STRUCTURAL AND SUBUNIT DISSOCIATION STUDIES OF A NEW HUMAN HEMOGLOBIN

by

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Dedication

To My Wife

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I. INTRODUCTION

Partly because of its important physiological function as an oxygen carrier, and partly because it is an easily obtainable protein in pure form, hemoglobin has been intensively studied for well over one hundred years. It was discovered in 1851 when Otto Funke isolated a red pigment from blood. In 1864 Hoppe-Seyler analyzed it and introduced the term "hemoglobin". This term is used to include the oxygen-carrying proteins of vertebrates. It has been shown in recent years that hemoglobins and myoglobins have quite similar structures and it is suggested that there is a phylogenetic relationship between these molecules and the transport and storage of oxygen. It is probable that these molecules have a common precursor in evolutionary development.

The structure of the intact molecule of hemoglobin was first subjected to study by physicists about 30 years ago with the application of x-ray diffraction to the problem. The introduction of isomorphous substitution into hemoglobin by Green et al. (1) and Perutz et al. (3) enabled rapid advances in x-ray studies on hemoglobin and four years later a model of the molecular structure of myoglobin was derived by Kendrew (2). A similar model for horse hemoglobin was available two years after that (Perutz et al., 3). An atomic model of sperm whale myoglobin at 2Å resolution was presented by Kendrew (4) in 1961.

Added interest in this field was kindled when Pauling et al. (5) in 1949 discovered hemoglobin S in studies on sickle-cell anemia. In 1958 Ingram (6) made the observation that the abnormality of this hemoglobin is caused by the exchange of a single amino acid and this observation opened a fascinating area in human genetics.

Primary structure work was started somewhat later and in 1960 sufficient correlation between chemical studies and x-ray data was achieved by Braunitzer et al. (7) and by Hill et al. (8) to give a partial picture of the structure of the total molecule. Shortly thereafter the complete primary structure had been determined (9,10,11).

These results correlated with a large number of earlier studies indicate that the structure and function of hemoglobin is probably better understood than that of any other macromolecule.

II. PHYSICOCHEMICAL PROPERTIES

A. Size and Shape

The iron content of hemoglobin was first accurately determinated as 0.35% by Engelhart in 1825 and later confirmed by Drabkin in 1957 (12), and the minimum molecular weight of hemoglobin was calculated to be 16,700. Osmotic pressure data of Adair (13) in 1925 gave a molecular weight between 60,000 and 68,000, four times that of the minimal molecular weight. Svedberg and Fahreus in 1926 (14) obtained a similar value of 66,800 using horse hemoglobin in the first ultracentrifugal analysis of a protein. In succeeding years the sedimentation constants of many vertebrate hemoglobins showed the molecular weights of vertebrate hemoglobins to be in the order of 64,000 to 68,000.

The hydrodynamic properties of the respiratory heme proteins also indicate that the molecules in solution are symmetrical, rather spherical particles, as would appear from the direct crystallographic study of hemoglobin and myoglobin.

Values from 1.1 to 1.3 for the frictional ratio have been determined and the difference from 1 is ascribed to the hydration of the molecule (94,96,118,119).

Under normal conditions the hemoglobins and myoglobins show small dependence of the sedimentation constant on concentration, the value of $s_{20,w}$ at 1% concentration being about 5% lower than the value extrapolated to zero protein concentration (20,60,94,119,120,121). Results of studies of dielectric dispersion (15), viscosity increment (16) and x-ray scattering at low angles (17,18) of myoglobin and hemoglobin are in good agreement with sizes and shapes of these molecules as determined by crystallographic analysis.

B. Molecular Weight Variation With State of Heme Iron

The effect of changes in the state of the heme iron on the molecular properties of respiratory proteins is important because the effect may be very significant for the understanding of the mechanism of the ligand equilibria of these pigments.

Thus far the available data are very inconclusive and show no definite differences under "normal conditions" in the particle size and sedimentation constant of the oxy, carbon monoxy, ferri, or deoxygenated derivatives of mammalian hemoglobins (75). The hemoglobins of lamprey (19) and some mollusk myoglobins are examples which exhibit

features of their ligand equilibria that are difficult to reconcile with molecular weight figures obtained.

These heme proteins are considered to consist of a single polypeptide chain bearing one heme group and having molecular weights of 17,000 to 24,000 (20,122,123). However, these proteins show sigmoid oxygen equilibrium curves which are consistent with heme-heme interactions but paradoxical in a monomeric molecule (19,124). An explanation of this can be found in an aggregation or higher polymerization state of the deoxygenated form with increase in hemoglobin concentration as observed by Briehl (19) where the presence of a large Bohr effect and apparent heme-heme interaction were confirmed.

C. Species Variation of Molecular Weight and Size

The heme proteins from different animal species have molecular weights from 17,000 to several million (125,126,127). There is great uniformity in particle size and shape of hemoglobins of mammals and other vertebrates. The classic studies of Svedberg (20), on the other hand, show great variability of molecular properties of the hemoglobins of invertebrates and lower vertebrates.

III. CHEMICAL STRUCTURE OF HEMOGLOBIN

A. Methods

1. Peptide Chain Separation

It was shown by the end-group analyses of Porter and Sanger (128) that hemoglobin A consisted of several polypeptide chains, and later studies by other workers (37,129) clearly indicated that two pairs of chains were present. Column chromatography was the first

important preparative procedure for the separation of the α and β chains (*vide infra*). Initial studies (21) showed the separation of peptide chains on cation-exchange resins using gradient elution with acidic urea. Better results of separation of these chains and the peptide chains of fetal globin were obtained with different conditions (22,23). Good results have been obtained with beef globin using stepwise elution with increasing urea gradient (24).

A highly successful technique to fractionate peptide chains is countercurrent distribution (25). A mobile phase of 2-butanol and a stationary phase of dichloro-acetic acid provides favorable partition coefficients. It is important that the heme group be previously removed from the globin. Yields are in the range of 70 to 80%.

Specific unique separations have been achieved by precipitation and by dialysis. Horse globin chains were separated by fractional precipitation with acid-acetone (21). Fetal hemoglobin peptide chains can be separated by dialysis as described by Matsuda et al. (26).

2. *Tryptic Peptides*

In the initial treatment to obtain tryptic peptides a fractionation is achieved by isoelectric precipitation in salt-free medium at pH 6.4 (6) resulting in a precipitated "core" fraction which contains a large part of the cysteine peptides.

The soluble supernatant is fractionated best by column chromatography as initially described by Hilse and Braunitzer (22) and by Rudloff and Braunitzer in 1961 (27). Detailed descriptions of

several column chromatographic methods have been given (30,31,32,33). It has been shown that conversion of the cysteinyl residues to the S-aminoethyl cysteinyl derivative (28) followed by tryptic hydrolysis results in completely soluble tryptic peptides with no formation of the "cores" (29) which are difficult to analyze.

3. Arrangement of Tryptic Peptides

Several ways are available to get a complete idea about the precise structure of a protein by analyzing the products of tryptic hydrolysis from the amino terminal to the carboxyl terminal ends of the chains.

A so-called overlapping technique (34) involves the hydrolysis with enzymes of different specificities, such as pepsin and chymotrypsin, yielding bridge peptides in which linkages that can be attacked by trypsin remain intact. If the amino acid sequence of the tryptic peptides is known, then it may be possible to arrange the order of the tryptic peptides from the composition of such lysine and arginine containing bridge peptides.

4. Amino Acid Sequence Analysis

Two main techniques are employed for sequence determination.

One method includes the early technique of Edman degradation, as developed by Fraenkel-Conrat et al. (35) and improved by Shelton and Schroeder (36). Under certain acid conditions partial hydrolysis of amide groups occurred. Also observed was that glycine peptides are relatively resistant to undergoing the required cyclization. It is presently limited to a peptide length of two to ten residues but has

the advantage that such a sequence can be established with very small amounts of an unknown peptide. As applied to intact proteins the method is useful for clear identification of up to no more than seven residues of a chain end (35).

Another method utilizes the principle of "one sequence-one analysis". Carboxypeptidase may be used and with carefully controlled conditions and by variation of the enzyme action time, accurate information about the position of amino acids can be determined by quantitative measurement of the amino acids which have been liberated. The rate of release of the C-terminal amino acid which must be the L configuration is significantly influenced by the nature of the side chains of the amino acids forming the susceptible bond and if the adjacent bond involves a proline the reaction is stopped.

B. Adult Human Hemoglobins

1. Normal Adult Hemoglobin A

The hemoglobin A molecule consists of 574 amino acids and four heme groups. The calculated molecular weight is 61,992 for globin and 64,458 for hemoglobin and these values are in excellent agreement with that of about 66,000 as obtained from most molecular weight determinations (13,14,130).

Early studies had shown that hemoglobin A was composed of several peptide chains and the further work clearly indicated that two pairs of chains were present (37). The four heme groups lie in four separate pockets on the surface of the molecule, each pocket being formed by the folds of one of the four polypeptide chains. Two chains

have a val-leu sequence at the N-terminus and are called the α chains, and the other two have val-his-leu at the N-terminus and are called the β chains. The common representation of the structure of hemoglobin A is $\alpha_2^A\beta_2^A$ and its overall amino acid composition is: Lys_{44}, His_{38}, Arg_{12}, Asp_{50}, Thr_{32}, Ser_{32}, Glu_{32}, Pro_{28}, Gly_{40}, Ala_{72}, Cys_6, Val_{62}, Met_6, Leu_{72}, Tyr_{12}, Phe_{30}, Try_6.

The molecule is composed of identical halves of αβ type units as indicated by the x-ray model (38).

The results for the sequence are from studies of Braunitzer et al. (9), Konigsberg et al. (30) and Schroeder et al. (39).

(a) It is shown that the α chain includes 141 residues with an amino acid composition as follows: lys_{11}, his_{10}, arg_3, asp_{12}, thr_9, ser_{11}, glu_5, pro_7, gly_7, ala_{21}, cys_1, val_{13}, met_2, leu_{18}, tyr_3 phe_7, try_1. The sequence is given in Scheme 1.

NH_2 10
val-leu-ser-pro-ala-asp-lys-thr-asp-val-lys-ala-ala-try-gly-lys-
20 30
val-gly-ala-his-ala-gly-glu-try-gly-ala-glu-ala-leu-glu-arg-met-
40
phe-leu-ser-phe-pro-thr-thr-lys-thr-tyr-phe-pro-his-phe-asp-leu-
50 NH_2 60
ser-his-gly-ser-ala-glu-val-lys-gly-his-gly-lys-lys-val-ala-asp-
NH_2 70 NH_2 80
ala-leu-thr-asp-ala-val-ala-his-val-asp-asp-met-pro-asp-ala-leu-
90
ser-ala-leu-ser-asp-leu-his-ala-his-lys-leu-arg-val-asp-pro-val-
NH_2 100 110
asp-phe-lys-leu-leu-ser-his-cys-leu-leu-val-thr-leu-ala-ala-his-
120
leu-pro-ala-glu-phe-thr-pro-ala-val-his-ala-ser-leu-asp-lys-phe-
130 140
leu-ala-ser-val-ser-thr-val-leu-thr-ser-lys-tyr-arg

Scheme 1.

Amino Acid Sequence of the α Chain in Human Hemoglobin A

(b) The β chain has 146 residues, thus longer than the α chain and has a different amino acid composition, as shown by the amino acid composition: lys_{11}, his_9, arg_3, asp_{13}, thr_7, ser_5, glu_{11}, pro_7, gly_{13}, ala_{15}, cys_2, val_{18}, met_1, leu_{18}, tyr_3, phe_8, try_2. The sequence of amino acids is shown in Scheme 2.

```
                                                    10
VAL-his-LEU-thr-PRO-glu-GLU-lys-SER-ALA-VAL-thr-ALA-leu-try-gly-
       NH2  20                                                30
lys-val-asp-val-ASP-GLU-VAL-gly-gly-glu-ALA-leu-gly-arg-leu-leu-
                          NH2  40
val-val-tyr-pro-try-thr-glu-arg-phe-phe-GLU-ser-phe-gly-ASP-leu-
     50                                NH2              60
ser-THR-PRO-ASP-ala-VAL-met-gly-asp-pro-lys-val-lys-ala-his-gly-
                        70                                      NH2
lys-lys-val-leu-GLY-ALA-PHE-SER-asp-GLY-LEU-ALA-his-leu-asp-ASP-
                                                90
leu-lys-gly-thr-phe-ala-THR-leu-ser-glu-leu-his-cys-asp-lys-leu-
             100     NH2                          NH2     110
his-val-asp-pro-glu-asp-phe-ARG-leu-leu-gly-asp-val-leu-val-CYS-
                                  120                        NH2
val-leu-ala-HIS-his-phe-gly-lys-glu-phe-thr-pro-PRO-val-glu-ala-
    130 NH2                                   NH2 140
ALA-TYR-glu-lys-VAL-val-ALA-gly-val-ala-ASP-ala-leu-ALA-HIS-LYS-

tyr-his
```

Scheme 2.

Amino Acid Sequence of the β Chain in Human Hemoglobin A

2. Minor Components

The presence of hemoglobin A_2 and hemoglobin A_3 was first shown by Kunkel and Wallenius (40) with starch block electrophoresis at pH 8.6. The slowest component was A_2 (2 to 3%), then the main component A_1 (85%) and hemoglobin A_3 (10 to 15%) with contamination by other proteins in the A_3 region.

Chemical investigations have shown the structure of hemoglobin A_2 to be that of $\alpha_2^{A}\delta_2^{A2}$. The differences in sequence between

the β and the δ chains were detected at eight positions by Stretton and Ingram (41,42) and in two more by Jones (33). The differences in the γ, β and δ chains are indicated in Table I.

The presence of hemoglobin A_2 and hemoglobin A_3 was first demonstrated by Kunkel and Wallenius (40) on starch block electrophoresis. Hemoglobin A_3 has been studied by Muller (44) who subjected the purified material to tryptic hydrolysis and used the fingerprint technique to compare the split products with those of hemoglobin A. The only difference between the peptide maps was a peptide from hemoglobin A_3 which was analyzed and shown to have the formula glu-gly-cys and had an R_f value corresponding to glutathione. It was found that oxidation of hemoglobin A_3 with performic acid would yield this peptide. It appears that hemoglobin A_3 is an addition product resulting from the oxidation of -SH groups with the formation of a -S-S- bond between the hemoglobin A and glutathione.

Other work indicates that the hemoglobin A_3 region is a mixture of hemoglobins (45) with several minor components detected by chromatography on IRC-50 and indicate these components as hemoglobins A_{Ia}, Ib, Ic, Id, Ie. The largest of these, hemoglobin A_{Ic}, present in 5% amounts, has the structure $\alpha_2{}^{A}\beta^{A}\beta^{AIc}$ where one of the β chains has a blocked N-terminal group (117). The blocking group is unknown but it has a molecular weight of the order of 250 (43) and it is possibly an aldehyde or ketone reacted with the amino group (118).

Table I.

Differences in Amino Acid Sequence in the β, γ and δ Chains*

Residue No.	β Chain	γ Chain	δ Chain
9	ser	ala	thr
12	thr	thr	$aspNH_2$
22	glu	asp	ala
50	thr	ser	ser
86	ala	ala	ser (?)
87	thr	$gluNH_2$	$gluNH_2$ (?)
116	his	ileu	arg
117	his	his	$aspNH_2$
124	pro	pro	
or			$gluNH_2$
125	pro	glu	
126	val	val	met

*Table taken from Schroeder and Jones (43).

C. *Fetal Human Hemoglobins*

1. *Hemoglobin F*

This hemoglobin is the major pigment found in the red cells during fetal life and like hemoglobin A consists of four polypeptide chains. Two of these are identical to the α^A chains (46) while the other two are definitely distinct from the β chains in 17 differences in amino acid composition and by 39 differences in sequence though the total number of residues is the same as the β chain. The second pair of chains has glycine in the N-terminal position and are called γ chains. Thus the structure of hemoglobin F is $\alpha_2{}^F\gamma_2{}^F$. The sequence of the α^A and α^F chains has been shown to be identical (47). The detailed sequence of the γ^F chain has been given by Schroeder et al. (48). It has been concluded that the β and γ chains both have 146 residues. The most distinctive difference between the β and γ chain amino acid composition is the presence of four isoleucine residues in the γ chains, whereas neither the α nor the β has isoleucine present. The sequence of the γ^F chain is given in Scheme 3.

```
                                        10
GLY-his-PHE-thr-GLU-glu-ASP-lys-ALA-THR-ILEU-thr-SER-leu-try-
           NH2 20                                          30
gly-lys-val-asp-val-GLU-ASP-ALA-gly-gly-glu-THR-leu-gly-arg-
                                    40      NH2
leu-leu-val-val-tyr-pro-try-thr-glu-arg-phe-phe-ASP-ser-phe-
                  50                          NH2          60
gly-ASP-leu-ser-SER-ALA-SER-ala-ILEU-met-gly-asp-pro-lys-val-
                                    70
lys-ala-his-gly-lys-lys-val-leu-THR-SER-LEU-GLY-asp-ALA-ILEU-
                  80                          NH2          90
LYS-his-leu-asp-ASP-leu-lys-gly-thr-phe-ala-GLU-leu-ser-glu-
                                   100  NH2
leu-his-cys-asp-lys-leu-his-val-asp-pro-glu-asp-phe-LYS-leu-
       NH2     110                                         120
leu-gly-asp-val-leu-val-THR-val-leu-ala-ILEU-his-phe-gly-lys-
```

NH_2 130 NH_2
glu-phe-thr-pro-GLU-val-glu-ala-SER-TRY-glu-lys-MET-val-THR-
140
gly-val-ala-SER-ala-leu-SER-SER-ARG-tyr-his

Scheme 3.

Amino Acid Sequence in the γ Chain of Human Hemoglobin F

2. Minor Fetal Hemoglobins

The largest of the minor hemoglobins in fetal blood is hemoglobin F_I described by Allen et al. (49) which amounts to about 10% of total hemoglobin. This has the structure $\alpha_2{}^A\gamma^F\gamma^X$, the γ^X chain differing from the γ^F chain by the acetylation of the amino group of the N-terminal residue (50).

D. Embryonic Human Hemoglobins

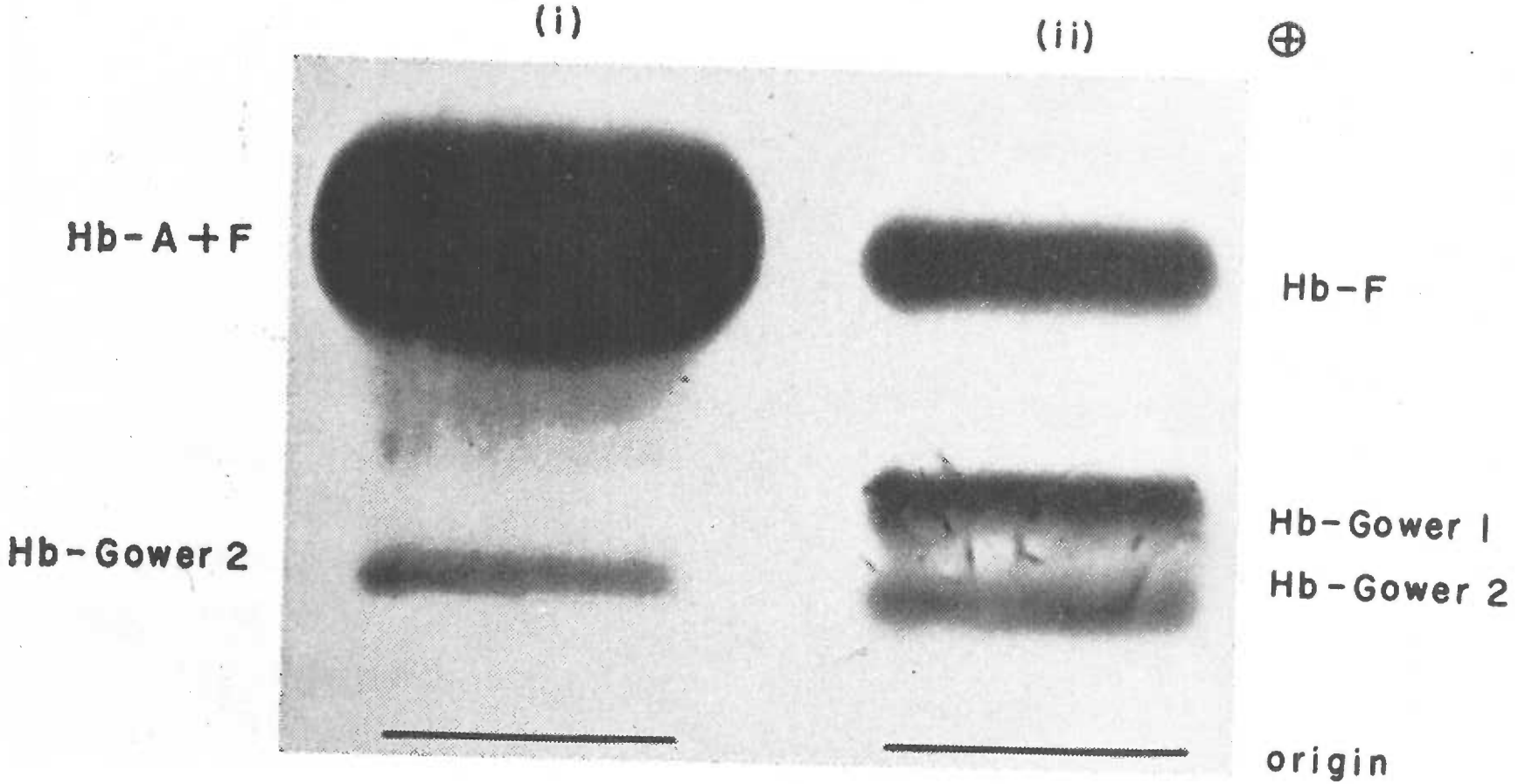
The possibility of the existence of a human embryonic hemoglobin was first suggested by Drescher and Kunzer (51).

It has been shown recently by Heuhns et al. (52,53) that there are two embryonic hemoglobins, HB Gower 1 and HB Gower 2 (Fig. 1).

Hemoglobin Gower 2 has a similar structure to the other normal human hemoglobins, Hb A, HB F and HB A_2. It consists of four polypeptide chains; two of these are α^A chains while the other two chains differ from the β, γ, and δ chains by more than one amino acid substitution and have been called the ε chains (53). The formula of Hb Gower 2 has been written $\alpha_2{}^A\varepsilon_2$ (54). Hemoglobin Gower 1 has not been as well studied as Hb Gower 2 but is suggested to consist solely of the non α chain of Hb Gower 2 which gives it the structural formula ε_4. The proportion of these hemoglobins was greatest in the smallest embryos examined and gradually decreased. After the 6.5 cm crown-rump

Figure 1

Starch Gel Electrophoresis of Hemoglobins Gower 1 and Gower 2. Starch gel analysis in the tris-citrate borate system at pH 8.6. [From: Huehns et al. (54)].


(i)
(ii)
⊕
Hb-A+F
Hb-Gower 2
Hb-F
Hb-Gower 1
Hb-Gower 2
origin

measurement stage only trace amounts were found and none was detected in specimens larger than 10 cm crown-rump measurement, which corresponds to a 90-day gestation period. Huehns concludes that Hb Gower 1 and Hb Gower 2 are normal embryonic hemoglobins.

It would thus appear that α^A chains are synthesized during all stages of human development with a non α chain specific for each stage. In the embryonic stage the non α chain is the ε chains of embryonic hemoglobin, while in the fetal stage the γ chains of fetal hemoglobin and in extra-uterine life the β and δ chains are found in adult hemoglobin A and hemoglobin A_2 respectively. This development of chains is illustrated in Figure 2.

IV. CONFORMATION OF HEMOGLOBIN

In recent years the three-dimensional structure of myoglobin and hemoglobin has been presented by the work of the Cambridge group (Kendrew et al., 2 , Perutz et al., 3 , Cullis et al., 57). These results represent a momentus step in the development of knowledge of biological structure at the molecular level. Many new and unsuspected details of myoglobin and hemoglobin have been shown as well as confirmation of structural features indicated by previous chemical and physicochemical work have been provided by the direct crystallographic evidence.

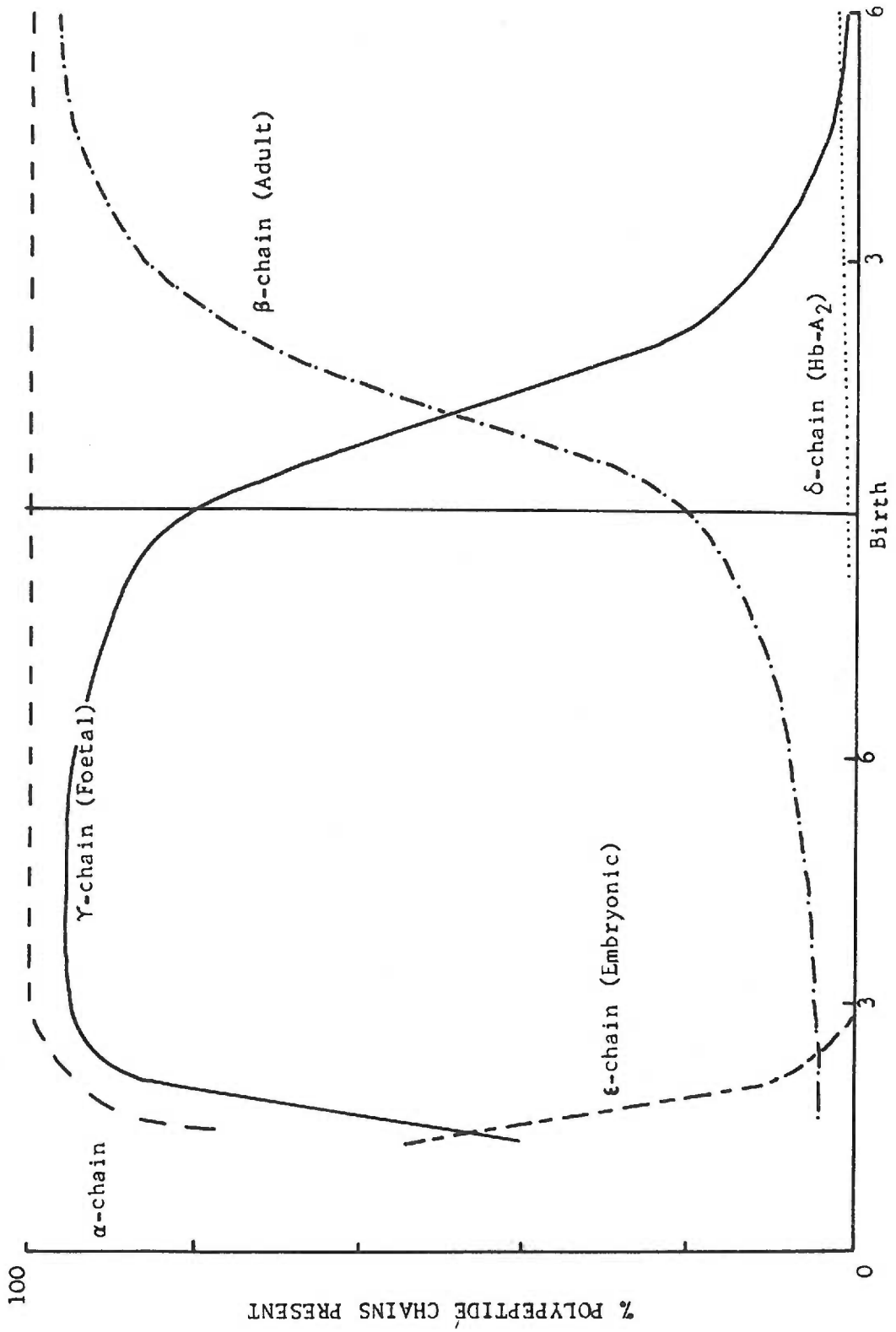
A. Crystallographic Models

Myoglobin is a heme protein of muscle which stores oxygen that has been transported by hemoglobin. Myoglobin consists of a single polypeptide chain containing only one heme group with a molecular

Figure 2

The Development of Human Hemoglobin Chains.

[From: Huehns et al. (131)].



The development of human hemoglobin chains.

weight of about 17,000 (132). It can be considered the approximate equivalent of a single hemoglobin chain. Myoglobin has been resolved to 2Å with x-ray diffraction (3) revealing some atomic positions. The similarity in the three-dimensional structure between the hemoglobin chain and the myoglobin chain allow conclusions to be drawn from the atomic model of myoglobin about the probable structural details existing in hemoglobin which are not discernible in the 5.5Å model of hemoglobin.

1. Sperm Whale Myoglobin

The structure of sperm whale metmyoglobin has been presented by Kendrew at a resolution of 6Å. The one polypeptide chain that makes up myoglobin possesses straight helical sections joined by segments of non-helical structure and is folded in a specific manner. The heme group is situated in a pocket of the folded chain structure and is near the surface of the molecule and in close contact with several sections of the chain (4).

The x-ray data (4,57) show that one of the coordination bonds of the heme iron is directed to the imidazole nitrogen of a histidine residue, the proximal histidine, at position number 92 in myoglobin, and that the other coordination binding site on the other side of the planar heme group is bound to water or to a ligand. A short distance from the water molecule is another side chain which is thought to be a second histidine residue, the distal histidine at position 63 in myoglobin. The carboxyl groups of the propionic acid side chains of the porphyrin appear to be important. The carboxyl

groups are charged and form salt linkages with basic groups of the protein and Kendrew's model (4) of myoglobin indicates a carboxyl group interacting with an arginine residue of the chain; however, these groups are replaced by non-polar amino acids in the analogous positions in hemoglobin (Kendrew, 61). The x-ray model of myoglobin shows the vinyl groups of heme to be directed toward the interior of the molecule where they may be available for π bonding with aromatic side chains (4). It is suggested by Kendrew (4) that there are 90 possible sites for van der Waal's interactions between the heme and the large number of non-polar residues which surround it. Similar findings are to be expected for hemoglobins.

Many more details appear at 2Å resolution and much of the amino acid sequence along the chain is revealed.

It is shown that about 70% of the chain has a right-handed α-helix structure. There are eight helical regions that make up straight segments (A-H) and eight non-helical regions, several interposed between helical regions and one at the carboxyl end of the chain. The corners of the chain correspond to the occurrence of a proline residue. The structure appears very compact with almost no liquid inside.

The amino acid sequence of sperm whale myoglobin (59) is presented in Scheme 4.

NH_2 10

val-leu-ser-glu-gly-glu-try-glu-leu-val-leu-his-val-try-ala-

20 NH_2 30

lys-val-glu-ala-asp-val-ala-gly-his-gly-glu-asp-ileu-leu-ileu-

40
arg-leu-phe-lys-ser-his-pro-glu-thr-leu-glu-lys-phe-asp-arg-
50 60
phe-lys-his-leu-lys-thr-glu-ala-glu-met-lys-ala-ser-glu-asp-
70
leu-lys-lys-his-gly-val-thr-val-leu-thr-ala-leu-gly-ala-ileu-
80 90
leu-lys-lys-lys-gly-his-his-glu-ala-glu-leu-lys-pro-leu-ala-
NH_2 100
glu-ser-his-ala-thr-lys-his-lys-ileu-pro-ileu-lys-try-leu-glu-
110 120
phe-ileu-ser-glu-ala-ileu-ileu-his-val-leu-his-ser-arg-his-pro-
NH_2 NH_2 130 NH_2
gly-asp-phe-gly-ala-asp-ala-glu-gly-ala-met-asp-lys-ala-leu-
140 150
glu-leu-phe-arg-lys-asp-ileu-ala-ala-lys-tyr-lys-glu-leu-gly-
NH_2
try-glu-gly

Scheme 4.

