CHROMATOGRAPHIC AND CHEMICAL STUDIES OF SOME ABNORMAL HUMAN HEMOGLOBINS AND SOME MINOR HEMOGLOBIN COMPONENTS

Thesis by

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In Partial Fulfillment of the Requirements

For the Degree of

Doctor of Philosophy

California Institute of Technology

Pasadena, California

1961

ABSTRACT

Major and minor components in hemoglobins A, C, D, F, H, and S were detected and compared with one another by chromatography on IRC-50 resin. A procedure utilizing radioactive hemoglobins was developed for differentiating two hemoglobins with very similar chromatographic movements.

Chemical studies including amino acid composition, N-terminal sequence analysis, peptide pattern analysis of tryptic hydrolysates, ultraviolet spectroscopy, and subunit hybridization were carried out on several of the components which were isolated by chromatography. Conclusions about the polypeptide chains present in each components were drawn from these studies. Molecular formulas in terms of the number and kind of polypeptide chains were deduced for some of the components.

As well as yielding information about the chemical structure of hemoglobin F and the two abnormal components associated with thalassemia hemoglobin H, subunit hybridizations of these hemoglobins and hemoglobin A indicated that the rate of the hybridization reaction is slow and that there is non-random recombination of the subunits of these hemoglobins.

ACKNOWLEDGEMENTS

I wish to express my appreciation to Dr. Walter A. Schroeder for his constant guidance and stimulating advice during the investigation and writing of this Thesis. Many phases of this work were greatly facilitated by the availability of information from his research program and the opportunity to collaborate with members of his group and with Dr. Jerome Vinograd. I am also grateful to Professor Linus Pauling for encouraging and helping me to enter into this graduate adventure.

It is with appreciation that I acknowledge the contribution of blood specimens from a multitude of people obtained through the generous help of Drs. Gail Anderson, William Bergren, Gurth Carpenter, Robert Koler, Hermann Lehmann, Darlene Powers, Samuel Rappaport, Demetrios Rigas, Philip Sturgeon, and Stanley Wright. The expert technical help of Mrs. Joan Balog Shelton is also appreciated.

I am indebted to the Commonwealth Fund, the Medical Division of the National Academy of Sciences-National Research Council, and the United States Public Health Service for fellowship support during three years of this work.

In conclusion, I dedicate this Thesis to my wife, Marilyn, who has made it possible by cheerfully enduring the life of a graduate student's wife for a second time.

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PREFACE

Hemoglobin is the predominant biochemical constituent of the erythrocyte. The functions and properties of this common protein have been objects of tremendous amounts of study. An active area of current investigation has developed from recognition that hemoglobin from higher animals is not a single molecular species but rather a heterogeneous mixture. The work of this Thesis is directed at the study of the heterogeneity of a variety of human hemoglobins by chromatographic methods and the correlation of heterogeneity with chemical structure.

The heterogeneity of human hemoglobins can be divided into three general types. The first type was discovered by Körber (1) in 1866, who demonstrated that the hemoglobin of fetal blood differs from that of adult blood by its greater resistance to denaturation by either alkali or acid. During the course of maturation of normal individuals this fetal hemoglobin disappears and is replaced by adult hemoglobin (2). This variance in molecular species of hemoglobin may be designated as heterogeneity of maturation. A second type of heterogeneity was discovered in 1949 by Pauling, Itano, Singer and Wells (3) when they determined that the hemoglobin associated with sickle-cell anemia is electrophoretically abnormal and that the hemoglobin in individuals heterozygous for the sickle-cell gene is a mixture of the abnormal form and the normal adult hemoglobin.

According to the genetic analysis of sickle cell disease by Neel (4), this abnormal hemoglobin results from a single abnormal gene. A large number of other genetically induced abnormal hemoglobins has been recognized since 1949. This type of variation of hemoglobins may be designated as genetic heterogeneity. The third type of heterogeneity was conclusively demonstrated by Kunkel and Wallenius (5) in 1955 with the separation of several hemoglobin components in minor amounts from the blood of normal adult individuals. Morrison and Cook (6) obtained confirmatory evidence of this heterogeneity by column chromatography. This third variability of hemoglobins will be designated as minor component heterogeneity. Because the nature and cause of minor component heterogeneity are poorly understood, their relationship to maturation and genetic heterogeneities is explored in this Thesis.

With the development of efficient procedures for the study of primary structure of proteins, the determination of the amino acid sequence of human hemoglobins has been pursued actively by several groups (7,8,9,10,11). In brief, it has been found that normal adult hemoglobin (hemoglobin A) is comprised of four single polypeptide chain subunits of two different sequences. One polypeptide chain begins with the N-terminal sequence valyl-leucyl (abbreviated as val-leu) and has been called the α^A chain while the other chain begins with the N-terminal sequence

valyl-histidyl-leucyl (val-his-leu) and has been named the β^A chain (7). Large portions of the internal sequence of both the α^A and β^A chains have now been determined.

As more exact structural information of normal adult hemoglobin became available, interest in the structural basis of the various heterogeneities has grown. Schroeder and Matsuda (12) demonstrated that fetal hemoglobin (hemoglobin F) is comprised of four subunits of two different N-terminal sequences: valyl-leucyl (val-leu) and glycyl (gly), which have been designated as the α^F and γ^F chains, respectively. These authors suggested that fetal and adult hemoglobins might have two identical α chains and differ in the other pair of chains. This was the first evidence that indicated a close relationship between these two different hemoglobins.

The first evidence of the relationship between genetically heterogeneous hemoglobins was obtained by Ingram (13,14) in 1958. He demonstrated by the "fingerprint" technique that sickle-cell hemoglobin (hemoglobin S) differed from hemoglobin A by the substitution of a valyl residue for a glutamyl residue in some part of the molecule. No other differences between these two hemoglobins were detected. The chemical basis of the genetic heterogeneity of several other hemoglobins has been shown to be analogous to that of the sickle-cell hemoglobin. Thus, genetic heterogeneity of hemoglobin appears to be due to single amino acid substitutions in either polypeptide chain.

Many questions may be asked about the structural basis of minor component heterogeneity. As a result of the observation of Allen, Schroeder and Balog (15) that minor component heterogeneity occurs in hemoglobin from cord blood and sickle-cell anemics as well as normal adults, one might ask what relation exists among minor components from these several sources. Are maturation and genetic heterogeneities also compounded by minor component heterogeneity, and if so, how are the minor hemoglobins related to the major or predominant molecular species? Are minor components as different as fetal and adult hemoglobin or as similar as normal adult and sickle-cell hemoglobin? Do some of these minor components differ from the major component in ways other than in amino acid sequence? These questions will be examined, at least in part, in this Thesis study.

Chromatographic procedures have been utilized almost exclusively in this study for the empirical characterization and isolation of components from hemoglobin mixtures. The chromatographic behaviors of several components have been studied in some detail in order to determine the reliability of this characterization and isolation technique. Several of the hemoglobin components which have been isolated have then been studied further by chemical procedures including amino acid analysis, N-terminal amino acid sequence

determinations, "finger printing," subunit hybridization, and ultraviolet spectroscopy, in order to correlate their structures to those of fetal and adult hemoglobins.

SUMMARY OF NOMENCLATURE

An outline of nomenclature and abbreviations is given below for reference purposes.

Terminology of Hemoglobins:--English capital letters have been used to designate different types of hemoglobins (16).

Examples:

A	normal adult hemoglobin
F	fetal hemoglobin
S	sickle-cell hemoglobin
C, D, H	other abnormal hemoglobins

Terminology of Hemoglobin Components:--Allen, Schroeder and Balog (15) introduced a temporary terminology to designate the various components which could be separated by chromatography on IRC-50 under their experimental conditions. Their original terminology has been expanded (17,18). The major or predominant hemoglobin component is designated by adding a Roman numeral II subscript to the symbol for the whole hemoglobin. The minor components are designated by Roman numeral subscripts I or III depending on whether they move more rapidly or slowly respectively than the major component.

Examples:

Major Hemoglobin Components

A_{II}	major adult component
S_{II}	major sickle-cell component
F_{II}	major fetal component

Rapidly Migrating Minor Components

A_{Ia}, A_{Ib}, A_{Ic}, etc.	fast adult minors
S_{Ia}, S_{Ib}, S_{Ic}, etc.	fast sickle-cell minors

Slowly Migrating Minor Components

A_{IIIa} and A_{IIIb}	slow adult minors
S_{III}	slow sickle-cell minor

Three hemoglobin components may be separated from normal adult hemoglobin by starch block electrophoresis: the main component, A_1, moves at a position intermediate between the most slowly moving component, A_2, and the most rapidly moving component A_3. Schnek and Schroeder have studied the relation between the minor components of whole normal human adult hemoglobin as isolated by chromatography and starch block electrophoresis (18). In summary their correlation is as follows:

Electrophoresis	Chromatography
A_1	A_{Ic}, A_{Id}, A_{Ie}, and A_{II}
A_2	A_{IIIb} and non-heme protein(s)
A_3	A_{Ia}, A_{Ib}, and non-heme protein(s)

Terminology of Polypeptide Chains:--These symbols are based upon the determination of the N-terminal amino acid sequences and certain pertinent internal sequences:

α	val-leu-
β	val-his-leu-
γ	gly-
δ	val-his-leu- (chain of A_2 component)

A superscript A, S, F, H, etc. is used to designate the type or source of the chain, thus the val-his-leu chain of hemoglobin S is β^S. Often no superscript is used when a chain is identical with the α^A, β^A, or γ^F chains.

