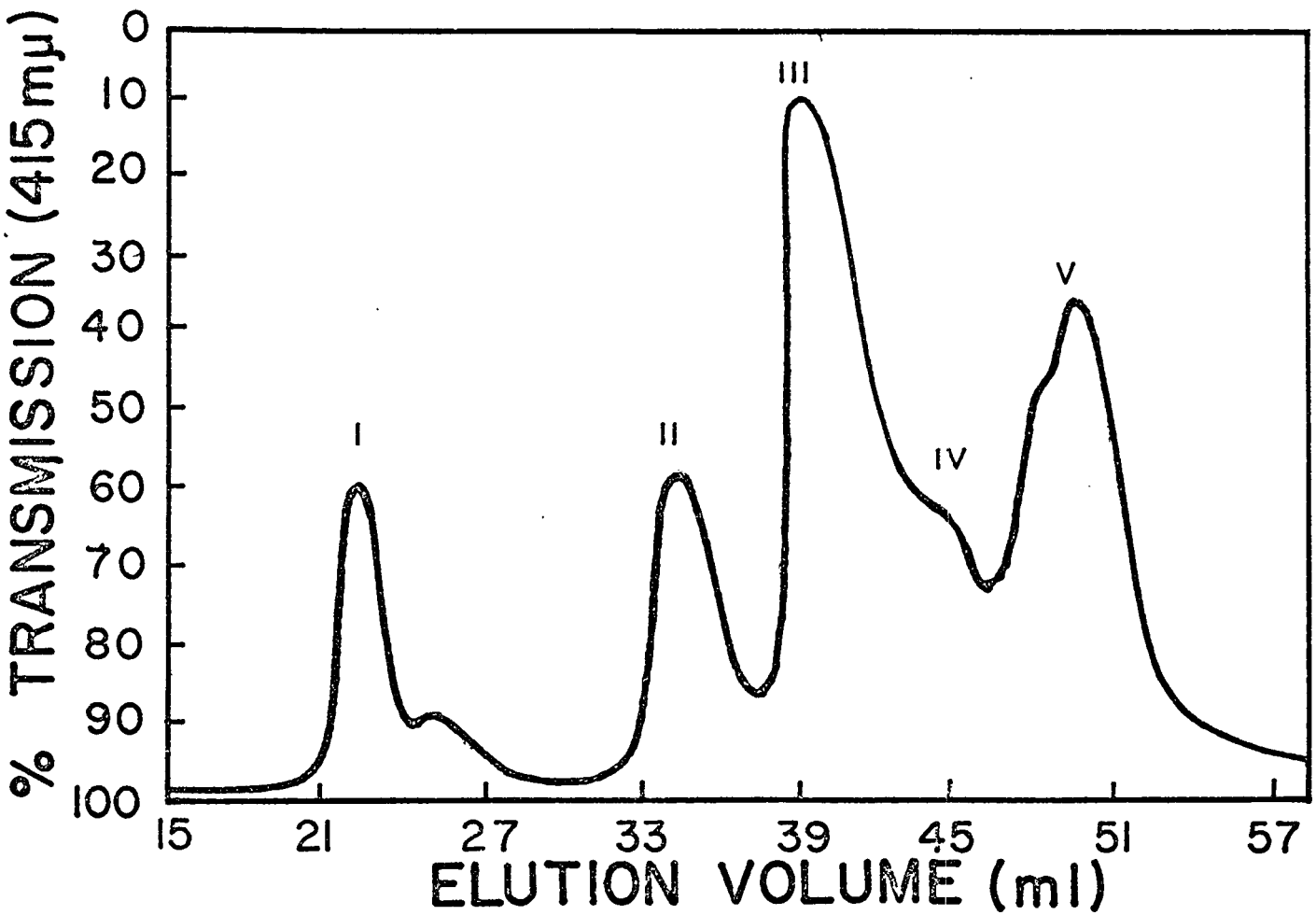

% TRANSMISSION (415mμ)
0
10
20
30
40
50
60
70
80
90
100
I
II
III
IV
V
15
21
27
33
39
45
51
57
ELUTION VOLUME (ml)

Figure 5. Elution profile on DEAE-cellulose of spot hemoglobin.