Amino Acid Sequence of Sperm Whale Myoglobin

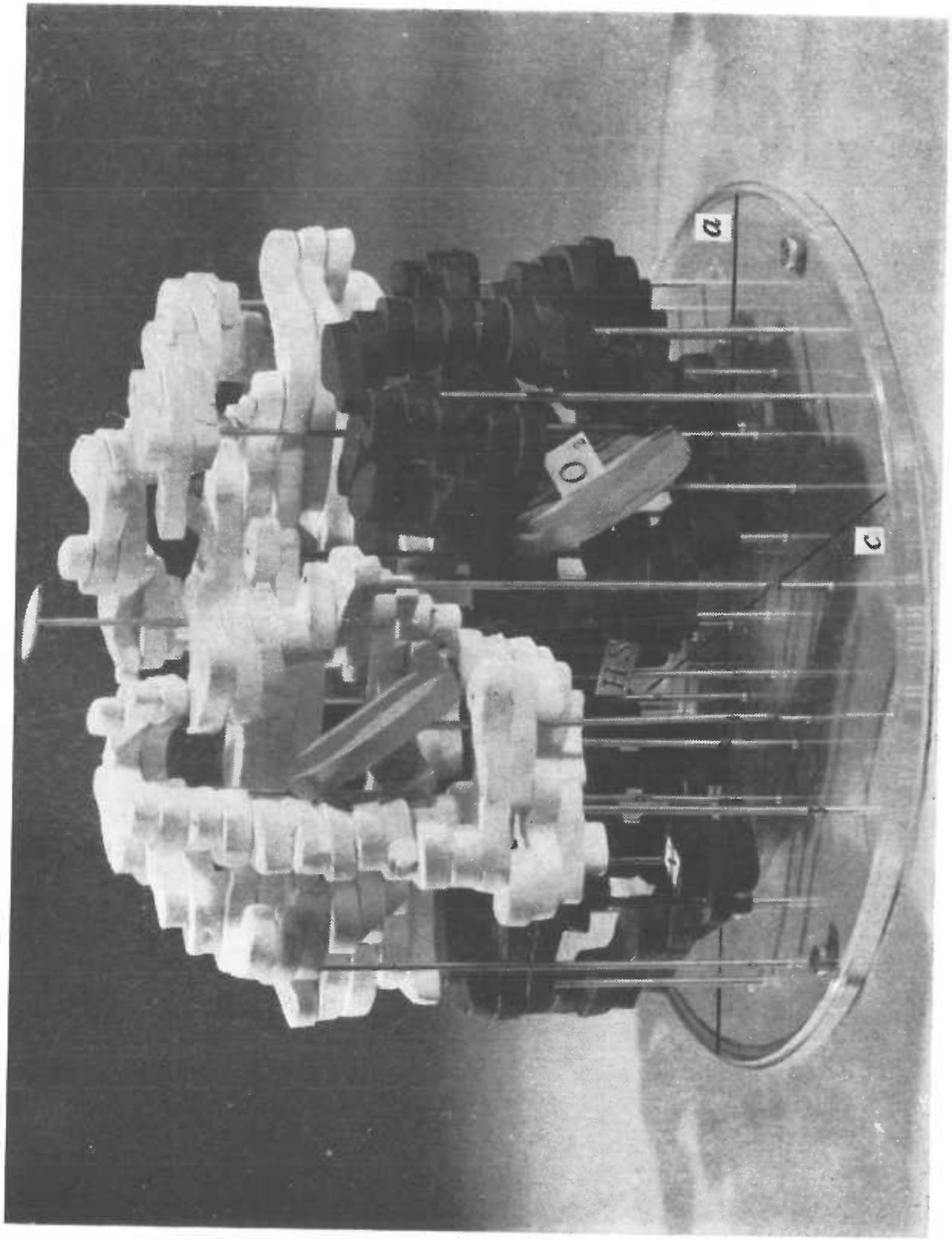
The chain is of 153 residues and is 12 residues longer than the α chain and 7 residues longer than the γ and β chains of hemoglobin. Comparison of the sequences of the α and β chains and of myoglobin shows that there are 26 segments which are common in all three chains (9,43,57). The general spatial arrangement of the α and β chains and myoglobin are the same and since myoglobin chain residues can be positioned in space, the relationships have been used to arrange the α and β chains in space.

2. *Horse Hemoglobin*

The analysis of horse hemoglobin has been completed at a resolution of 5.5A (56,57). Figure 3 pictures a model of the horse hemoglobin molecule. The hemoglobin molecule appears to be made up of four subunits, in identical pairs with the arrangement of a nearly regular tetrahedron. The α and β chain have similar structures and

Figure 3

Three-Dimensional Model of Horse Hemoglobin. Complete hemoglobin model showing the heme groups as grey disks. [From: Cullis et al. (57)].


a
c

are similar to the myoglobin chain. Both hemoglobin chains, like myoglobin, appear to have eight helical regions. These α helical segments contain 75% of the amino acid residues. These values are supported by optical rotatory-dispersion measurements of Beychok and Blout (58). Both the α and β chains appear to have a short non-helical region at the amino end and terminate with a helical region at the carboxyl end. It is noteworthy that there is relatively little contact between identical subunits but there is complimentary structure between the α and β pairs.

The heme groups are located in pockets on the surface of the four subunits and present extensive contacts with the polypeptide chain (*vide supra*). The iron atoms of an α and a β chain are the closest and are 25Å apart; thus the heme groups are remote from each other.

B. Secondary and Tertiary Structure

The primary structure refers to the amino acid sequence; the secondary structure refers to the arrangement in helical or non-helical regions and tertiary structure refers to the arrangement of the secondary structures.

It is possible to draw conclusions about which residues of the α and β chains are in helical arrangement by comparing the sequences of the α and β chains and of myoglobin. A summary of comparative information as presented by Edmundson (59) and tabulated by Schroeder and Jones (43) is shown in Table II.

Helical*	Non-Helical*	Residue Numbers			Length of helix (No. of residues)
		α chain	β chain	myoglobin	
	Pre-A	1–2	1–3	1–2	
A		3–18	4–19	3–18	16
	AB	19	—	19	
B		20–35	20–34	20–35	16
	BC	—	—	—	
C		36–42	35–41	36–42	7
	CD	43–51	42–49	43–50	
D		—	50–56	51–57	7
	DE	—	—	—	
E		52–71	57–76	58–77	20
	EF	72–79	77–84	78–85	
F		80–88	85–93	86–94	9
	FG	89–93	94–98	95–99	
G		94–112	99–117	100–118	19
	GH	113–118	118–123	119–124	
H		119–141	124–146	125–148	24
	Post-H	—	—	149–153	

* Beginning at the N-terminus, the helices are designated by capital letters and the interhelical regions by the letters of the two helices that they connect.

Table II.

Probable Residues in Helical and Non-Helical Arrangement in the α and β Chains of Hemoglobin and in Myoglobin. [From: Schroeder and Jones (43)]

Complete and detailed discussions of comparative features and specific individual features of the chains are presented by Cullis et al. (57), by Braunitzer (60) and by Schroeder and Jones (43).

A perspective drawing as presented by Schroeder and Jones (43) of the β chain of hemoglobin A is given in Figure 4 and is helpful to get a three-dimensional view of the helical and non-helical segments of a hemoglobin chain.

With reference to the three-dimensional view of whole hemoglobin (Fig. 3) and by use of Figure 4, along with the data in Figure 5 and Table II, and perhaps reference to information about comparative features of the chains (Cullis et al., 57, Braunitzer, 60), the reader should be able to locate and relate positions of substituted residues in variant hemoglobins.

C. Quaternary Structure

The arrangement of the protein subunits in three-dimensions is called the quaternary structure.

The assumption can be made that the characteristic, symmetrical organization of the four subunits is essentially identical in hemoglobins with a molecular weight of 64,000. The arrangement of the two α and β subunits approaches a tetrahedral form, although not a regular tetrahedron although on the dyad axis there is a plane of symmetry.

It has been pointed out that there is relatively little contact between the two α chains or the two β chains but that there are fairly large areas where α chains touch β chains. By examination

Figure 4

A Perspective Drawing of the β Chain of Hemoglobin A. Each filled point corresponds to the approximate position of a residue. The helices correspond to the letters of helices in Table II. [From: Schroeder and Jones (43)].

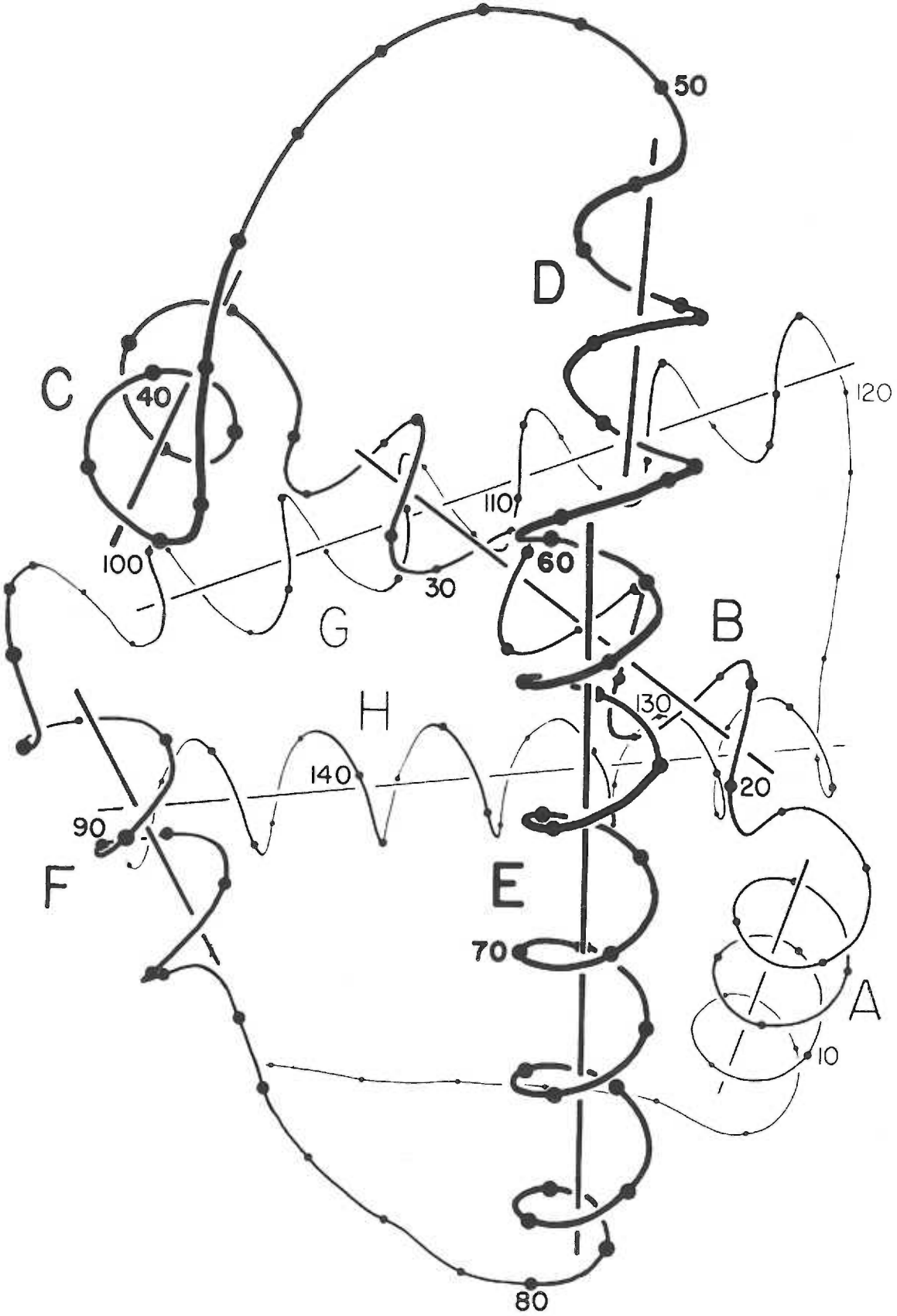
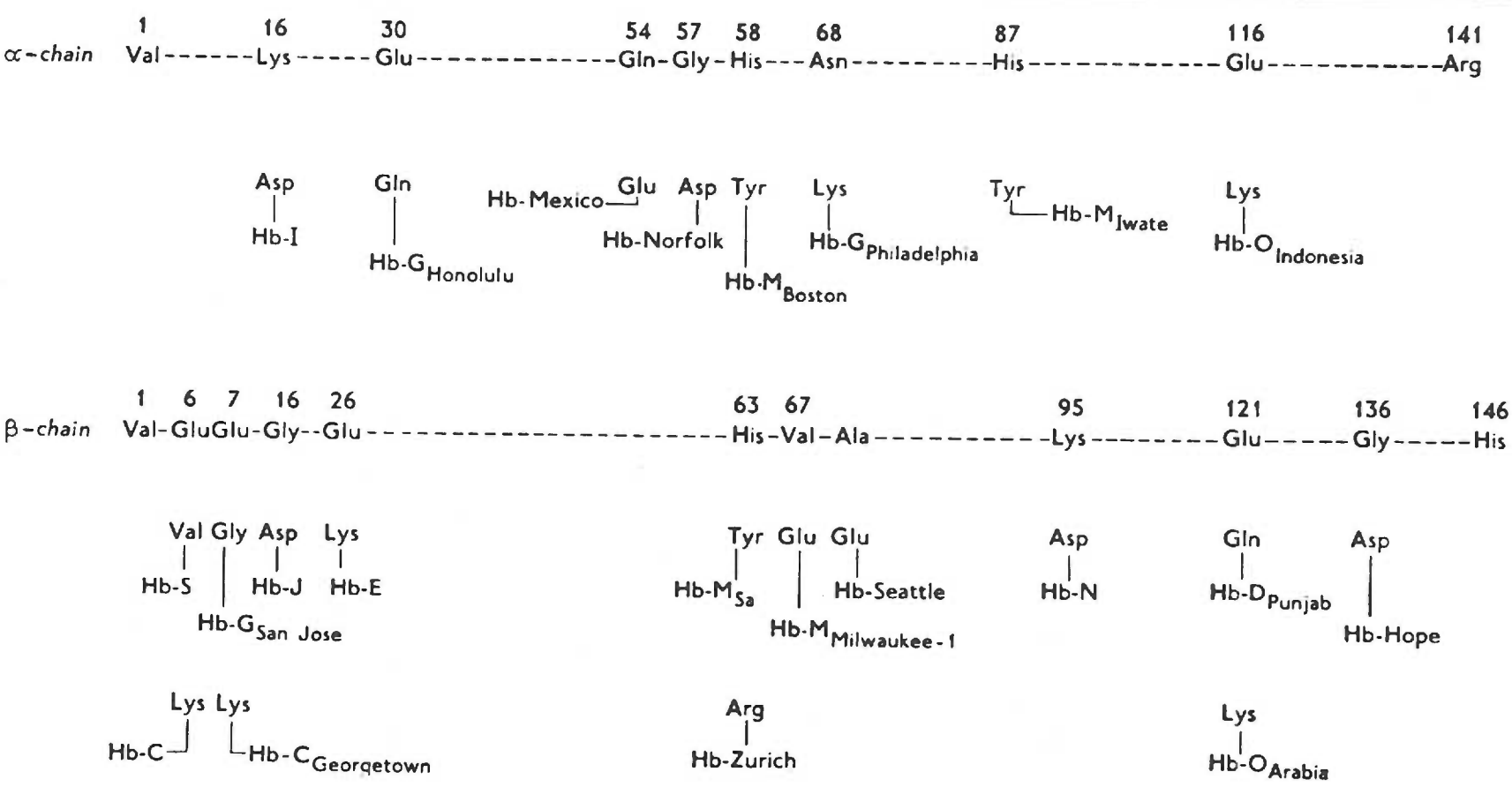

50
D
C
40
120
110
100
60
30
G
B
H
130
140
20
90
F
E
70
A
10
80

Figure 5

Amino Acid Substitutions in Some Known Hemoglobin Variants. [From: Huehns, E.R. and Shooter, E.M., Sci. Progress 52, 353 (1964)].



The amino acid substitutions in the haemoglobin variants.

of models it is seen that the α and β chains meet differently at the top of the model as compared to the contact at the bottom of the model.

Chemical studies (137,138) on hemoglobin seem to exclude covalent linkages such as disulfide bonds between cysteine residues and from the fact that the molecule is easily split into four subunits, it is most probable that the four peptide chains are held together by interpeptide salt linkages and various non-ionic interactions. Even under mild so-called normal conditions partial dissociation of hemoglobin has been indicated. Evidence comes from studies on the effects of dilution (20) and from thin membrane studies (89) at neutral pH and low ionic strength. The dissociation of hemoglobin in urea (90,91,93,94) has been ascribed to an effect on hydrogen bonding which would imply that hydrogen bonds play an important role in maintaining the structure of the molecule. The effect of urea may also be due to rupture of hydrophobic bonds (139,140) which Kendrew (61) has shown to play an important role in stabilizing the hemoglobin structure. Facts about the dissociation of hemoglobin at acid and alkaline pH values (94,96,97) suggest that the subunits are also held together by electrostatic forces associated with the ionization of acidic functions such as carboxyls and histidines and by basic functions such as amino groups. The effects of salt (99,100) on hemoglobin may be linked to electrostatic effects where shielding effects of the salts on charges is important. At very high ionic strength there may also be rupture of hydrophobic bonds which contributes to splitting of the molecule.

D. Allosteric Transition

The first evidence for the changes in hemoglobin crystals upon reversing the oxygenation reaction were made by Haurowitz in 1938 (62).

Evidence of a configurational change was obtained by Muirhead and Perutz (63) in x-ray studies of human deoxyhemoglobin compared to horse oxyhemoglobin. The arrangement of the subunits alters during oxygenation and when oxygen is removed the β chains move apart, increasing the distance between their iron atoms by 7Å. The α chains do not alter their relative positions.

In later work (64) horse (rather than human) deoxyhemoglobin was compared to horse oxyhemoglobin with similar results obtained as in studies with human deoxyhemoglobin. Thus it is not a species difference that causes the alteration and indeed the conformations of horse and human hemoglobin must be very similar.

There are indications that both α and β chains, or at least two kinds of chains, are necessary for hemoglobin to exhibit the characteristic sigmoid oxygen equilibrium curve and the related properties of heme-heme interaction and the Bohr effect. It has been shown by Horton and Huisman et al. (147) for Hb Bart's and by Benesch (141) for hemoglobin H (β_4) that there is an absence of a Bohr effect, which concerns the change in oxygen affinity of hemoglobin with a change in pH of the medium, and an absence of "heme-heme interactions" which concerns the indirect interaction between the four heme groups as the presence of a ligand on some sites modifies the affinities of

other sites for the same ligand. The high overall oxygen affinity of hemoglobin H suggests a decreased interaction between the chains, hence between the hemes (141). Recent findings of Perutz and Mazzarella (65) indicate an absence of structural changes in hemoglobin H upon oxygenation-deoxygenation as shown for hemoglobin A ($\alpha_2\beta_2$). This supports the hypothesis that a change in conformation is associated with the characteristic sigmoid oxygen equilibrium curve of normal hemoglobin (142).

This is the first definitive demonstration of a molecular rearrangement within a protein molecule following the binding of a substrate and in this instance is directly related to the interactions between heme groups.

The change in the positions of the hemoglobin subunits in the molecule on oxygenation illustrates a wider phenomenon which Monod, Changeux and Jacob (66) suggest plays a key role in the regulation of cell metabolism.

V. HUMAN HEMOGLOBIN VARIANTS (ABNORMAL)

A. Methods of Identification of Abnormal Hemoglobins

A pure form of an abnormal hemoglobin is required to study the structure and when a pure form is available specific techniques can be used to identify certain properties.

1. Electrophoretic techniques are widely used for both detection and isolation of abnormal hemoglobins. A discussion of electrophoretic methods is presented by Huisman (68).

2. There are chromatographic techniques to separate

hemoglobins on ion-exchange materials such as Amberlite IRC-50, CM-cellulose and DEAE-cellulose and descriptions of these methods are given by Huisman (69) and Jones and Schroeder (70).

3. The hybridization technique of Itano and Singer (71) is used to locate the abnormality in one or the other or both types of chains. A mixture of an unknown and reference hemoglobin are subjected to acid or alkaline pH where subunit dissociation occurs. Neutralization and reassociation follows with random recombination and transfer of chains as shown by the following example:

$$\alpha_2^X\beta_2^X + \alpha_2^Y\beta_2^Y \longrightarrow \alpha_2^Y\beta_2^Y + \alpha_2^X\beta_2^Y + \alpha_2^Y\beta_2^X + \alpha_2^X\beta_2^X$$

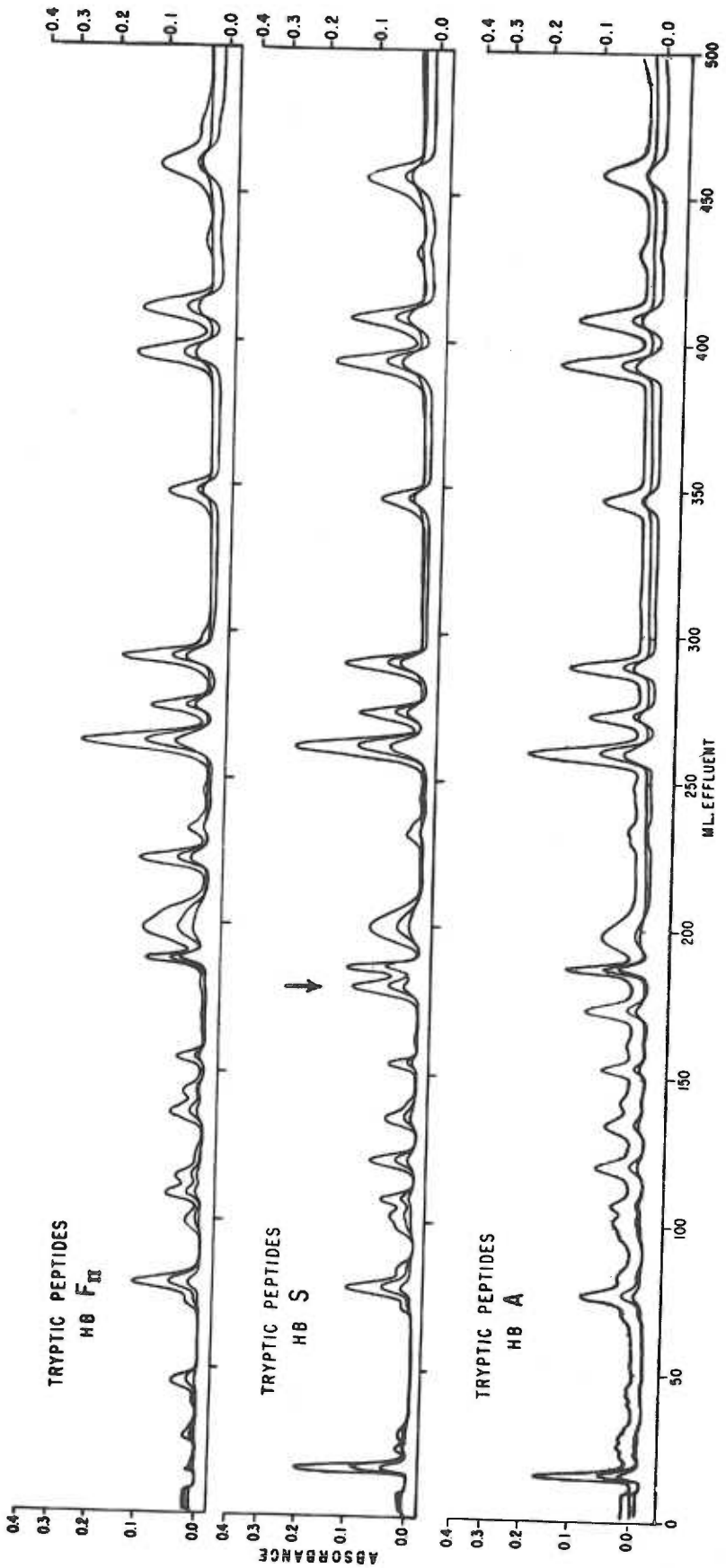
When the relative charges on the two reacting species and the new species are different, the resulting four species can be resolved by electrophoresis.

4. A study of the tryptic digest peptides by the "finger-print" or "peptide map" technique of Ingram (72) yields further localization of the abnormality.

5. Column chromatography techniques with automatic recording of the pattern of enzymatic digests have been described by Jones (33). Figure 6 shows a comparison of tryptic digest patterns of hemoglobin A and hemoglobin S where the peak indicated by the arrow has moved relative to the equivalent peak of hemoglobin A. It is then necessary to isolate the peptide and determine its sequence for final identification.

Figure 6

Peptide Patterns of Tryptic Hydrolysates of Hemoglobins A, S and F. [From: Jones, Cold Spring Harbor Symp. Quant. Biol. 29, 297 (1964)].


TRYPTIC PEPTIDES
HB F_{II}
TRYPTIC PEPTIDES
HB S
TRYPTIC PEPTIDES
HB A
ABSORBANCE
ML. EFFLUENT
0.0
0.1
0.2
0.3
0.4
0
50
100
150
200
250
300
350
400
450
500

B. Relationship of Abnormal Hemoglobins and Disease

1. Impairment of Physical Properties of a Hemoglobin

The first and classic hemoglobinopathy is sickle cell hemoglobin disease. The hemoglobin S under conditions of low oxygen tension produces sickle-shaped forms of the red cell which are very easily broken, thus resulting in severe anemia. The findings in sickle-cell disease are explained by the physical properties of reduced hemoglobin S and particularly its low solubility (143). Deoxygenation of sickle-cell hemoglobin results in the intracellular gelling of hemoglobin resulting in sickle-shaped tactoids which can be made to disappear on reoxygenation. The single amino acid substitution (5,73) in hemoglobin S thus causes a disease by a change in the physical properties of the hemoglobin molecule with no alteration in its ability to carry oxygen.

2. Impairment of Oxygen Transport Ability

The group of hemoglobinopathies where the ability to carry oxygen is impaired is the hemoglobin M group. A characteristic is the unusual stability of the oxidized ferric heme iron which leads to a hereditary methemoglobinemia. The methemoglobin does not combine with oxygen. Though not due to an unusual oxidized heme iron, the hemoglobins H and Bart's have such high oxygen affinities as to render them of little use in oxygen transport (141,147).

Very recently an abnormal hemoglobin associated with a polycythemic condition has been shown to have a greatly increased oxygen affinity (148). A second such abnormal hemoglobin linked with a condition of polycythemia and having an increased oxygen affinity is

currently being studied by Koler et al. (149).

3. Unstable Hemoglobin Diseases

A number of hemoglobinopathies are known which result from the production of an abnormal molecule which is less stable than the normal form. An example is hemoglobin Zurich which is very unstable and precipitates in the cell in the presence of the administered drug sulphanilamide. Not all members of this group require certain drugs be present. Another specific example is the highly unstable hemoglobin H (Rigas et al., 116) which results in formation of intraerythrocytic inclusion bodies which represent the denatured and less soluble precipitate of the hemoglobin.

4. Thalassemias (Chain Synthesis Failure)

In the thalassemias the rate of synthesis of hemoglobin is reduced but the resulting protein has no abnormal amino acid sequence. Either the α or β chain may be affected so that abnormal hemoglobins lacking α chains appear in α thalassemia. The rate of synthesis of α chains is decreased and there is an excess of non α chains which then form tetramers. Hemoglobin H (116) consists solely of β chains (144,145) and hemoglobin Bart's consists of four γ chains (145). Recently a hemoglobin of only δ chains has been described (74). It appears that these hemoglobins have such a high affinity for oxygen that they are of very little use in oxygen transport.

For a detailed discussion of the various abnormal hemoglobins, the related clinical symptoms and the relevant structural-functional features and genetic studies, the reader is referred to

pertinent references (43,60,67).

VI. EQUILIBRIUM OF HEMOGLOBIN WITH OXYGEN

There is a substantial configurational change of the hemoglobin molecule upon oxygenation (63). The evidence points to different bonding in the two forms (oxy- and deoxyhemoglobin) of the protein both between unlike chains and like chains (142). It is of interest to mention some aspects of hemoglobin function which are connected to oxygen equilibrium in view of the configurational change associated with oxygenation.

The oxygenation curve of hemoglobin has a sigmoid shape whereas a rectangular hyperbolic curve would be expected as a result of mass action for equilibrium reaction between deoxyhemoglobin and oxygen to form oxyhemoglobin. It has been shown by Roughton et al. (150) that strong interaction exists between the four heme groups which is termed "heme-heme interaction". The affinities of the other sites for ligand binding are modified by the presence of a ligand on some sites and in this case there is an increased affinity for oxygen at successive sites.

Another point of interest is the relationship between a change in acid strength and the change in oxygen affinity. This phenomenon is the Bohr effect (151) which is suggested by Coryell and Pauling (152) to be to alteration of the acid strength of different dissociating groups, two of them the histidines involved in the heme-globin linkage. They suggested that the acid strength changes were due to alterations in the electronic structure of hemoglobin during

oxygenation. More recent explanations of the Bohr effect by Wyman (153) suggest that configurational changes that led to alterations in the positions and environments of certain acid groups produced a change in their acid strength. This proposal fits into present concepts of allosteric proteins. Chemical studies by reactions with sulfhydryl groups have been done to elucidate the Bohr effect (82,141,142). The two reactive sulfhydryl groups (79,82) are the cysteine residues at position 93 of the β chain (81) and since they are adjacent to the proximal histidine at position 92 of the β chain it is likely that they are involved in the processes of reversible oxygenation of hemoglobin.

VII. CHEMICAL MODIFICATIONS OF HEMOGLOBIN

A. Sulfhydryl Group Treatment

It was first shown by Anson and Mirsky (136) that mammalian hemoglobins contain sulfhydryl groups, some active and some not reactive. Investigations of the number and properties of SH groups in different hemoglobins have been discussed in many papers. Many analytical results have been considered in a review by Cecil and McPhee (77).

There are six SH groups in human hemoglobin with one in each α chain and two in each β chain (9,78). Two of the six are reactive (79) toward SH reagents and these two are located in the β chain (80). It was shown by Goldstein et al. (81) that the reactive SH group of the human β chain is at the cysteine residue at β_{93} which is adjacent to the proximal histidine residue.

There determination was based on sequence studies of tryptic peptides of the β chain to locate the cysteine residues as derivatives of iodoacetamide. Following reaction with iodoacetamide the hitherto inaccessible lysine of the βT-13,14 dipeptide which contained one of the sulfhydryl groups was cleaved and yielded βT-13 and βT-14 tryptic peptides with carboxymethylcystine in βT-13 and none in βT-15. The reactive sulfydryl group of the βT-13 has been shown by x-ray to be next to the histidine which is coordinated with the heme iron atom, thus there is close proximity between the reactive sulfhydryl and proximal histidine bound to the heme group.

It has been found by Riggs (81) and by Benesch and Benesch (83) that the sulfhydryl of β_{93} has reactivity toward SH reagents in the oxyhemoglobin form but not in the deoxyhemoglobin form. The role of SH groups in oxygen equilibrium as far as the Bohr effect (pH effect on oxygen dissociation curve) is concerned has been discussed by Benesch and Benesch (83) and they concluded this effect is not directly related with the presence of free SH groups. The heme-heme interactions and participation of free SH groups in this phenomenon have been considered by Taylor et al. (84) and it is indicated that the absence of free SH groups does not necessarily modify the shape of the O_2 dissociation curve.

A detailed study of the reactions of the SH groups with N-ethylmaleimide and iodoacetamide has been made by Guidotti and Konigsberg (85) where they conclude in part that the N-terminal group of the β chain is involved in the α and β chain interaction.

B. Covalently Linked Foreign Groups

Introduction of foreign groups into the polypeptide chain produce substantial changes in the O_2 equilibrium of hemoglobin. Antonini (86) has recently done studies on hemoglobin bound with fluorescein isothiocyanate which is probably attached to the amino groups. There are great changes in the properties with O_2 affinity much greater and the Bohr effect nearly eliminated.

C. Other Reactions

It has been shown that the ferrous heme iron can combine with a number of small ligands, such as CO and NO, as well as oxygen. When in the ferric form the iron can combine with cyanide.

Acetylation reactions with acetic anhydride has been studied by Bucci et al. (87) but this system is not well described.

Also titration curve data of Wyman et al. (15) indicate histidine imidazole groups linked to the heme iron as indicated by x-ray data.

VIII. ASSOCIATION-DISSOCIATION PHENOMENA IN HEMOGLOBIN

A. Introduction

The tendency of hemoglobin to dissociate into lower molecular weight subunits even under mild conditions is a very notable property of hemoglobin. This dissociation is directly related to the non-covalently linked teteramer of polypeptide chains. The actual forces responsible for the integral structure are not completely known as yet but some discussion of these forces has been given in Section IV, A and C.

B. Dissociation Studies

1. Normal Conditions

It has been observed that horse and human hemoglobin may be partially dissociated at concentrations below 1% even under mild, so-called normal conditions (20). In contrast to these observations is the data of Schumaker and Schachman (88) which suggests no splitting of hemoglobin at concentrations of 0.05 mg/ml. Also the light scattering studies of Rossi Fanelli do not indicate any appreciable dissociation below 0.2 mg/ml. Recent work by Guidotti et al. (89) presents evidence for the presence of an equilibrium between the tetramer and its subunits at neutral pH and low ionic strength.

2. Special Conditions

Urea solutions and their effects on hemoglobin have been studied a great deal. The early work of Burk and Greenberg (90) and Wu and Yang (91) showed that horse hemoglobin had a molecular weight half that of the tetramer in 6.66 M urea. They observed species differences of hemoglobins exposed to urea. Steinhardt (92) did a detailed study of the effects of urea and other amide solutions on the sedimentation and diffusion constants.

More recent work includes that of Gutter et al. (93) of the effects of 4 M urea on horse, human and dog carboxy hemoglobins. The effects of urea and guanidine hydrochloride were again investigated by Kuihara and Shibata (94) with results similar to those of Steinhardt (92). The oxygen equilibrium of hemoglobin in concentrated urea solutions has been examined by Rossi Fanelli et al. (95). These

studies show that in urea, hemoglobin undergoes conformational changes with altered oxygen equilibrium and increased susceptibility to irreversible denaturation.