Structural Formulas of Hemoglobin Components.--The gross structural formulas of hemoglobin components can be expressed in terms of the kind and number of polypeptide chains present per molecule.

Examples:

A_{II} $\alpha_2^A\beta_2^A$ or $\alpha_2\beta_2$

F_{II} $\alpha_2^F\gamma_2^F$ or $\alpha_2\gamma_2$

S_{II} $\alpha_2^A\beta_2^S$ or $\alpha_2\beta_2^S$

$A_{IIIb}=A_2$ $\alpha_2^A\delta_2^{A_2}$ or $\alpha_2\delta_2$

SECTION I. CHROMATOGRAPHIC BEHAVIOR OF HUMAN HEMOGLOBINS

INTRODUCTION

Although the separation of two different hemoglobins was first achieved by electrophoretic methods (3), chromatographic procedures have become more useful for the detection of small differences between hemoglobin components and for their preparative isolation. Chromatographic separation of hemoglobins has been achieved mainly on two different types of ion exchangers, namely the carboxylic acid resin IRC-50 and carboxymethylcellulose. The first successful chromatograms of hemoglobin on IRC-50 were obtained by Boardman and Partridge (19) who were able to separate the maternal and fetal hemoglobins of sheep as well as sheep fetal and bovine maternal hemoglobins. Huisman and Prins (20,21) effected separations of hemoglobins F, A, E, S, and C with IRC-50 columns. The first demonstrations of minor component heterogeneity of hemoglobins from normal adult and umbilical cord bloods were also carried out on IRC-50 (6,22). Observations of genetic heterogeneity and minor component heterogeneity have also been achieved by Huisman, Martis, and Dozy (23) and Gutter, Peterson, and Sober (24) on carboxymethylcellulose.

Schroeder and his co-workers (15,17,18) have utilized IRC-50 exclusively for their chromatographic studies of the heterogeneity of human hemoglobin. In place of a gradient elution technique utilized by others (6), these authors

employed a series of developer buffers of different ionic strength and pH and therefore of different eluting strengths. Allen, Schroeder and Balog (15), in establishing the original conditions, were able to separate normal adult hemoglobin into two zones using a fairly strong developer (No. 1). These zones were designated A_I and A_{II} in their order of elution from the column. The A_I zone, which comprised 10 to 12% of the adult hemoglobin, was resolved further into three hemoglobin components (designated A_{Ia}, A_{Ib}, and A_{Ic}) by rechromatography with a weaker developer (No. 4) on a second column of IRC-50 resin. These authors were able also to separate the hemoglobins from umbilical cord blood of normal infants into two zones which were designated F and A_{II}. The F zone could be resolved further into four zones by rechromatography with weaker developers (15). These zones are now designated as f_3, f_4, F_I, and F_{II} (25).

Clegg and Schroeder extended the methods of Allen, et al. (15) by devising a single developer with which normal adult hemoglobin could be separated into 6 hemoglobin zones by one chromatographic procedure (17). Their developer (No. 5) was similar to Allen's weak buffers and effected a resolution of most of the rapidly moving minor components (A_I components) at a temperature of 6°. This temperature had been chosen by Allen (15) in the hope of maintaining the native state of hemoglobin during the separation procedure. At 6° Clegg and Schroeder found that the rapidly moving minor components (A_I's) could be eluted with

Developer No. 5 before the major component (A_{II}) reached the bottom of the chromatographic column. When the A_{II} zone reached the end of the column however, Clegg and Schroeder warmed the system to 28° and thereby effected an elution of the remaining components at a more rapid rate. With this procedure, Clegg and Schroeder were able to demonstrate the presence of a minor component, A_{III}, which moved more slowly than the main component, A_{II}. Schnek and Schroeder (18) were able to separate this A_{III} zone into two components and to correlate each component from the entire chromatographic separation of normal adult hemoglobin with the fractions obtained by starch block electrophoresis.

A study of the heterogeneity of sickle-cell hemoglobin by chromatographic methods was suggested by Professor Linus Pauling and Dr. Walter Schroeder in 1957. Their suggestion was stimulated by Dr. David Allen's observations of minor component heterogeneity of normal adult hemoglobin (15). Because minor component heterogeneity of sickle-cell hemoglobin was possible, the question of its existence and relation to the chromatographic difference between hemoglobins A and S was raised. This Thesis work is an outgrowth of their suggestion; Section I not only includes a study of the minor component heterogeneity of sickle-cell hemoglobin but also detailed examination of the chromatographic behavior of a number of other hemoglobins including

C, D, F, and H. Particular attention has been given to the problem of identifying hemoglobins on the basis of their chromatographic behavior. The latter has involved a study of the influence of several factors upon the migration rate and the formation of double zones of several, apparently single, component hemoglobins. A procedure based upon the use of hemoglobins labelled with C-14 containing amino acids was developed and applied to detecting small differences between hemoglobins with similar chromatographic movement.

SECTION I. EXPERIMENTAL PROCEDURES

Preparation of Hemoglobin Solutions.--Normal adult blood was obtained from the author and workers in the laboratory as freshly drawn venous specimens. Either heparin (1 drop of a 50 mg. per ml. solution per 10 ml. blood) or 3.2% sodium citrate dihydrate (1 ml. per 5 ml. blood) was used as an anticoagulant. Blood samples of abnormal hemoglobins were anticoagulated and transported in Alsever's Solution^{R} (26) or in Sequestrene^{R} tubes (27). Cord blood samples were obtained at the time of delivery and anticoagulated with heparin. Whenever possible these whole blood samples were kept at refrigeration temperatures (ice bath or refrigerator at 2-6^{o}) during transportation and storage.

The erythrocytes were separated from the plasma by centrifugation for 5-10 minutes at 4,000 rpm (about 1,200xg) in a refrigerated centrifuge and were then washed from 3 to 5 times with 1 to 5 times their volume of chilled (0 to 10^{o}) 0.9% sodium chloride solution. The cells were separated from the saline each time by centrifugation for 5 to 10 minutes at 4,000 to 6,000 rpm (about 1,200 to 3,000xg). Attempts to remove the white cell layer from the top of the erythrocytes were made only when this separation did not result in significant losses of red cells. Hemolysis was effected by frequent, vigorous shaking of the washed red cells with 1 to 3 volumes of distilled water and 0.3

to 0.6 volumes of toluene for 10 to 20 minutes at room temperature. The hemolysate was then centrifuged for 30 to 60 minutes at 25,000 xg at 0 to 5°, the aqueous hemoglobin layer (middle) was removed by means of a long hypodermic needle and syringe and again centrifuged at 25,000 xg for at least 1 hour at 0 to 5°. Hemoglobin solutions cleared of stroma in this way were then immediately dialyzed for 12 or more hours at 0 to 5° against at least 100 times their volume of one of the chromatographic buffers. These dialyzed hemoglobin solutions were then stored, generally in the oxyhemoglobin form, at 0 to 5° in well closed containers.

Determination of Concentration of Hemoglobin Solutions.--The concentrations of the hemoglobin solutions were determined with a Beckman Spectrophotometer, Model DU, at 542, 522, and 563 mμ. The extinction coefficient of oxyhemoglobin and carbonmonoxyhemoglobin are approximately equal at 542 mμ. The 522 mμ wavelength is an isobestic point of mixtures of carbonmonoxyhemoglobin and ferrihemoglobin cyanide whereas 563 mμ is an isobestic point of oxyhemoglobin and ferrihemoglobin cyanide (28). The concentration of hemoglobin in mg. per ml. was calculated by multiplying the optical density for a 1-cm. path length appropriately corrected for dilutions by the factor 1.14 for 542 mμ and 1.77 for 522 and 563 mμ readings. In some cases the hemo-

globin solutions were diluted with 6×10^{-4} M potassium ferricyanide in Developer No. 4 (see Table I) in order to convert all hemoglobin to ferrihemoglobin cyanide and then the concentration was estimated using the factor 1.44 with the optical density at 540 mμ (29).

Preparation of Chromatographic Columns.--Amberlite IRC-50 synthetic cation-exchange resin as prepared by Allen, et al. (15) was used exclusively for the hemoglobin chromatography. Wet resin in the hydrogen form was sifted through a series of wire sieves. Two different ranges of resin sizes were isolated and employed for column work. One range was that fraction of resin that passed a 200-mesh sieve (200 mesh-per-square-inch) but was retained by a 250-mesh sieve and the other range was that passed by the 250-mesh sieve but retained by a 325-mesh sieve. These two ranges in resin particle size were not mixed in spite of the observation that the separations of hemoglobins appear to be identical on both. The sized resin was washed with 3 N hydrochloric acid and then water until white in color. In several cases resin was stored for extended periods of time in this form at 4°.