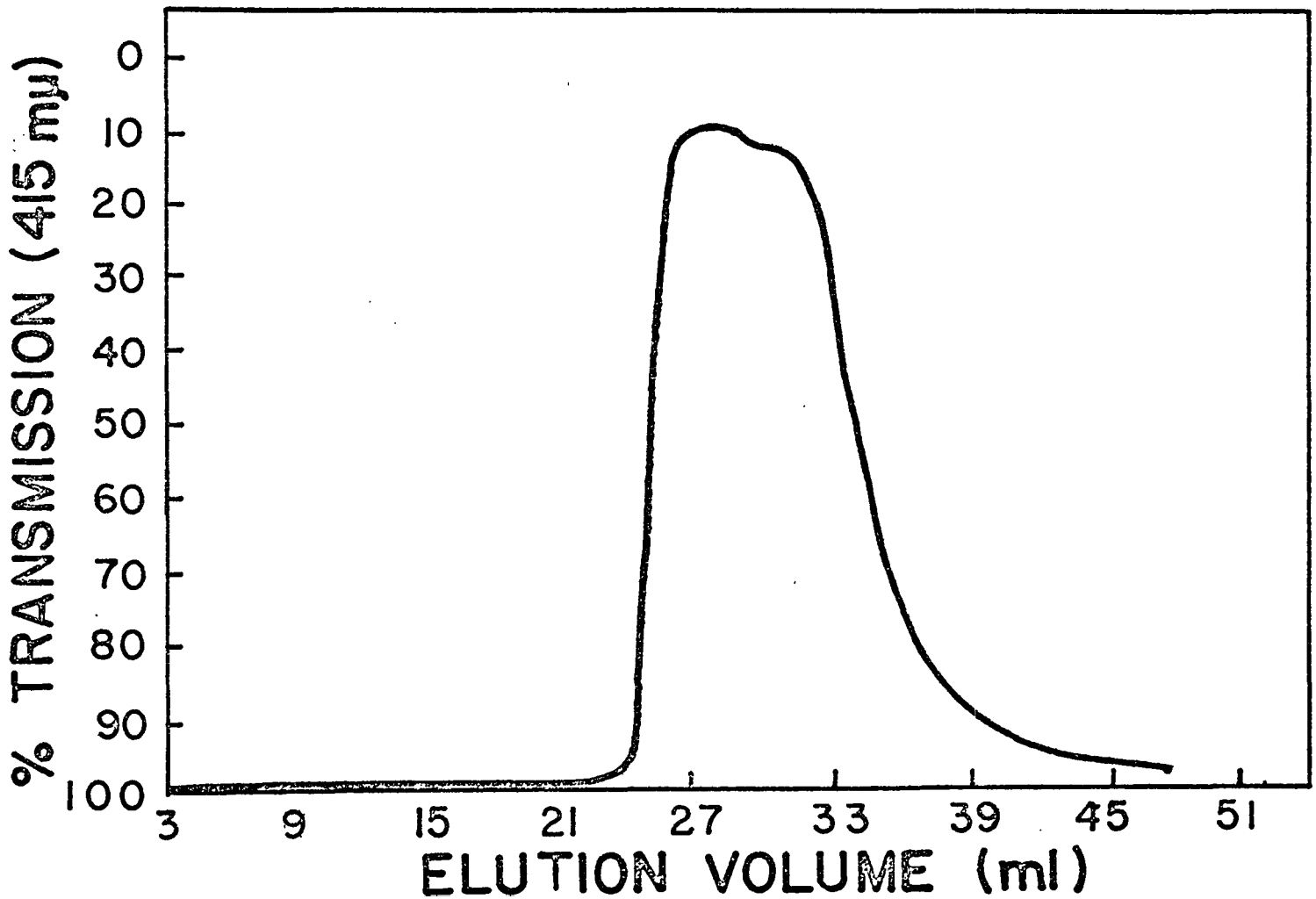

% TRANSMISSION (415 mμ)
0
10
20
30
40
50
60
70
80
90
100
3
9
15
21
27
33
39
45
51
ELUTION VOLUME (ml)

Figure 6a. Electrophoretic properties of fractions from major peaks, I-VI of croaker hemoglobin phenotype B separated on DEAE-cellulose.

Figure 6b. Electrophoretic properties of fractions from major peaks, I-V of croaker hemoglobin phenotype C separated on DEAE-cellulose.

FIGURE 6a

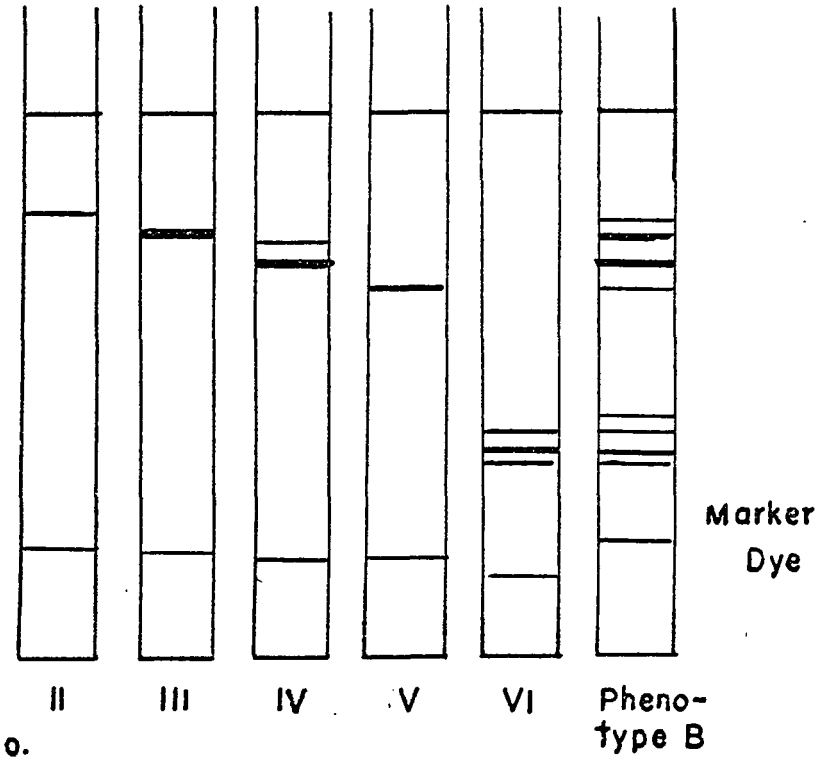
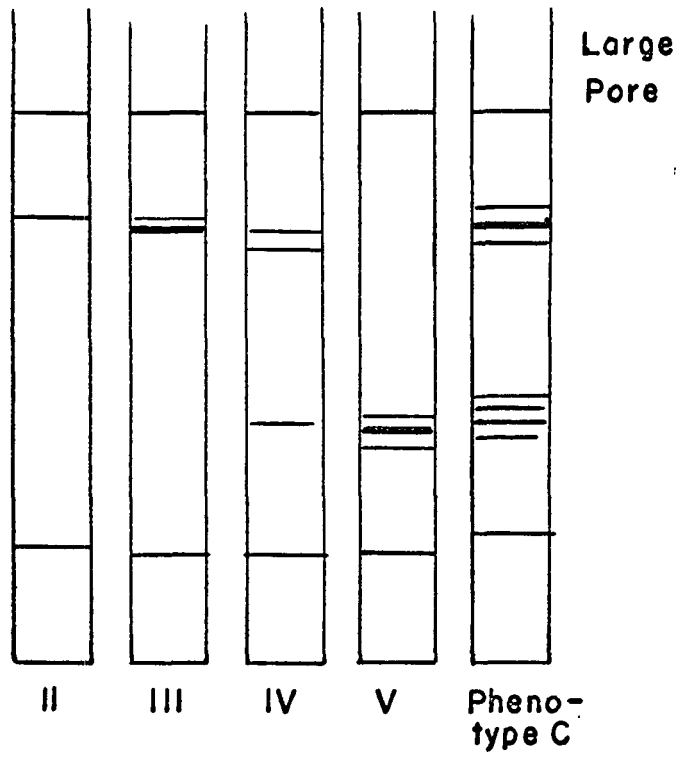


FIGURE 6b



start of DEAE chromatography and which is contained in the first major peak.