Many investigations on dissociation of hemoglobin under conditions of low and high pH have been performed.

There appears to be a stability zone of hemoglobin between pH 6 to 10. Careful studies of hemoglobin at pH values below 6 have been made by Field and O'Brian (96), by Hasserodt and Vinograd (97) and by Wyman et al. (75). The effects of temperature seem to be negligible (75). In the range from pH 6 to 45 it is revealed that there is a dissociation into subunits, most likely of αβ subunits, with molecular weight value half of the normal value and a rapid association-dissociation equilibrium exists.

Below pH 4.5 a further dissociation of hemoglobin into quarter molecules occurs (98) but a completely reversible and rapid association-dissociation equilibrium is not revealed.

At alkaline pH hemoglobin dissociates (20) into subunits and data (97) indicates that the sedimentation coefficient of human hemoglobin decreases at above pH 9.5 to a minimal 3 S at pH 11, thus a molecular weight of about half the whole molecule. Other data on horse hemoglobin by Kurihara and Shibata (94) indicate no decrease in molecular weight as the sedimentation coefficient decreases from pH 9.5 to 10.5.

Concentrated salt solutions also have been used to effect dissociation of hemoglobin into subunits (99,100,101). There does

not appear to be the tendency to denature in salt solutions as is the case in solutions at extreme pH. The dissociation appears to be readily reversible and the molecular weight approaches a value half the normal value in the most concentrated salt solutions. The comparative effects of salt solutions upon oxyhemoglobin and deoxyhemoglobin have been observed (99,101).

C. Mechanism of Dissociation

The data reported demonstrate that hemoglobin is a tetramer of four chains that undergoes reversible dissociation into double chain and single chain subunits. Salt solutions and extreme pH solutions can produce such dissociation of hemoglobin though it is also considered that with physiological conditions an equilibrium exists between the tetrameric, the dimeric and the monomeric forms of the molecule.

The problem that arises is as to how the hemoglobin dissociates, whether it is symmetrical ($\alpha\beta$ type subunits) or asymmetrical (α_2 and β_2 type subunits). Consideration of the possible dissociation schemes that can be written for the reactions to form dimers and monomers from tetramers is given:

$$\begin{array}{ccccccc} 2\alpha & + & 2\beta & \rightleftharpoons & \alpha_2\beta_2 & \rightleftharpoons & \alpha_2 + \beta_2 \\ & & & & \downarrow\uparrow & & \downarrow\uparrow \\ & & & & 2\alpha\beta & \rightleftharpoons & 2\alpha + 2\beta. \end{array}$$

Hybridization studies (71) were used to make interpretations about the mechanism of dissociation. When a mixture of two hemoglobins which differ in the composition of opposite chains are exposed to acid pH for some time and then neutralized, new hybrid species can be observed.

This can be illustrated by the following overall reaction in formation of new hybrids between human and canine hemoglobins.

$$\alpha_2^{A}\beta_2^{A} + \alpha_2^{Can}\beta_2^{Can} \longrightarrow \alpha_2^{A}\beta_2^{Can} + \alpha_2^{Can}\beta_2^{A} + \alpha_2^{A}\beta_2^{A} + \alpha_2^{Can}\beta_2^{Can}$$

with the parent species reformed as well.

The first proposed mechanism by Singer and Itano (102) was that of asymmetric dissociation into α_2 and β_2 subunits and was written as

$$\alpha_2\beta_2 \rightleftharpoons \alpha_2 + \beta_2$$

It was observed that the rate of exchange to form hybrids is slow but that the initial dissociation of hemoglobin is rapid. A second proposal was made by Vinograd and Hutchinson (103) which is represented by the following reaction:

$$\alpha_2\beta_2 \rightleftharpoons 2\alpha\beta \rightleftharpoons 2\alpha + 2\beta$$

One assumption was that an initial rapid symmetrical dissociation into αβ subunits occurred with a subsequent slower equilibrium reaction into α and β chains. Evidence of a stable chain monomer in solution (104) supports this mechanism. Another assumption was that only like two-chain subunits as αβ subunits could unite. This assumption was to explain the absence of a hybrid of the type $\alpha_2^{A}\beta^{A}\beta^{S}$.

An explanation of the above phenomena by Guidotti and his colleagues (89) has been recently presented. The important aspect of their argument is that for the preceding reaction with four chain structures in equilibrium with the two chain and single chain subunits that in the separation process the rate of separation is slower than

the rates of the above equilibria. Thus the species $\alpha_2{}^A\beta^A\beta^S$ will dissociate into $\alpha^A\beta^A$ and $\alpha^A\beta^S$ subunits which then are converted into the species $\alpha_2{}^A\beta_2{}^A$ and $\alpha_2{}^A\beta_2{}^S$

$$2\ \alpha_2{}^A\beta^A\beta^S \rightleftharpoons \alpha_2{}^A\beta_2{}^A + \alpha_2{}^A\beta_2{}^S$$

as represented in the above reaction.

The studies of Guidotti et al. (89,105) stress the concept of "dynamic equilibrium" which appears to explain most phenomena of hemoglobin dissociation, although there are still questions that can be answered only with qualification (43), such as the observation that an asymmetric hemoglobin such as A_{Ic} ($\alpha_{2\beta\beta}{}^X$) and F_I ($\alpha_{2\gamma\gamma}{}^{N\text{-acetyl}}$) where a non-alpha chain has a blocking group at the N-terminus. If the rate of dissociation ($\alpha_{2\gamma\gamma}{}^{FI} \longrightarrow \alpha_\gamma + \alpha_\gamma{}^{FI}$) into half molecules is slow or if the form $\alpha_2\ \gamma_2{}^{FI}$ is less stable than $\alpha_{2\gamma\gamma}{}^{FI}$ then the isolation of these hemoglobins by chromatography may be consistent with the dynamic equilibrium.

It does not necessarily follow that the dissociation pathway is identical to the reassociation pathway. In addition to the asymmetric mechanism of Itano and the symmetric mechanism of Vinograd with the dynamic equilibrium model of Guidotti there is another proposed dissociation-association scheme (43) as illustrated by the reaction scheme

$$\begin{array}{ccc} \alpha_2\beta_2 & \rightleftharpoons & 2\alpha\beta \\ \downarrow\uparrow & & \downarrow\uparrow \\ \alpha_2 + \beta_2 & \rightleftharpoons & 2\alpha + 2\beta. \end{array}$$

It is called the "circular mechanism" and considers that dissociation

and reassociation may involve a different set of reaction steps.

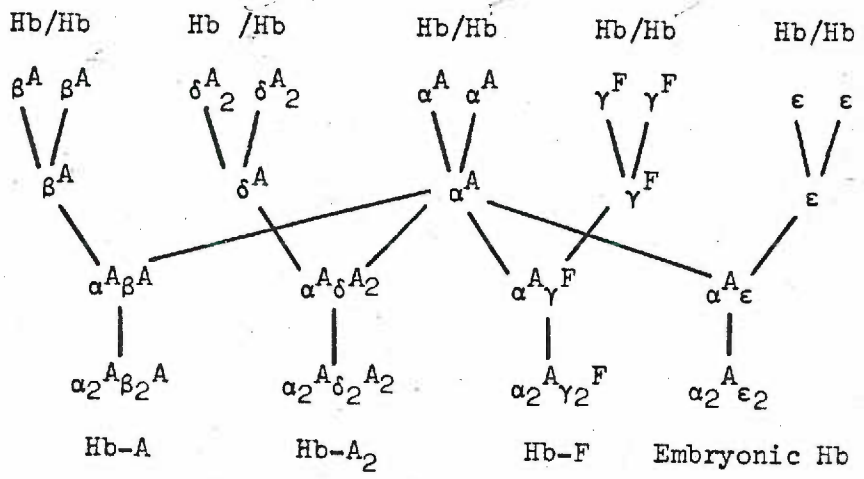
IX. BIOSYNTHESIS OF HEMOGLOBIN

A great amount of data is available about both the structure of hemoglobin and of family studies of inheritance of variants of hemoglobin, thus it is a productive area for studies on genetic control of protein synthesis.

It is thought that the primary structure of proteins is determined genetically and that there is a linear correspondence existing between codons or triplets of the DNA bases and the amino acid sequence in the polypeptide.

It has been concluded that the α and β chains of hemoglobin are controlled by separate genetic loci (106,107). The α chains of the normal human hemoglobins are identical and it has been suggested that they are controlled by the same genetic locus (108,109). Because of structural differences between the γ and β chains, it is assumed that there is a separate genetic locus for the control of the γ chain synthesis. The chemical evidence also suggests that the genetic control of the sequence of the γ and δ chains are independent of the α and β chain genes (67,109,110).

It has not been possible to perform genetic studies on the embryonic hemoglobins (52,53,54) but it has been postulated by Huehns (67) that there is a separate genetic control for the ε chain. The outline for the control of synthesis of the normal hemoglobins would then be as represented in the following diagram:



There must be a genetic control of the change-over from synthesis of embryonic hemoglobin to synthesis of fetal hemoglobin and then to adult hemoglobin. This control may be explained (154,155) through the concept of controller and regulator genes as proposed by Jacob and Monod (111).

The hemoglobins of humans are synthesized intracellularly at specific stages before the erythroid cells develop into mature erythrocytes which are non-nucleated in mammals. The unnucleated erythrocyte does not carry on hemoglobin synthesis. An intermediate between the nucleated erythroid cell and the mature erythrocyte is the reticulocyte which carries out to some extent hemoglobin synthesis and these reticulocytes have served as the main system for hemoglobin synthesis studies. The reticulocytes used for studies have often been obtained from rabbits after treatment with phenylhydrazine to produce severe anemia.

The mature red cell can accumulate constituent amino acids (158) and the reticulocyte can concentrate these amino acids even more (159).

Amino acids enter the biosynthesis of proteins by means of an activation and coupling with s-RNA (160). The s-RNA's are low molecular weight ribonucleic acids (RNA) and there is a specific s-RNA for each specific activated amino acid.

The genetic information of a hemoglobin polypeptide chain would presumably be transcribed from the deoxyribonucleic acids (DNA) in the nucleus into the messenger ribonucleic acid (m-RNA) (161). The m-RNA in turn carries the transcribed genetic information to the ribosomes (245) where active hemoglobin synthesis takes place.

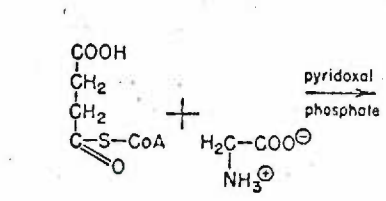
The amino acid residues are incorporated into polypeptide chains consecutively, and as shown by Dintzis (163) and by Naughton and Dintzis (164) with radioactive amino acids in rabbit reticulocytes, the synthesis is initiated at the N-terminal end and progresses by sequential addition towards the C-terminal end where it terminates. Studies of Dintzis et al. (163,164) have indicated that the synthesis of the α and β chains are independent of one another. The mechanism for the final release of a completed hemoglobin polypeptide chain is not understood but appears to involve a specific release reaction (165) that is independent of the chain formation process.

The heme prosthetic group of hemoglobin is synthesized independently (114,166,178,179) and a summary of the biosynthetic steps are shown in Figure 7.

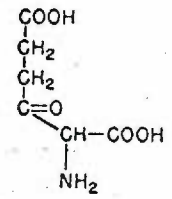
It is as yet not known when the protein achieves its final secondary, tertiary and quarternary structures nor is it known at what point the heme and a polypeptide chain are assembled.

Figure 7

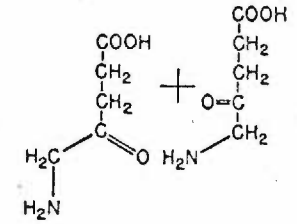
Biosynthetic Steps of Heme from Succinyl-Coenzyme A and Glycine. [From: Schemin, D. (179) as presented by Schroeder and Jones (43)].



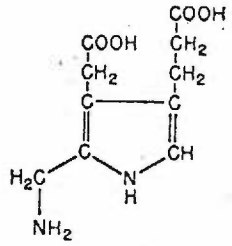
(1.) Succinyl-coenzyme A.



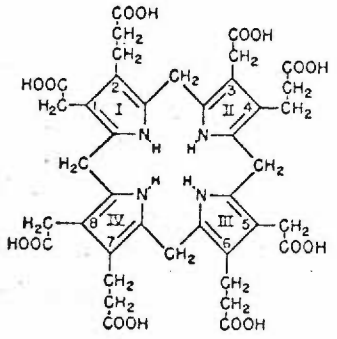
(2.) α-Amino-β-keto-adipic acid.



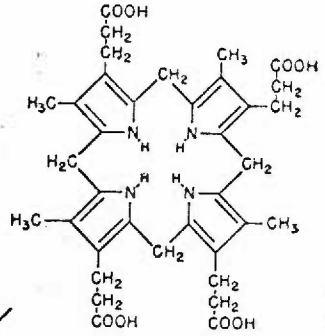
(3.) δ-Aminolevulinic acid.



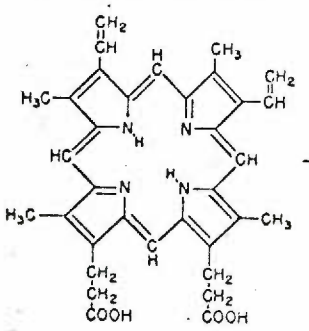
(4.) Porphobilinogen



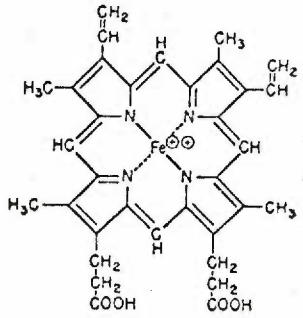
(5.) Uroporphyrinogen III.



(6.) Coproporphyrinogen III.



(7.) Protoporphyrin-9.



(8.) Heme.

With the α chains of the normal human hemoglobins identical it has been suggested that these α chains arise from a common metabolic pool. The production and release of δ, γ and β chains appears to be independent of the release of α chains since the existence of molecules such as β_4 and γ_4 is known. These and other findings have led to the suggestion that β, γ or δ chains are necessary for free release of α chains from their site of synthesis (115) to form an intermediate αβ type subunit with combination of two of these to form a tetramer in the presence of heme.

X. STATEMENT OF PROBLEM

Previous work of Huehns (54) has demonstrated the existence of embryonic hemoglobins and in particular the persistence of embryonic hemoglobin Gower 2 ($\alpha_2\varepsilon_2$) in the D-trisomy disease where increased amounts of hemoglobin Bart's (γ_4) were also observed. These observations have been interpreted in terms of the operator-regulator control mechanism of Jacob and Monod (66) with a proposed mechanism that leads to an excess of ε and γ chains. Other observations of undescribed hemoglobin components in hemoglobin H (β_4) cases, where hemoglobin Bart's (γ_4) occurs as well, have been reported. The isolation of the hemoglobins which included hemoglobin Bart's (γ_4) from a possible D trisomy case revealed a new component designated as hemoglobin X. It was proposed to study the physical and chemical properties of hemoglobin X and to compare it to known hemoglobin structures in terms of properties and of possible mechanisms for its production.

Information about the forces which hold the subunits of proteins can be deduced by studies of the dissociation into lower weight subunits under certain conditions. Very little information is available about the dissociation of hemoglobins other than normal adult hemoglobin and horse hemoglobin. It was proposed to examine the subunit dissociation of hemoglobin X, Bart's, H and A to detect differences and similarities in the nature of the forces holding together the subunits of abnormal and normal hemoglobins.

EXPERIMENTAL

I. HEMOGLOBIN SOURCES

A. Hb A

The samples of normal Hb A were isolated from normal adult human blood samples. Samples were drawn in a heparinized syringe.

B. Hb H

The blood samples containing Hb H were obtained from two sources. One patient, H.D., was a 42-year old female of Chinese ancestry. The second patient was a male, R., about 22 years old of Thai ancestry. Samples from H.D. were drawn in this laboratory with a heparinized syringe. The sample R. was drawn in Thailand, preserved in Alsever's vial and transported by airmail within five days.

C. Hb Bart's

The sample of Hb Bart's was secured from a 20-day old female infant Chinese, M.K.D., immediately after the baby expired. The diagnosis included multiple congenital anomalies, Trisomy-E syndrome and possibly mosaicism of Trisomy-D.

D. Hb X

This sample of hemoglobin was isolated from the same patient, M.K.D., as described for Hb Bart's source.

II. ISOLATION OF HEMOGLOBIN SAMPLES

A. Hemolysate Preparation

A given blood sample was centrifuged at low speed for 15 minutes to separate the red cells from the plasma. The red cells were then washed three times with cold 0.9% saline solution by

centrifuging in the cold (4°C) and removing the resultant supernatant each time (79). The washed red cells were hemolyzed by adding an equal volume of distilled deionized water and toluene added in the amount of 0.1% of the volume of cells. The mixture was shaken vigorously for five minutes, then centrifuged in the Servall, SS-34 at 20,000 rpm (4°C) for 30 minutes. The clear hemolysate was removed from the precipitate with a syringe. If any stroma remained a second centrifugation at 20,000 rpm was performed.

B. Concentration of Hemoglobin Samples

The concentration of any hemolysates or hemoglobin containing effluents was achieved by use of an apparatus specially designed by Dr. D.A. Rigas of the University of Oregon Medical School (167). It consisted of a long-necked, round-bottom piece of glassware with ground glass outside fittings at top and bottom and a drain plug in the bottom. A selected Visking dialysis tubing 8/32" (HMC, 52 Goucester Place, W.I.) was placed between an inside ground glass fitting at the top and a small bulb 0.25 to 1.0 ml with outside ground glass fitting at the bottom. This allowed the tubing to be moderately stretched between top and bottom with an attachment point at the top where a tygon tubing could be connected and extended to the sample flask. A side arm on the long neck was attached to a vacuum line and when vacuum was applied the dialysis sack stretched and ultrafiltration proceeded as water and small molecules passed through the membrane but protein was retained. The entire apparatus was placed in a cold room (4°C) when in operation.

C. Starch Block Electrophoresis

Separation of some hemoglobin species was done on starch block electrophoresis by the general procedure of Kunkel (168). The procedure employed the use of an E-C Apparatus (E-C Apparatus Co., Swarthmore, Pa.) complete with power supply. The starch used was soluble potato starch (Baker). An amount of 300 g of starch was placed in a 1 liter beaker and buffer was added to make a slurry to which was added 0.15 ml 1 N NaOH. The mixture was stirred with a magnetic stirrer for half an hour and filtered on a Buchner suction filter and rinsed with a liter of buffer, then allowed to dry for about half an hour.

The starch was then suspended in buffer by slow addition of the desired buffer to produce a thick batter-like consistency. This mixture was then poured onto the level plate of the apparatus and allowed to stand overnight at a temperature of 10 to 15°C.

In several cases a synthetic granular material, Pevicon, was used in place of starch with very similar results. Pevicon is a copolymer of polyvinyl chloride and polyvinyl acetate.

The buffer used in all cases was a barbital buffer pH 8.6, .05 M prepared with sodium diethylbarbiturate and diethylbarbituric acid (Merck & Co., Inc.) and distilled deionized water.

To load the hemolysate a 1 ml syringe fitted with a #27 needle was employed. A maximum of eight evenly spaced spots were made by addition of 0.2 to 0.4 ml hemolysate per spot.

A voltage of up to 800 V, 80 ma was then applied until the desired separation was achieved.

The desired portions of the separated fractions were then cut from the block with a spatula and placed in fritted glass suction filters and the hemoglobin carefully eluted with buffer and slight suction carefully avoiding the passage of any air into the starch.

An alternate elution method employed glass cylinders with a fritted glass disc midway and an air vent just below the disc. The upper cylinder section was filled with the desired fraction of starch and then placed into a centrifuge tube and centrifuged to effect elution of the sample. The eluted sample was then concentrated by the ultrafiltration procedure as described.

D. Column Chromatography

1. DEAE Cellulose

This procedure was used for isolation of Hb H from patients H.M.D. and R. The resin was DEAE type 40 #72, Lot #1338, capacity 0.98 meq/g distributed by C. Schleicher and Schuell Co., Keene, N.H.

An amount of 80 g of DEAE was suspended in 1 liter of 1 M K_2HPO_4, pH 9.18 and mixed with a mechanical stirrer for one hour. The mixture was then filtered on a Buchner funnel through Whatman #5 paper. The resin was washed with five liters of H_2O to obtain a pH of 7.4 of wash filtrate. The resin was suspended in 3 vols of H_2O and the fines were removed after settling. The resin was then resuspended in 1 vol of .003 M phosphate buffer, pH 7 and allowed to settle and the fines were removed with an aspirator. This was repeated five times. A final resuspension in 3 vols of .003 M phosphate buffer was done and the pH of the suspension was adjusted to pH 7.0 with approximately 1.6 M (1:10) H_3PO_4.

Before repouring the column this resin was regenerated by washing with 0.969 M KCl (one liter) and then washed with one liter of 0.1 N acetic acid followed by rinsing with 500 ml of H_2O. It was subsequently mixed with two liters of 1 M K_2HPO_4, pH 9.18, filtered and washed with five liters of H_2O until the pH of the filtrate reached 7.8. It was then washed with 1.5 liters of .003 phosphate buffer, pH 7.0 and resuspended two times with removal of fines. A final adjustment to pH 7 was made by dropwise addition of approximately 1.6 M (1:10) H_3PO_4 to the stirred suspension.

Two columns were used for this procedure. One was a jacketed 0.9 x 25 cm column which was packed with 40 ml of a 1:3 suspension of DEAE in pH 7, .003 M phosphate buffer. The slurry was added with an extension tube above the column and packed with five lbs. p.s.i. of air pressure to a resin height of 20 cm. The column was then equilibrated with 60 to 100 ml pH 7, .003 M phosphate buffer.

A load of between 600 and 750 mg of Hb (H.M.D.) in a total volume of 15 ml was placed on the column and forced into the resin at five lbs. p.s.i.

A flow rate of 20 ml per hr. was maintained and fractions were collected at 20 min. intervals in a refrigerated collector. After 12 fractions had been collected a three-vessel gradient was started which consisted of 100 ml each of pH 7, .003 M phosphate; pH 7, .03 M phosphate; and 0.5 M KCl.

A second column was used which was a 2.5 x 25 cm jacketed column which was packed with a 1:4 suspension of DEAE. A load of

500 to 800 mg of hemoglobin (H.M.D.) was placed on the column, followed by elution with pH 7, .003 M phosphate at 35 ml per hr. with fractions collected at 12 min. intervals. After 175 ml effluent was collected the eluent was changed to pH 7, .03 M phosphate and after 210 ml of effluent, the eluent was changed to 0.5 M KCl. This stepwise elution method was done with a second set of conditions using a flow rate of 22 ml per hr. and 18 min. fractions with an initial buffer of pH 7, .003 M phosphate, followed by pH 7, .03 M phosphate and then 0.5 M KCl. Still a third modification for the method included the same column but utilized a gradient of 100 ml each of pH 7 phosphate buffer at .0015 M, .003 M, and .03 M, respectively, with a flow rate of 20 ml per hr. and fractions collected at 20 min. intervals.

2. *IRC-50 Chromatography*

The separation of hemoglobin components on IRC-50 was done by methods described in detail by Jones and Schroeder (70).

Two different jacketed columns were used with a 0.9 x 38 cm column used for analytical runs and a large 5 x 38 cm column used for the preparative work. Each was poured with IRC-50 ion exchange resin (Lot DS-2654, B-1251, 200 to 300 mesh). Before use the large column was equilibrated at 6°C with six liters of developer 6 with a composition given by Schnek and Schroeder (45), which was a phosphate buffer pH 6.70 $\pm$ .02, .05 M Na^+ and .01 M KCN, while the small column was equilibrated with three liters of this developer.

The technique employed for application of a sample was to first stir the top of the resin with a smooth glass rod to a depth of

3 to 5 cm, allow the resin to resettle to a smooth, even surface, then immediately layer the sample by means of a syringe fitted with a flexible plastic tip onto the resin beneath the developer which was maintained on the column. A sample load of up to 3.5 g of hemoglobin was applied to the large column and up to 150 mg of hemoglobin to the small column.

Elution was effected with developer 6 throughout each run while maintaining flow rates of 60 ml per hr. and 6 ml per hr., respectively, on the large and small columns by means of either a Milton-Roy or Spinco pump. The temperature of the column jacket was changed from 6°C to 40°C when it was judged that the fast fractions had been eluted, which usually corresponded to 18 to 20 hours on the large column and 35 to 40 hours on the small column.

Fractions were collected in a refrigerated collector and the absorbance was measured at 540 mμ and then inspected fractions were pooled and concentrated by ultrafiltration.

III. DETECTION AND CONCENTRATION DETERMINATIONS OF HEMOGLOBIN

A. Starch Gel Electrophoresis

This procedure was carried out using the horizontal gel technique of Smithies (168) with a Tris-EDTA-borate system of pH 8.15, 0.025 M. Samples were applied by saturating paper wicks (6 x 10 mm) cut from Whatman #5 filter paper carried out at a voltage of 175 V (measured at the ends of the 13 x 25 cm gel) for 4 to 8 hours. Then the gel was sliced and stained with Amido-Schwartz stain or by the benzidine reaction.

B. Absorption Spectra

The absorption spectra of hemoglobin samples were examined and automatically recorded with either a Cary Model 14 or Model 15 recording spectrophotometer.

C. Hemoglobin Concentration Measurement

The cyanmethemoglobin method (169) was used for determination of hemoglobin concentrations. The reagent was made of 1 g $NaHCO_3$, 200 mg $K_3Fe(CN)_6$, and 50 mg KCN diluted to one liter with distilled deionized water. A mixture of 20 µl hemoglobin solution in 5 ml reagent was measured at 540 mµ with a Zeiss spectrophotometer. Adjustment for dilution and molar extinction coefficient (absorbance x 360) gave a mg/ml value for the hemoglobin solution.

An alternate method to determine concentrations was to read the absorbance of the hemoglobin solution directly and then utilize the molar extinction coefficient with necessary dilution factor to obtain a concentration value.

IV. PHYSICOCHEMICAL STUDIES

A. Sedimentation Velocity Studies

The measurement of sedimentation coefficients was carried out in a Spinco Model E analytical ultracentrifuge employing the Schlieren optical system. The An-D rotor fitted with one or two of the following cells was used: 12 mm filled-Epon double sector, 2-1/2° sectors; 12 mm aluminum single sector valve synthetic boundry, 40° sector; 12 mm aluminum single sector, 4° sector. When two cells

were employed, one was fitted with a 1° positive wedge quartz window. For a complete description of the ultracentrifuge see the Model E Instruction Manual (170).

Samples of concentrations 0.5 to .8% were dialyzed against the desired solution, or otherwise treated as indicated, prior to centrifugation and added to the ultracentrifuge cell with a small syringe.

The inorganic salt solutions were prepared with reagent NaCl (MC&B) in distilled deionized water. The buffered urea solutions employed were prepared by making stock solutions of .15 M NaH_2PO_4 (A) and .15 M Na_2HPO_4 (B) using .1 M NaCl as diluent. Then 80 ml of A and 120 ml of B plus 240 g urea were diluted with .1 M NaCl to final concentrations of .038 M PO_4 and 8 M urea with a final pH 7.4.

The other buffers used in studies of the effect of pH on the sedimentation coefficient were prepared according to tables in Methods in Enzymology, Vol. I (178) with the modification of using .15 M stock solutions in place of .2 M and using .1 M NaCl as a diluent for a final concentration of .138 M.

Dialysis of samples was done using Visking 8/32" tubing and was done at 6°C usually for 10 to 15 hours.

All pH values were made with either a Beckman Model GS or a Beckman Model 76 pH meter.

B. Measurement of Sedimentation Coefficients

Sedimentation coefficients were obtained by measurement of Schlieren patterns photographed on Kodak 1-D spectroscopic plates

utilizing a Gaertner two-dimensional microcomparator and in some cases feeding the data into a digital computer utilizing a specially written program (173) for calculation of the $s_{20,w}$ values.

The sedimentation coefficient is defined as the sedimentation velocity per unit of centrifugal field strength, $s = \frac{dx/dt}{\omega^2 x}$, and has the dimensions cm/sec/dyne/g or sec. Sedimentation coefficients are reported in svedberg units (S) where 1 S = 10^{-13} sec. The correction of observed s to $s_{20,w}$, standard conditions, i.e. in water at 20°C, is done by use of the equation:

$$s_{20,w} = s \frac{\eta}{\eta_{20,w}} \frac{1-\bar{V}\rho_{20,w}}{1-\bar{V}\rho}$$

The term η is the viscosity of the solvent at the temperature of the experiment and $\eta_{20,w}$ is the viscosity of water at 20°C. The $\rho_{20,w}$ is density of water at 20°C and ρ is the density of the solution at the temperature of the experiment (20).

C. Diffusion Coefficient Determination

The diffusion coefficients were obtained with the use of the Spinco Model E analytical ultracentrifuge utilizing the An-J rotor and 12 mm aluminum plug synthetic boundary cell with Hb concentrations of 0.7 to 1.0%. The technique is described in detail by Schachman (172).

Photographs were taken with the Schlieren optical system on Kodak 1-D spectroscopic plates. The plates were placed in an enlarger, emulsion side up, and the curves traced at a fivefold magnification. The areas were then measured by planimetry and the $D_{20,w}$ values calculated.

D. Hybridization Method

The procedure employed was essentially as described by Huehns (104). A mixture of two cyanmet-hemoglobins was dialyzed against acetate buffer, pH 4.7, .1 M for 12 to 14 hours and then dialyzed for 12 to 15 hours against a starch gel buffer, e.g. Tris-EDTA-borate, pH 8.15, .025 M.

Visking (8/32") dialysis tubing was used and the dialyses were done in the cold at 4 to 6°C.

Analysis of samples was done on starch gel electrophoresis (Tris-EDTA-borate, pH 8.15, .025 M) and the gels stained by Amido-Schwartz stain and the benzidine reaction.

V. CHEMICAL STRUCTURE ANALYSIS

The chemical procedure used in the structural analysis of hemoglobin chains was that described by Jones (33).

A. Globin Preparation

The purified hemoglobin components were converted into globin by a deheming procedure with cold acid-acetone (180).

A sample of hemoglobin (80 mg), dialyzed against distilled water overnight, was sprayed from a small syringe and #27 needle into a cold, rapidly stirred mixture of 100 ml acetone + 1 ml 1 N HCl chilled in a dry ice-cellosolve bath. The preparation was stirred for an additional one-half hour and then centrifuged at high speed in a Servall centrifuge, precooled to -20°C, utilizing a SS-34 rotor. The packed precipitate was quickly washed twice with a cold (dry ice-cellosolve) acid-acetone solution. The precipitate was then dissolved

in water, filtered through glass wool and the filtrate was lyophilized.

B. Aminoethylation and Desalting

1. Aminoethylation of globin was effected by dissolving five to 100 mg of protein in 20 ml of solution which was 8 M in urea (Merck, Reagent) and .05 M in 2-mercaptoethanol (Calbiochem). Solid Tris [2-amino-2-(hydroxymethyl)-1,3-propanediol] (MC&B) was added to make the solution 1 M in Tris (2.42 g/20 ml) and the pH of the solution was adjusted to 9.2 with concentrated HCl. Ethylenimine (MC&B) was added to make 0.5 M (0.5 ml/20 ml) and the mixture stood at room temperature for 2.5 to 3 hours. The pH was then adjusted to 3.0 with concentrated HCl.

2. The desalting procedure was used to free the aminoethylated protein from urea and other reagents by passing the mixture through a 3.5 x 35 cm column of Sephadex G 25 equilibrated with 0.2 M acetic acid. Development was with 0.2 M acetic acid and the effluent was automatically monitored at 280 mμ utilizing a Beckman DB spectrophotometer fitted with a continuous flow cuvette and readings were recorded with a continuous chart recorder. The selected protein zone was then lyophilized.

C. Chain Separation

The chains of hemoglobins X and F were separated by a simple 14 transfer train using a solvent system prepared from 36 ml 0.5 M acetic acid, 4 ml of 10% dichloroacetic acid and 40 ml of sec-butanol. The solvent components were mixed several times in a

separatory funnel and separated into aqueous and organic phases.

The transfer vessels were 10 ml glass stoppered graduated conical tubes. To each vessel was added 2 ml of the aqueous phase and 25 mg of AE-globin was dissolved in the first vessel by combining it with 2 ml of the organic phase. Successive transfers of the organic phase were performed with a syringe and needle. Each time a transfer was made of the organic phase to each succeeding vessel, the first vessel was replenished with 2 ml of fresh organic phase.

After the final transfer 0.5 ml aliquots were taken from each phase in each vessel and mixed with 0.1 ml methanol. The absorbance of each mixture was read at 280 mμ. The aliquots were returned to their vessels followed by a cyclohexane extraction and collection of the aqueous phase. The aqueous phase was evaporated to a smaller volume using a rotary evaporator, then dialyzed against water overnight utilizing acetylated dialysis tubing.

Acetylation of the dialysis tubing was done simply by immersing the tubing in a solution of 10 ml acetic anhydride plus 90 ml of pyridine and allowing it to stand at room temperature overnight.