Prior to pouring of a chromatographic column, portions of the washed resin were then suspended in the appropriate developer and the suspension stirred continuously while the pH was adjusted back to that of the original developer by the gradual addition of concentrated sodium hydroxide

(20 to 40%). The resin was then placed on a Büchner funnel and washed with approximately 2 liters of developer per 100 grams of wet resin. The resin was then suspended in a volume of developer such that the volume of the supernatant fluid was three times that of the settled resin. Thicker suspensions generally resulted in poor chromatographic results. All columns were poured in 4 to 8 sections either at room temperature or at 6°. The most satisfactory results were obtained by pouring developer into the tube to form a column of liquid 5 to 10 cm. high and then pouring the slurry of resin into the column rather than directly onto the fritted disc of the chromatographic tube. After each section had settled, the top $\frac{1}{2}$ to 2 cm. was resuspended in about 5 cm. of developer by stirring with a glass rod and then the next section was poured before the resin settled again. Columns 1 x 35 cm. in dimension were used for analytical work and were jacketed so that water at 6° could be circulated around the resin bed. Several large preparative columns 1.5, 2.5, and 3.5 by 35 cm. in dimensions were also employed in a cold room at 4 to 6°. Before using each newly poured column or when converting a column from one chromatographic developer to another, the appropriate buffer was passed through at 6° C at a rate of 3 to 12 ml. per $cm.^2$ of cross sectional area per hour until 1 to 1.5 liters per $cm.^2$ of cross sectional area of resin had passed.

The preparation of several of the chromatographic columns and all of the resin used for these studies was made by Drs. Allen, Clegg, and Schnek.

Chromatographic Developers.--As has been noted by Allen et al. (15) the chromatographic behavior of hemoglobins on IRC-50 resin is extremely sensitive to slight changes in sodium ion concentration and pH. Therefore, the developers were prepared exactly as described by Allen et al. (15) using anhydrous Na_2HPO_4 and $NaH_2PO_4 \cdot H_2O$. Substitution of these reagents with other hydrates of these sodium phosphate salts was found to yield unreliable results. The composition and pH of the developers which were employed are listed in Table I. This table includes those developers described by Allen et al. (15) and Clegg and Schroeder (17) as well as several newly devised developers.

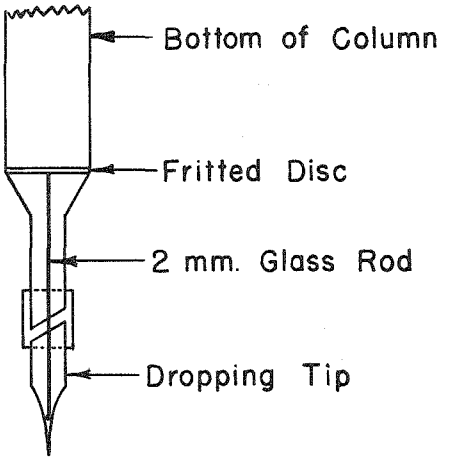
TABLE I

COMPOSITION OF CHROMATOGRAPHIC DEVELOPERS

No.	pH at 25°	Conc. Na^+ M	Grams required for 4 l. of buffer		
			$NaH_2PO_4 \cdot H_2O$	Na_2HPO_4	KCN
1	7.22±0.02	0.075	13.80	14.20	2.60
2	7.18±0.02	.0625	13.80	10.65	2.60
3	7.02±0.02	.050	13.80	7.10	2.60
4	6.91±0.02	.050	16.56	5.68	2.60
5	6.85±0.05	.055	16.56	7.10	2.60
6	6.66±0.04	.050	18.40	4.70	2.60
6a	7.10±0.05	.030	9.94	3.40	2.60
6b	6.20±0.05	.050	27.60	0.00	2.60
6c	6.50±0.05	.050	22.08	2.84	2.60
6e	6.65±0.05	.050	19.32	4.26	2.60
6f	6.40±0.05	.050	20.24	2.60	2.60

Operation of Column.--The resin in the chromatographic column is supported on top of a fritted disc placed near the bottom of the chromatographic tube. A space of about 2 ml. is present between this disc and the dropping tip at the end of the chromatographic tube. Air in this space can be replaced by effluent during the course of a chromatogram and thereby can result in poorer resolution of components in the fractions which are collected because of mixing of dense hemoglobin solutions. The replacement of the air by effluent can be prevented by inserting a glass rod 2 mm. in diameter into the space so that one end of the rod touches the fritted disc and the other end is in the dropping tip (see diagram).

The glass rod provides a route by which the droplets of effluent from the fritted disc can flow directly to the dropping tip without forming large drops which can occlude the tube below the fritted disc.



Prior to starting each chromatogram, the column was carefully adjusted to a vertical position with a level. The top 1 to 3 cm. of the resin bed was then stirred by even circular motions with the tip of a long glass rod. The suspended resin was allowed to settle and the supernatant

was removed with a long, bent-tip pipet. Chromatograms were started only if the surface of the freshly settled resin was even and horizontal. Small irregularities in the surface often resulted in the formation of distorted zones and therefore poor resolution of components.

The hemoglobin, which had been dialyzed against the developer, was pipetted slowly down the side of the glass tube onto the resin bed with a bent-tip pipet while 1 to 3 mm. of developer remained above the surface of the column. This method of application resulted in the least disturbance of the top of the resin bed and effected a cooling of the sample to the temperature of the column. In the event that the surface of the resin was disturbed during pipetting of the sample, the surface was carefully re-stirred before the sample of hemoglobin had run in. This latter procedure produced even zones. Either the sample was allowed to flow in under gravity and then the sides of the tube were washed with several 0.2 ml. portions of developer or the sample was covered carefully with a layer of developer. A sharp interface between the relatively dense hemoglobin solution and the lighter developer could be obtained provided the buffer was added very slowly onto the wall of the tube about 5 mm. above the existing fluid surface. The overlayering procedure had the advantage of resulting in more even zones and more rapid application of samples. The hemoglobin samples not exceeding 100 mg. per ml. were applied in a volume of 0.5 to 2.0 ml. per $cm.^2$ of cross

sectional area of resin.

The development of the chromatograms was essentially as described by Allen et al. (15) and Clegg and Schroeder (17). A developer flow rate of either 5 or 6 ml. per hour was generally used at a temperature of $6.0 \pm 0.2^{\circ}$ for the 1 x 35 cm. columns. This flow rate was often doubled for the elution of slowly moving hemoglobins. When the columns were warmed to 28°, the flow rates of developer were maintained in the range of 8 to 10 ml. per hour. One to 10 ml. fractions were automatically collected in a time-interval fraction collector. When zones were to be re-chromatographed or subjected to certain chemical procedures, the effluent was collected in vessels chilled in ice-water and then refrigerated. The effluents that were rechromato-graphed were first dialyzed against the new developer in the cold.

After all zones had been washed from a column, it was used again without further treatment provided the tempera-ture had remained at 6° and the same developer had been used throughout. In the event the column had been warmed or the developer changed, reequilibration was effected by passing at least 1000 ml. per $cm.^2$ of cross sectional area of resin of developer through the column at 6°.

Spectrophotometry and Chromatographic Figures.--The optical density of each fraction was measured in a 1-cm. cuvette either in a Beckman spectrophotometer, model DU at

280 and 415 mμ, or in a Beckman model B at 415 mμ. In certain cases, optical density at 522, 542, or 563 mμ was also measured. In order to obtain reliable spectrophotometric measurements it was necessary to dilute some concentrated fractions with appropriate amounts of distilled water or developer. As suggested by Allen et al. (15), readings with the Beckman model DU were simplified by using the hydrogen lamp at both 280 and 415 mμ, although this introduces an error of about 3% at the higher wave length setting. Because most proteins contain aromatic amino acids, light absorption at 280 mμ is a general characteristic of proteins. Absorptions in the regions of 415, 522, 542, and 563 mμ are fairly specific for heme containing proteins (30).

Chromatographic figures were constructed by plotting the optical density of each fraction against the effluent volume of that fraction. The percentage of any component present in a chromatogram was estimated from these figures by determining the ratio of the area of that component to the area under the entire curve.

Measurement of Radioactivity.--The radioactivity of materials containing C-14 labelled amino acids was measured with one of two different counter systems. Some radioactive countings were made with Nuclear Chicago Model C100B Automatic Sample Changer equipped with a Model D-47 gas flow geiger counter. Other countings were made with a Nuclear

Chicago Model C115 Low Background Automatic Sample Changer. "Micromil" windows were used in both detector systems.

Volumes of 0.5 or 1.0 ml. of hemoglobin solution were pipetted onto aluminum, stainless steel, or copper sample pans. These samples were then evaporated to dryness with a 250-watt infrared lamp. In general, less than 0.5 mg. of protein was placed on these sample pans so that self absorption corrections were unnecessary (28). Special care was taken to be sure that the sample was evenly distributed on the surface of the pans and did not touch the side walls. Corrections were not made for the absorption by the buffer salts or for the absolute counting efficiency of the system. However, the same kind and volume of buffer and same counting equipment were used for any one experiment. Therefore, the relative activity of all samples within any given experiment was comparable.