Sedimentation studies of individual hemoglobin solutions of both the croaker and the spot reveal that both hemoglobins are homogenous as regards size and shape of the molecules, as only one symmetrical peak is observed for both hemoglobins. Figure 7a shows the Schlieren diagram of the sedimentation of croaker hemoglobin from which the $s_{20,w}$ value was calculated. Considering the marked heterogeneity of croaker hemoglobin demonstrated by acrylamide gel electrophoresis, it appears that such heterogeneity is not a result of molecular aggregates, but a reflection of the presence of molecules of different charge. The value of $s^{o}_{20,w}$ was calculated for croaker hemoglobin to be 4.3×10^{-13} sec^{-1}, which is within the accepted value of $4.2\text{-}4.4 \times 10^{-13}$ sec^{-1} for hemoglobins of various fish determined by Svedberg and Pedersen (1940). Svedberg and Pedersen also report only one sedimentation component for fish hemoglobin.

One of the most striking properties of hemoglobin is its tendency to dissociate into lower molecular weight subunits when subjected to a variety of conditions, sometimes without denaturation of the pigment. Concentrated salt solutions at pH values near neutrality have been shown to have a dissociating effect on human and horse hemoglobin. Results of Benhamou et al. (1957, 1960) and of Rossi-Fanelli et al. (1961) indicate a decrease in the molecular

Figure 7a. Schlieren diagram of sedimentation pattern of croaker hemoglobin (phosphate buffer pH 6.85) at 80 minutes after reaching a speed of 56,100 r.p.m.

Figure 7b. Schlieren diagram of sedimentation pattern of croaker hemoglobin (2M NaCl, phosphate buffer) at 20 minutes after reaching a speed of 56,100 r.p.m.

Figure 7c. Schlieren diagram of sedimentation pattern of croaker hemoglobin (Glycine-NaOH buffer, pH 11.4) at 32 minutes after reaching a speed of 56,100 r.p.m.

Figure 7a

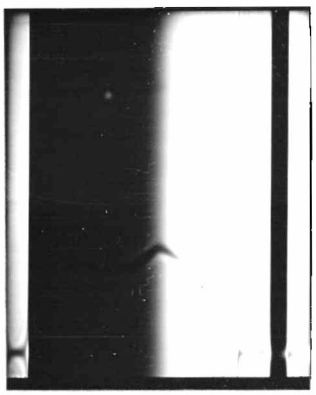


Figure 7b

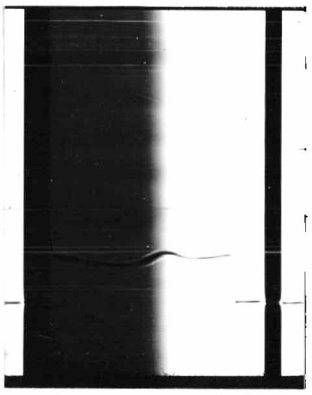
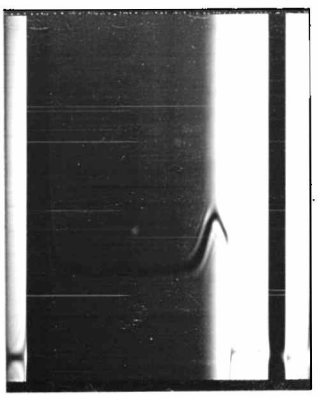


Figure 7c



weight of hemoglobin in NaCl solutions above 0.5M. In concentrated salt solutions, hemoglobin shows no signs at all of denaturation, even after several days of exposure. This is not the case for hemoglobin solutions of extreme pH. Rossi-Fanelli et al. (1961) have shown that the sedimentation constant decreases from a value of 4.4 to about 3.3 in 2M NaCl. An analysis of the sedimentation behavior of croaker hemoglobin in 2M NaCl was carried out. The value of $s_{20,w}$ for the hemoglobin so treated was found to be 3.4. The fact that a single peak was observed in the ultracentrifuge during this analysis would indicate that there is a rapid reversible equilibrium between tetramer and dimer.

Field and O'Brien (1955) have made studies of sedimentation of human CO-hemoglobin at pH values between pH 4.5 and pH 5.0, and have shown that the hemoglobin undergoes a dissociation into subunits at these pH values, Rossi-Fanelli et al. (1964) state that hemoglobin does not show spectral changes at pH values between 4.5 and 6.0. In this regard it was found that croaker hemoglobin was very susceptible to acid denaturation, and that even at pH 5.15, observable spectral changes occurred, indicating denaturation. Such a condition is similar to the situation of human hemoglobin which undergoes denaturation below pH 4.5, and does not show a completely reversible association-dissociation equilibrium. The value of $s_{20,w}$ for croaker hemoglobin at pH 4.8 was calculated to be 3.5.

Data in the early literature indicate that hemoglobin dissociates at alkaline as well as acid pH. Hasserodt and Vinograd (1959) have established that the sedimentation constant decreases above pH 9.5, reaching a value of about 3.5 at pH 11. Above pH 11 there is a further decrease in the sedimentation constant, the hemoglobin however, being rapidly denatured at this high pH. Croaker hemoglobin at pH 10.6 did not show any spectral changes, however at pH above 11, it was found that spectral changes did occur. The value of s calculated for croaker hemoglobin at pH 11.4 was 2.5 indicating dissociation. A rapid association-dissociation equilibrium must be taking place as only one symmetrical peak is observed. Moreover the spreading of the peak as a function of time indicates that the diffusion constant has increased, and a dissociation into subunits rather than an unfolding of the molecule is taking place. Schlieren diagrams of the sedimentation of croaker hemoglobin in 2M NaCl and in Glycine-HaOH buffer, pH 11.4, are shown in Figure 7b and 7c, respectively.