D. Tryptic Hydrolysis

Tryptic hydrolysis was carried out by dissolving 20 mg globin in 5 ml H_2O (0.1 ml aliquot removed for amino acid analysis) and then adjusting the pH to 8.5 to 9 with trimethylamine. Trypsin was added to provide a protein to trypsin ratio of 50 to 1 and the mixture was allowed to stand at room temperature for three hours.

Adjustment to pH 6.5 with 1 N HCl was followed by standing for one hour, subsequent removal of any precipitate by centrifugation, and gradual adjustment of the clear solution to pH 2 with 1 N HCl and adjustment of the volume to 1 to 2 ml by rotary evaporation.

E. Peptide Analysis

The analysis of peptides was carried out by methods described by Jones (33). A block diagram of the overall apparatus is given in Figure 8.

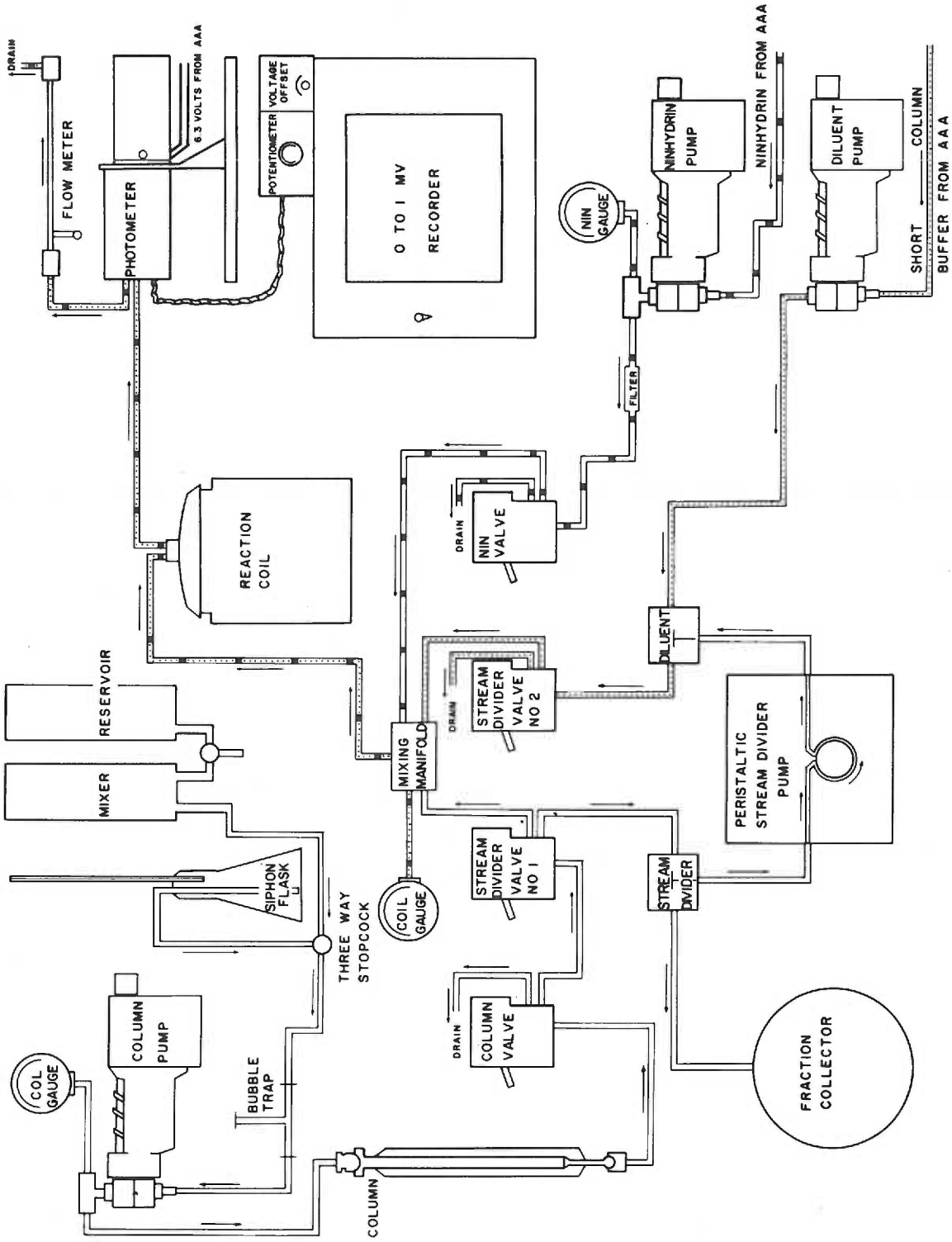
1. Analytical Chromatography

Analytical chromatograms were made with 1 mg amounts of complete tryptic hydrolysates of hemoglobin, AE-hemoglobin, AE-globin and AE-globin chains.

The column was a 0.6 x 13 cm, Spinco type 15A resin at a temperature of 50°C. The load was a soluble tryptic peptide digest of 1 mg of protein applied to 1 ml or less of solution adjusted to pH 2.0 with 1 N HCl. Initial development was a linear gradient (250 ml) of pyridine-acetic acid beginning at pH 3.1, 0.2 M pyridine and ending at pH 5, 2.0 M pyridine. The gradient was produced by placing 125 ml of pH 3.1, 0.2 M pyridine buffer in the first mixing chamber of a Varigrad (Buchler Instruments, Fort Lee, N.J.) and 125 ml of pH 5.0, 2.0 M pyridine buffer in the second chamber. Following the gradient a pH 5.0, 2.0 M pyridine buffer was passed through as a limiting buffer before re-equilibrating the column with pH 3.1, 0.2 M pyridine buffer. The flow rates of the column pump and ninhydrin pump were set at 15.0 ml per hour each, thus a total of 30 ml per hour of reaction mixture was

Figure 8

Diagramatic Illustration of Peptide Analysis Apparatus. (From: Dr. R.T. Jones with permission)


DRAIN
FLOW METER
PHOTOMETER
6·3 VOLTS FROM AAA
POTENTIOMETER
VOLTAGE OFFSET
0 TO 1 MV RECORDER
NIN GAUGE
NINHYDRIN PUMP
NINHYDRIN FROM AAA
DILUENT PUMP
SHORT COLUMN BUFFER FROM AAA
FILTER
REACTION COIL
DRAIN
NIN VALVE
DILUENT
RESERVOIR
MIXER
MIXING MANIFOLD
STREAM DIVIDER VALVE NO 2
PERISTALTIC STREAM DIVIDER PUMP
SIPHON FLASK
THREE WAY STOPCOCK
COIL GAUGE
STREAM DIVIDER VALVE NO 1
STREAM DIVIDER
COL GAUGE
COLUMN PUMP
BUBBLE TRAP
COLUMN VALVE
FRACTION COLLECTOR
COLUMN

passed through a normal length of reaction coil. The light path of the photometer flow cell was 20 mm long (174) and a recorder range of 0 to 1.0 mv per first 10 inches of scale was necessary with only the absorbance at 570 mμ being recorded at a chart speed of three inches per hour.

2. *Preparative Chromatography*

Preparative chromatograms were made with 25 mg loads of tryptic hydrolysates of AE-globin using the procedure illustrated in Figure 8.

The column was a 0.9 x 17 cm, Spinco type 15A resin at 50°C. The load was applied in a volume of 2 ml or less of solution adjusted to pH 2 with 1 N HCl. Development for the first 500 ml was a linear gradient of pyridine-acetic acid buffer beginning at pH 3.1, 0.2 M pyridine and ending at pH 5, 2.0 M pyridine. The gradient was formed as described above but 250 ml rather than 125 ml of the two buffers was placed in their respective chambers of the Varigrad.

The flow rate of the column pump was 30 ml per hour and that of the ninhydrin pump was 15 ml per hour.

A stream divider flow rate of 3 ml per hour was used, resulting in the collection of 27 ml per hour or 9/10 of the column effluent to the fraction collector which was set for a 6 min. fraction interval. The peptides were then recovered by pooling the selected zones and removing of the volatile pyridine-acetic acid buffer by evaporation.

A diluent pump was used with a flow rate of 27 ml per hour to supply a total of 45 ml per hour of reaction mixture to the coil.

The flow cell, recorder range, monitored wavelength and recorder chart speed were the same as in Section V, E, 1.

3. Rechromatography

Some of the peaks isolated from tryptic hydrolysates contain mixtures of peptides, most of which were separated by rechromatography on a 0.9 x 45 cm column of Bio-Rad Laboratories AG 50W-X2, 270 to 325 wet mesh or in some runs 80 to 200 wet mesh.

The load was applied in a volume of 2 ml or less of a zone from a preparative chromatogram and adjusted to pH 2 with 1 N HCl.

Development for the first 500 ml was a linear gradient of pyridine-acetic acid buffer as described in Section V, E, 2. A variable volume of pH 5, 2.0 M pyridine buffer was passed through the column as described above before re-equilibrating with pH 3.1, 0.2 M pyridine buffer.

The flow rate of the column pump was 30 ml per hour and the ninhydrin pump was 15 ml per hour. The stream divider pump flow rate was either 3 or 6 ml per hour depending on the estimate sample amount with a corresponding diluent pump setting for 27 or 24 ml per hour for a 30 ml per hour flow of reaction mixture through the coil.

The flow cell, recorder sensitivity and monitored wavelength were the same as Section V, E, 1 but a recorder chart speed of 1.5 inches per hour was used instead of 3 inches per hour.

4. Resin

The Spinco type 15A resin (a sulfonated styrene-8% divinyl benzene copolymer resin) used for analytical and preparative chromatography and the Bio-Rad Laboratories AG 50W-X2, (a sulfonated polystyrene-2% divinyl benzene copolymer resin) 270 to 325 wet mesh range or 80 to 200 wet mesh range were all regenerated by the same procedure. The resin was placed in a scintered glass suction filter and washed as follows:

1. 200 ml dd H_2O
2. 250 ml 4 N HCl
3. 200 ml 1 N NaOH
4. 200 ml dd H_2O
5. 200 ml pH 3.1, 0.2 M pyridine-acetic acid buffer

The resin was then suspended in the pH 3.1 pyridine buffer, allowed to settle and any fines removed. A 1:3 suspension in pH 3.1 pyridine buffer was then poured with the aid of a column extension. The resin was packed and equilibrated before use with pH 3.1, 0.2 M pyridine buffer.

5. Buffers

The pyridine-acetic acid buffers used for development in peptide chromatography were those described by Jones (33). The pH 3.1, 0.2 M buffer was prepared by dilution of 32.2 ml of distilled pyridine (Merck) to a 2 liter volume with diluent made of 5,103 ml glacial acetic acid (Merck) plus 13,147 ml dd H_2O. The pH 5.0, 2.0 M buffer was prepared by dilution of 322 ml of distilled pyridine to a 2 liter volume with diluent composed of 2,578 ml glacial acetic acid plus 13,230 ml dd H_2O.

The pyridine was treated by distilling 5 pints of pyridine (Merck) over 3 g of solid ninhydrin.

A summary of the steps for the preparation of hemoglobin and its chains for peptide analysis is shown in Figure 9.

F. Amino Acid Analysis

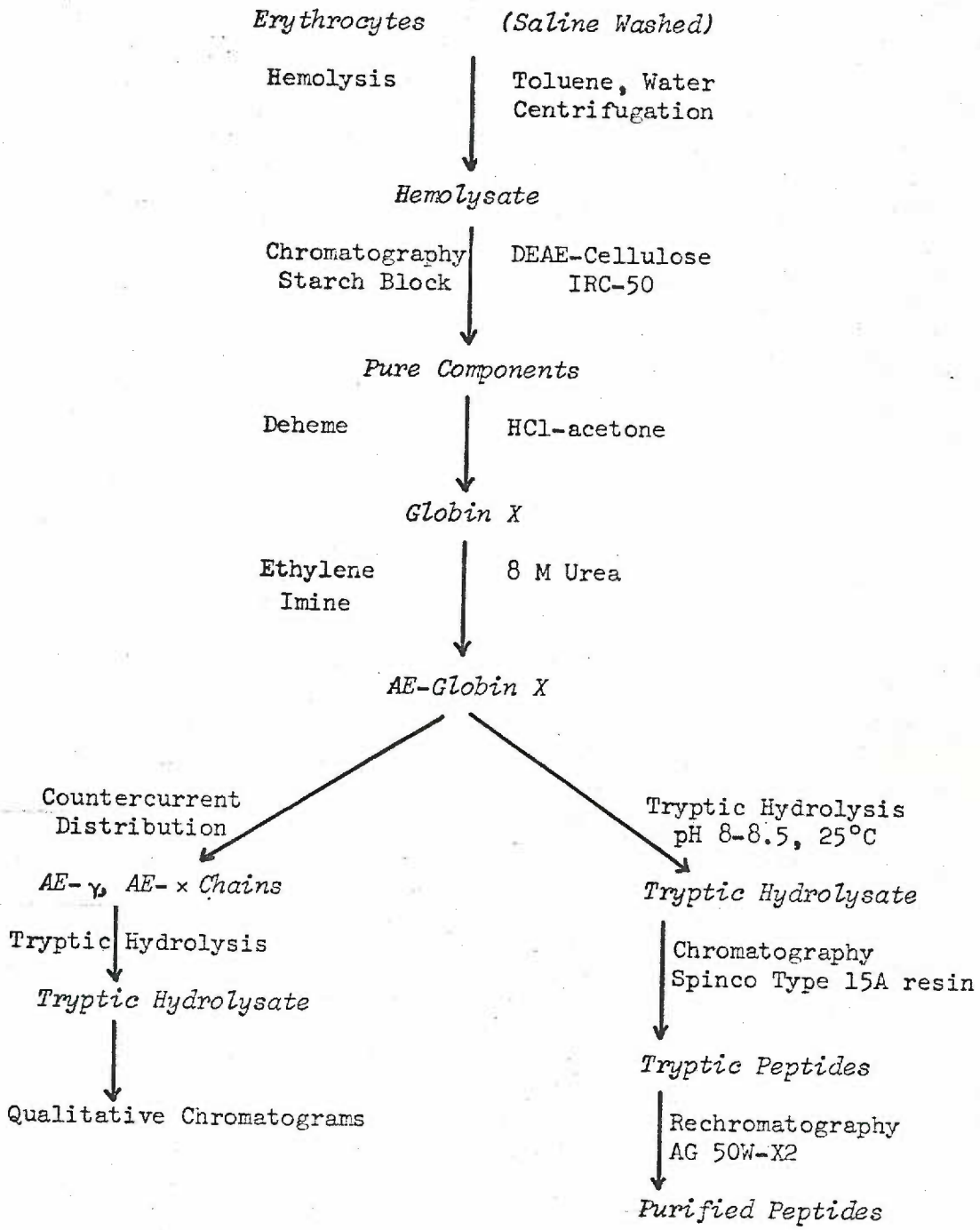
The amino acid analyses were performed with an automatic amino acid analyzer (Beckman Instruments, Inc., Spinco Div., Palo Alto, Calif.) modified to an accelerated system now commercially available.

The samples were prepared by drying in a round bottom flask with a rotary evaporator, then adding 2 ml of doubly distilled 6 N hydrochloric acid. The acid solution was placed in an ampoule which was evacuated, sealed and then heated at 110 $\pm$ 0.5°C for 22 hours. Following hydrolysis the sample was evaporated to dryness and re-dissolved in sample buffer prior to loading on the analyzer.

The analyzer was fitted with an automatic integrator for automatic integration of the peak areas for each amino acid. The integrator was a Model CRS-12A B, Infotronics, Houston, Texas.

Figure 9

Summary of Preparation of Tryptic Peptides. The peptides were prepared from aminoethylated globin of hemoglobin X. AE is used for an abbreviation of aminoethyl derivatives.


Erythrocytes (Saline Washed)
Hemolysis
Toluene, Water Centrifugation
Hemolysate
Chromatography Starch Block
DEAE-Cellulose IRC-50
Pure Components
Deheme
HCl-acetone
Globin X
Ethylene Imine
8 M Urea
AE-Globin X
Countercurrent Distribution
AE-γ AE-x Chains
Tryptic Hydrolysis
Tryptic Hydrolysate
Qualitative Chromatograms
Tryptic Hydrolysis pH 8-8.5, 25°C
Tryptic Hydrolysate
Chromatography Spinco Type 15A resin
Tryptic Peptides
Rechromatography AG 50W-X2
Purified Peptides

RESULTS

I. ISOLATION OF HEMOGLOBIN H

A. DEAE-Cellulose Chromatography

The isolation of hemoglobin H was effected by chromatography on DEAE cellulose. Figure 10 illustrates a chromatogram of 760 mg of hemolysate using stepwise elution with 78 ml of 0.003 M phosphate, pH 7 followed by 338 ml of 0.03 M phosphate, pH 7 then by 0.5 M KCl. There were no readily detectable amounts of hemoglobin removed after the 0.03 M phosphate elution and it can be seen that separation into two distinct zones was accomplished.

Zone I represents 625 mg (82%) and Zone II represents 125 mg (16%) of the hemoglobin loaded on the column.

Starch gel mobilities demonstrated that Zone I was the hemoglobin A fraction and Zone II was the hemoglobin H fraction.

B. Starch Block Electrophoresis

Starch block electrophoresis was used in some cases for further purification following DEAE cellulose chromatography and in some cases as the primary isolation technique with good results.

Figure 11 is a photograph of the results of the electrophoresis of hemoglobin H. The material in region 1 represents about 25 mg (0.6 ml) of hemoglobin H already once run on starch block and the material in region 2 represents 30 mg (0.5 ml) of the original hemolysate. It can be seen that hemoglobin H migrates much more rapidly toward the anode at pH 8.6 and good resolution of the zones is obtained.

Figure 10

Chromatogram of 760 mg of Hemoglobin on DEAE-Cellulose Column (2 x 25 cm) at 6°C. Zone I represents 82% and Zone II represents 16% of the sample. The insert (b) represents the same chromatogram using a different scale and Zones I and II of the insert are the same as for the large chromatogram.

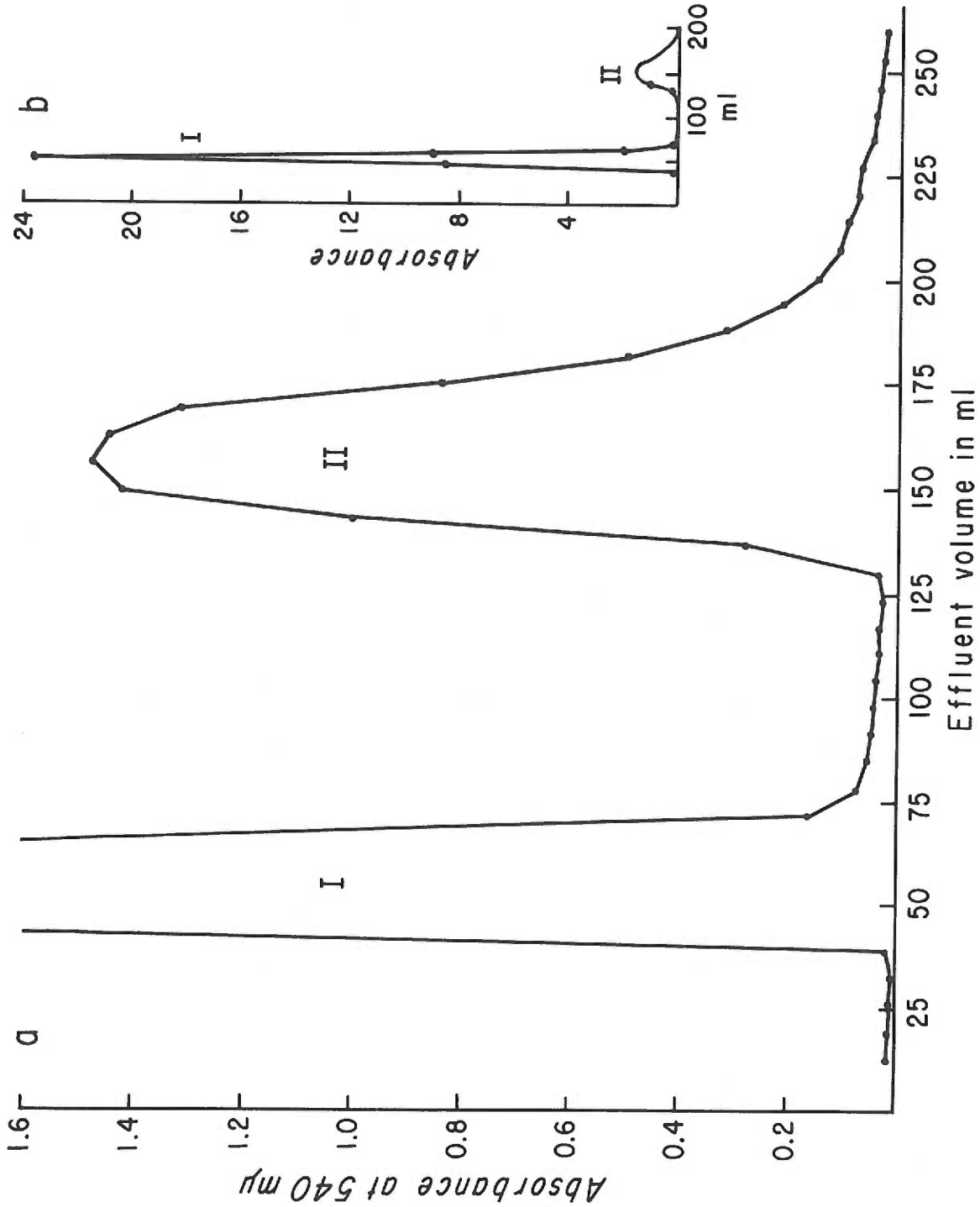
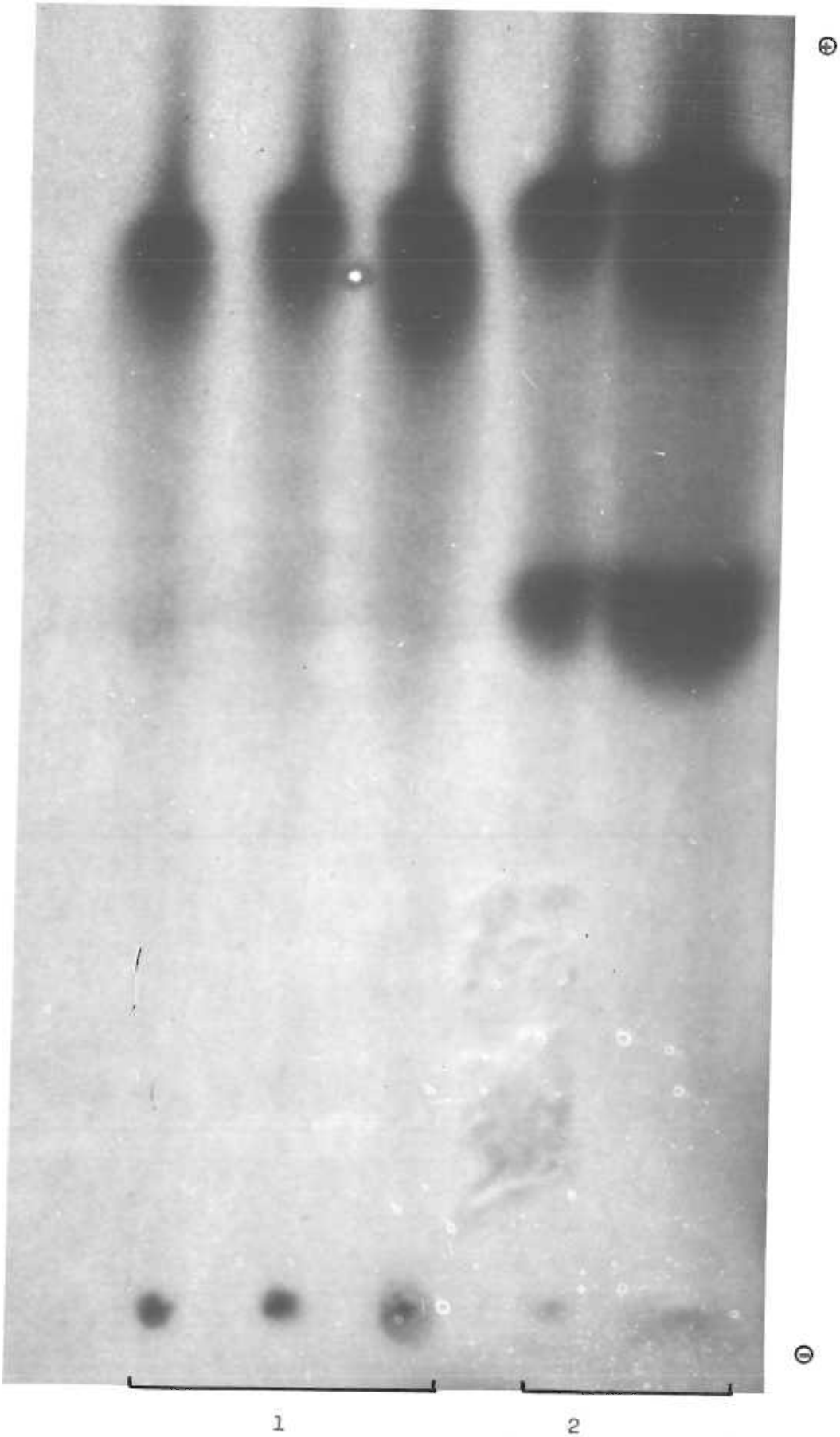

Absorbance at 540 mμ
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
a
I
II
25
50
75
100
125
150
175
200
225
250
Effluent volume in ml
b
I
II
24
20
16
12
8
4
Absorbance
100
200
ml

Figure 11

Electrophoresis on Starch Grain (Barbital Buffer, pH 8.6, 0.5 M) for 855 ma-hrs. (3 hr., 500V, 45 ma.; 18 hr., 450V, 40 ma.). Section 1 is 25 mg of Hb H, Section 2 is 30 mg of a mixture of Hb A + Hb H.


⊕
⊖
1
2

II. ISOLATION OF HEMOGLOBIN X

A. IRC-50 Chromatography

Figure 12 is a graphic illustration of a typical preparative chromatogram of 3 g of hemoglobin containing a certain percent of hemoglobins Bart's (γ_4) and X. The resolution obtained with Developer No. 6 is quite good for the major four Zones I, II, III and IV and practically excludes any significant contamination of Zone I by Zones II, III or IV. Zone I contained a mixture of hemoglobins Bart's, H and X; Zone II is the fetal hemoglobin F_I; Zone III is the fetal hemoglobin F_{II} and Zone IV is the major adult hemoglobin A_{II} (49).

A comparison of the relative amounts of the fractions indicates that Zone I is 12%, Zone II is 11%, Zone III is 55% and Zone IV is 22%.

The chromatogram was run at 6°C until the point indicated in Figure 12 when the temperature was raised to 40°C to effect rapid elution of the F_{II} and A_{II} components.

B. Starch Grain Electrophoresis

The desired components contained in Zone I were not separated on the column under these conditions; therefore, this zone was further resolved by starch grain electrophoresis as shown in Figure 13. Two major components were resolved on starch block at pH 8.6. There was also some indication of the existence of a third minor component migrating ahead of the other two. Comparison by starch gel electrophoresis of these components with hemoglobins Bart's and H indicated that the slow component (number 1) was the unknown component

Figure 12

Chromatogram (IRC-169) of 3 g of Hemoglobin (MKD) With a Column (5 x 38 cm) of IRC-50 Resin. Elution throughout was with Developer 6 with a temperature change from 6°C to 40°C at point indicated. Zone I represents a mixture of hemoglobins X, Bart's and H; Zone II represents hemoglobin F_I; Zone III represents hemoglobin F_{II}; Zone IV represents hemoglobin A_{II}.

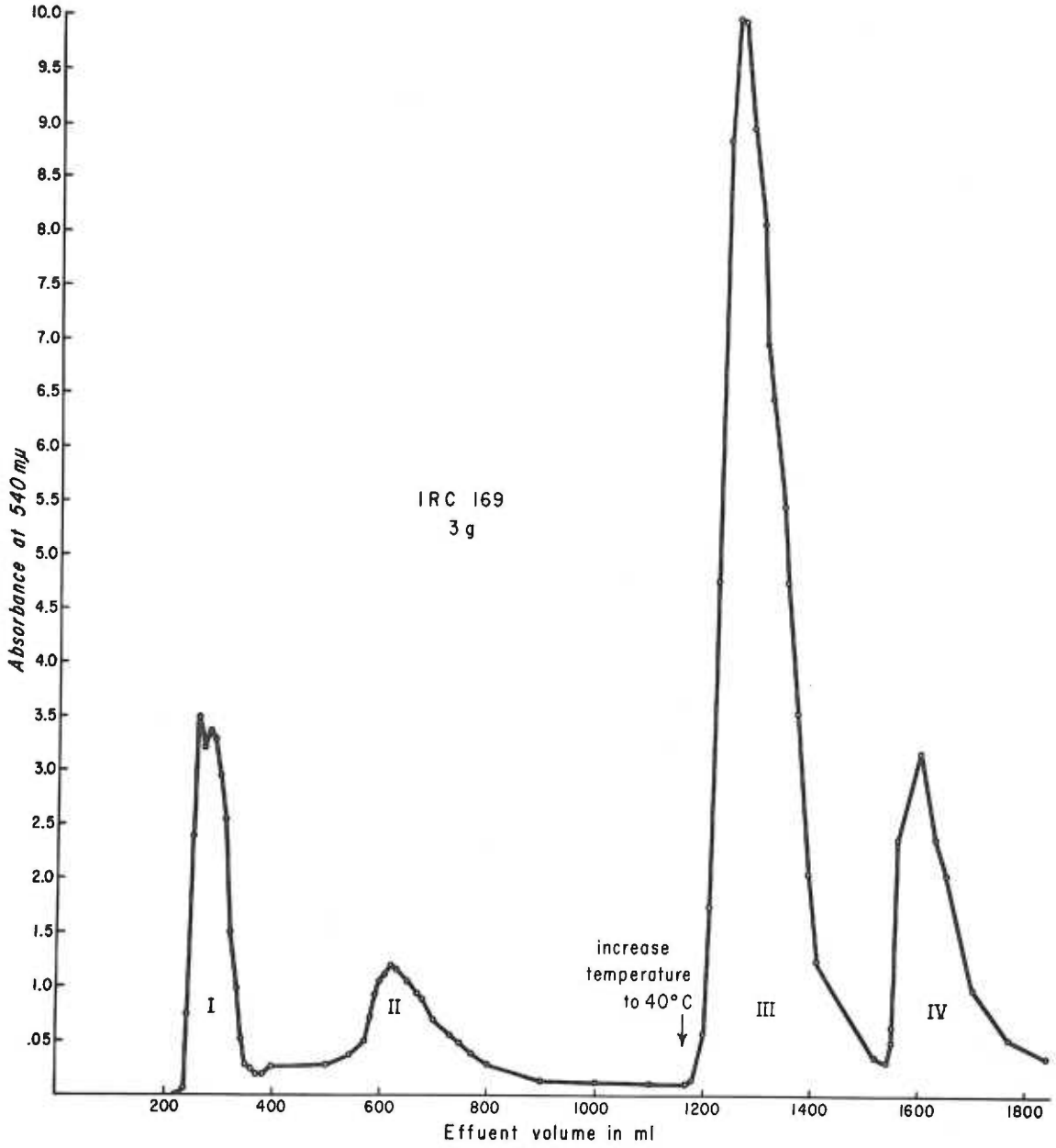
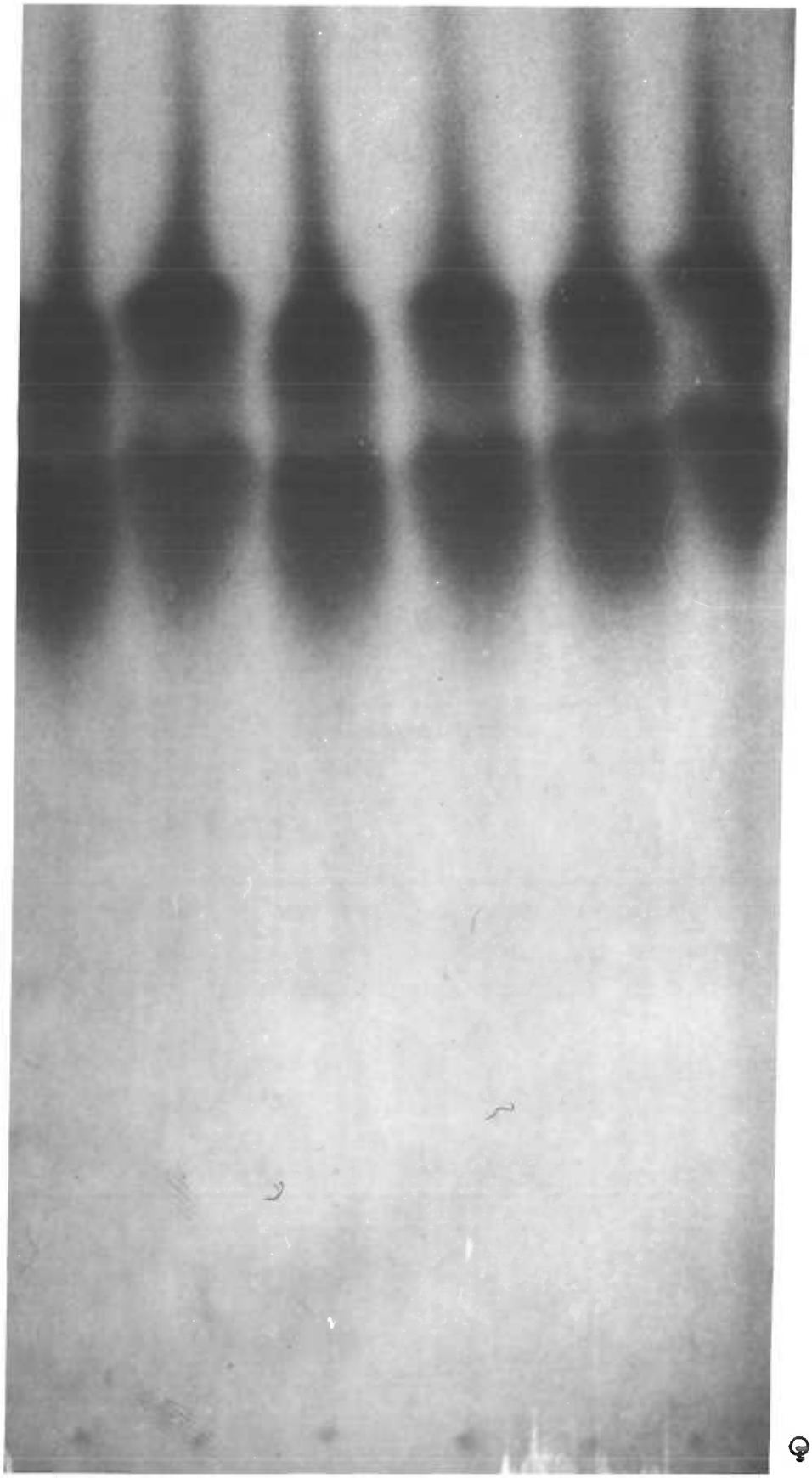

Absorbance at 540 mμ
10.0
9.5
9.0
8.5
8.0
7.5
7.0
6.5
6.0
5.5
5.0
4.5
4.0
3.5
3.0
2.5
2.0
1.5
1.0
.05
IRC 169
3 g
increase
temperature
to 40°C
I
II
III
IV
200
400
600
800
1000
1200
1400
1600
1800
Effluent volume in ml

Figure 13

Starch Grain Electrophoresis (800V, 75 ma., 18 hrs.) of Approximately 160 mg of Hemoglobin from Zone I (Hb X, Bart's, H) of IRC-50 Chromatogram as Shown in Figure 12 with Barbital Buffer, pH 8.6, .05 M. Migration is towards the anode.