The Concentrating of Hemoglobin Solutions.--Three different procedures were used for concentrating dilute solutions of hemoglobin. In the first procedure, 5 to 25 ml. volumes of hemoglobin solution were placed in dialysis bags and suspended over concentrated sulfuric acid in a closed, evacuated container at 4°. Solutions could be concentrated at a rate of about 1 ml. per hour. These concentrated solutions were then dialyzed against fresh developer after a volume of 1 to 3 ml. was obtained. The second concentration procedure was to lyophilize the hemoglobin solutions

to dryness after first dialyzing thoroughly against distilled water. The third and most useful procedure was developed by Vinograd and Hutchinson (31). The hemoglobin solution was concentrated by centrifuging in the No. 40 rotor of a "Spinco" model L preparative ultracentrifuge at 40,000 rpm for 18 to 24 hours at 6 to 10°. The concentrated solutions were withdrawn from the bottom of the centrifuge tubes through a 4-inch, 20-gauge hypodermic needle into a 1-ml. tuberculin syringe. These concentrates were always re-dialyzed against developer before rechromatography. The third procedure was found to be the most satisfactory and was used almost exclusively for the experiments described below.

Preparation of Radioactive Hemoglobins.--Hemoglobins containing C-14 labelled amino acids were either obtained from Dr. Jerome Vinograd and Dr. William Hutchinson or prepared by incubating blood samples containing reticulocytes in the presence of C-14 L leucine according to procedures outlined by these investigators (31). After the incubations, the hemoglobin was treated as previously described.

SECTION I. RESULTS AND DISCUSSIONS

A. Chromatography of Human Hemoglobins

Normal Adult Hemoglobin.--Figure 1 illustrates a typical chromatogram of 15 mg. of normal adult carbonmonoxyhemoglobin with Developer No. 1. This chromatogram is also typical of ferrihemoglobin cyanide and oxyhemoglobin as previously observed by Allen et al. (15). It is apparent from Figure 1 that the separation of zone A_I from zone A_{II} may not be complete. Substantial improvement in the resolution of these zones was achieved by the introduction of a glass rod in the space below the fritted disc of the column assembly.

The A_I zone was not adsorbed strongly by the resin in equilibrium with Developer No. 1 and emerged at about 18 ml. of effluent volume. The maximum of the A_{II} zone varied between effluent volumes of 22 to 28 ml. for similar chromatograms. This variation in the emergence of the A_{II} zone appeared to be dependent upon several factors, including the amount of hemoglobin applied to the column, the exact composition of the developer, and the temperature of the column.

The percentages of A_I and A_{II} components in blood samples from three different normal adults were determined from Developer No. 1 chromatograms and are listed in Table II. These results indicate that the percentages of A_I and A_{II} may be relatively constant in samples from different

Figure 1. Chromatogram of 15 mg. of normal adult carbonmonoxyhemoglobin on IRC-50 with Developer No. 1. Optical density measured at 415 mμ.

Figure 2. Rechromatography on Zone A_I from 50 mg. of normal adult hemoglobin with Developer No. 4. Optical density at 415 mμ is represented by the solid line, optical density at 280 mμ is represented by dashed line.

Figure 3. Chromatogram of 50 mg. of normal adult oxyhemoglobin with Developer No. 5. The representation of optical densities is the same as for Figure 2.

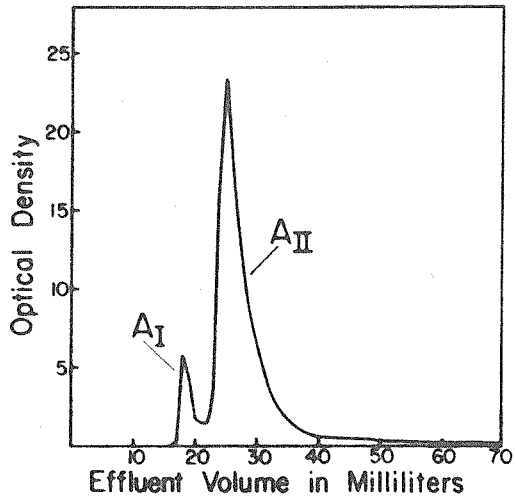


Figure 1

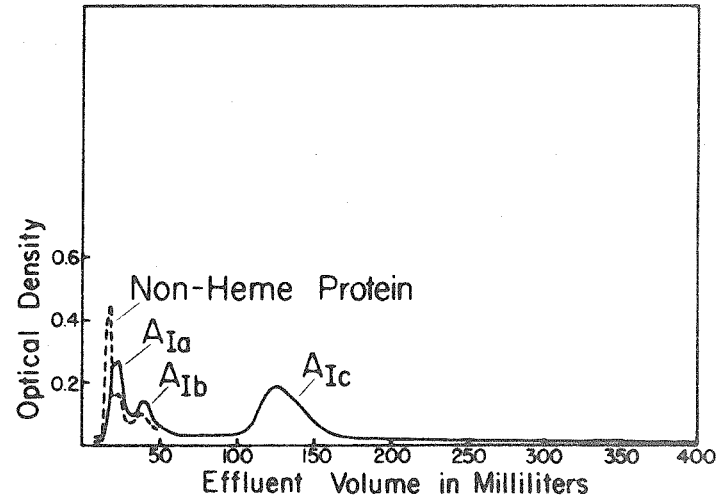


Figure 2

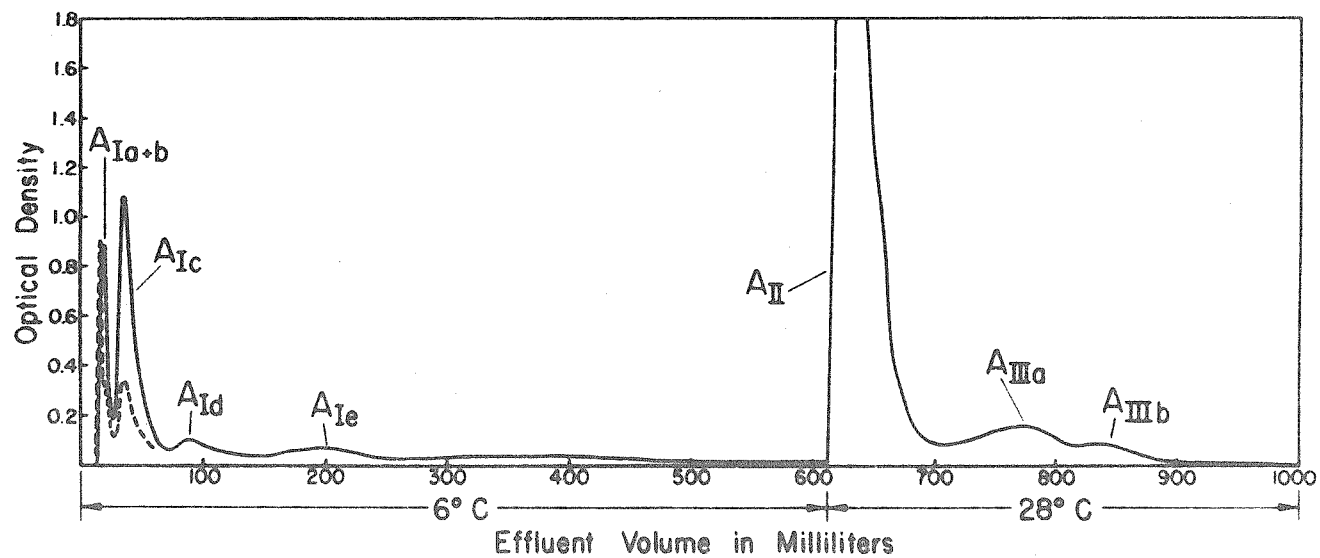


Figure 3

TABLE II

Percentage of Components in Normal Adult Hemoglobin with Developer No. 1

Sample	Date Run	Chrom. No.	Amount in mgs.	Percentage of A_I	Percentage of A_{II}
M.D.C.	10/4/1957	B-1	15	10.0	90.0
	10/9/1957	B-3	15	9.0	91.0
R.T.J.	10/23/1957	B-9	50	9.4	90.6
	10/25/1957	B-10	55	9.6	90.4
	10/31/1957	B-11	25	9.6	90.4
W.A.S.	12/6/1957	B-21	50	9.5	90.5
	12/8/1957	B-22	50	10.1	89.9

normal adults. This observation has been confirmed by more extensive studies with Developer No. 5 made by Schroeder and his co-workers (17,18).

Figure 2 illustrates a typical chromatogram of the A_I zone from 50 mg. of normal adult hemoglobin with Developer No. 4. This result is essentially identical with the observation of Allen et al. (15) except that zones A_{Ib} and A_{Ic} emerged slightly later than did those of Allen. A comparison of the optical density curves for 280 and 415 mμ indicates the presence of an appreciable amount of protein that does not contain heme groups near the solvent front of the chromatogram represented in Figure 2. This conclusion is based upon the observation of a decrease in the ratio of the optical density at 415 mμ to the optical

density at 280 mμ. This ratio is of the order of 3.5 for pure hemoglobin solutions. Non-heme proteins will decrease this ratio because they absorb strongly at 280 mμ but not at 415 mμ.

During the course of this work, a new chromatographic procedure was devised by Clegg and Schroeder (17) in order to improve the quantitation of each component and to eliminate the necessity of rechromatography of A_I minors. Figure 3 represents a chromatogram of 20 mg. of normal adult oxyhemoglobin with Developer No. 5. The chromatography was carried out at 6° for the first 600 ml. and then the column was warmed to 28° to elute the remaining hemoglobin. The results, shown in Figure 3, are in agreement with the published studies (17,18). The most efficient procedure for the chromatographic separation of the hemoglobin components from normal adult blood is with the Developer No. 5 system. However, this procedure does not resolve zones A_{Ia} and A_{Ib}, nor is it applicable to the study of most abnormal hemoglobin components that move more slowly than A_{II}.