The visible absorption spectrum of croaker CO-hemoglobin is shown in Figure 8 (% transmission as a function of wavelength), and in Figure 9 (absorbance as a function of wavelength). On the basis of differences in this absorption spectrum, the separate phenotypes A and B, could not be distinguished, nor could the separate hemoglobin components obtained by DEAE chromatography, be differentiated. The

Figure 8. Absorption spectrum of croaker CO-hemoglobin (% transmission as a function of wavelength).

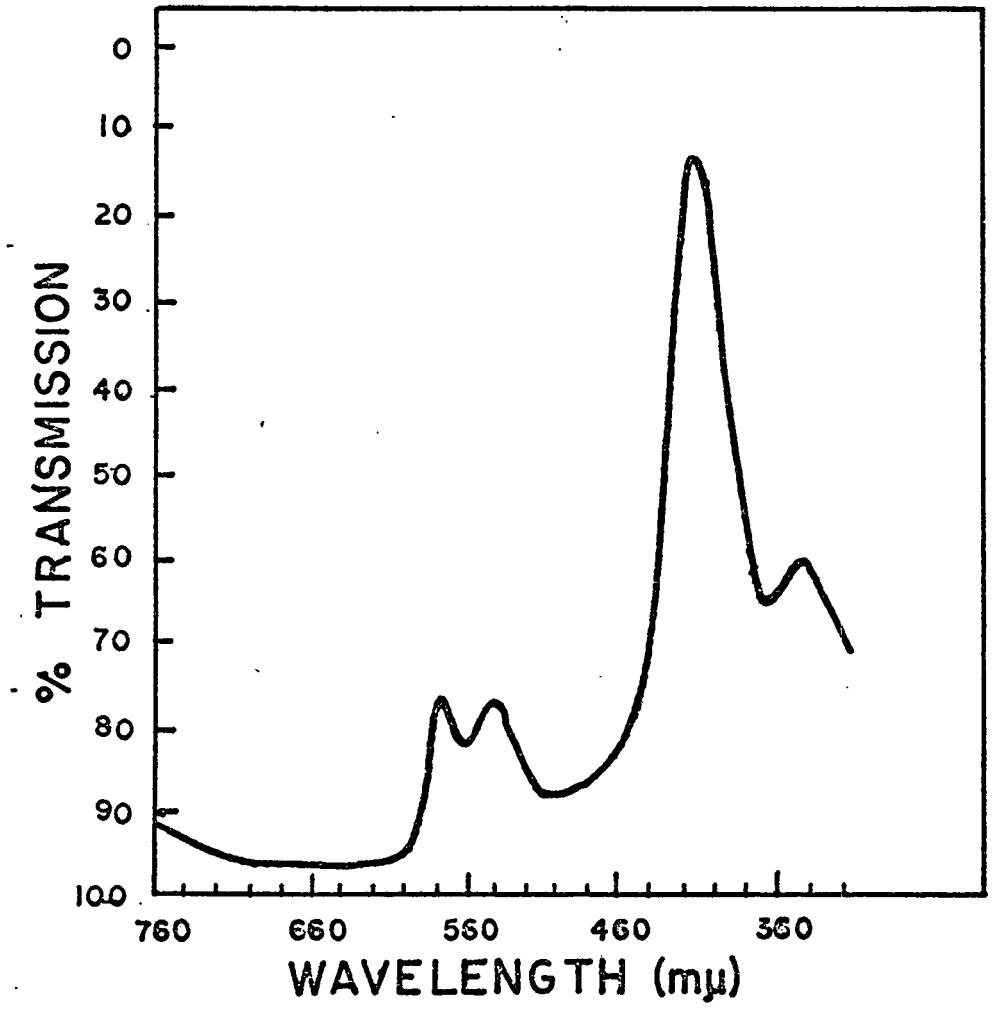
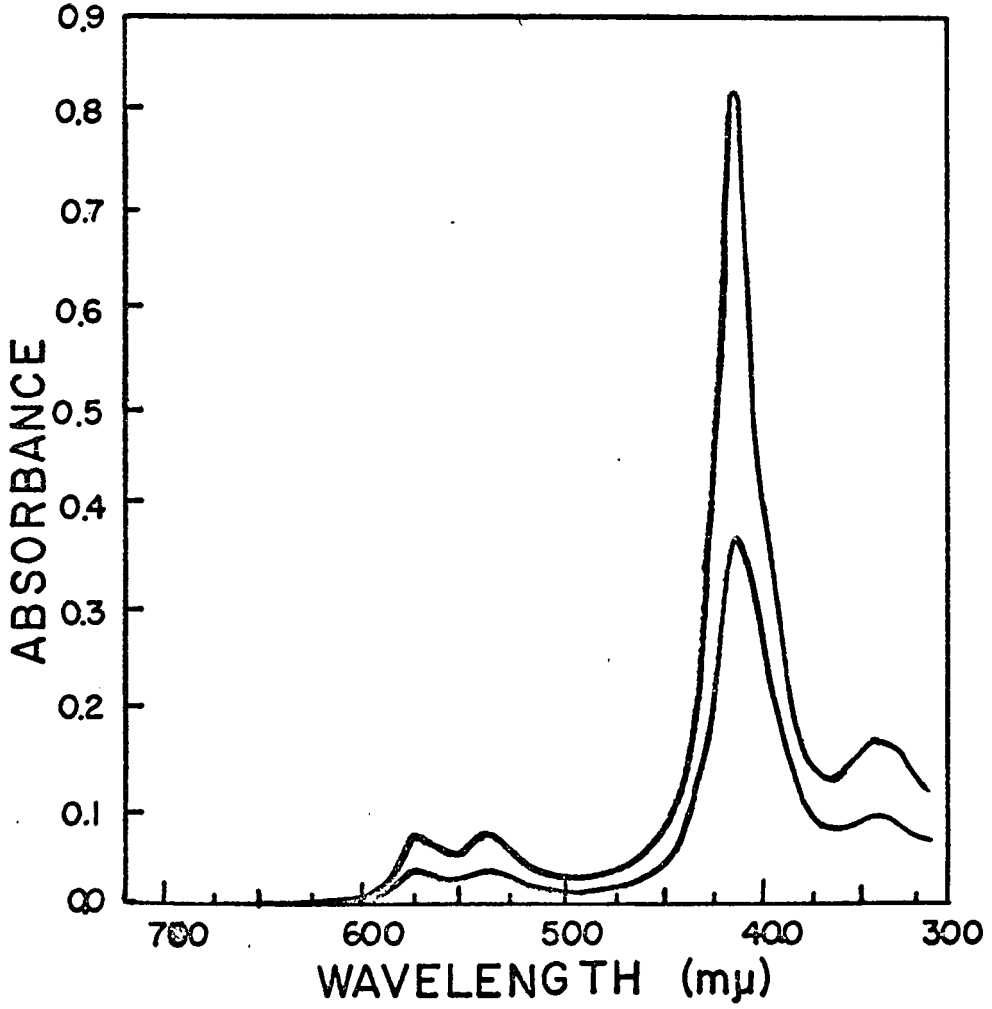