⊕
3
2
1
⊖

here referred to as hemoglobin X, component number 2 was hemoglobin Bart's, while component number 3 probably contained some hemoglobin H.

A starch gel analysis as shown in Figure 14 illustrates the distinctly different mobilities of hemoglobins A, X, Bart's and F with the new component being faster than A but slower than Bart's.

A starch gel pattern shown in Figure 14 illustrates the relative electrophoretic mobilities of the original sample and the isolated components.

The improvement in resolution between hemoglobins A, X and Bart's when these components are run separately is due to the increased concentration of the minor components X and Bart's after isolation and concentration as opposed to their relatively low concentration in the original hemolysate.

The sequence from IRC-50 chromatography to the starch gel electrophoretic mobilities of the isolated components indicates the presence of a distinct component differing in chromatographic and electrophoretic behavior from hemoglobins A, F, H and Bart's.

III. PHYSICOCHEMICAL PROPERTIES

A. Sedimentation and Diffusion Coefficients

The sedimentation coefficients for hemoglobins A, H, Bart's and X were determined at various pH values and in some cases at varying ionic strength of NaCl. The pH effect on these components is graphically presented in Figures 15 and 16. At near neutral pH each component gave only a single peak sedimenting in the ultracentrifuge and their $s_{20,w}$ values indicate molecular weights of the

Figure 14

Starch Gel Electrophoresis With Tris-EDTA-Borate System: (1) normal Hb A control, (2) high Hb F control, (3) MKD case hemolysole mixture, (4) same as (3) but lower concentration, (5) Hb Bart's control, (6) Zone II (F_I) from IRC-50, (7) Zone III (F_{II}) from IRC-50, (8) Zone IV (A_{II}) from IRC-50, next three bands represent components from Zone I IRC-50 after starch block electrophoresis (9) hemoglobin X, (10) hemoglobin Bart's, (11) mixture of hemoglobins Bart's and H. Migration is towards the anode.

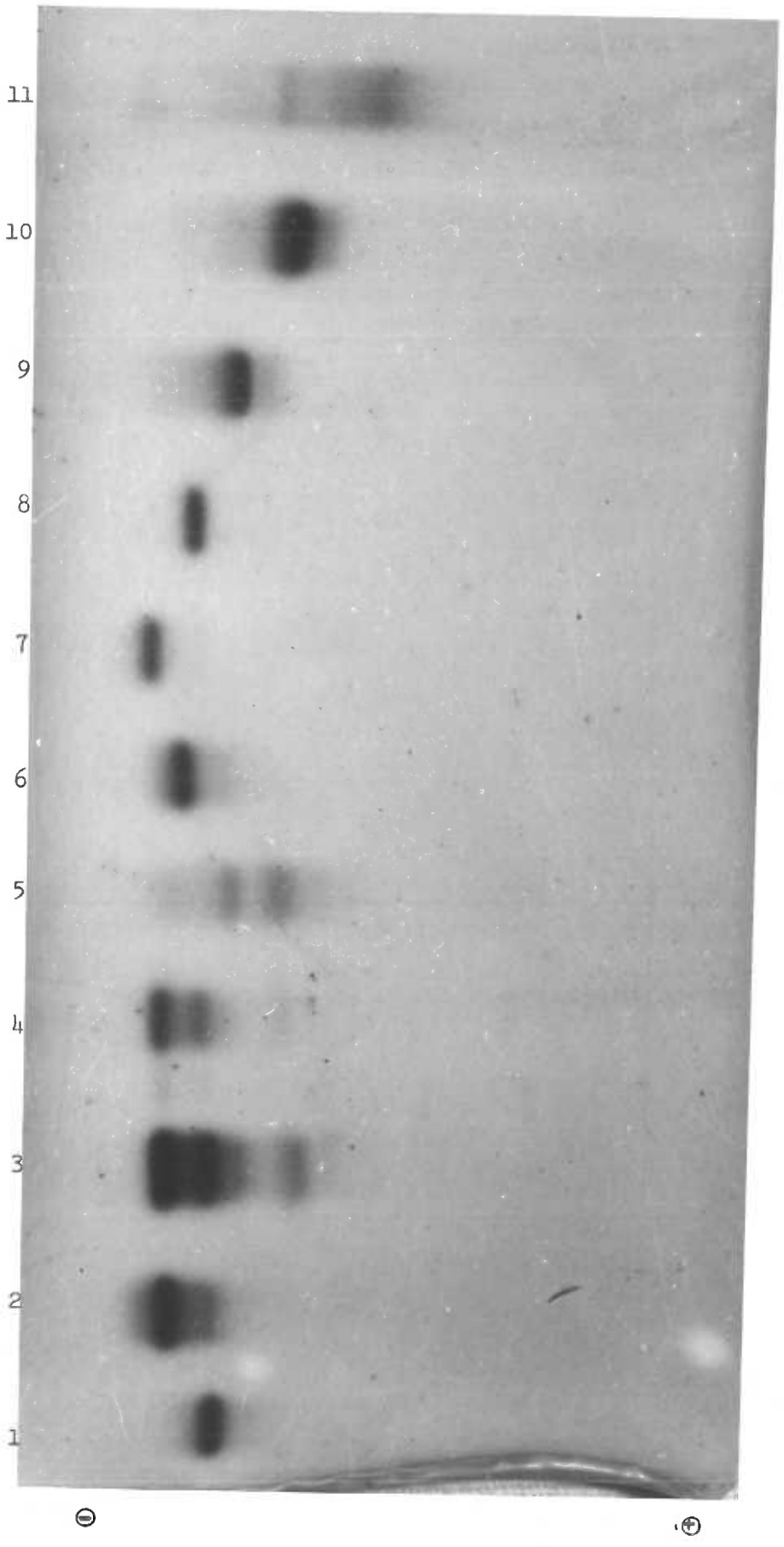

11
10
9
8
7
6
5
4
3
2
1
⊖
⊕

Figure 15

Sedimentation Coefficients Versus pH. Sedimentation coefficients of human hemoglobin Bart's (15,A) and hemoglobin H (15,B) in the pH range 4.5 to 10.6. Hemoglobin concentration was 0.6 to 0.8%. In two duplicate experiments with hemoglobin H at pH 4.5 two sedimenting components were observed. The triangular symbols represent the slow component and the square symbols represent the fast component.

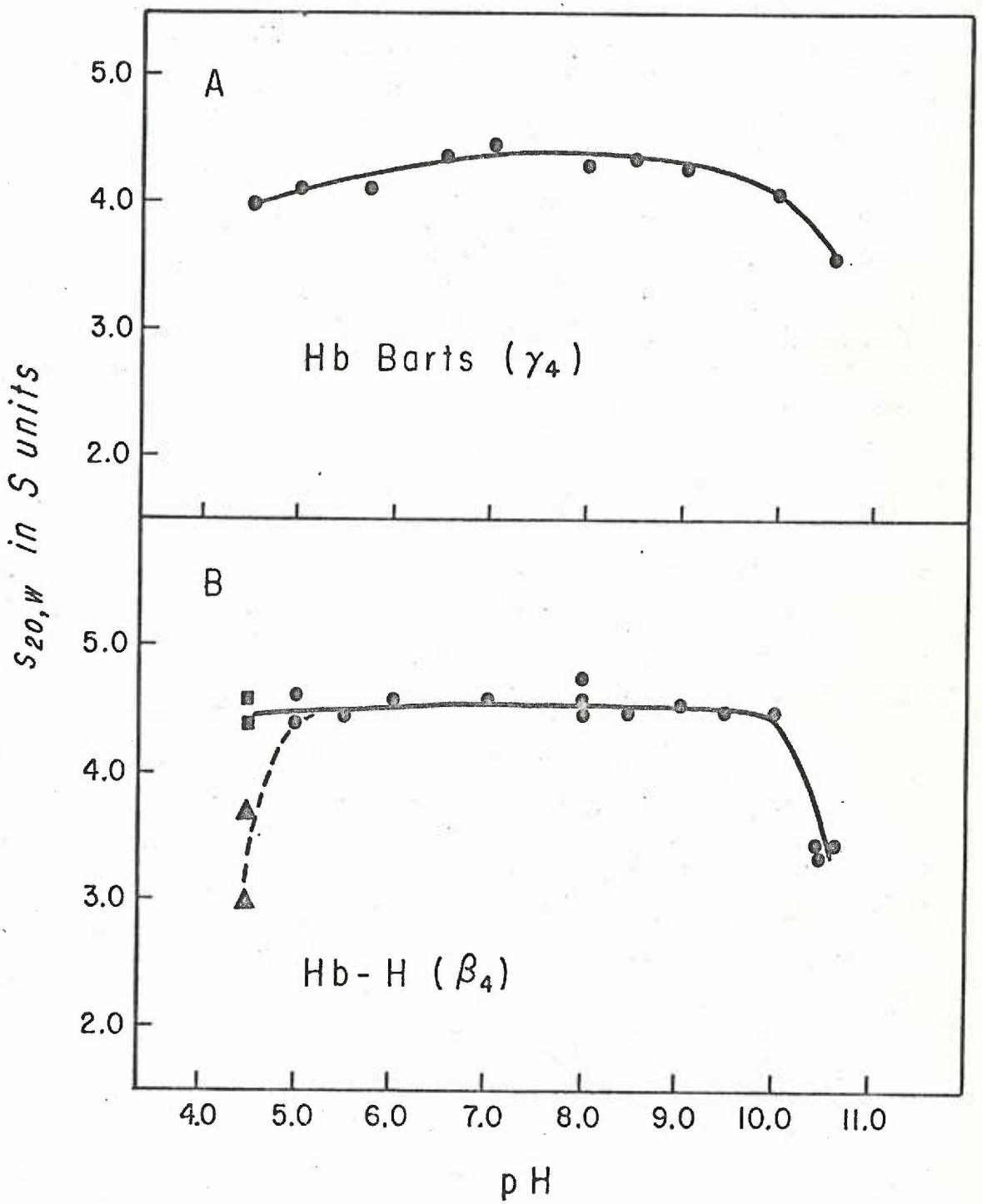
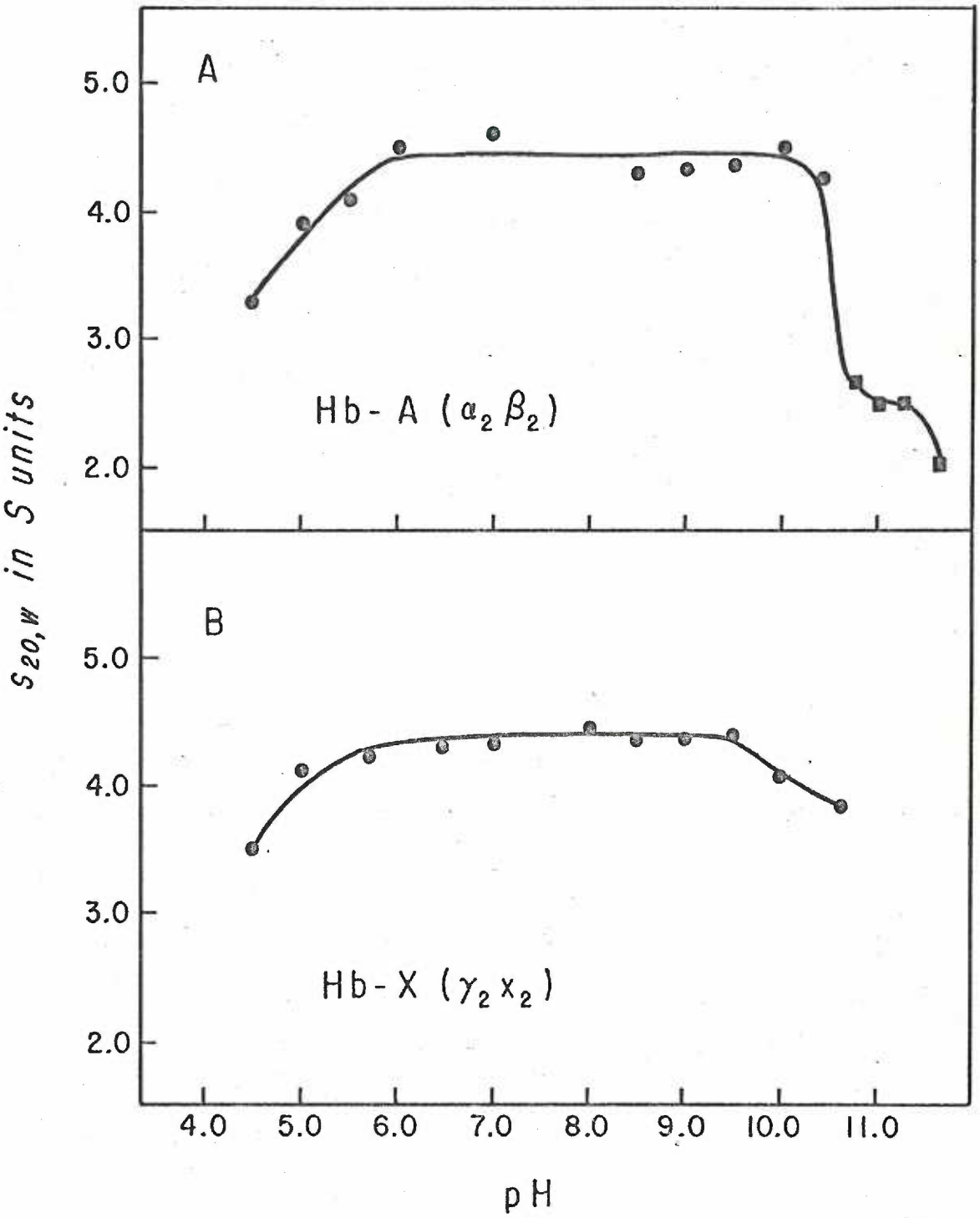

A
Hb Barts (γ_4)
B
Hb-H (β_4)
$s_{20,w}$ in S units
5.0
4.0
3.0
2.0
4.0
5.0
6.0
7.0
8.0
9.0
10.0
11.0
pH

Figure 16

Sedimentation Coefficients Versus pH. Sedimentation coefficients of human hemoglobin A (16,A) and hemoglobin X (16,B) in the pH range 4.5 to 11.6. ● this study, ■ from Hasserodt and Vinograd (97).


A
B
5.0
4.0
3.0
2.0
Hb - A ($\alpha_2 \beta_2$)
Hb - X ($\gamma_2 x_2$)
$s_{20,w}$ in S units
4.0
5.0
6.0
7.0
8.0
9.0
10.0
11.0
pH

order of 60,000 to 70,000. The $s_{20,w}$ values for hemoglobin H are on the order of 3% higher than the values of hemoglobin A which is in agreement with results reported by Rigas et al. (116). The effects of salt concentration on hemoglobin H are shown in Figure 17.

The $s_{20,w}$ (4.62) at zero concentration of hemoglobin X (Fig. 17) was obtained by computer extrapolation of $s_{20,w}$ values versus concentration.

The ultracentrifuge patterns shown in Figure 18 illustrate boundaries obtained for hemoglobin A at pH values 7, 4.5 and 10.5. Included is a diffusion boundary of hemoglobin A in 0.1 M NaCl.

Figure 19 shows the schlieren patterns obtained for hemoglobin H at pH 7, 4.5 and 10.5. Note worthy are the pictures at pH 4.5 showing the presence of two peaks, hitherto unobserved for this hemoglobin.

Results of sedimentation at pH 6.5, 4.5 and 10.5 for hemoglobin Bart's can be seen in Figure 20.

Included in Figure 21 is the diffusion pattern obtained for hemoglobin X in 0.1 M NaCl as well as sedimentation patterns at pH 7, 4.5, 10.5 and at pH 4.5 in the presence of 0.1 M mercaptoethanol. At pH 4.5 in the absence or presence of mercaptoethanol two peaks are in evidence. Also shown in Figure 21 is the pattern obtained following reversal of the pH of a sample of hemoglobin X from 4.5 to 6.9 demonstrating the replacement of the bimodal peak by a single sedimenting peak.

The physicochemical properties ($s_{20,w}$, $D_{20,w}$, M_{SD}) of

Figure 17

A. Sedimentation Coefficients Versus NaCl Concentration. Sedimentation coefficients of hemoglobin H at various molar concentrations of NaCl.

B. Sedimentation Coefficients Versus Concentration of Hemoglobin X. Sedimentation coefficients obtained at varying concentration of hemoglobin X. Concentration determined by the cyanmethemoglobin method. Extrapolated value at zero concentration is 4.62 S.

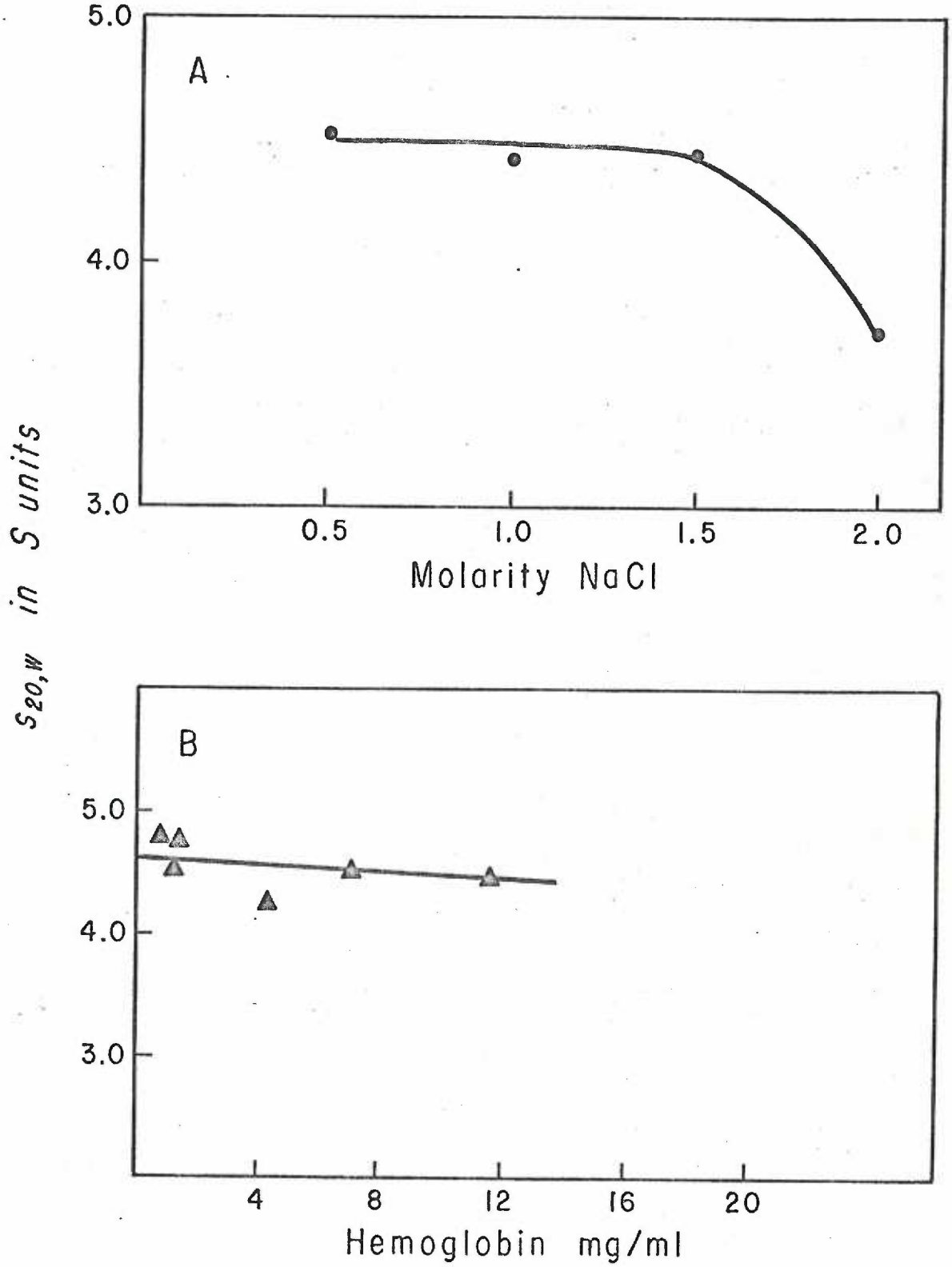

A
5.0
4.0
3.0
0.5
1.0
1.5
2.0
Molarity NaCl
$s_{20,w}$ in S units
B
5.0
4.0
3.0
4
8
12
16
20
Hemoglobin mg/ml

Figure 18

Ultracentrifuge Patterns for Sedimentation and Diffusion of Hemoglobin A.

a. Diffusion pattern of hemoglobin A in 12 mm synthetic boundary cell at 3,189 r.p.m. Pictures taken at top speed, then at 16, 32 and 40 min. and at 8 min. intervals thereafter. Conc. 7 mg/ml; 0.1 M NaCl, 20°C. Bar angle at 60° throughout.

b. Sedimentation boundary of hemoglobin A. Conc. 10 mg/ml, lower sample at pH 7, upper sample at pH 8. Run at 59,780 r.p.m., 20°C. Pictures at 8, 32, 64, 96, 108 and 160 min. after attainment of top speed. Bar angle at 65° for first four pictures and 55° for last two.

c. Sedimentation boundary at pH 4.5 (lower pattern). Conc. 6.5 mg/ml. Run at 59,780 r.p.m., 20°C. Pictures taken at 14, 46, 78, 110, 142 and 174 min. after attainment of top speed. Bar angle at 65° for first picture and at 60° thereafter.

d. Sedimentation boundary at pH 10.5 (lower pattern). Conc. 9.5 mg/ml. Run at 59,780 r.p.m., 20°C. First picture approximately 12 min. after attainment of top speed; pictures at 32 min. intervals thereafter. Bar angle at 65° for first picture and 60° thereafter.

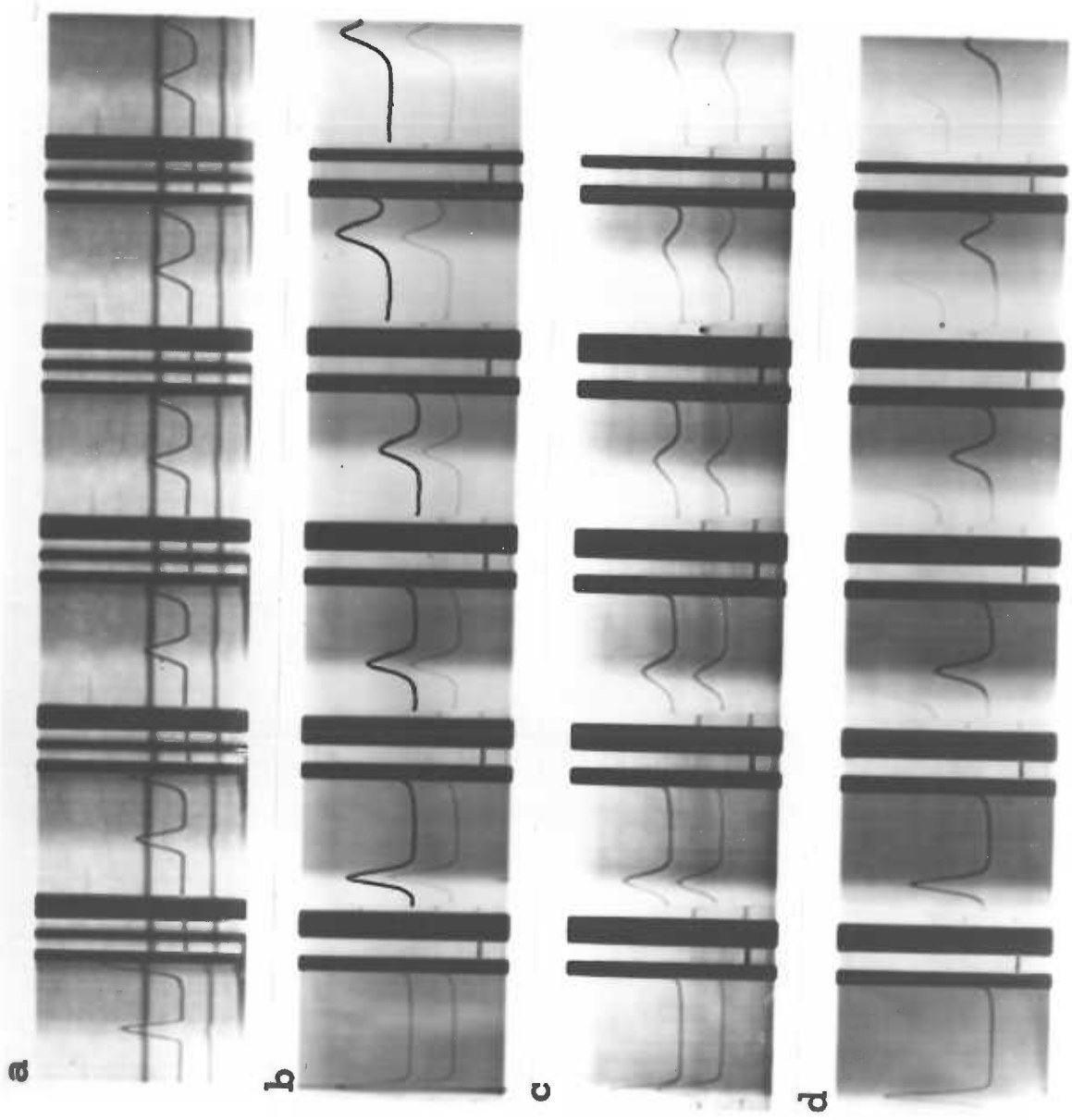

a
b
c
d

Figure 19

Ultracentrifuge Patterns of Hemoglobin H.

a. Sedimentation boundary at pH 7, upper peak 8.2 mg/ml; pH 6, lower peak 8.4 mg/ml. Run at 59,780 r.p.m., 20°C. First picture at 9 min. after attainment of top speed; pictures at 32, 64, 96, 128 and 144 min. thereafter. Bar angle at 65°, 65°, 60° and 55° thereafter.

b. Sedimentation boundary at pH 4.5. Conc. 7.8 mg/ml. Run at 59,780 r.p.m., 20°C. First picture approximately 26 min. after attainment of top speed; pictures at 32, 48, 64, 96 and 128 min. thereafter. Bar angle at 50°, 50°, 50°, 40°, 35° and 30°.

c. Sedimentation boundary at pH 10.5 (upper pattern); pH 10 (lower pattern). Conc. 8.2 mg/ml. Run at 59,780 r.p.m., 20°C. First picture at 6 min. after attainment of top speed; pictures at 32 min. intervals thereafter. Bar angle at 65° for first three pictures and 45° thereafter.

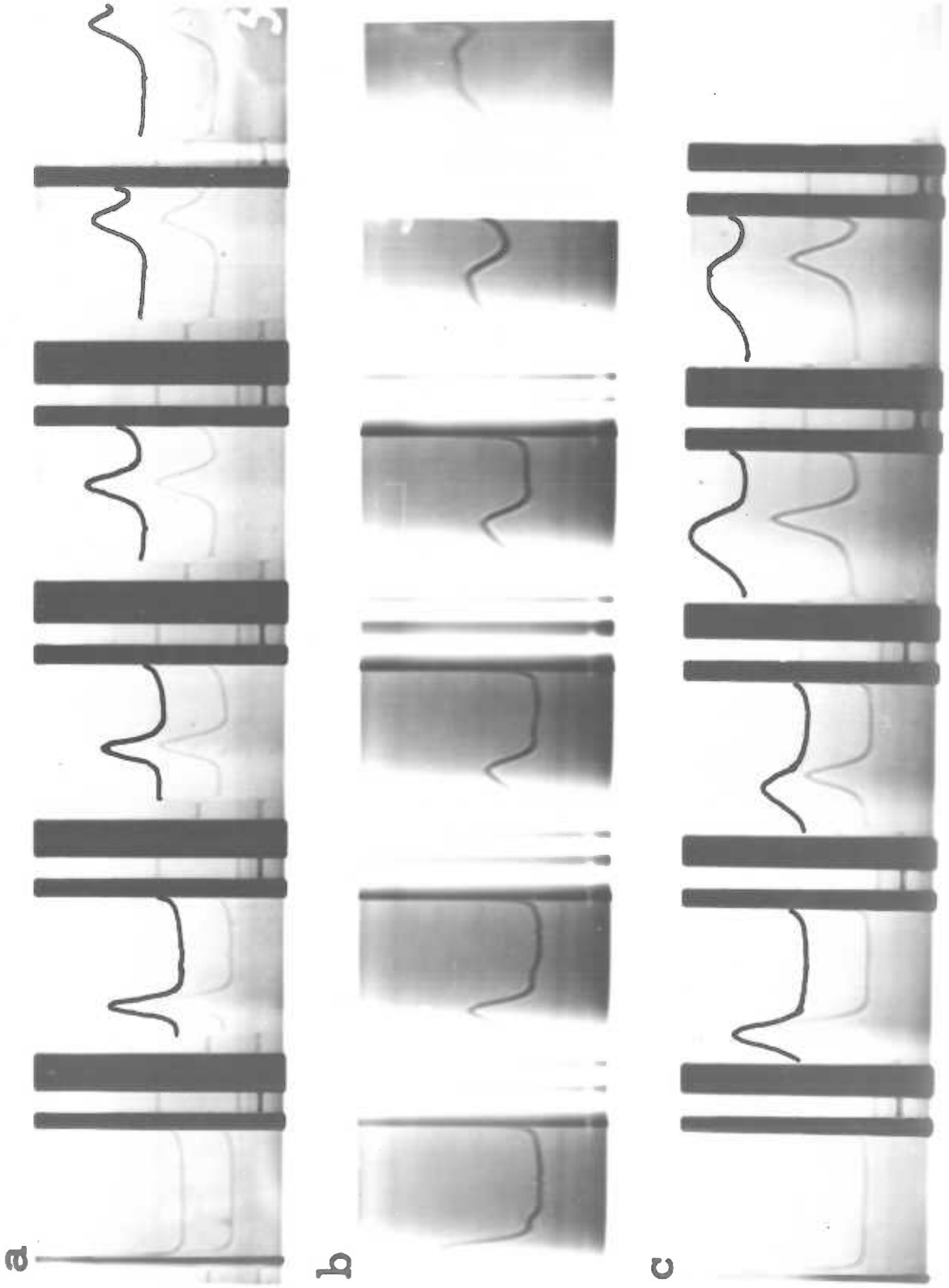

a
b
c

Figure 20

Ultracentrifuge Patterns of Hemoglobin Bart's.

a. Sedimentation at pH 6.5 (upper pattern). Conc. is approximately 6 mg/ml. Run at 59, 780 r.p.m., 20°C. First picture at 9 min. after attainment of top speed; pictures at 32, 48, 64, 80 and 96 min. thereafter. Bar angle at 60° for first picture and 65° thereafter.

b. Sedimentation at pH 4.5. Conc. 7 mg/ml. Run at 59,780 r.p.m., 20°C. First picture at 5 min. after attainment of top speed; pictures at 16, 32, 48, 64 and 96 min. thereafter. Bar angle at 65° throughout.

c. Sedimentation at pH 10.5 (upper pattern). Conc. is approximately 7 mg/ml. Run at 59,780 r.p.m., 20°C. First picture at 9 min. after attainment of top speed; pictures at 16 min. intervals thereafter. Bar angle at 65° throughout.