The A_{III} components which can be detected with the Developer No. 5 system probably move in the tail region (descending limb) of the A_{II} zone during chromatography with Developer No. 1. This conclusion is based upon the observation of Schnek and Schroeder (18) who found that A_{III} components were more concentrated in the tail region of A_{II} zone from Developer No. 2 chromatograms than in

other regions of the chromatogram. Apparently the relative amounts of A_{II} to A_{III} in the normal adult and the resolving power of the Developer No. 1 and 2 systems are such that these components are not separated well enough to yield a third maximum point on the chromatographic curves.

Normal Cord Blood Hemoglobins.--When 15 mg. of hemoglobin from umbilical cord blood was chromatographed with Developer No. 1, the result illustrated in Figure 4 was obtained. The separation of F and A_{II} zones with Developer No. 1 was not as satisfactory as with Developer No. 2 (15). Further separation of the F zone from chromatograms with Developer No. 1 or 2 could be effected with Developer No. 4 as shown in Figure 5. Since the early chromatographic studies of cord blood hemoglobin were done, it has been found that whole cord blood can be chromatographed more effectively with either Developer No. 4 or 5. A chromatogram of 100 mg. of cord blood hemoglobin is illustrated in Figure 6. This chromatogram was developed at 6° until most of the F_{II} zone had been eluted and then the system was warmed to 28° in order to elute the A_{II} and slowly moving minor component. Better resolution of F_I and F_{II} is obtained when smaller amounts of hemoglobin are chromatographed. The separations resulting with Developer No. 4 are better than with Developer No. 5 because the fast minors f_3 and f_4 are resolved from F_I; however, the migration of A_{II} is much slower with Developer No. 4 than with Developer No. 5. These procedures employing

Figure 4. Chromatogram of 15 mg. of whole cord blood hemoglobin on IRC-50 with Developer No. 1. Optical density measured at 415 mμ.

Figure 5. Rechromatography of approximately 5 mg. of Zone F from a chromatogram similar to Figure 4. Developer No. 4 was used for the rechromatography. Optical density at 415 mμ is represented by the solid line, optical density at 280 mμ is represented by the dashed line.

Figure 6. Chromatogram of 100 mg. of whole cord blood hemoglobin on IRC-50 with Developer No. 5. The representation of optical densities is the same as for Figure 2 although the scale is different.

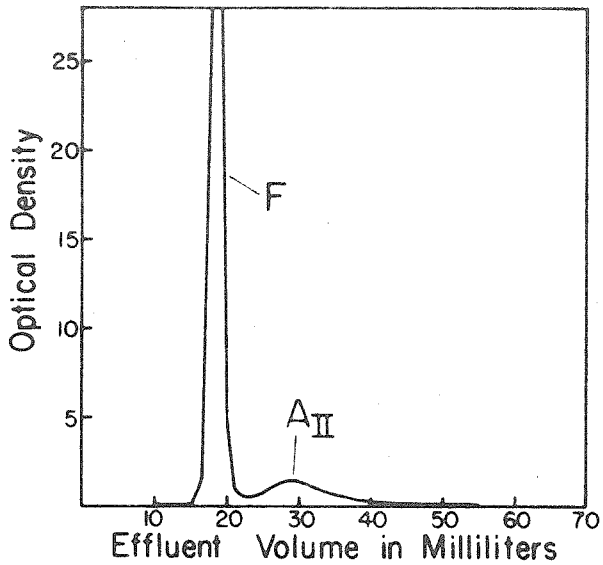


Figure 4

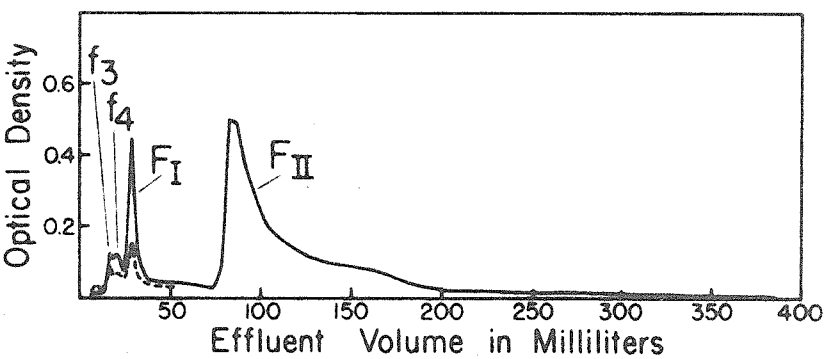


Figure 5

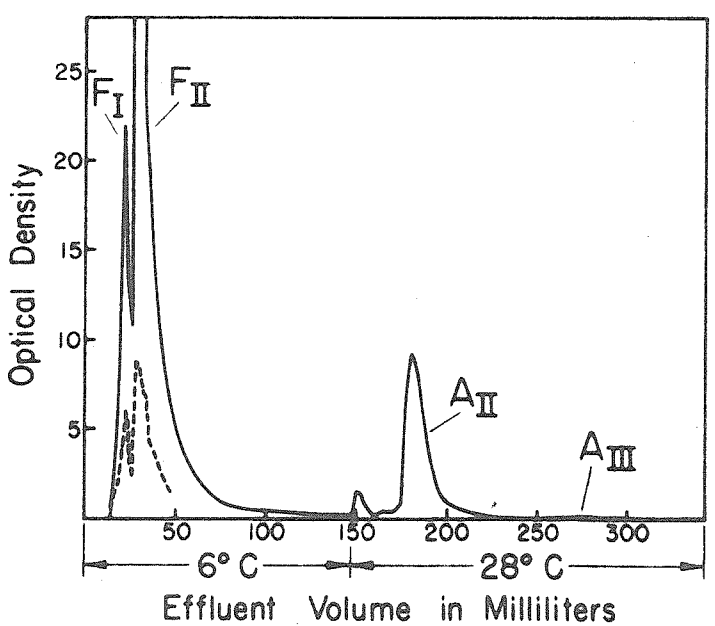


Figure 6

Developer Nos. 4 and 5 with whole cord blood hemoglobin are extensions of the chromatographic techniques described by Matsuda et al. (32) and Clegg and Schroeder (17).

Unlike the results of rechromatography of A_I zone from normal adult hemoglobin (fig. 2), chromatography of the F zone with Developer No. 4 revealed the presence of only a small amount of non-heme protein near the front of the chromatogram.

Sickle-Cell Hemoglobin.--The hemoglobin from individuals with sickle-cell anemia can be resolved into three well-separated zones by chromatography with Developer No. 1. Figure 7 is a graphic representation of a chromatogram of 50 mg. of oxyhemoglobin from a 57 year-old male with sickle-cell anemia. Although only the fast minor components, S_I, and the main component, S_{II}, are shown, a very slowly moving third zone, S_{III}, was also observed on the column during the chromatography. The peak of this third component generally was eluted at about 500 ml. at 6° C but could be eluted more rapidly by warming the column to 28° C.

Chromatograms very similar to Figure 7 were obtained for sickle-cell hemoglobin as carbonmonoxyhemoglobin and ferrihemoglobin cyanide. A more careful comparison of the chromatographic differences of oxyhemoglobin and ferrihemoglobin cyanide forms of several hemoglobins, including hemoglobin S, will be given later in this section.