0
10
20
30
40
50
60
70
80
90
100
% TRANSMISSION
760
660
560
460
360
WAVELENGTH (mμ)

Figure 9. Absorption spectrum of croaker CO-hemoglobin (absorbance as a function of wavelength).

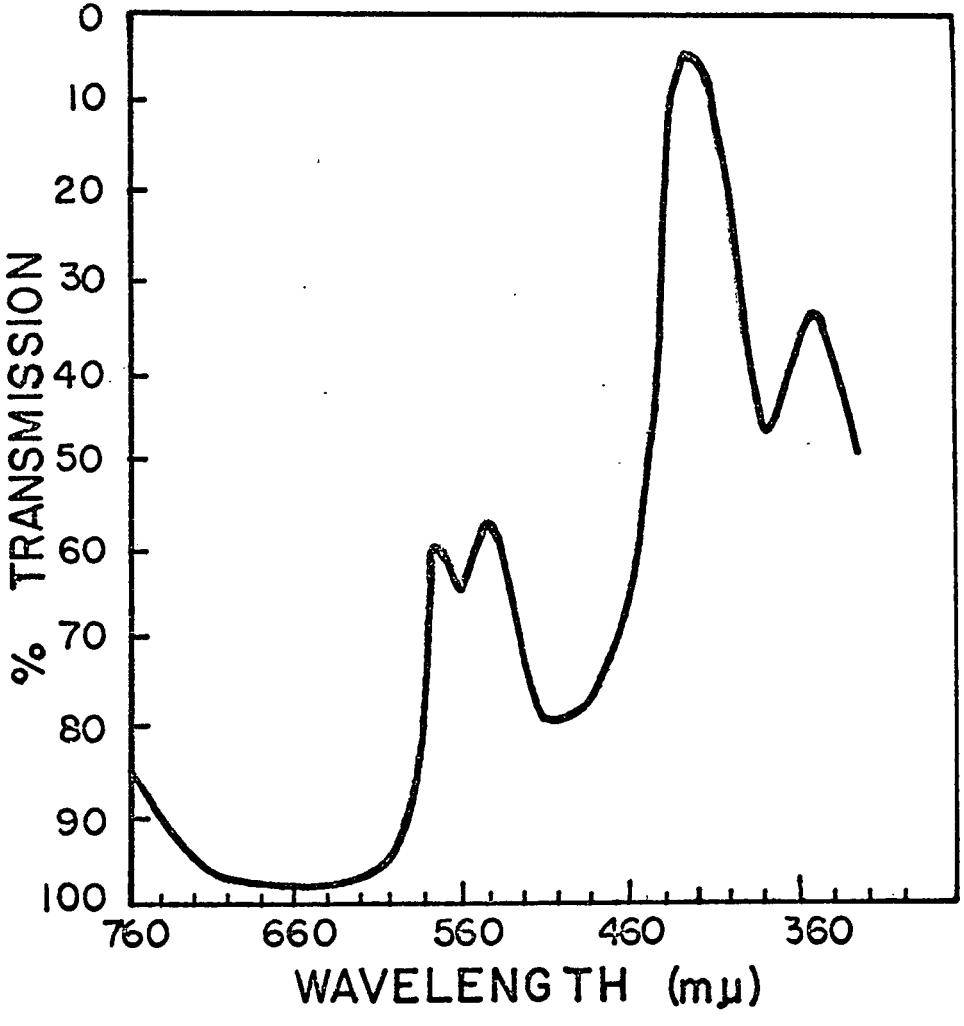

0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
ABSORBANCE
700
600
500
400
300
WAVELENGTH (mμ)

spectrum of croaker CO-hemoglobin is nearly identical to that of rat CO-hemoglobin whose visible absorption spectrum is shown in Figure 10.

Adult and fetal mammalian hemoglobins can be differentiated on the basis of the rate of alkaline denaturation at pH 11 through pH 13 (Brinkman and Jonxis, 1936). Manwell (1957) investigated the alkaline denaturation of the hemoglobin of adult and post-larval specimens of the teleost fish Scorpaenichthys marmoratus. Post-larval hemoglobin was found to have an alkaline labile component that denatured faster than that of the adult hemoglobin. This difference is alkaline denaturation between adult and post larval fish was consistently observed at both pH 11.0 and 12.0.