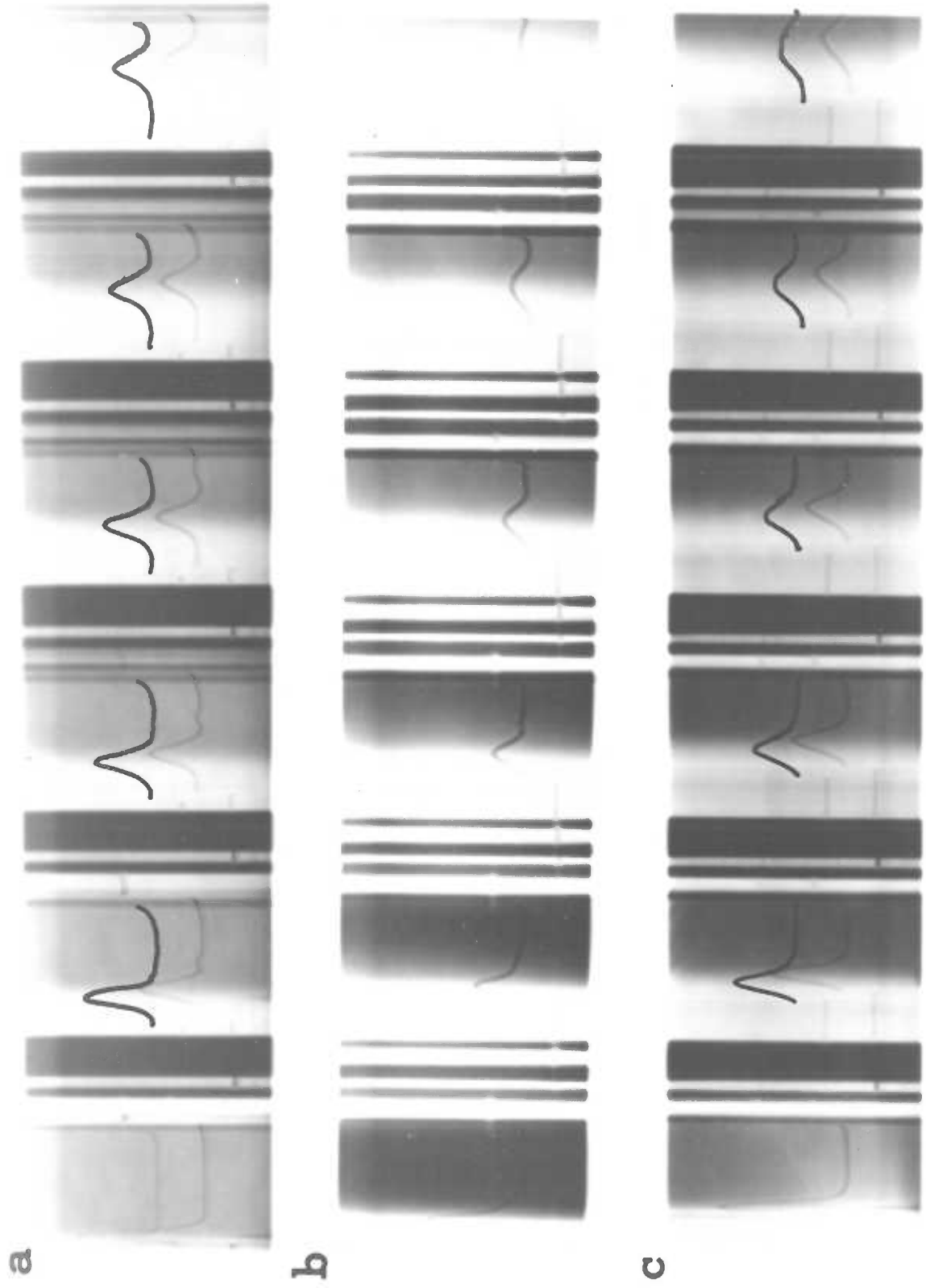

a
b
c

Figure 21

Ultracentrifuge Patterns of Hemoglobin X.

a. Diffusion pattern in 12 mm synthetic boundary cell at 3,189 r.p.m. Conc. 10.4 mg/ml; 0.1 M NaCl; 20°C. Pictures at 12, 20, 24, 28, 32, 36, 52, 68, 84, 100, 116 and 132 min. after attainment of speed. Bar angle at 70° throughout.

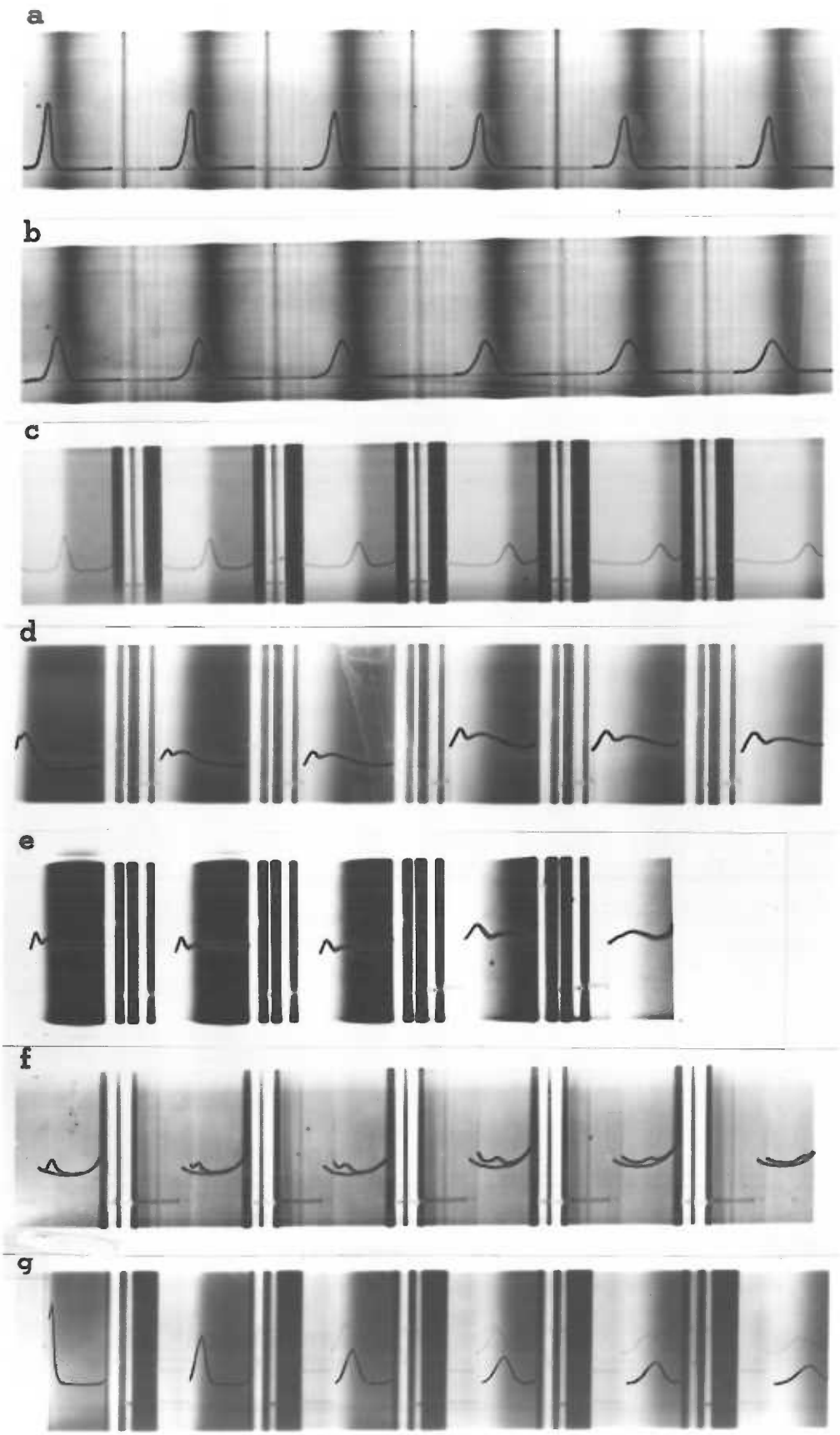
b. Sedimentation at pH 6.9. Conc. 6.5 mg/ml. Run at 59,780 r.p.m., 20°C. Pictures at 3, 11, 27, 43, 59 and 67 min. after attainment of top speed. Bar angle at 70° throughout.

c. Sedimentation at pH 4.5. Conc. 7.4 mg/ml. Run at 59,780 r.p.m., 20°C. Pictures at 3, 11, 15, 19, 23 and 27 min. after attainment of top speed. Bar angle at 65° for first three pictures and at 50° thereafter.

d. Sedimentation at pH 4.5 in the presence of 0.1 M mercaptoethanol. Conc. 7.3 mg/ml. Run at 59,780 r.p.m., 20°C. Pictures at 8, 12, 16, 32 and 64 min. after attainment of top speed. Bar angle at 50° for first three pictures and at 40° thereafter.

e. Sedimentation boundary of hemoglobin X after exposure to pH 6.9. Conc. is approximately 2 mg/ml. Run at 59,780 r.p.m., 20°C. Pictures at 7, 15, 23, 39, 55 and 71 min. after attainment of top speed. Bar angle at 55° for first picture and 50° thereafter.

f. Sedimentation at pH 10.5. Conc. approximately 7 mg/ml. Run at 59,780 r.p.m., 20°C. First picture at 9 min. after attainment of top speed and at 16 min. intervals thereafter. Bar angle at 65° throughout.


a
b
c
d
e
f
g

hemoglobins A, H, Bart's and X are tabulated in Table 3. A summary of the $s_{20,w}$ values including those of the slow and fast components of hemoglobin H and hemoglobin X at pH 4.5, at various pH values with these same hemoglobins is presented in Table 4.

B. Ultraviolet Spectra

The spectra for oxyhemoglobins A, H, Bart's and X are represented in Figure 22 with the region from 280 mμ to 300 mμ illustrated to characterize the samples by utilizing the resolution of the "tryptophan notch" region at hemoglobin concentrations sufficient to give an optical density of 0.8 to 0.9 at 290 mμ. The other features of the spectra for the samples were identical but distinct differences are apparent in the 290 mμ region. The fractional resolution of hemoglobin F was 0.24×10^{-2} and for hemoglobin Bart's it was 1.8×10^{-2}. The resolution for hemoglobin X was 0.66×10^{-2} which is intermediate to hemoglobin F and hemoglobin Bart's.

C. Subunit Hybridization of Hemoglobin X and Bart's with Hemoglobin A

Hybridization of hemoglobins X and Bart's, respectively, with hemoglobin A was done by mixing small amounts (2 mg of each sample) with 2 mg of hemoglobin A in a volume of about 0.5 ml. The hemoglobins were hybridized at pH 4.7 at 6°C for 15 hours. After dialysis against the starch gel buffer for 21 hours a starch gel electrophoresis was obtained as illustrated in Figure 23.

Figure 23(2) illustrates that when hemoglobins A and X are hybridized, four new zones appear. In the recombination of A + Bart's, two new zones appear [Fig. 23(3)].

Table 3

Sedimentation and Diffusion Coefficients and Molecular Weights of Hemoglobins A, H, Bart's and X.

	pH	Sedimentation Coefficient $s_{20,w}$	Diffusion Coefficient $D_{20,w} \times 10^7$ $cm^2\ sec^{-1}$	Molecular Weight M_{sD}*
Hb A	7	4.51 S	6.35	67,000
Hb H**	7	4.89 S	6.75	69,100
Hb Bart's	6.5	4.44 S	-	68,000†
Hb X	6.5	4.45 S	6.54	66,000

*The M_s is a molecular weight calculated from sedimentation and diffusion.

**Value determined by Rigas (personal communication).

†Estimated value reported by Lehman (175).

Table 4

Sedimentation Coefficients at Various pH Values for Hemoglobins A, H, Bart's and X.

	$s_{20,w}$ in S Units		
	pH 7	pH 4.5	pH 10.5
Hb A	4.51	3.29	4.27
Hb H	4.60	slow 2.99 fast 4.62	3.47
Hb Bart's	4.44	3.98	3.56
Hb X	4.45	slow 3.63 fast 11.01	3.86
Hb X treated with mercapto-ethanol at pH 4.5.		slow 3.33 fast 11.31	
Hb X treated at pH 4.5; reversed to pH 6.9 by dialysis.	4.61		

Figure 22

Absorption Spectra of Four Different Oxyhemoglobins Showing the Resolution of the Tryptophan Fine Structure Band in the 280 to 300 mμ Region. Figure 22(a) = Hb X; (b) = Hb A; (c) = Hb Bart's; and (d) = Hb F. Concentrations between 5×10^{-6} M and 6×10^{-6} M in hemoglobin. Spectra measured through 1 cm light path cuvette.

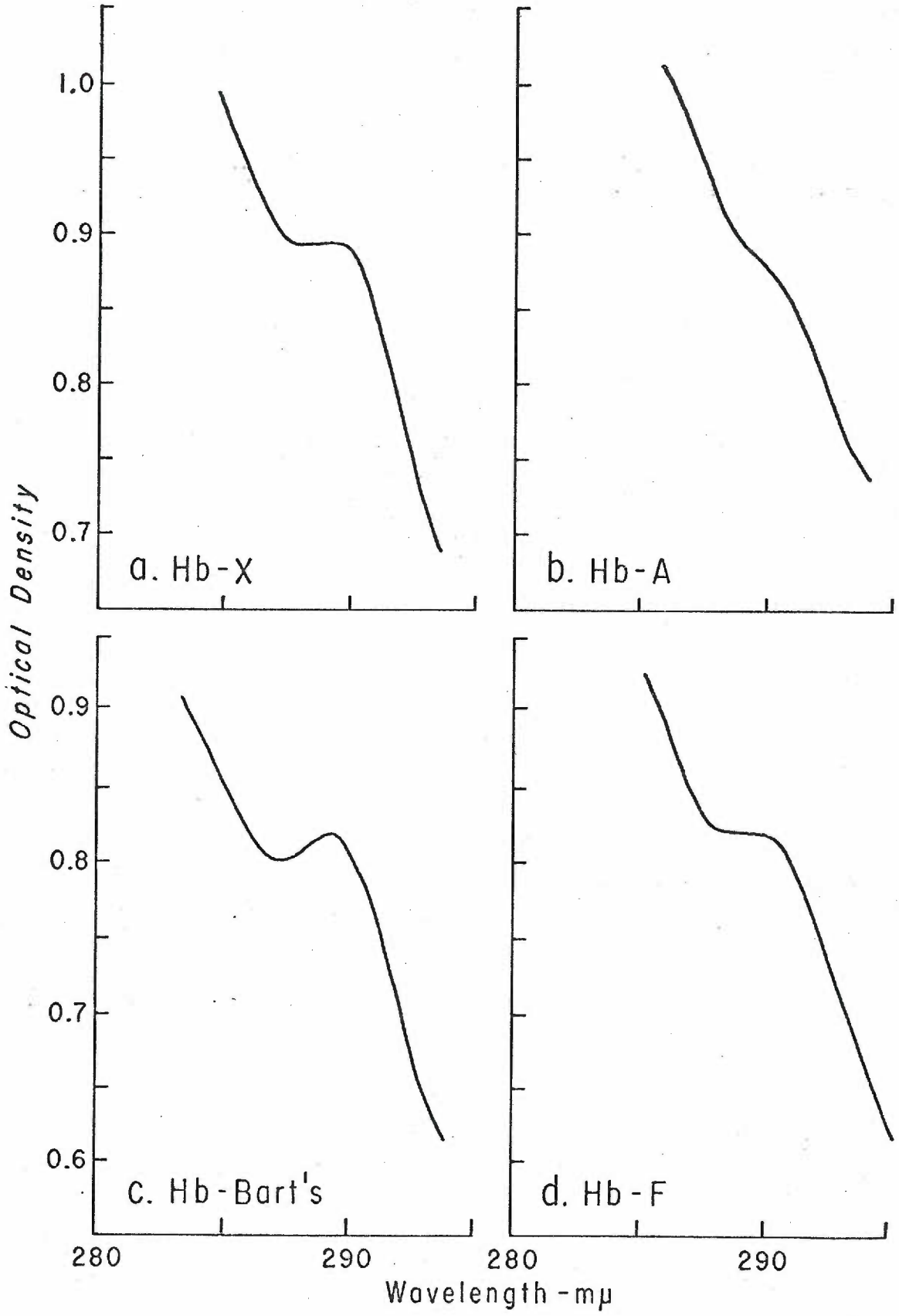
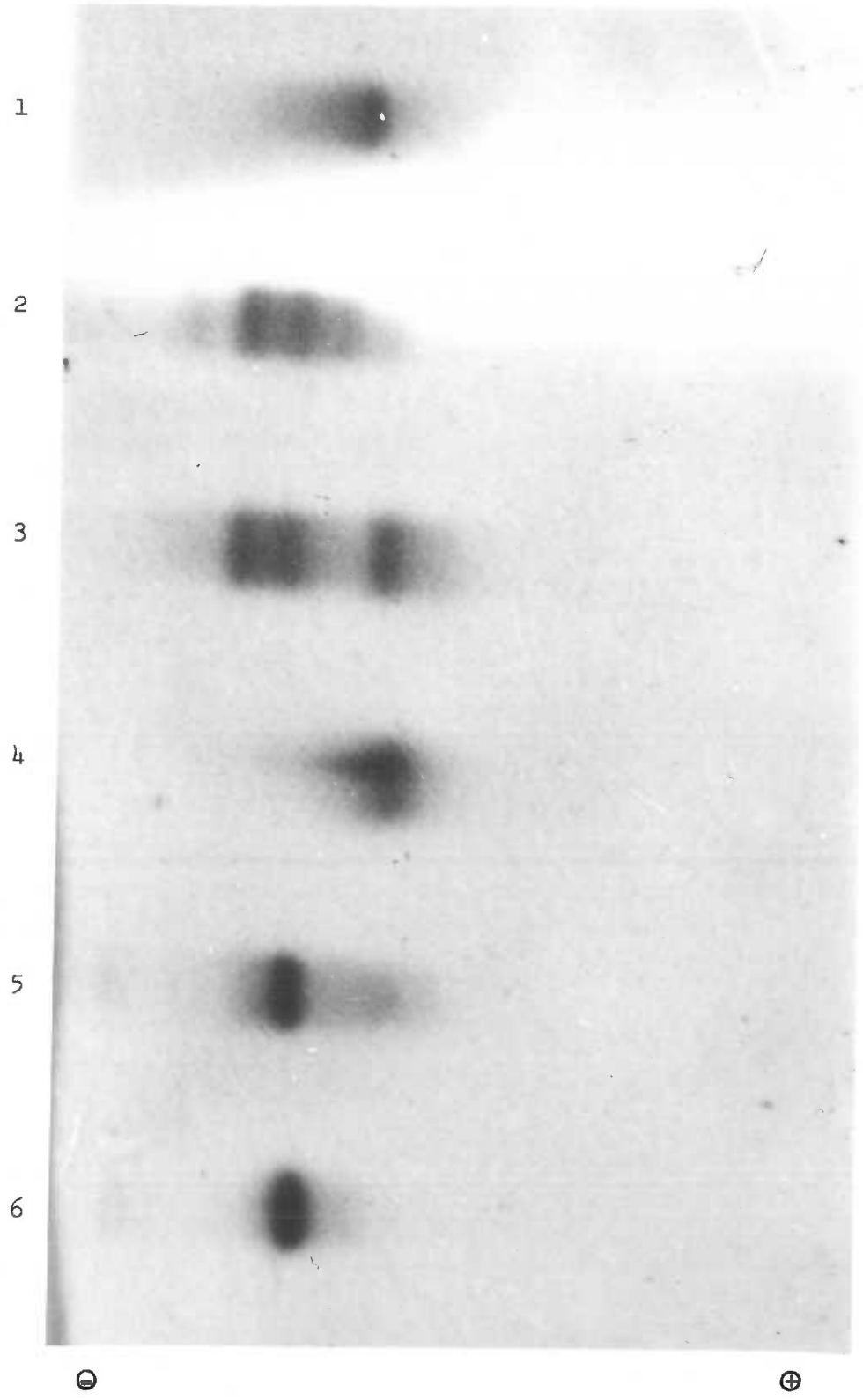

1.0
0.9
0.8
0.7
a. Hb-X
b. Hb-A
0.9
0.8
0.7
0.6
c. Hb-Bart's
d. Hb-F
Optical Density
280
290
280
290
Wavelength - mμ

Figure 23

Recombination of Hb A With Hb X And of Hb A With Hb Bart's Shown on Starch Gel at pH 8.12. (1) hemoglobin X control, (2) hemoglobin X + A hybrid, (3) hemoglobin Bart's + A hybrid, (4) hemoglobin Bart's control, (5) hemoglobin H (plus Bart's and Hb A) control, and (6) hemoglobin A control.


1
2
3
4
5
6
⊖
⊕

IV. CHEMICAL STRUCTURE

A. Aminoethylation and Desalting of Globin

The globin preparation proceeded smoothly and after aminoethylation and desalting procedures were carried out, yields of 90 to 100% were obtained for the aminoethylated globins F, Bart's and X.

Figure 24 illustrates the typical plots for desalting subsequent to aminoethylation. Figure 24(a) represents a desalting plot of approximately 20 mg of globin F, Figure 24(b) represents 24 mg of AE-globin Bart's, and Figure 24(c) represents 90 mg of AE-globin X. The protein is eluted first and is represented by the front zone. Since the exclusion volume of Sephedex G-25 is 5,000 no fractionation of the globin could occur which would predict the front zone to be the protein and indicates that the second "salt" zone could not be protein. A test for the presence of salt with silver nitrate gave negative tests for the first zone but positive salt reaction for the second zone which includes the buffer salts and the urea. The test was performed by mixing one drop of the contents of a tube plus one drop of silver nitrate and visually inspecting for precipitate.

B. Chain Separation

Separation of the chains of hemoglobins F and X was done using a countercurrent method with a butanol-acetic acid-dichloacetic acid solvent system. Figure 25 indicates the separation of 25 mg of aminoethylated globin F with a yield of 7.2 mg from the aqueous phase and 9 mg from the butanol phase for a 70% recovery. Figure 26 represents the separation of 24 mg of AE-globin X with a yield of 10 mg

Figure 24

Desalting Chromatograms of Aminoethylated Globins on Column of Sephedex (G-25). Elution with 0.2 M acetic acid. Automatic recording at 280 mμ. Figure 24(a) represents 20 mg of AE-γ^F chain globin; (b) represents 24 mg of AE-Bart's globin (from MKD); and (c) represents about 90 mg of AE-X globin.

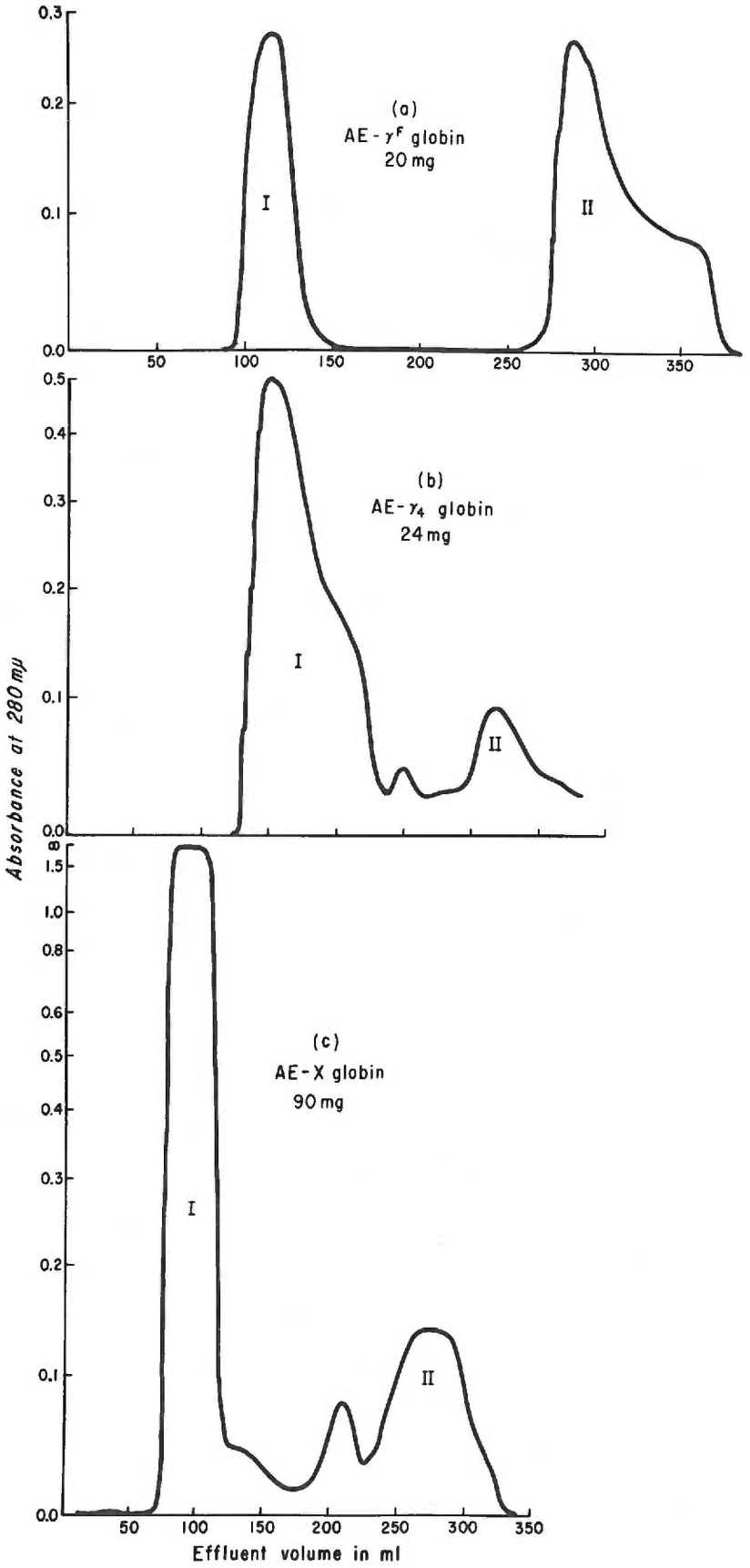

(a)
AE-γ^F globin
20 mg
I
II
(b)
AE-γ4 globin
24 mg
I
II
(c)
AE-X globin
90 mg
I
II
Absorbance at 280 mμ
Effluent volume in ml

Figure 25

Countercurrent Distribution Pattern of 25 mg of AE-Globin From Hemoglobin F.

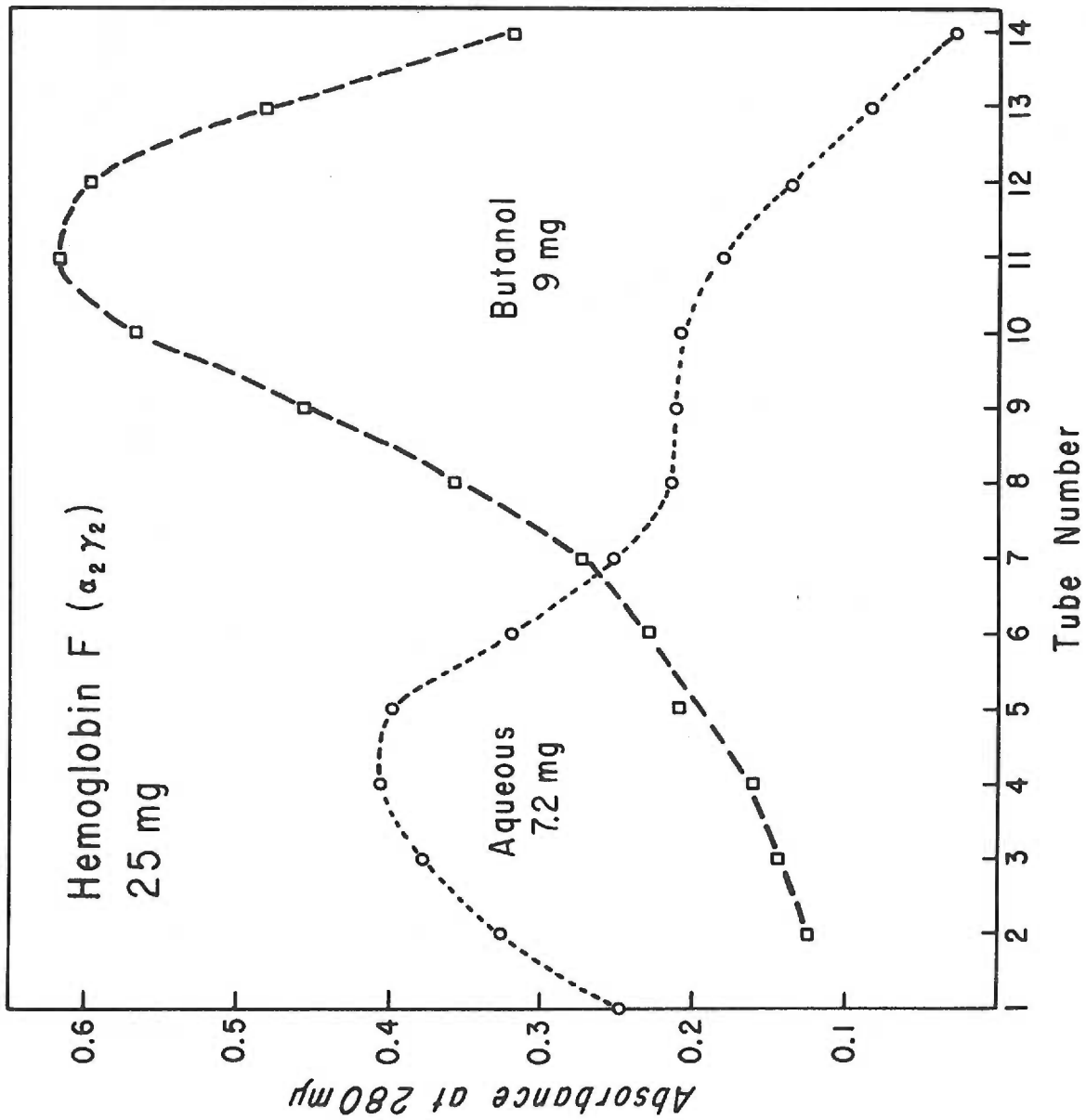
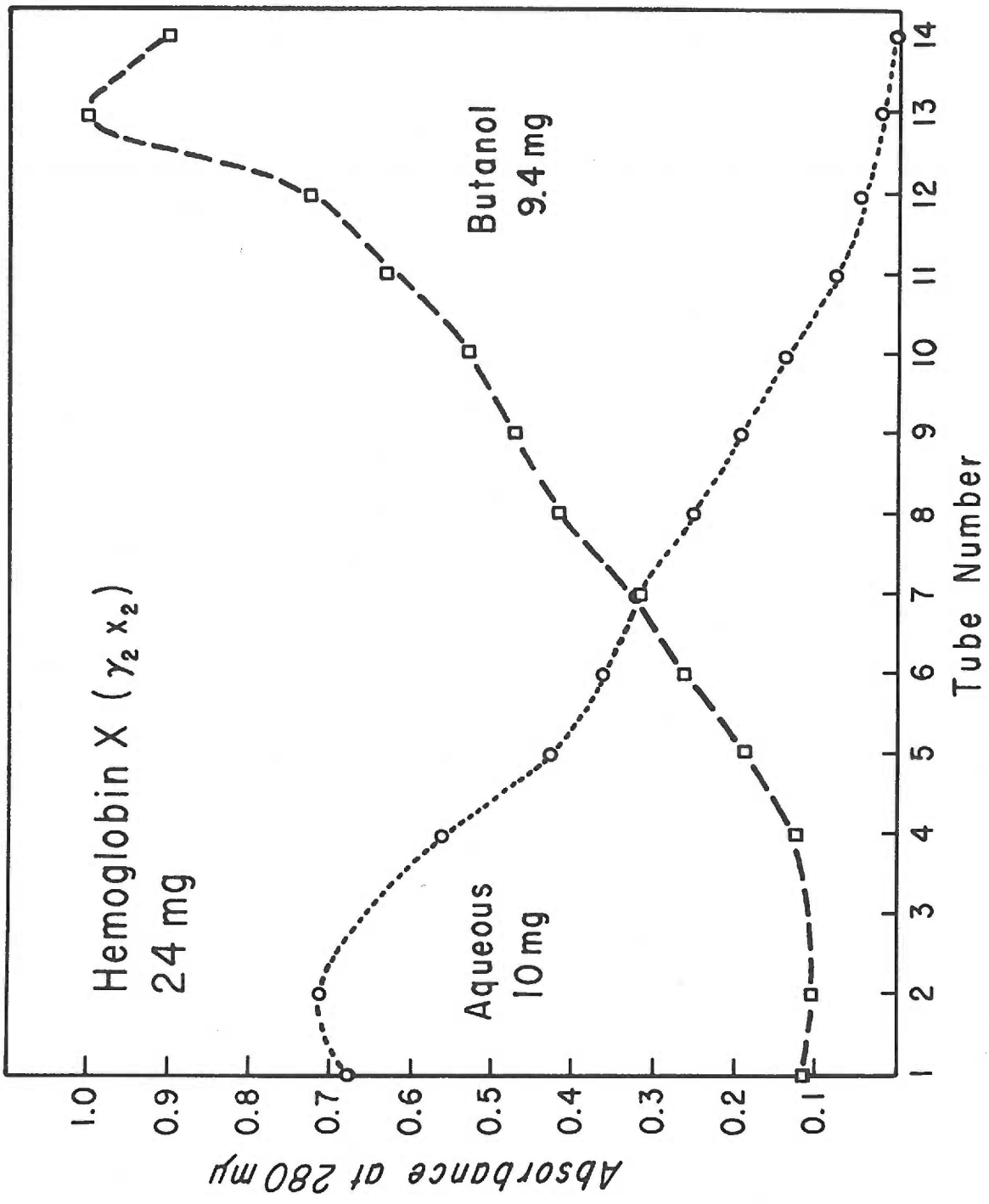

Hemoglobin F ($\alpha_2\gamma_2$)
25 mg
Butanol
9 mg
Aqueous
7.2 mg
Absorbance at 280mμ
0.6
0.5
0.4
0.3
0.2
0.1
1
2
3
4
5
6
7
8
9
10
11
12
13
14
Tube Number

Figure 26

Countercurrent Distribution Pattern of 24 mg of AE-Globin From Hemoglobin X.