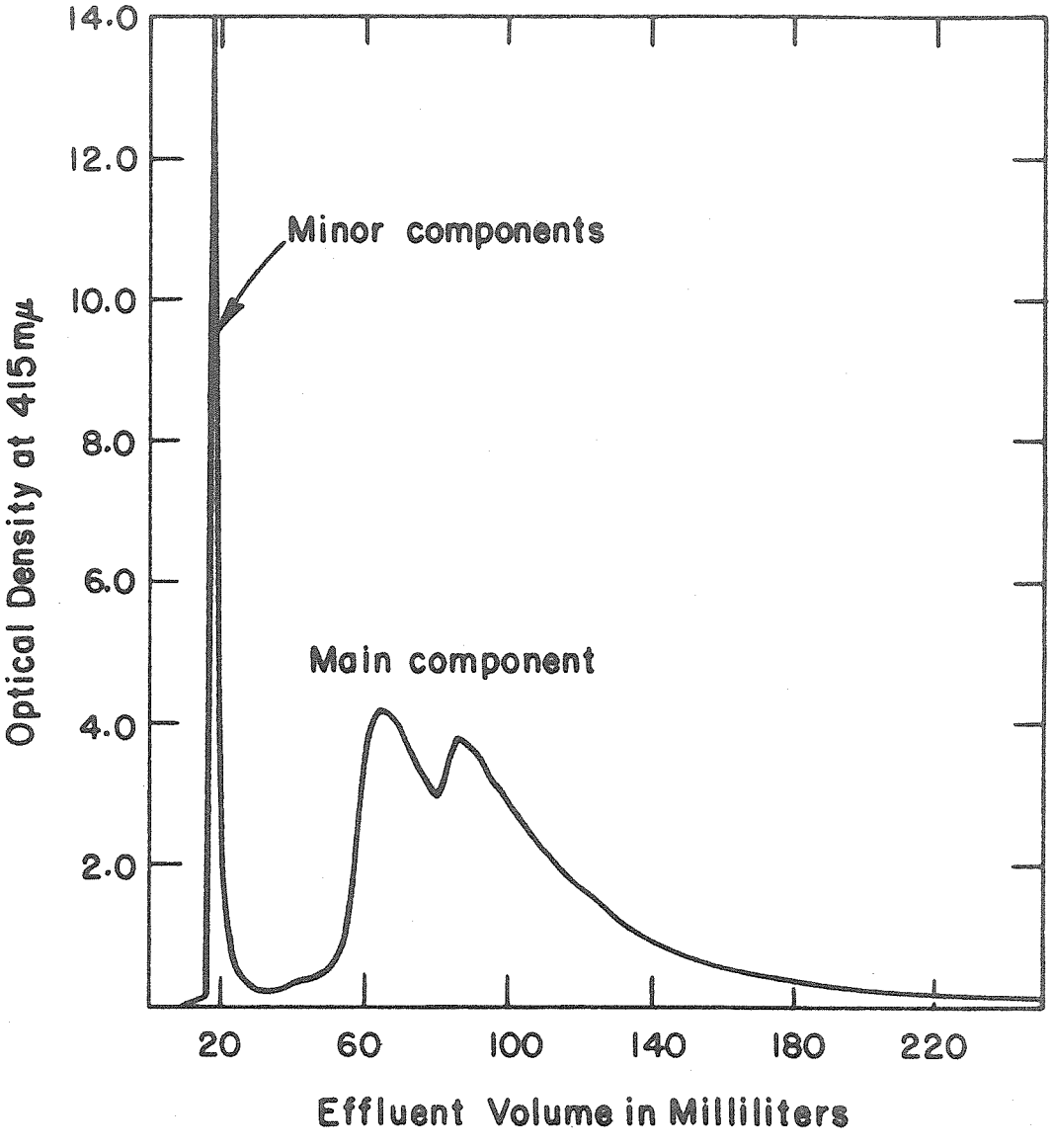


Figure 7

Chromatogram of 50 mg. of oxyhemoglobin S from a subject with sickle-cell anemia, Developer No. 1 was used for the separation and the optical density was measured at 415 mμ. The Minor Components are designated as S_I and the Main Component, is designated as S_{II}. A slowly moving Minor Component, S_{III}, is not shown.

It is apparent from Figure 7 that the main component, S_{II}, is partially resolved into two peaks; this phenomenon is discussed in Appendix I. In summary, however, the two peaks in the S_{II} zone appear to be different chromatographic forms of the same hemoglobin component.

Hemoglobin specimens from about 30 different sickle-cell anemia patients were chromatographed with Developer No. 1. The results of 10 representative chromatograms are listed in Table III. Only the S_I and S_{II} zones are eluted and quantitatively measured for the chromatograms listed in the top half of the table. For those chromatograms in the lower half of Table III, the S_{III} zone was also collected and included in the calculation of the percentage of each zone.

A comparison of the relative amounts of the S_I zones listed in Table III indicates that the variation of S_I among these sickle-cell anemia individuals is much greater than the variation of the A_I zone among different normal adult subjects. (See Table II and References 17 and 18.)

Duplicate chromatograms were run on sample LACH-9 and gave very similar results as shown in Table III. These and other duplicate studies indicate that the precision of the estimation of the components present in sickle-cell hemoglobin is of the order of $\pm$ 10% of the total percentage of S_I zone present and $\pm$ 2% of the total percentage of S_{II} component present.

TABLE III

Percentage of Components in Sickle-cell Hemoglobin Separated by Chromatography with Developer No. 1

Sample	Date of Sample	Chrom. No.	Percentage of S_I	S_{II}	S_{III}
1037	10/27/1957	B-12	10.8	89.2	*
1047	11/19/1957	B-17	10.7	89.2	*
LACH-1	12/18/1957	B-23	18.7	81.3	*
1011	12/17/1957	B-25	17.0	83.0	*
LACH-2a	1/8/1958	B-47	7.3	92.7	*
2b	6/25/1958	B-94	5.0	95.0	*
2c	8/6/1958	B-106	6.7	93.0	*
LACH-3	1/8/1958	B-31	17.2	82.8	*
1124	1/17/1958	B-39	11.6	88.4	*
LACH-9	4/23/1958	B-60	11.5	78.2	9.9
		B-62	10.9	77.3	11.6
LACH-60	12/13/1960	B-373	11.2	76.8	12.0
Ob-6a	11/14/1958	B-151	92.3	7.7	
6b	1/28/1959	B-178	52.6	42.0	5.4
6c	4/1/1959	B-206	37.7	53.9	8.4
6d	1/3/1961	B-370	20.2	68.2	11.6

*The S_{III} zone was observed to be present on the chromatographic column but was not collected in the effluent fraction nor included in the calculation of percentages of S_I and S_{II}.

Chromatographic studies of repeated specimens obtained from subject LACH-2 (Table III) revealed the lowest percentages of S_I component found in any of the sickle-cell patients. Although the percentage of S_I in this individual was only about one half the amount of A_I components found in the typical normal adult, it was stable over a 7 month period that included an episode of sickle-cell crisis (LACH-2c) (26).

Subject Ob-6 was 1 day old when his hemoglobin was studied for the first time (sample Ob-6a, Table III). Hemoglobin in the S_I region from this child was shown by other chromatographic studies to be comprised mainly of F_{II} and F_I components. A decline in the fetal components after birth is well known for normal infants (2) and is apparently the explanation for the changes observed in the percentage of S_I in subsequent specimens from subject Ob-6. The other sickle-cell anemia individuals listed in Table III were well above the age at which little or no fetal hemoglobin can be detected in normal individuals with adult hemoglobin.

A series of chromatograms was run on the same sample of sickle-cell hemoglobin (1037) over a prolonged period of storage in order to assess the effect of ageing on the stability of the S_{II} component. The results of repeated chromatographic analyses of the whole oxyhemoglobin stored in Developer No. 1 at 4-6° C for 169 days are listed in Table IV. From these results, it is apparent that the percentage

TABLE IV

Stability of Sickle-cell Hemoglobin with Storage

Duration of Storage in Days	Chrom. No.	Amount in mgs.	Percentage of S_I	Percentage of S_{II}
1	B-12	50	10.8	89.2
8	B-14	50	9.7	90.3
13	B-15	100	12.0	88.0
28	B-19	25	9.4	90.6
34	B-20	50	9.1	90.9
169	B-56	50	11.3	88.7

of the S_{II} component did not change appreciably with time. Although the S_{III} zone was observed to be present in approximately constant amounts on all of these chromatograms, it was not estimated in the effluent.

In Table V are listed the results of chromatographic studies of specimens of blood most of which appeared to contain hemoglobin A_{II} in addition to hemoglobin S_{II}. These studies were also made with Developer No. 1.

Sample LACH-4 was found to contain about 8% rapidly moving minor components, 60% A_{II} component, and 32% S_{II} component. A band that was apparently the S_{III} component was judged to be less than about 3% of the total hemoglobin. This specimen was from a subject who is known to have a sickle-cell trait and is therefore heterozygous for the

TABLE V

Chromatography of Special Individuals with Hemoglobins A and S

Sample	Date of Sample	Chrom. No.	S_I	Percentage of A_{II}	Percentage of S_{II}	S_{III}
LACH-4	1/17/1958	B-35	8.6	59.8	31.6	*
LACH-19	10/7/1958	B-143	20.7	17.9	52.6	8.8
LACH-17	9/29/1958	B-137	10.7	5.0	74.2	10.1
1121	1/9/1958	B-36	9.0	7.0	84.0	*
CH-9	4/4/1959	B-216	25.4		61.6	13.0
LACH-35	6/3/1959	B-274	19.0	16.5	64.5	*

*The S_{III} zone was observed to be present but was not collected in the effluent fraction nor included in the calculation of percentages of S_I, A_{II}, and S_{II}.

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sickle-cell anemia gene (34). The ratio of the percentages of hemoglobin A_{II} and S_{II} is similar to ratios reported to be typical for most sickle-cell trait individuals (35).

The subjects from whom samples LACH-19 and LACH-17 were obtained were reported to have typical sickle-cell anemia. The presence of small amounts of component A_{II} may be due to the fact that both individuals had received blood transfusions. Specimen LACH-19 was obtained 2 months after a blood transfusion whereas sample LACH-17 was obtained 4 months after a transfusion. Although the life span of the normal

red cell is estimated to be 120 days (33), the survival of normal donor cells may be longer in the sickle-cell anemia patient. Transfusion data on subject 1121, also reported to have sickle-cell anemia, were not available.