Alkaline denaturation of croaker hemoglobin was attempted to determine whether selective differences in the denaturation process between the separate hemoglobin phenotypes could be found. Denaturation was followed by changes in the visible absorption spectrum as a function of time. At pH 10.6, no spectral changes of croaker CO-hemoglobin were evidenced. When the pH was raised to 10.9, denaturation occurred within 2-3 hours. At pH 11.2, denaturation occurred rapidly and hemoglobin solutions were used immediately for alkaline denaturation experiments. Both phenotype A and phenotype B were analyzed for the presence of resistant components at pH 11.2. At this pH, denaturation was uniform and complete, and no indication of resistant components was found. Obvi-

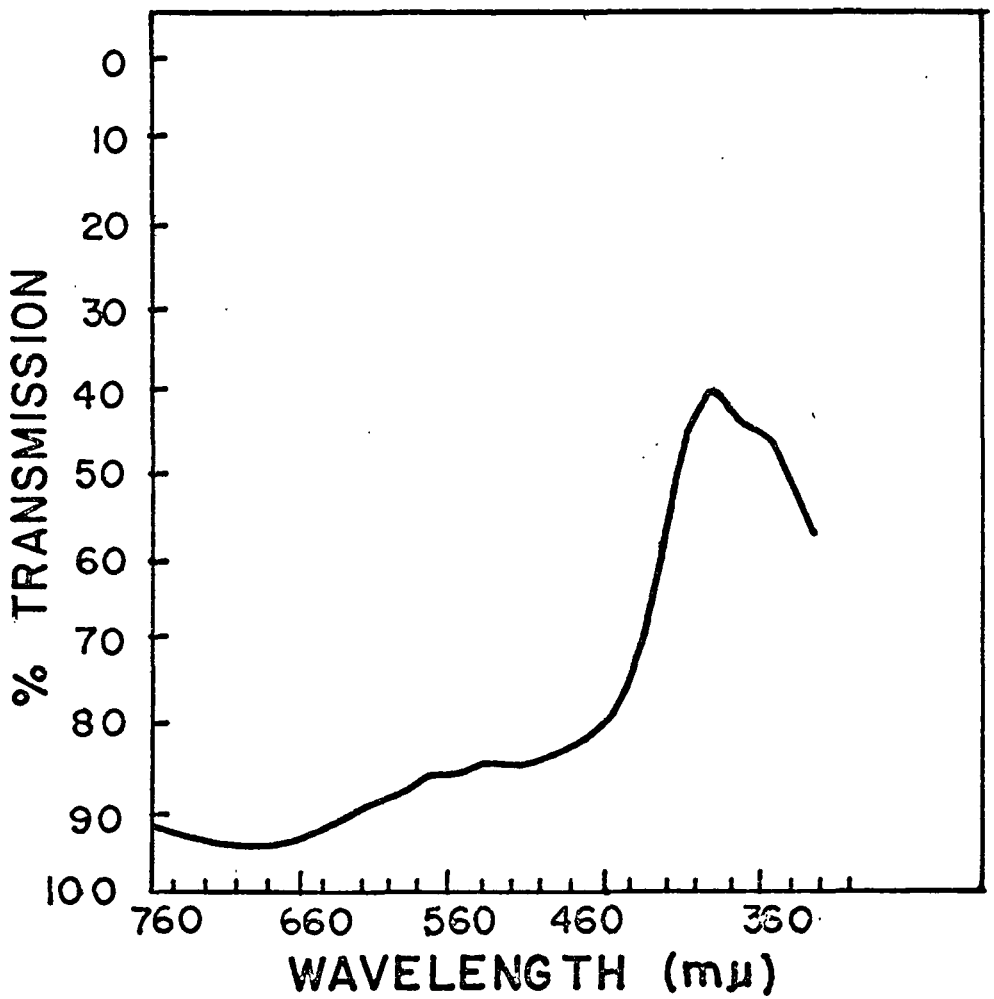
Figure 10. Absorption spectrum of rat CO-hemoglobin.


0
10
20
30
40
50
60
70
80
90
100
% TRANSMISSION
760
660
560
460
360
WAVELENGTH (mμ)

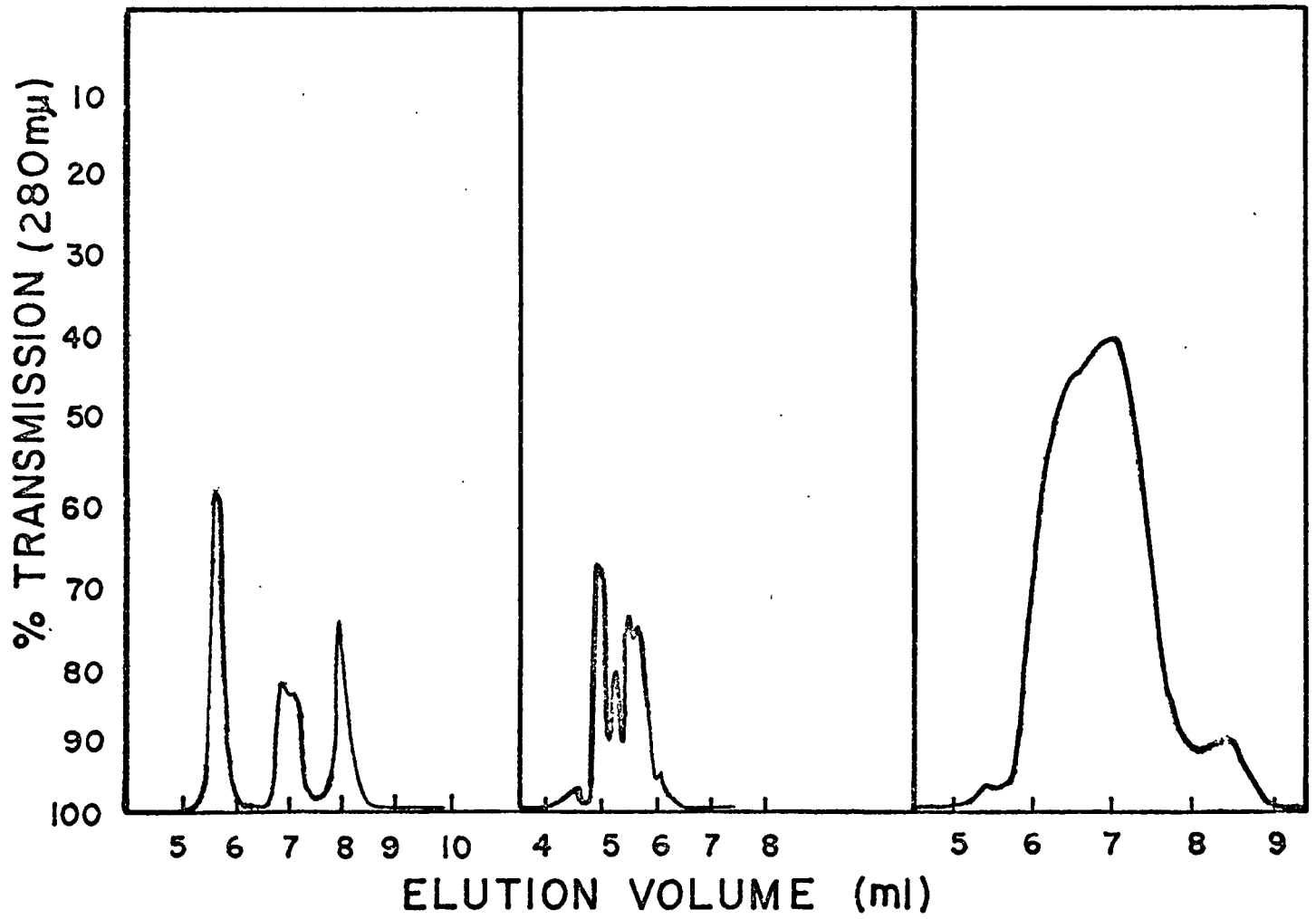
ously this could be re-examined using the separate fractions from DEAE-cellulose chromatography. Rates of denaturation of the separate phenotypes or the individual hemoglobin components were not compared. Figure 11 shows a typical absorption spectrum of croaker CO-hemoglobin in Glycine-NaOH buffer, pH 11.2, after a period of 30 minutes.