Hemoglobin X ($\gamma_2 x_2$)
24 mg
Butanol
9.4 mg
Aqueous
10 mg
Absorbance at 280 mμ
Tube Number
1.0
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
1
2
3
4
5
6
7
8
9
10
11
12
13
14

in the aqueous phase and 9.4 mg in the butanol phase for an 80% recovery.

C. Peptide Chromatography of Tryptic Hydrolysates

Qualitative peptide chromatograms of hemoglobins X, Bart's and globins Bart's, F, and A are represented in Figure 27. These analytical peptide patterns were obtained by applying the soluble tryptic peptides from 1 mg of each sample to a column (0.6 x 13 cm) of Spinco 15A resin and eluting with a linear gradient of pyridine-acetic acid developer.

Figure 28 represents the qualitative chromatograms obtained on a preparative column (0.9 x 17 cm) of Spinco 15A resin from tryptic hydrolysates of 2 to 3 mg of aminoethylated globins of Hb X, $\varkappa^X$ chain, γ^X chain, and γ^F chain, respectively. The column was regenerated prior to this series of runs and the runs were made successively under the same conditions with a linear gradient of pyridine-acetic acid buffer.

Preparative chromatograms of the tryptic hydrolysates of 24 mg of aminoethylated globin X and 25 mg of globin Bart's* are represented in Figure 29. The zones are numbered and Table 5 presents a tabulation of the zone number, the fractions pooled and the average pH for the zone indicated.

The volatile part of the effluent corresponding to each zone in Figure 29 (Hb X) was removed by rotary evaporation and the peptide residues were either subjected to amino acid analysis directly

---

* Isolated from same hemolysate as hemoglobin X.

Figure 27

Analytical Peptide Patterns of Tryptic Hydrolysates of 1 mg Each of Soluble Tryptic Peptides From: (A) hemoglobin X, (B) hemoglobin Bart's (MKD), (C) globin Bart's, (D) hemoglobin F, and (E) globin A. Column was 0.6 x 13 cm Spinco 15A resin; temperature 50°C. Development with a linear gradient of 250 ml of pyridine-acetate buffer beginning at pH 3.1, 0.2 M pyridine and ending at pH 5.0, 2.0 M pyridine (125 ml of each). Column flow of 15 ml per hr. and ninhydrin flow of 15 ml per hr.

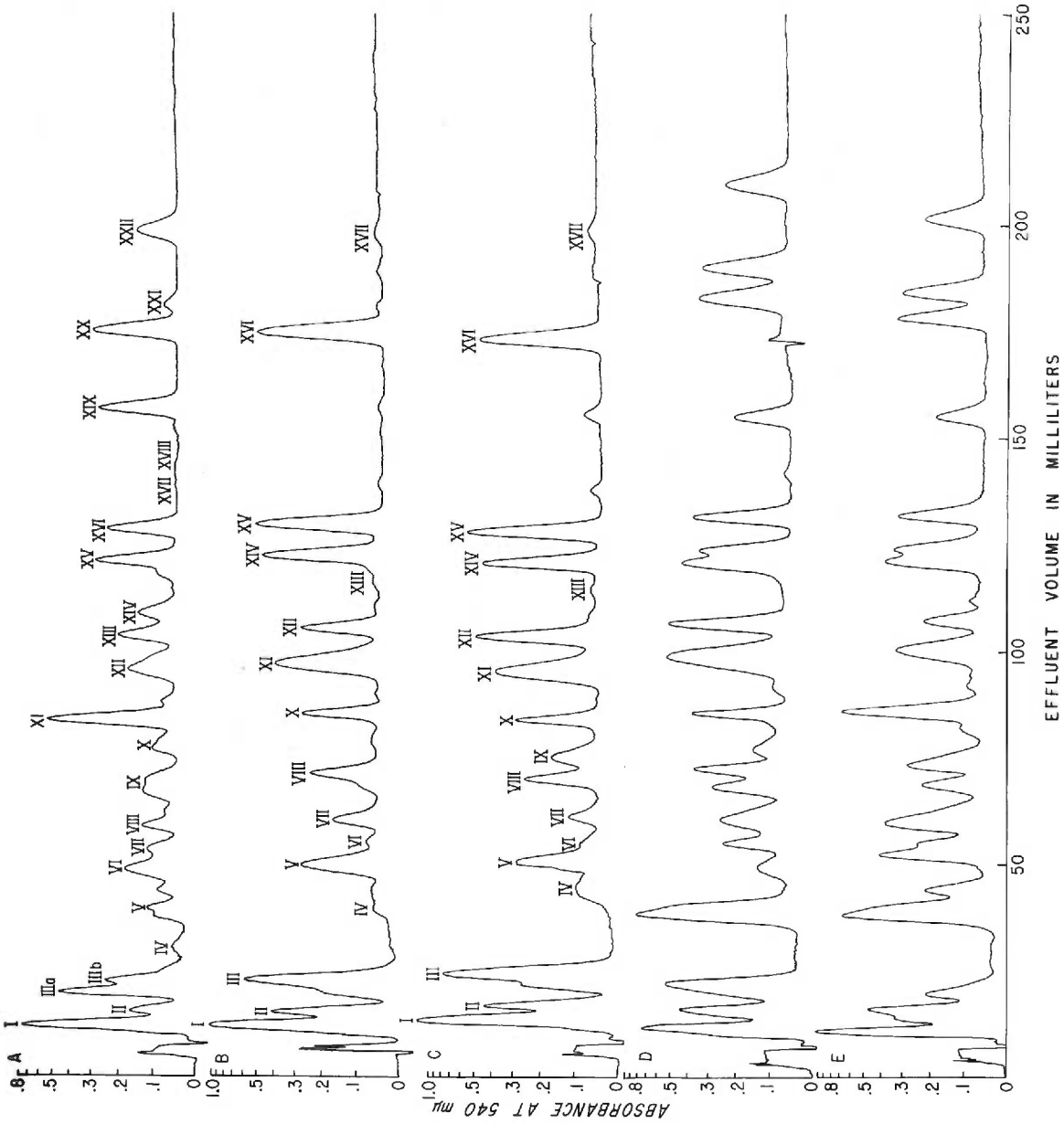

A
B
C
D
E
ABSORBANCE AT 540 mμ
EFFLUENT VOLUME IN MILLILITERS
0
50
100
150
200
250

Figure 28

Peptide Patterns of Complete Tryptic Hydrolysates: (A) 3 mg of AE-Hb X, (B) 2.4 mg of AE-γ Chain and (C) 2.2 mg of AE-× Chain of Hemoglobin X and (D) 2 mg of the AE-γ^F Chain of Hemoglobin F. Loads of soluble tryptic peptides were: (A) 3 mg; (B) 2.4 mg; (C) 2.2 mg; and (D) 2 mg. Column was 0.9 x 17 cm Spinco 15 A resin; temperature 50°C. Development was with a linear gradient of 500 ml of pyridine-acetate buffer beginning at pH 3.1, 0.2 M pyridine and ending at pH 5.0, 2.0 M pyridine (250 ml of each). Column flow of 30 ml per hr. and ninhydrin flow at 15 ml per hr.

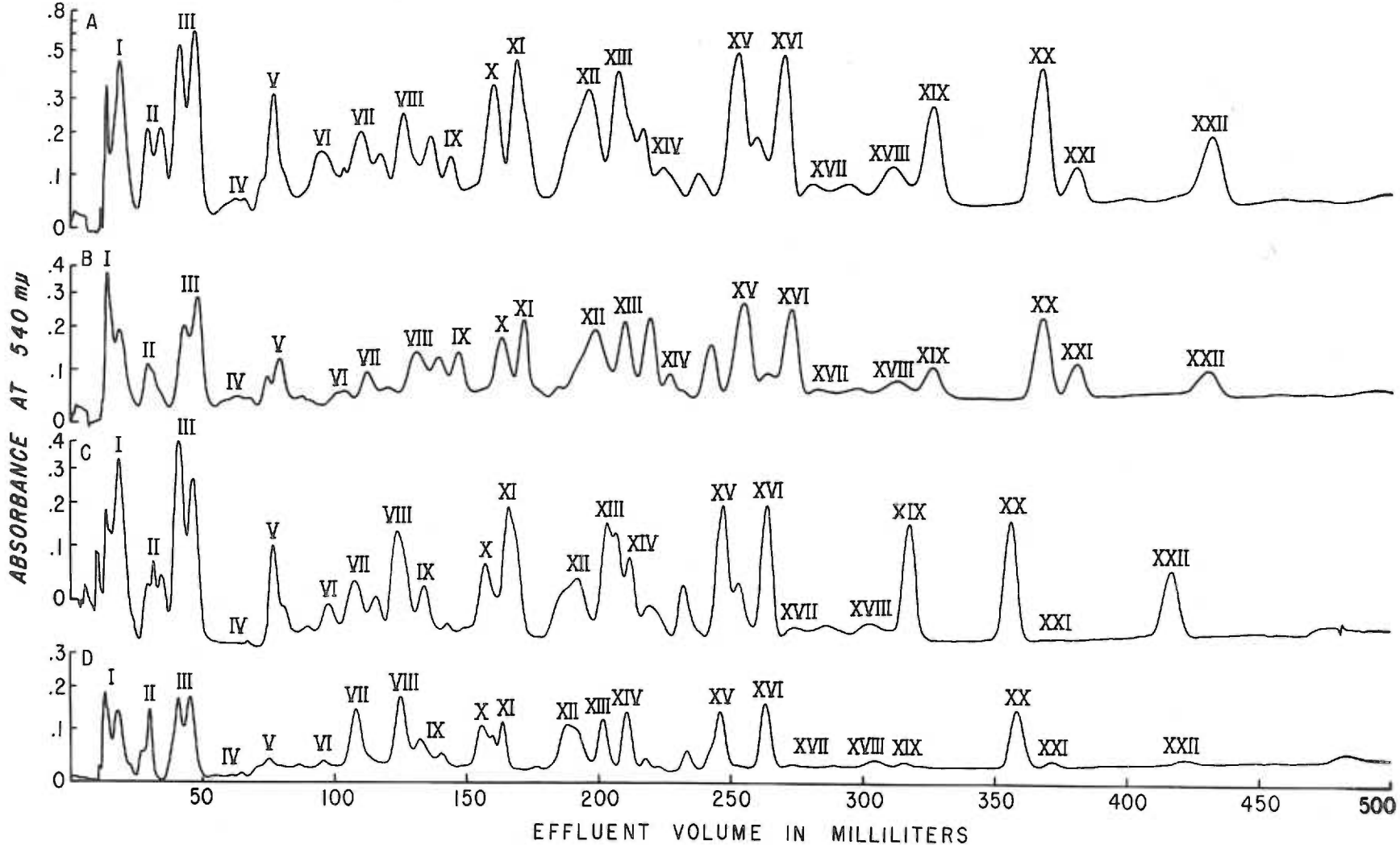

A
B
C
D
I
II
III
IV
V
VI
VII
VIII
IX
X
XI
XII
XIII
XIV
XV
XVI
XVII
XVIII
XIX
XX
XXI
XXII
ABSORBANCE AT 540 mμ
0
.1
.2
.3
.4
.5
.8
50
100
150
200
250
300
350
400
450
500
EFFLUENT VOLUME IN MILLILITERS

Figure 29

Peptide Patterns of Tryptic Hydrolysates of (A) AE-Globin Bart's and (B) AE-Globin X. Loads of 25 mg of tryptic peptides were used on a 0.9 x 17 cm column of Spinco type 15A resin. Development was the same as described for Figure 28.

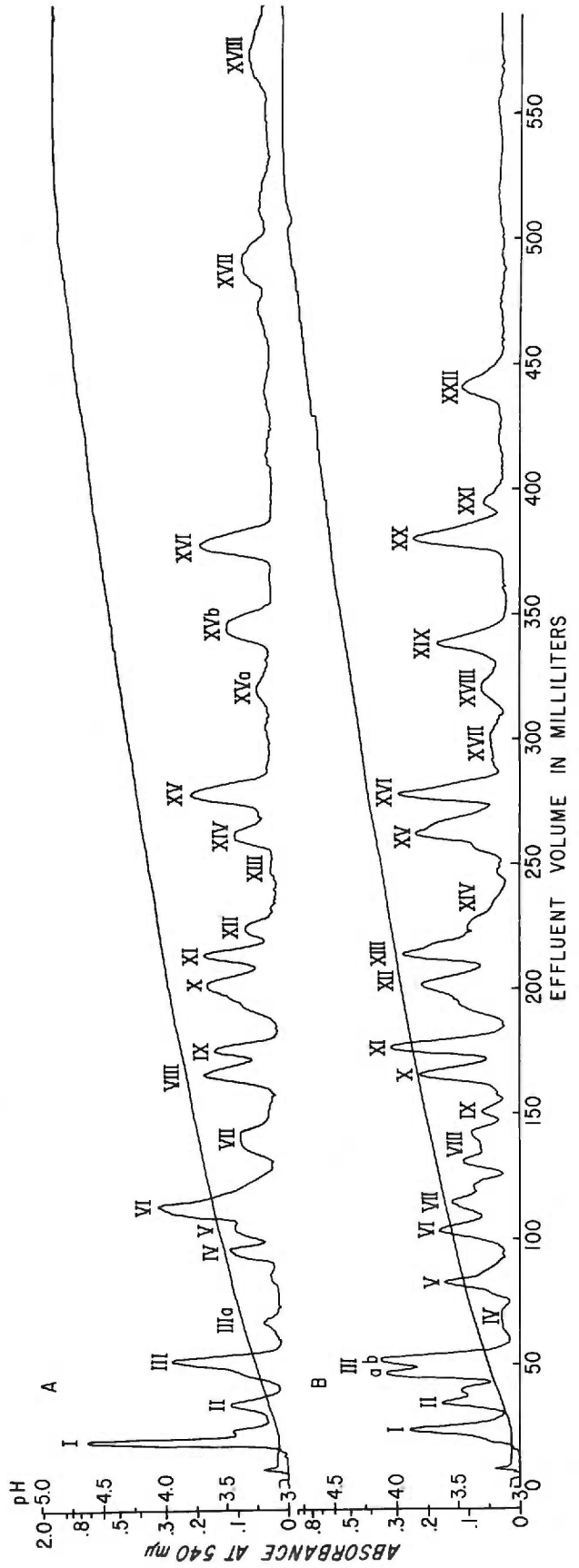

A
B
I
II
III
IIIa
IV
V
VI
VII
VIII
IX
X
XI
XII
XIII
XIV
XV
XVa
XVb
XVI
XVII
XVIII
IIIb
XIX
XX
XXI
XXII
pH
2.0
5.0
.8
4.5
.5
4.0
.3
3.5
.2
.1
0
3
ABSORBANCE AT 540 mμ
0
50
100
150
200
250
300
350
400
450
500
550
EFFLUENT VOLUME IN MILLILITERS

Table 5

Tabulation of Zone Number, Fractions Pooled and pH of Where Zone was Eluted for Preparative Tryptic Peptide Chromatography Shown in Figure 29.

Hb X						Hb Bart's					
Zone	Ml. Effluent	pH	Zone	Ml. Effluent	pH	Zone	Ml. Effluent	pH	Zone	Ml. Effluent	pH
I	10-23	3.08	XIII	200-211	4.00	I	16-26	3.07	XIII	243-254	4.19
II	23-35	3.15	XIV	212-242	4.07	II	28-40	3.18	XIV	256-268	4.23
IIIa	36-44	3.23	XV	245-264	4.17	III	41-57	3.29	XV	271-287	4.36
IIIb	45-51	3.26				IIIa	58-72				
IV	52-66	3.36	XVI	265-277	4.23	IV	89-99	3.37	XVa	312-328	4.42
V	67-85	3.44	XVII	278-302	4.27	V	100-108	3.52	XVb	337-355	4.55
VI	86-100	3.55	XVIII	303-320	4.36	VI	109-128	3.60	XVI	370-388	4.86
VII	103-116	3.60	XIX	321-338	4.43	VII	130-148	3.72	XVII	480-502	4.95
VIII	117-137	3.70	XX	366-382	4.54	VIII	160-171	3.81	XVIII	560-588	
IX	138-143	3.76	XXI	382-394	4.57	IX	172-181	3.86			
X	150-162	3.81	XXII	427-441	4.71	X	188-208	3.92			
XI	163-174	3.88				XI	209-219	4.02			
XII	177-199	3.96				XII	220-230	4.06			

or purified further by rechromatography on columns of AG 50W-X2.

Several new peptides were isolated and the recordings of the rechromatography of the zones where these peptides were found are illustrated in Figure 30. It can be seen that more than one peptide could be contained in a given zone.

D. Amino Acid Analyses

The amino acid compositions of the isolated peptides were obtained and Table 6 presents a tabular summary of the preparative chromatogram zone, the corresponding rechromatogram number with its specific zone, pooled fractions and the pH of the rechromatography chromatogram zones. Amino acid compositions identified the presence of all the readily detectable γT-peptides, four αT-peptides in low yield and ten possible new peptides. The location of these peptides relative to the chromatographic zones is indicated in Table 6.

The amino acid compositions of the nine possible new peptides (×T-peptides) of hemoglobin X are presented in Table 7. These are compositions which are not represented by any peptide of the known hemoglobin chains. The empirical formulas for the possible ×T-peptides, along with the zone of the preparative chromatogram from where the peptide arises, are shown in Table 8.

In Table 9 are presented the overall amino acid compositions of hemoglobin Bart's, hemoglobin X and the $\times^{X}$ and γ^{X} chains. The reported composition of the γ^{F} chain is included for comparison to the experimentally obtained data.

A comparison of the amino acid compositions of hemoglobin X

Figure 30

Rechromatography Patterns of Zones From Preparative Chromatograms Shown in Figure 29 and Tabulated in Table 5 and Table 6. These patterns correspond to the zones which contained new peptides. A column (0.9 x 50 cm) of AG-50W - X2 at 50°C and linear gradient of pyridine-acetic acid buffer as described in Figure 28. The preparative chromatogram zone is indicated on each pattern with the zones resulting from rechromatography indicated by Roman numerals. The figures shown correspond to rechromatograms as follows: A (735), B (736), C (740), D (744), E (745), F (747), G (749), H (751). Numbers in parentheses correspond to the rechromatogram number.

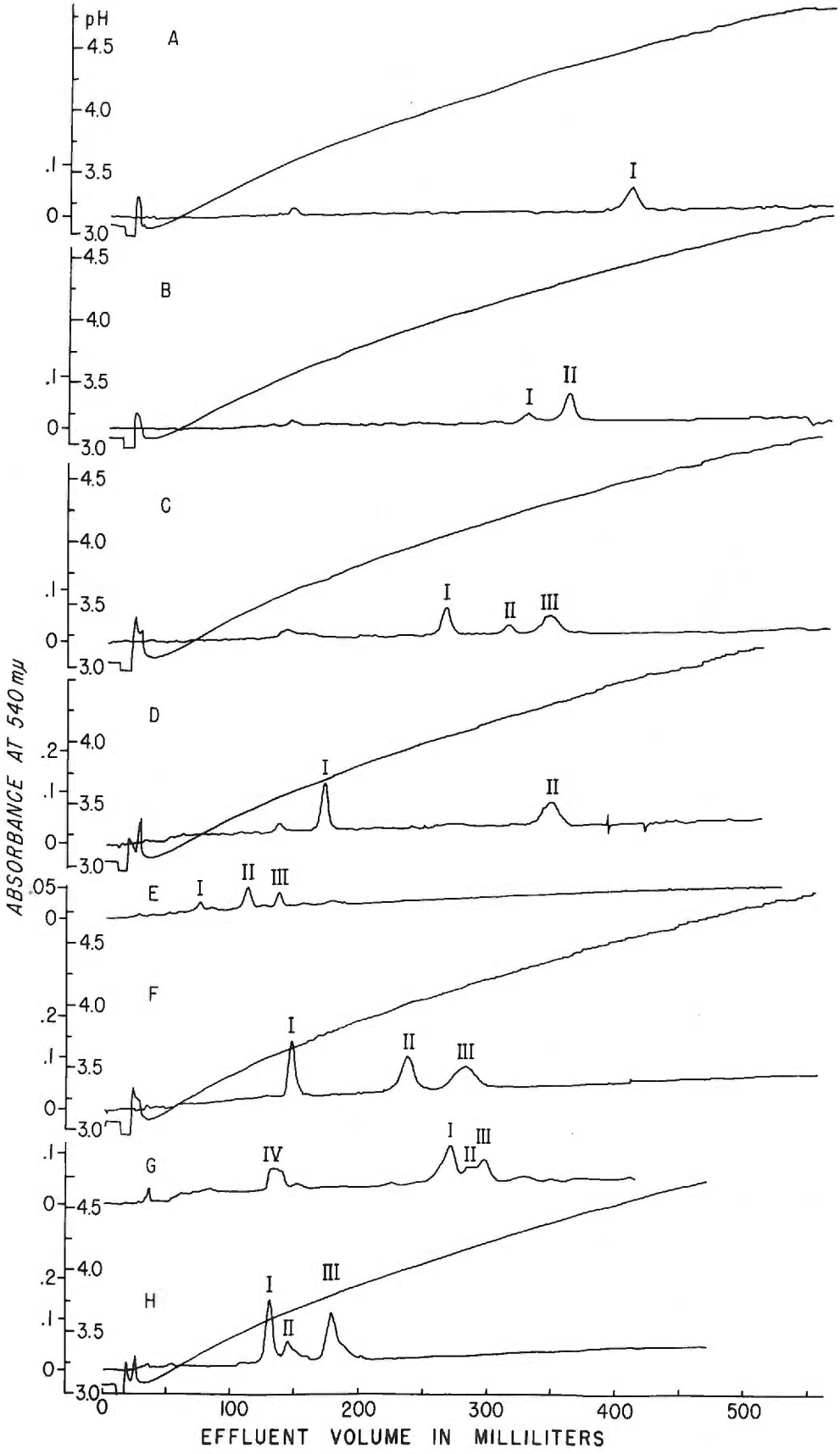

pH
A
B
C
D
E
F
G
H
I
II
III
IV
ABSORBANCE AT 540 mμ
EFFLUENT VOLUME IN MILLILITERS
0
100
200
300
400
500

Table 6

Peptides Isolated from Tryptic Hydrolysate of Aminoethylated Globin of Hb X.

Preparative Chromatogram		Rechromatography				Isolated Peptides		
Zone No.	Ml. of Effluent	Run No.	Zone No.	Pooled Fractions	pH	γ-Peptides	×-Peptides	α-Peptides
I	16-26	745	I	11-12	3.25		×T-A	
			II	17-19	3.41		×T-B	
II	25-35	751	I	21-23	3.61	γT-3		
			III	29-33	3.81		×T-C	
IIIa	36-44	755	I	22-24	3.60			
			III	27-28	3.73	γT-9		
			IV	29-30	3.76	γT-15		
			V	31	3.78	γT-15		
			VI	32-35	3.85	γT-14		
IIIb	45-51	756	II	26-27	3.69	γT-9		
			III	29-30	3.75	γT-14,15		
			IV	31-32	3.81	γT-14		
IV	52-66							
V	67-85	737	I	29-30	3.72			αT-11

Table 6 (Cont'd)

Preparative Chromatogram		Rechromatography				Isolated Peptides		
Zone No.	Ml. of Effluent	Run No.	Zone No.	Pooled Fractions	pH	γ-Peptides	×-Peptides	α-Peptides
VI	86-100	753	I	36-42	3.99	γT-4		
VII	103-116	749	I	42-47	4.13	γT-12	×T-D	
VIII	117-137	747	II	37-40	4.06		×T-E	
			III	43-50	4.19	γT-8,9		αT-4
IX	138-143							
X	150-162	746	I	47-50	4.14	γT-12		
XI	163-174	744	I	27-30	3.72	γT-8		αT-8
			II	55-61	4.28		×T-F	
XII	177-199	742	I	57-61	4.37	γT-11		
			II	64-67	4.48	γT-1,2		
XIII	200-211	741	III	44-49	4.16	γT-10		
XIV	212-242							
XV	245-264	740	I	42-46	4.09	γT-6		
			II	51-54	4.21		×T-G	
			III	56-60	4.34	γT-7	×T-H	

Table 6 (Cont'd)

Preparative Chromatogram		Rechromatography				Isolated Peptides		
Zone No.	Ml. of Effluent	Run No.	Zone No.	Pooled Fractions	pH	γ-Peptides	×-Peptides	α-Peptides
XVI	265-277	738	I	47-50	4.11	γT-16		
XVII	278-302							
XVIII	303-320							
XIX	321-338	736	II	56-59	4.34		×T-I	
XX	366-382	739	I	60-64	4.43	γT-7		
XXI	383-394							αT-7
XXII	427-441	735	I	65-70	4.50		×T-J (=αT-14)	

Table 7

Amino Acid Composition of New Tryptic Peptides (×T Peptides) of Aminoethylated Chains of Hb X.

Amino Acid	×T-A	×T-B	×T-C	×T-D	×T-E	×T-F	×T-G	×T-H	×T-I	×T-J
	I[a]	I	II	VII	VIII	XI	XV	XV	XIX	XXII
Lysine	0.98		1.01	1.00		1.07	0.07	0.93	0.91	
Histidine					1.03	1.03		0.96	1.07	
Arginine		1.00					1.07			1.09
Aspartic Acid	0.21	1.08	0.36							
Threonine	0.99	3.79	1.21	1.72		1.01				
Serine	1.00	0.96	2.93	1.07	1.09	1.01	0.12	1.15	1.05	
Glutamic Acid	0.21	3.08	0.86		1.09	1.02				
Proline	0.07	0.08				0.93				
Glycine	0.19	1.12	0.41	1.04	0.24	0.15	0.12	1.11	1.05	
Alanine	0.21	1.08	0.67	2.19	1.00	0.13		1.52	0.88	
S-Aminoethylcysteine								0.76		
Valine	0.14	0.13	2.12							
Methionine										
Isoleucine	0.09	1.87		0.59			0.84			
Leucine	1.03	1.04	2.09	1.00	1.86	1.97	0.93	1.80		
Tyrosine					0.91					0.91
Phenylalanine	0.06		0.96			0.97				

[a]Roman numerals indicate zone from preparative chromatogram shown in Figure 29(A).

Table 8

Empirical Formulas of xT Peptides of Aminoethylated Chains of Hemoglobin X.

Preparative Chromatogram Zone		Rechromatogram No. and Zone		Peptide Composition
I	xT-A	745	I	lys, thr, ser, leu
I	xT-B	745	II	asp, thr_3, ser, glu_2 gly, ala, $Ileu_2$, leu, thr, glu, arg
II	xT-C	751	III	lys, thr, ser_3, glu, ala, val_2, leu_2, phe
VII	xT-D	749	I	lys, thr_2, ser, gly, ala_2, leu
VIII	xT-E	747	II	his, ser, glu, ala, leu_2, tyr
XI	xT-F	744	II	lys, his, thr, ser, glu, pro, leu_2, phe
XV	xT-G	740	II	arg, Ileu, leu
XV	xT-H	740	III	lys, his, ser, gly, ala_{1-2}, cys, leu_2
XIX	xT-I	736	II	lys, ala, his, gly, ser
XXII	xT-J	735	I	arg, tyr

Table 9

Amino Acid Composition of Hemoglobins Bart's and X and of the $\varkappa^X$ and γ^X Chains.

Amino Acid	γ Chain*	Hb Bart's(a)	Hb X(b)	$\varkappa^X$ Chain(c)	γ^X Chain(c)
Lysine	12	11.2	11.4	9.9	11.0
Histidine	7	6.8	7.7	7.1	7.6
Arginine	3	2.9	4.7	4.4	3.5
Aspartic Acid	13	13.6	13.4	12.1	13.8
Threonine	10	10.1	11.5	11.0	9.9
Serine	11	10.3	12.6	11.4	10.4
Glutamic Acid	12	12.2	12.2	11.1	11.0
Proline	4	4.4	5.5	4.7	5.6
Glycine	13	13.0	11.8	10.2	12.0
Alanine	11	12.4	16.2	14.0	14.9
S-Aminoethyl-cysteine	1	0.8	0.6	0.6	0.5
Valine	13	12.8	13.5	11.4	13.0
Methionine	2	0.9	1.6	1.2	1.1
Isoleucine	4	3.1	5.0	4.8	3.0
Leucine	17	17.9	19.1	17.8	18.0
Tyrosine	2	2.1	2.7	2.5	2.4
Phenylalanine	8	8.4	8.7	7.9	8.1
Tryptophan	3	3**			

*Values from the literature.

**Not experimentally determined and value is from literature.

(a)Values from a 22 hr. and 70 hr. hydrolysis of AE-globin (MKD).

(b)Values extrapolated from a 22 hr. and 70 hr. hydrolysis on one sample (AE-globin X) and from a 22 hr., 36 hr., 48 hr., and 70 hr. hydrolysis on a sample of hemoglobin X.

(c)Values from a 22 hr. and 70 hr. hydrolysis of AE-globin of each chain.

and the γ and α chains is presented in Table 10. Also given is the average composition of hemoglobin X obtained from the compositions of the individual chains of that hemoglobin. In addition the composition of the × chain is given as obtained by doubling the value of each residue for hemoglobin X, subtracting the corresponding value of the γ^X chain and assigning the resultant difference to the × chain of hemoglobin X.

The comparison of amino acid compositions in Table 9 of the $\times^X$ and γ^X chains illustrates differences between the two chains, particularly for lysine, arginine, threonine, serine, glycine, and valine. Evaluation of each of the chains versus the literature values for the γ^F chain reveal that though similarities exist between the $\times^X$ and γ^F chains there are many more similarities between the γ^X and γ^F chains. There is experimental variance between amino acid analyses but these comparisons just mentioned show a clear preference to state that the γ^X is like the γ^F whereas the $\times^X$ is unlike the γ^F chain.

There were more determinations made in hemoglobin X than either of its separated chains and although, as already pointed out, variance exists between two given analyses, the comparison in Table 10 of the average of $\times^X$ and γ^X versus hemoglobin X is quite good and indicates overall consistency of these results.

Another approach to the composition of the $\times^X$ chain is shown in Table 10 where the resultant values of $2X-\gamma^X$ are assigned to the × chain. This procedure generally diminishes the influence of α contaminants which would be expected to be directly reflected in

Table 10

Comparison of Hemoglobin X and the α^X and γ^X Chains.