Careful genetic studies of the subject from whom specimen CH-9 was obtained indicate that this individual is heterozygous for the sickle-cell gene and heterozygous for a thalassemia gene. Although a large fraction (25.4%) of rapidly moving minor components, S_I, was observed in sample CH-9 (Table V), no hemoglobin A_{II} could be detected. The sensitivity of the studies was such that as much as 2 or 3% A_{II} might have been present in the S_I zone. In spite of this reservation, it is interesting that this individual, who is judged by genetic studies to have one gene for hemoglobin A, apparently has little or no hemoglobin A in circulation. Similarly, sample LACH-35 was from a person known to be heterozygous for sickle-cell anemia because her mother was found by chromatographic studies to have only the normal adult hemoglobins. The compositions of neither samples CH-9 nor LACH-35 resemble the composition of sample LACH-4. The latter, as previously mentioned, was from a subject with a typical sickle-cell trait.

From studies such as those listed in Table V, it is apparent that chromatographic analysis of hemoglobins without careful clinical and genetic data is not sufficient to diagnose heterozygosity and homozygosity for the sickle-cell anemia gene.

According to Fessas (36) hemoglobin A_2, which corresponds to component A_{IIIb} (18), can be detected by starch block electrophoresis in blood from individuals with either sickle-cell anemia or sickle-cell trait. As noted earlier, the A_{III} components cannot be separated from the A_{II} zone in normal adult hemoglobin chromatographed with Developer No. 1. Presumably in the chromatography of sickle-cell hemoglobin, the A_{III} component should become evident, provided its chromatographic mobility is greater than that of the S_{II} zone. Careful examination of Figure 7 reveals a small rise in the baseline at an effluent volume of about 40 ml. This rise has been observed also with other sickle-cell specimens and may represent the A_{III} component. Further study of this problem is necessary before one can be certain of the presence or absence of the A_2 (A_{IIIb}) component in sickle-cell hemoglobin as judged by chromatography.

The S_I zone from chromatography of sickle-cell hemoglobin can be separated into a number of heme and non-heme components by rechromatography with Developer No. 4. Figure 8 illustrates the result of further separation of the S_I zone originally isolated from 100 mg. of sickle-cell hemoglobin (LACH-2b). Seven separate hemoglobin components were observed. Two of these minor sickle components (S_{If} and S_{Ig}) moved so slowly with Developer No. 4 that it was necessary to

Figure 8. Rechromatography of zone S_I from 100 mg. of whole oxyhemoglobin S with Developer No. 4. Optical density at 415 mμ is represented by the solid line, optical density at 280 mμ is represented by the dashed line. This chromatogram is typical of most of the sickle-cell hemoglobin samples that were examined.

Figure 9. Rechromatography of zone S_I from 200 mg. of whole oxyhemoglobin S with Developer No. 4. Representation of the optical densities is the same as in Figure 8 except for the scale. This chromatogram is representative of an occasional "atypical" sickle-cell hemoglobin sample.

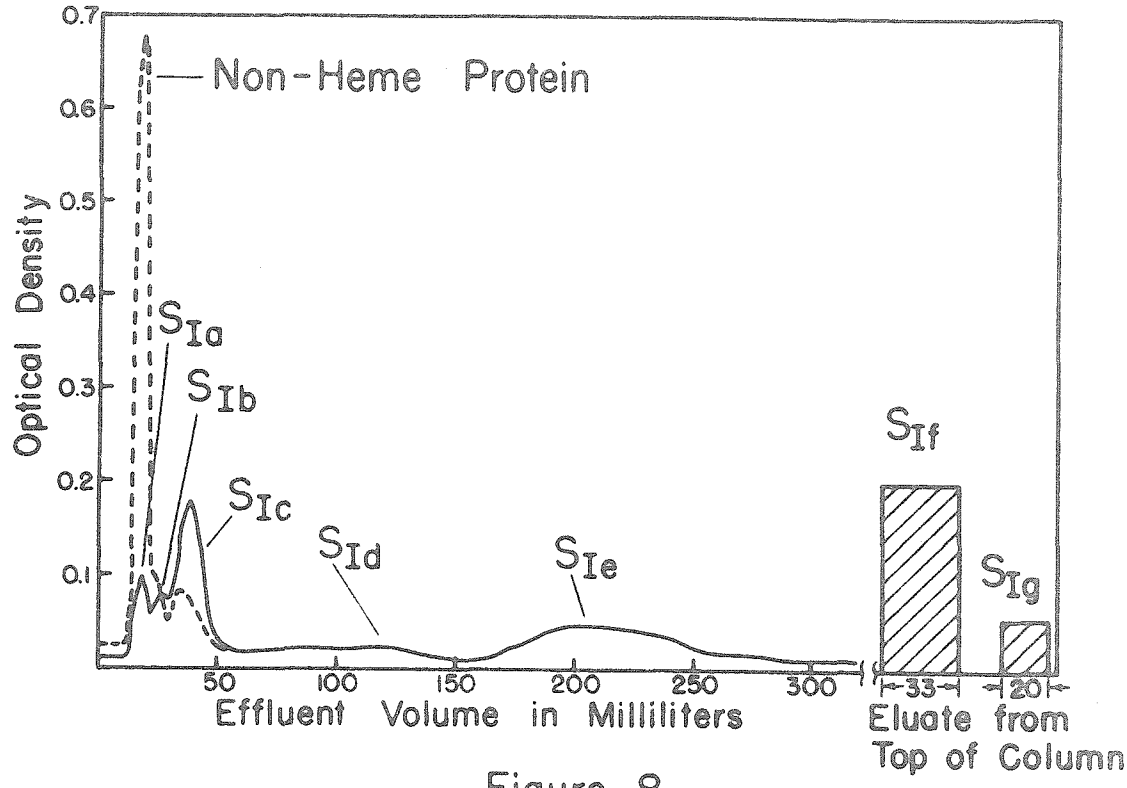


Figure 8

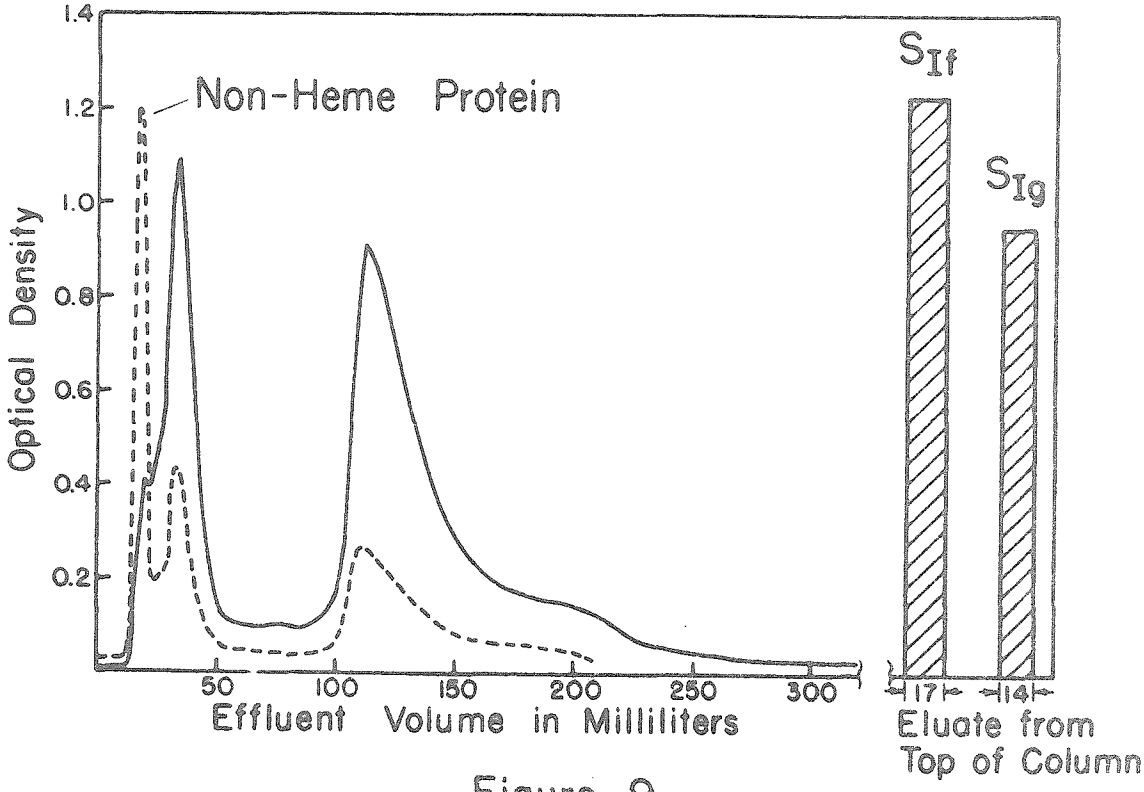


Figure 9

remove the resin containing these bands from the top of the column and elute each component separately with Developer No. 1 at room temperature. The movements of components S_{Ia} and S_{Ib} resembled the movements of f_3 and f_4 from umbilical cord blood chromatograms. The S_{Ic} zone appeared to move more slowly than the F_I component of cord blood, whereas S_{Id} appeared to be eluted in a region typical of component F_{II}. As judged by these preliminary chromatograms, components S_{Ie}, S_{If}, and S_{Ig} did not appear to be comparable in chromatographic behavior to any zones in either cord blood or normal adult hemoglobin.

Results very similar to those shown in Figure 8 were obtained using Developer No. 4 for the separation of S_I zones from the hemoglobin of 15 other sickle-cell anemia patients. However, the separation of S_I zones from 3 other sickle-cell anemia individuals appeared to resemble the chromatography of fetal components from umbilical cord blood except for the two slow zones, S_{If} and S_{Ig}. Rechromatography of one of these three exceptions is illustrated in Figure 9.