Preparation and chromatography of globin from pooled CO-hemoglobin of the croaker was performed three times. The results are shown in Figures 12a, 12b, and 12c. In Figures 12a and 12b at least three and possibly four globin chains have been resolved. In Figure 12c the resolution of the globin chains is poor, but the shoulder at the leading edge of the peak and the shoulder at the trailing edge indicate the presence of at least three globin types. As the globin chains were eluted almost immediately as the gradient elution began, it is evident that a new gradient system must be designed in order to increase the resolution of the globin chains of the fish hemoglobin.

Figure 11. Absorption spectrum of croaker CO-hemoglobin in Glycine-NaOH buffer, pH 11.2, after a period of 30 minutes.


0
10
20
30
40
50
60
70
80
90
100
% TRANSMISSION
760
660
560
460
360
WAVELENGTH (mμ)

Figures 12a, 12b, and 12c. Elution profile of globin chains from pooled croaker CO-hemoglobin on CM-cellulose (urea-mercaptoethanol-phosphate buffer).


% TRANSMISSION (280mμ)
10
20
30
40
50
60
70
80
90
100
5 6 7 8 9 10
4 5 6 7 8
5 6 7 8 9
ELUTION VOLUME (ml)

DISCUSSION

Previous studies in other laboratories have shown that the hemoglobins of many species are electrophoretically heterogenous, and fish hemoglobins demonstrate a complex heterogeneity not commonly found in other classes. An important difference exists between situations in which all individuals of a species possess the genome for the several hemoglobin components, and those in which there is a difference in the number of kinds of hemoglobin components possessed between individuals of a species. In the former case it can be assumed that the components are synthesized under independent genetic control, and the organisms are homozygous for the genes concerned. In the latter case a genetic polymorphism does exist, and allelic genes are segregating in the population (Gratzer and Allison, 1960).

Intraspecific variation of hemoglobin in the croaker (Micropogon undulatus) does exist. Such a variation may be the result of a genuine genetic polymorphism or it may result from some biological modification of hemoglobin synthesis. Indeed, as regards the latter case, the effect of environmental conditions and the physiological state on hemoglobin phenotype is raised frequently. As protein synthesis is genetically controlled, barring any drastic mutational changes, environmental conditions should not alter the proteins directly. However, the synthesis of hemoglobin, may by some mechanism, be influenced direcely by environmental factors such as temperature or oxygen availability.

In sheep, although traces of Hb C may be present in nonanemic sheep, this component becomes the sole form in animals severely anemic from blood loss or chemically induced anemia (Naughton et al., 1966). In humans there have been suggestions (Allen and Jandl, 1960; Thomas et al., 1960) that the change in the oxygen tension at birth activates chain synthesis. Such a mechanism however seems hardly able to account for the intraspecific variation seen in the croaker, because all three phenotypes were observed in fish which were caught in the same geographical area and exposed to the same environmental conditions. The sex of the fishes was not determined; however, Manwell et al. (1963) and Tsuyuki et al. (1965) have shown that the hemoglobin pattern in fish does not vary from male to female. Furthermore, less than 3% of croakers caught in the bay have fully developed gonads. No correlation between season and hemoglobin phenotype could be detected, as all phenotypes were evidenced throughout the period of study.

Evidence has accumulated in recent years which indicates that hemoglobin undergoes ontogenetic change throughout a large part of life cycle in some species of fish. Hashimoto and Matsuura (1960) have reported an increase in the Hb S fraction of Chum salmon, which is correlated with an increase in length of individual fish. Vanstone et al. (1964) have described size dependent changes in the starch gel electrophoretic pattern of the Pacific salmon. Wilkins and Iles

(1966) have found that intraspecific variation of herring hemoglobin exists, and that intermediate patterns are stages in the development of the adult pattern from the pattern at metamorphosis. Juvenile-adult hemoglobin polymorphism has also been detected in the Sprat.