Amino Acid	Hb X	$\frac{\alpha^X + \gamma^X}{2}$	$2X - \gamma^X$	γ	α
Lysine	11.4	10.5	11.8	12	11
Histidine	7.7	7.4	7.8	7	10
Arginine	4.7	4.0	5.9	3	3
Aspartic Acid	13.4	13.0	13.0	13	12
Threonine	11.5	10.5	13.1	10	9
Serine	12.6	10.9	14.8	11	11
Glutamic Acid	12.2	11.1	13.4	12	5
Proline	5.5	5.2	5.4	4	7
Glycine	11.8	11.1	11.6	13	7
Alanine	16.2	14.5	17.5	11	21
S-Aminoethylcysteine	0.6	0.6	0.7	1	1
Valine	13.5	12.2	14.0	13	13
Methionine	1.6	1.2	2.1	2	2
Isoleucine	5.0	3.9	7.0	4	0
Leucine	19.1	17.9	20.2	17	18
Tyrosine	2.7	2.5	3.0	2	3
Phenylalanine	8.7	8.0	9.3	8	7
Tryptophan	-	-	-	3 (1)	1

the γ^X composition since the γ^X and α components were present in the same phase after chain separation. When the assigned values of the κ^X chain obtained this way are compared to literature values of the γ^F chain, the overall composition is quite dissimilar to that of the γ^F chain. Differences exist for the values of arginine, threonine, serine, valine, isoleucine and leucine.

DISCUSSION

This study includes the analysis of primary structure and it is important to establish that the materials under investigation were clearly resolved. That this was accomplished is indicated by criteria presented in the Results Section and specifically established that hemoglobin X is a distinct and clearly isolatable component as demonstrated particularly by its chromatographic and electrophoretic behavior.

In reference to the electrophoretic behavior of hemoglobin X, it is of interest that in the studies of hemoglobin H disease a second slower component has been observed on starch block electrophoresis which has a mobility less than hemoglobin Bart's but slightly greater than hemoglobin A (146). At this time it is not known if there is a relationship between that component and hemoglobin X.

It has been shown that hemoglobin H can exist in two isomeric forms which are electrophoretically distinguishable (134) but have no significant differences in their amino acid compositions. The possibility that hemoglobin X could bear a similar isomeric relationship to hemoglobin Bart's is ruled out by the evidence from chain separation and subunit hybridization, both of which demonstrate the presence of two types of chains. These results plus the fact that no δ chains were revealed by peptide analysis rule out the possibility of another abnormal hemoglobin consisting solely of δ^{A2} chains and thought to be a δ_4^{A2} structure (133).

One of the striking properties of hemoglobin is its tendency to dissociate into lower molecular weight subunits under a variety of fairly mild conditions with no hydrolysis or denaturation of the protein. This type of behavior is of course dependent on the fact that the complete normal mammalian hemoglobin molecule is composed of four polypeptide chains held together by noncovalent links. The precise nature of these forces is as yet not completely known but it is hopeful that pertinent information about them can be deduced by studies of the dissociation.

The information currently available in the literature is mostly about the dissociation of normal human and horse hemoglobins (75) and there may be important differences between these normal components and the abnormal components and between these and other normal components for that matter.

Physicochemical studies of hemoglobin X indicate that it is a tetrameric structure with a molecular weight of 66,000 and in view of the dissociation phenomena observed for other hemoglobins discussed in the preceeding paragraph, it was felt that it would be instructive to examine in particular the dissociation properties of hemoglobins A, H, X and Bart's by exposure to various pH conditions in both acidic and alkaline ranges. Ultracentrifuge patterns for these samples at values of pH 4.5, pH 7 and pH 10.5 are shown in Figures 18, 19, 20 and 21. From the data obtained at various pH values a pH versus $s_{20,w}$ diagram has been formulated for each one of these components (Fig. 15, Hb Bart's and Hb H; Fig. 16, Hb A and Hb X).

The "stability zone" of hemoglobin was first established by Svedberg and Nichols (121) to exist in dilute buffer solutions between pH 6 to 10. It is thought that the state of the hemoglobin molecule or the dynamic equilibrium between subunits as indicated by sedimentation coefficients or diffusion coefficients and molecular weights determinations is independent of pH in this range from pH 6 to 10. Recent studies (135) indicate that the dissociation equilibrium involves tetramers, dimers and monomers at all pH values. At pH values below 6 and above 10 there is a shift in the equilibrium behavior and the hemoglobin undergoes reversible dissociation into subunits and the molecular weight of the protein approaches one-half its normal value with a corresponding $s_{20,w}$ of the order of 2.55.

The data from the schlieren patterns (Figs. 18 to 21) and the graphs of $s_{20,w}$ versus pH (Figs. 15 and 16) suggest the presence of incomplete dissociation at the extreme pH values although for the hemoglobin A component at pH 11 the plateau suggests complete dissociation into two subunits (97). The spreading and skewing of the boundaries are in accord with the conclusion of incomplete dissociation or a slowly attained equilibrium.

The data in Figures 15 and 16 indicate that both hemoglobin A and H exhibit the independence of $s_{20,w}$ versus pH at pH 6 to 10 and hemoglobin H seems to maintain this independence to pH 5. For hemoglobin Bart's the results suggest a less drastic dependence on low pH although the $s_{20,w}$ values decrease in the range of pH 6 to 6.5. At alkaline pH the dissociation of hemoglobin Bart's begins at pH 10 with

a less noticeable break in the curve between pH 10 to 10.5. For hemoglobin X the acid dissociation resembles the hemoglobin A data and the dissociation of hemoglobin X is reversible (Table 4) upon neutralization by dialysis from pH 4.5 to 6.9. At alkaline pH the dissociation of hemoglobin X starts at pH 10 but at pH 10.5 the $s_{20,w}$ has not decreased as significantly as for hemoglobins Bart's, H or A. This is reminiscent of the alkali resistance of foetal hemoglobin which seems to be possibly a function of the γ chain in combination with an unlike chain (e.g. $\alpha_2\gamma_2$).

The graphic illustrations of pH effects on the sedimentation coefficient in Figures 15 and 16 provide certain information into the dissociation mechanism. From the knowledge of protein structure in general and that of hemoglobin specifically it is known that ionizable groups such as carboxyl groups, histidine residues, guanidyl groups and amino groups play an important role in maintaining the integral tertiary structure of the proteins through electrostatic forces and participation in hydrogen bonding. One of the major disruptive influences upon the native conformation of the protein is from the repulsion of like charges at extreme pH values. If a curve as shown in Figures 15 and 16 demonstrates a break around pH 5.5 then a weak acidic function such as the histidine residues may be involved. At lower pH values the continued decrease in the curve indicates involvement of carboxyl groups which become protonated and no longer supply an oppositely charged function necessary for electrostatic bonding to positively charged groups which, due to repulsion, cause disruption of

the conformation. The involvement of weak electrostatic bonds is also indicated by the near independence of dissociation upon temperature (75). The mechanism of alkaline dissociation is possibly similar to that of acid dissociation and above pH 9.5 the dissociation could result from suppression of the ionization of basic amino groups essential for electrostatic bonds between subunits with disruption resulting from repulsive negatively charged carboxyl groups. The influence of guanidyl groups is probably not reflected much here since their pK value is so high and only little suppression of ionization would occur at these pH values.

The acid dissociation schlieren patterns for hemoglobin X and hemoglobin H exhibit two skewed peaks instead of a single skewed peak (Fig. 19). In the case of hemoglobin H the $s_{20,w}$ values were 3.0 S for the slow peak and 3.7 S for the fast peak. The 3.0 S component would correspond to a near half value for the molecular weight of the whole molecule and the 3.7 S component would correspond to a molecular weight of about 55,000 assuming the frictional ratio to be the same as at neutral pH. For hemoglobin X at pH 4.5, the schlieren pattern (Fig. 21) indicates a slow component with $s_{20,w}$ of 3.6 and a fast component with $s_{20,w}$ of 11.0.

The effects of concentrated salt solutions at near neutral pH values have been studied (99) and at 2 M NaCl concentration hemoglobin A molecular weight approaches a value half that under normal conditions. The sedimentation coefficient ($s_{20,w}$) correspondingly decreases from a value of 4.4 to about 3.3 S in 2 M NaCl.

It has been shown that hemoglobin H is clearly more resistant to salt splitting than hemoglobin A (101) which is surprising in view of its great instability in other respects (116). Figure 17(A) illustrates the effects of NaCl on the $s_{20,w}$ for hemoglobin H and the 3.72 S value obtained at 2 M NaCl corresponds to the molecular weight of 55,700 reported by Benesch (101) for this hemoglobin under these conditions.

If calculations are made with assumptions for a spherical molecule by using the formula $n = \sqrt[3]{\left(\frac{s^0_{20,w}}{s^0_{20,w}}\right)^3}$ (177) then the 11.3 S component has n = 4, thus 4 molecules of hemoglobin appear to be aggregated. It must be pointed out that at pH 4.5 the frictional ratio may be different than at pH 7 so the subunit number calculation is approximate.

In the case of lamprey hemoglobin, it has been shown that two peaks occur in schlieren patterns with samples at neutral pH and such a second peak is associated with incompletely deoxygenated solutions according to Briehl (19).

Studies on human hemoglobin at pH below 5 with deoxyhemoglobin give evidence of two peaks in the schlieren diagrams (76). It appears that the dissociation at acidic pH is dependent on oxygenation.

It is not clear at present as to the relationship between the observations just indicated and the dissociation features of hemoglobin H and hemoglobin X. Both samples were equilibrated at atmospheric pressure which was assumed to be adequate to provide saturation of oxygen of the samples. The possibility of disulfide linkages exists with the alternatives of either intra- or intermolecular bridges or perhaps both types. When the hemoglobin X sample was exposed

to a buffer solution at pH 4.5 which was 0.1 M in mercaptoethanol to prevent possible disulfide linkages, the double peak pattern was observed again with both the 3.3 S and an 11.3 S component present. The conclusion is that there may be intramolecular disulfide bonds but that there are no intermolecular type links since the mercaptoethanol would be expected to interfere with such bonds and no such effects were observed.

The ultraviolet spectrum of hemoglobin A, F, Bart's and X were compared for the resolution of the tryptophan fine-structure band just below 290 mμ (Fig. 22). The fractional resolution of the tryptophan fine structure is helpful in characterizing hemoglobins and is calculated with the following formula:

$$R = \frac{E_{max} - E_{min}}{E_{max}} \qquad (51)$$

The fractional resolution for hemoglobin F was 0.24×10^{-2} and for hemoglobin Bart's it was 1.8×10^{-2} which is within the reported range for hemoglobin Bart's (51). The resolution for hemoglobin X was 0.66×10^{-2} which is intermediate to hemoglobin F and Bart's. The resolution for hemoglobin Gower 2 was not measured by Huehns (54) but on visual comparison of the data there was slightly less resolution of this region for hemoglobin Gower 2 than for hemoglobin X. The structure of hemoglobin Gower 2 is reported to be $\alpha_2\varepsilon_2$. The relative resolution of the tryptophan fine-structure band for these hemoglobins follows the order: Hb Bart's > Hb X > Hb F > Hb Gower 2 > Hb A. It indicates that the greater the γ chain-like structure, the greater

the resolution of the tryptophan fine-structure band with Hb Bart's containing the highest γ structure and Hb Gower 2 the least γ like structure and suggests a greater proportion of γ like structure in Hb X than in Hb F.

Dissociation and recombination experiments of hemoglobin X with hemoglobin A and of hemoglobin Bart's with hemoglobin A (Fig. 23) indicated that in addition to the parent species, there were four new zones formed, two faster than either parent species and two slower. The two faster components were indicated to correspond to hemoglobin Bart's and H, while one slower zone, just slower than hemoglobin A, was indicated to be hemoglobin F. The slowest zone is suggested to be a hybrid of α chains from hemoglobin A and $\times$ chains from hemoglobin X. It is instructive to recall the relative mobilities for hemoglobin A, F and hemoglobins Gower 1 (ε_4) and Gower 2 ($\alpha_2\varepsilon_2$) under similar conditions as presented by Huehns (54).

The position of the slowest zone, indicated $\alpha_2{}^{\times}{}_2$, is near where hemoglobin Gower 2 ($\alpha_2\varepsilon_2$) or hemoglobin A_2 ($\alpha_2\delta_2^{A2}$) would be expected to occur. This suggests that the $\times$ chain may be identical or a variant of the ε chain. However, the data presented here do not allow a definite conclusion on this point. The possibility of the new zone being hemoglobin A_2 or the unknown hemoglobin a $\gamma_2\delta_2^{A2}$ structure is eliminated since peptide chromatography demonstrates that no δ chains are present in hemoglobin X. Allowing for a slight contamination of hemoglobin X by hemoglobin Bart's, the amount of hemoglobin F formed is too great to be accounted for except by a hybrid formed from α chains

of hemoglobin A with γ chains of hemoglobin X. The recombination reaction would then proceed as follows:

$$\alpha_2\beta_2 + \gamma_2 x_2 \longrightarrow \alpha_2 x_2 + \alpha_2\beta_2 + \alpha_2\gamma_2 + \gamma_2 x_2$$

Other theoretically possible recombinants resulting from this reaction include: β_4, γ_4, $x_2\gamma_2$ and $\beta_2 x_2$. The presence of a structure consisting solely of α chains is also possible since *in vitro* occurrence of α monomers has been shown, although free α chains would be expected to recombine with β or γ chains to form $\alpha_2\beta_2$ type structures. The β_4 and γ_4 species are well known and both were detected in this experiment with a high yield of the γ_4 and very low yield of the β_4. The $\beta_2\gamma_2$ and $\beta_2 x_2$ species have never been reported and their presence was not indicated here. These structures could possibly occur and then go undetected if the rate of dissociation of such species is very much faster than association to form them.

In retrospect it is possible to speculate that the species $\gamma_2\varepsilon_2$ would have a mobility on starch gel faster than $\alpha_2\beta_2$ but slower than γ_4 which places it near the position observed for hemoglobin X. The rational for this comes from the observation that combination of two non-like chains results in a species with a mobility intermediate to either parent species, e.g. α chains remain near the origin in starch gel at pH 8.2 whereas a β chain structure moves very rapidly and the composite of these two chains ($\alpha_2\beta_2$) moves with an intermediate mobility. A similar application can be illustrated for α chains and γ chains compared to $\alpha_2\gamma_2$. Also the same relationships can be shown for α chains and ε chains (Gower 1) compared with a $\alpha_2\varepsilon_2$ (Gower 2)

structure. This type of analogy would predict the mobility of a $\gamma_2\varepsilon_2$ structure to be intermediate to that of the mobilities of ε chains and γ chains.

Analytical peptide analyses as illustrated in Figure 27 revealed certain differences between those samples compared. Some difficulty was encountered in maintaining exact duplication of flow rates for all these runs but comparison of the chromatograms by the reader does reveal certain similarities and dissimilarities. It is possible to indicate in the chromatogram for hemoglobin X that certain features are unique, e.g. in the region numbered II and III, also peaks XV, XIX and XXII indicate unusual characteristics.

The hybridization studies indicated the presence of two types of chains and following aminoethylation the sample of globin from hemoglobin X was subjected to countercurrent distribution which also revealed two types of chains.

The qualitative peptide patterns shown in Figure 28 illustrate several interesting features of the separated chains (α^X,γ^X) of hemoglobin X when compared with the parent species and with γ^F chain. Inspection of the four chromatograms reveals common qualities for X, α^X and γ^X. It can be seen that particular features are shared by X and α^X but not by γ^X. On the other hand, γ^X and γ^F seem to be nearly identical indicating the γ^X to be a normal γ^F chain. In order to point out features of these chromatograms, they all have been numbered according to the position of the zones as they appear in the chromatogram of X. Comments about the zones are as follows: Zone II illustrates

a greater resolution common to X and $\times^X$; Zone V is similar for X and $\times^X$ but different in γ^X; Zone VIII of $\times^X$ indicates a greater relative yield than in X or γ^X, which is thought to be due to removal of the γ^X component; Zone XI indicates a shoulder on the peak in X and $\times^X$ but not in γ^X; Zone XIII points out the additivity of $\times^X$ and γ^X to yield X; Zone XV of X and $\times^X$ is in greater yield than in γ^X and appears to have a small zone following the main peak not prominent in γ^X; Zone XIX is in relatively large yield in X and $\times^X$ but in small yield in γ^X; Zone XXI is present in X and γ^X but not in $\times^X$ which may represent removal of small amounts of α chain contaminants into the aqueous phase where they are expected; Zone XXII is in relatively large yield in X and $\times^X$ but in small yield in γ^X where it is probably due to contamination of γ^X by $\times^X$ in addition to a small residual amount of α chain contamination. Comparison reveals that the small amount of α chain peptides present as revealed in amino acid analyses of the individual peptides are carried into the aqueous phase with the γ^X chain. The large yield of Zone XXII in $\times^X$ from the butanol phase indicates that this zone, which represents a peptide identical in composition with αT-14, is an integral segment of the $\times^X$ chain. That the α chain contamination is present in the aqueous phase with the γ^X chain is revealed again in the overall amino acid compositions shown in Table 9 where the influence of α peptides upon the composition of the γ^X chain can be seen.

These amino acid analyses and chromatograms indicate that hemoglobin X is a composite of $\times^X$ plus γ^X and that γ^X appears to be the same as γ^F while $\times^X$ is dissimilar to the γ^F chain and appears to be a new chain.

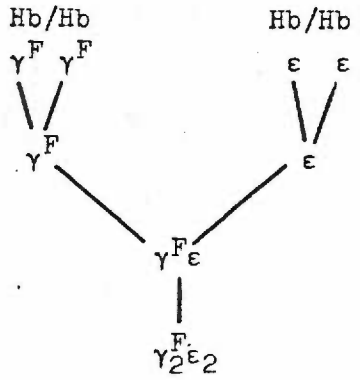
Results from preparative chromatography and rechromatography with amino acid analyses on isolated peptides indicated the presence of a normal γ chain plus nine new peptides which appear to belong to a new chain. The yields of the new peptides are good and with the exception of ×T-E, each has a lysine or arginine which is considered to be the C-terminal residue in each peptide. In ×T-E there is neither lysine nor arginine but there is a tyrosine residue and it is reported to be possible for a cleavage to occur at tyrosine or tryptophan (176).

Although no direct sequence work was performed, an attempt will be made to speculate as to some of the sequence of the new chain by analogy to the γ chain. By comparison of the ninhydrin color yield for the peptides an estimate may be made as to where quantitative and qualitative gaps might occur in the analogous sequence. It is pointed out that some peptides are produced in better yields than others so any estimate of this sort is clearly speculation. These estimates suggest quantitatively that the γT peptides 3, 4, 6, 7, 8, 10 and 16 are present in amounts sufficient to account for occurrence in both chains. The γT peptides 1, 2, 9, 11, 12, 14 and 15 may be the ones which are replaced in the analogous × chain.

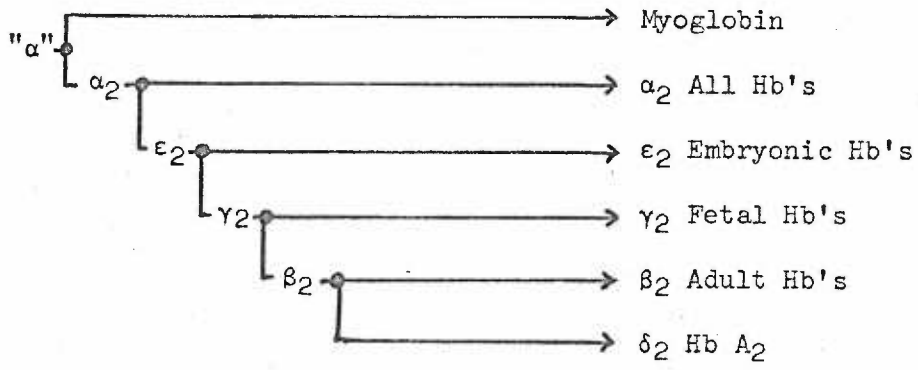
Of particular significance is the $\gamma_2 x_2$ structure where neither of two dissimilar chains is an α chain. This is the first report of an *in vivo* occurrence of such a hemoglobin structure. Until now a hemoglobin structure has been found to be either the type $\alpha_2\beta_2$ or γ_4 or β_4, i.e. an α chain plus a non-α chain or a combination of like non-α chains. A recent personal communication from Dr. E.R. Huehns

relates the occurrence *in vitro* of such hemoglobin structures as $\beta_2^{A}\delta_2^{A_2}$, $\beta_2^{A}\beta_2^{C}$, and $\beta_2^{S}\beta_2^{C}$ produced in hybridization experiments.

As mentioned in the introduction (p. 26), it is postulated that there is a separate genetic control for the ε chain and an outline was presented to illustrate it. The outline as presented by Huehns could be modified to include the following figure:



The following illustration shows evolutionary relationships for the known chains of human hemoglobin and myoglobin as proposed by Ingram (110). The five dots or branching points are assumed to coincide with a gene duplication. Following gene duplication the two initially equivalent genes have evolved independently.



Presumably the most ancient duplication resulted in a chain gene (shown here as "α") giving rise to myoglobin and another gene which gave rise,

by repeated duplications, to the α, β, γ, δ and ε chain genes of hemoglobin. As each chain gene evolved it eventually achieved the property of dimerization to form dimers and sooner or later evolved sufficiently to be able to form tetramers. It is generally considered that the fetal and embryonic proteins are more primitive which is the reason for the order of the ε, γ and β chains. Relatively very little is known about the ε chain so its placement is uncertain and it is not clear if it is produced before or simultaneously with the α chain in the embryo (131). If the × chain described here is the same as the ε chain then it appears to have similarities to the γ chain and based on this as well as the reasons stated regarding the more primitive nature of fetal proteins it is placed between the α and γ chain in the evolutionary scheme.

The occurrence of the hemoglobin X may have never been previously detected due to its being masked on starch gel electrophoresis by the hemoglobin A_3 zone since most qualitative gels are not run more than four hours and the A_3 zone and the X zone are not resolved prior to five or six hours on the gel. IRC-50 chromatography followed by starch block electrophoresis is necessary to yield clear resolution of hemoglobin X. This procedure was not used by Huehns in his work on the hemoglobin Gower 1 and Gower 2 (53).

It cannot be directly demonstrated that the × chain is an ε chain since it was not possible in this study to make a peptide "finger-print" for direct comparison to one presented by Huehns (53) for the ε chain. Neither was it possible to obtain a suitable sample of hemoglobin Gower 2 for a starch gel electrophoresis control to compare

to the new hybrid species. However, on the basis of the electrophoretic mobilities of species arising from subunit hybridization, the presence of ten possible new peptides, and the fact that the sample was obtained from a possible D-trisomy, it is suggested that the × chain of hemoglobin X corresponds to the ε chain which would allow the structure of hemoglobin X to be written as $\gamma_2\varepsilon_2$.

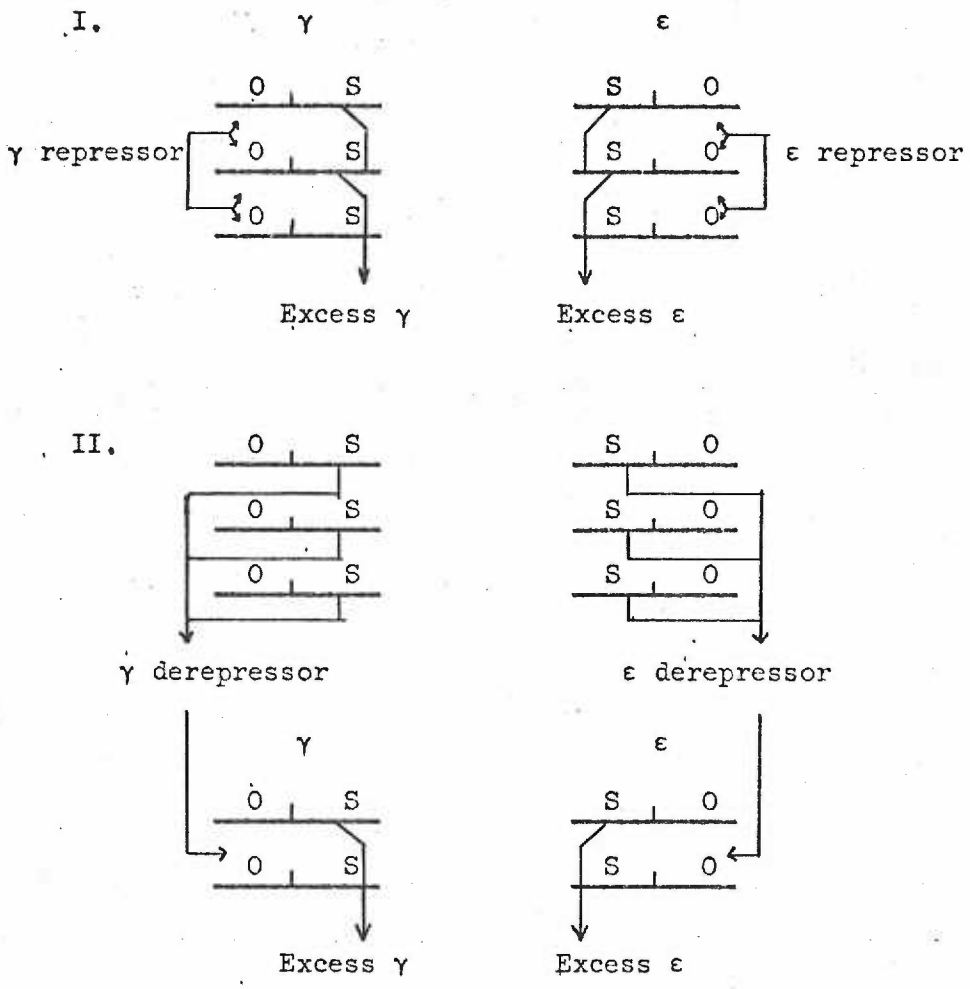
If this component is common in the D-trisomy, its occurrence may be explained by mechanisms as proposed by Huehns (54). It is suggested that the mechanism may involve triplication of the genetic loci (regulatory or structural) which control the synthesis of γ or ε chains.

If an imbalance exists between the amount of structural genetic material and the amount of repressor or regulatory substance formed, then variable increases of γ and ε chain synthesis could occur. Huehns presents two mechanism schemes (Fig. 31) to illustrate this. In hypothesis I there is triplication of the structural gene and the normal amount of repressor would be insufficient to inactivate the three genes. The second hypothesis (II) would suggest triplication of the genetic loci indirectly regulating the γ and ε chain synthesis by a resultant excess in derepressor (inducer) available which leads to excess ε and γ chain synthesis.

If such an excess of γ and ε chains occurs, the presence of a $\gamma_2\varepsilon_2$ structure would be plausible and expected as long as affinities for recombination were suitable.

Figure 31

Scheme to Explain Excess Production of ε and γ Chains in D-Trisomy. (From: Huehns, E.R. et al. Proc. Natl. Acad. Sci., 1964. 51, 89.)

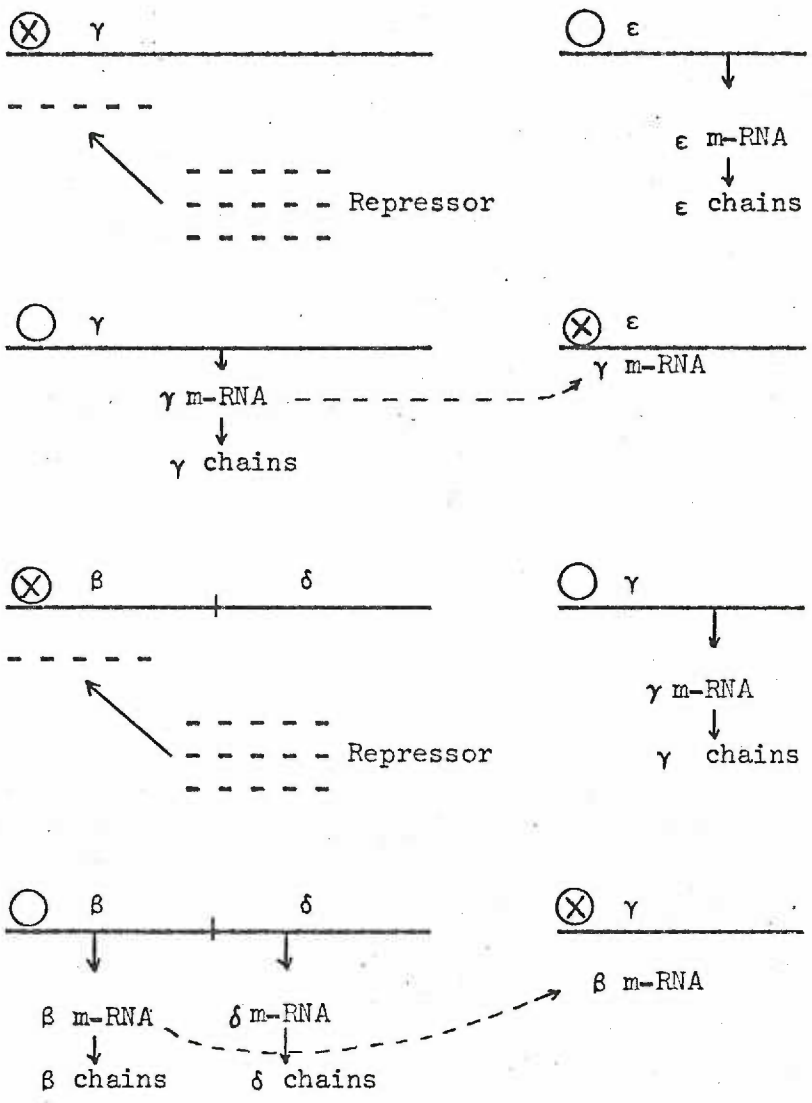

I.
γ
ε
O S
S O
γ repressor
ε repressor
Excess γ
Excess ε
II.
γ derepressor
ε derepressor
Excess γ
Excess ε

Explanations for the fetal-adult hemoglobin switch mechanism have been proposed. A scheme proposed by Baglioni (109) is based on differentiation of the "stem cells" in the erythropoetic tissues of fetus and adult. Ingram (110) extended Baglioni's scheme in terms of the Jacob and Monod operator and repressor hypothesis (66) to explain the switch from γ chain synthesis to β chain synthesis. Extending this idea one step more (Fig. 32) might include the possible switch mechanism from ε chains to γ chains. The postulate is that in the youngest stem cells of the embryo there is a repressor capable of repressing the operator locus which controls the γ gene. As the stem cell continues to divide the first repressor is either unstable or too dilute and no repression of the γ chain occurs any longer, i.e. the γ locus is induced. It is assumed that a new repressor for the ε operator is now made or perhaps the γ m-RNA acts as a repressor on the ε operator for a type of feedback situation. This series of events could be similarly carried out with a different genetically controlled repressor acting on the β and δ loci. As the stem cells continue to differentiate the disappearance or ineffectiveness of the β and δ loci repressor results and the β and δ loci are induced. Once again the β m-RNA may be acting as a repressor on the γ operator or a new repressor may be made at this point.

The fact that $\alpha_2 \times_2$ was not detectible in the hemolysate of the patient (MKD) suggests that the relative affinities of the γ and β chains for α chains are greater than that of the × chains for α chains or alternatively the relative affinity of × chains for γ chains is greater than for either α or β chains. The occurrence of $\times_4$ would be expected only if there is an excess of × chains.

Figure 32

Postulated Scheme for the Switch Mechanism of Embryonic and Fetal Hemoglobins to Adult Hemoglobins.


γ
ε
ε m-RNA
ε chains
Repressor
γ
ε
γ m-RNA
γ m-RNA
γ chains
β
δ
γ
γ m-RNA
γ chains
Repressor
β
δ
γ
β m-RNA
β m-RNA
δ m-RNA
β chains
δ chains

SUMMARY

The study of a new hemoglobin component (hemoglobin X) occurring in a possible D-trisomy has been characterized with physicochemical and primary structure properties determined.

The new component migrates as a distinct band slightly faster than hemoglobin A and slightly slower than hemoglobin Bart's on starch gel electrophoresis.

The $s_{20,w}$ and $D_{20,w}$ values are consistent with a tetrameric hemoglobin structure and a calculation for molecular weight was 66,000.

The presence of two different types of chains is indicated by both countercurrent distribution chain separation and subunit hybridization which yields hemoglobin F and A slower species in addition to the parent species.

The ultraviolet spectrum suggests that the hemoglobin X has structure features in common with the γ^F chain with characteristic absorption features in the 290 mμ region.

Amino acid analysis of purified tryptic peptides reveals nine new peptides not found in the α, β, γ, or δ hemoglobin chains, indicating the presence of a different type of chain in hemoglobin X which is designated as × chain.

The structure $\gamma_2 x_2$ is proposed for the hemoglobin X and this unique structure is the first reported of the *in vivo* occurrence of a tetrameric hemoglobin composed of two unlike chains, neither one being the α chain.

It has been tentatively suggested that the × chain referred to may be the ε chain of Huehns, and the possible relationship of a D-trisomy to the production of excess ε and γ chains with the occurrence of a $\gamma_2\varepsilon_2$ species is discussed. In addition a possible switch mechanism for change of production from ε chains to γ chains preceding the γ to β chain switch mechanism is proposed.

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