These studies emphasize the necessity of investigating hemoglobin polymorphism at different growth and development stages, particularly before frequency determinations based on such polymorphisms are used as indices of genetic variation for the differentiation of populations. In the present study no gross corellation could be made between hemoglobin phenotype and size of fish, and no intermediate patterns were observed. These observations indicate that a genetic polymorphism exists, and therefore, the existence of three reproductively isolated subpopulations.

Considerable intermixing must exist between these populations, if they are present, during this stage of their life cycle, because no geographical or hydrographical barrier exist in the area where these fish were caught. According to Sick (1965), forces which tend to level out differences between adjacent subpopulations of marine fishes are relatively large, so that significant differences in the frequencies of the hemoglobin alleles between them can only be shown when breeding subpopulations are separated by some distance. In the one case where differences of hemoglobin gene frequencies has been demonstrated between adjacent populations, Sick

assumes that the situation can be explained by previous isolation of the populations.

As intraspecific variation of croaker hemoglobin is restricted to three phenotypes, with the absence of any intermediate patterns, it seems that there must be some mechanism to restrict gene flow between subpopulations of the croaker. Data on the sexual maturity of croakers collected from inshore waters show that 97% had undeveloped gonads and the remainder were ripening or ripe (Roithmayr, 1964). As these immature fish are not physically separated, it is possible that members of the subpopulations segregate at spawning time in the deeper offshore waters of the Gulf, and remain isolated from one another, be it isolation in time, isolation in space, or ecological isolation.

Evidence indicates that the croaker demonstrates genetically polymorphic hemoglobins and is divided into at least three reproductively isolated subpopulations. In order to confirm this conclusion two studies must be further pursued. First, an analysis of the globin subunits of each separate hemoglobin components must be carried out, with the aim of being able to distinguish between the separate phenotypes by demonstrating corresponding differences in the polypeptide chains of the components. Second, the genetics of the polymorphism, if it exists, must be determined in order to ascertain that the different hemoglobins are controlled by alternative alleles rather than independent, non-allelic

genes.

Investigation of the subunit structure of each of the hemoglobin components of individual croakers was initiated by the separation of these components on DEAE-cellulose. Further studies on the globin chains of these hemoglobins were not feasible because the preparation of native globin by the method of Anson and Mirsky (1930) required a greater amount of hemoglobin than could be obtained from individual fish. As a result, globin analysis of pooled hemoglobin from the croaker was performed in order to determine whether different globin chains could be detected. Results indicate the existence of at least three, and possibly four, globin chains in the pooled hemoglobin sample. It must be noted, however, that the globin chains were eluted almost immediately, even before the gradient had a chance to operate. The method of column chromatographic separation used for this work was that of Clegg et al. (1965), designed especially for the separation of human globin chains. It is quite probable that this technique is not well suited for the resolution of fish globin chains, and it is imperative for this study, that a new gradient system be developed in future studies of fish globins. When this is accomplished it is very possible that analysis of fish globins through the use of column chromatography will reveal the presence of several more globin chains.

In addition to the intraspecific variation of croaker hemoglobin, quite a difference exists between the hemoglobin phenotype demonstrated by the croaker and the spot (Leiostomus

xanthurus). The croaker shows a complex heterogeneity, while the spot, a member of the same family, demonstrates a consistent electrophoretically homogenous pattern.

Often no reason for hemoglobin heterogeneity is apparent, but on general grounds it can be assumed that the synthesis of several hemoglobin components would not have persisted in the course of evolutionary development unless there were some advantage to such a system even though quite subtle (Gratzer and Allison, 1960).

Hashimoto (1959) has found that the two components of the hemoglobin of Chum salmon show considerable differences in their oxygen dissociation curves, as a function of changes in phophate concentration or temperature. These two hemoglobins might have different physiological functions allowing adequate oxygen carriage under widely different environmental conditions. The multiple hemoglobins S and D of the turtle Chrysemys picta show great physiological difference in oxygen equilibria. The S-component possesses a much higher oxygen affinity, greater heme-heme interaction, and slightly larger Bohr effect (Manwell, 1966a). In the turtle apparently three hemoglobin cistrons are present in all adults of the species. A species of tortoise appears to have four different hemoglobin chains (Dozy et al., 1964). Great variation in the number of multiple hemoglobins is observed among fish species (Manwell, 1964; Manwell et al., 1963; Tsuyuki, 1965; Buhler and Shanks, 1959; Chandrasekhar, 1959), and it is believed

that such heterogeneity may result from both a multiplicity of polypeptide chain types, coupled with the ability to form tetramers with more than two kinds of chains (Manwell, 1966a).

Both Glueksohn-Waelsch (1960) and Manwell (1964) attribute hemoglobin heterogeneity to gene or chromosomal duplication resulting in several non-allelic yet homologous genes which code for several molecular species of hemoglobin. Such a condition might confer an adaptive advantage on the carrier since these hemoglobin species may differ slightly in regards to their physiological properties, and there would be available a variety of hemoglobins to meet various specific requirements.

In order to account for the electrophoretic and chromatographic homogeneity of spot hemoglobin, it can be hypothesized that non-allelic, homologous loci do not exist, or else these do exist but code for peptide chains which do not differ in net charge. It is difficult to experimentally determine which of these two cases apply. However, using the model of LDH (Kaplan, 1964), it can be seen that duplication of genes can take place independently in closely related species. Duplicate loci in the spot may have been selected against in a particular environment, or it may be that the species was exposed to a stable environment, where functionally nearly neutral amino acid substitutions were not advantageous, and not subjected to favorable selection pressure.

The intraspecific hemoglobin variation in the croaker is indeed genuine; however, further studies on the genetics of

the polymorphism, as well as the geographical distribution of the observed phenotypes are necessary before such a polymorphic system can be used to differentiate subpopulations. A promising extension to this study would be to investigate the hemoglobin phenotypes of individual croakers in other geographical areas in order to establish the existence of additional phenotypes if they exist and more importantly, determine if and how individuals segregate at spawning time in the offshore waters.

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