Thus, the chromatographic patterns obtained from further chromatography of the S_I zones indicate that the hemoglobin from sickle-cell anemia individuals possesses minor component heterogeneity. Although some of the fast sickle-cell minor components may be identical to some fetal and normal adult components, several sickle-cell components appear to be different from any observed in either cord blood or normal

adult hemoglobin. Further characterization of components S_{Ic}, S_{Ie}, and S_{If} appears to be of considerable interest. The chromatographic identity of S_{Ie} and F_{II} will be discussed in the last part of this section.

Hemoglobin C.--Figure 10 represents the results of the chromatography with Developer No. 1 of the hemoglobin from an 8-year-old patient with homozygous hemoglobin C disease. The solid line in Figure 10 was obtained from a chromatogram made 1 day after obtaining the blood specimen. A second chromatogram represented by the dotted line, was made on the same specimen 53 days after the first study. The striking differences between these two studies were concluded to be due to instability of the main hemoglobin C component, C_{II}, which under normal conditions is strongly adsorbed to the top of the column in equilibrium with Developer No. 1. The C_{II} zone of these chromatograms was isolated by removing the resin containing it from the top of the column and eluting this hemoglobin in a short chromatographic tube at 28° C.

Further separation of the first zone (C_{Ia+b+c}) from the initial separation of hemoglobin C (fig. 10) was carried out chromatographically with Developer No. 4. Three heme and one non-heme components were observed as illustrated in Figure 11. Unlike the separation of the S_I with Developer No. 4, the first zone from the chromatogram of hemoglobin C shown in Figure 10 did not contain minor components other than those shown in Figure 11.

Figure 10. Chromatograms of 100 mg. of oxyhemoglobin C on IRC-50 with Developer No. 1. The optical density was measured at 415 mμ. The solid line represents a chromatogram run 1 day after obtaining the sample; the dotted line represents part of a chromatogram run 53 days later.

Figure 11. Rechromatography of zone C_{Ia+b+c} from first chromatogram represented in Figure 10. Developer No. 4 was used for the separation. The optical density at 415 mμ is represented by the solid line, whereas the optical density at 280 mu is represented by the dashed line. No zone other than those shown in the figure was noted on the column during the chromatography.

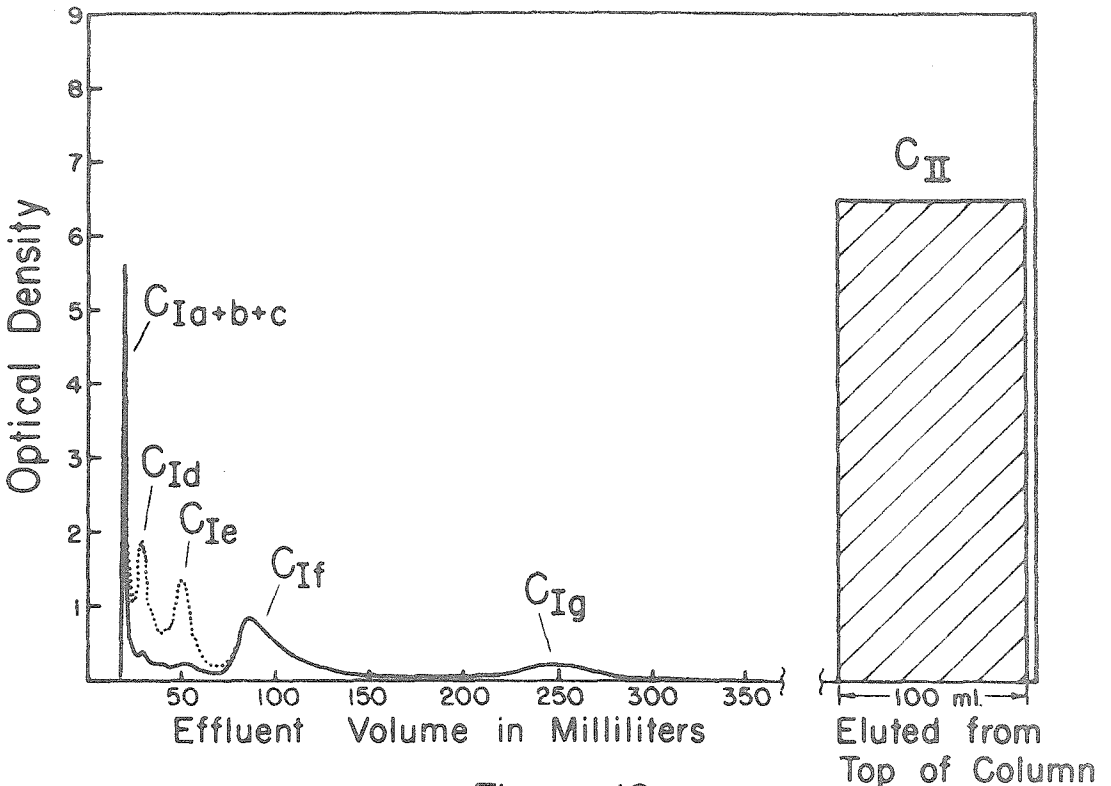


Figure 10

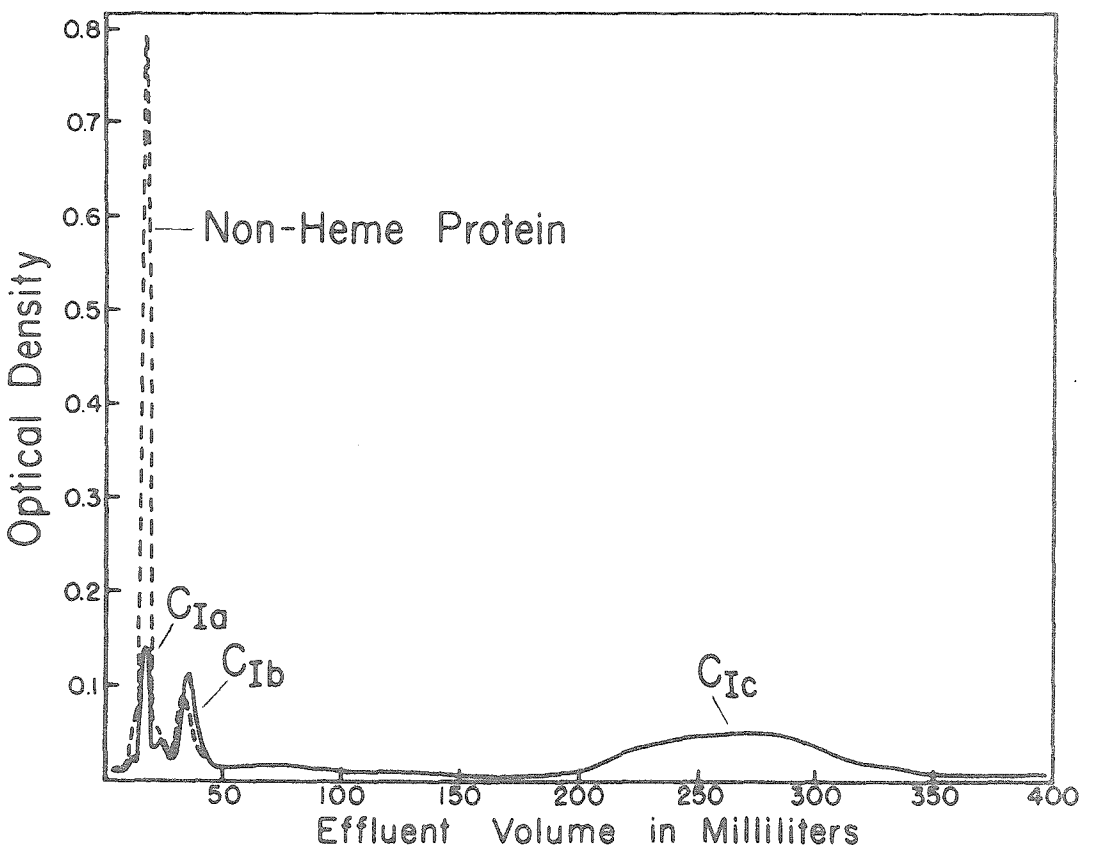


Figure 11

Hemoglobin D.--The original hemoglobin D was discovered and studied by Itano and co-workers (37,38). They found this abnormal hemoglobin in a family in which hemoglobin S was also present. Hemoglobin D was characterized by noting that it has the electrophoretic properties of hemoglobin S, but the solubility property of hemoglobin A.

Specimens of blood were obtained from members of this family, including two subjects with sickle-cell hemoglobin D disease and two subjects who are heterozygous for the hemoglobin D gene and the normal hemoglobin A gene. Chromatographic studies with Developer No. 1 revealed that the fast minors, A_{II}, and major D zones could be separated from one another in blood from the heterozygous individual. A typical chromatogram of hemoglobin from one of the AD subjects revealed 11% rapidly moving minor components, 51% A_{II}, and 38% D_{II}. The major abnormal component, D_{II}, was very similar to S_{II} in its chromatographic movement. When mixtures containing S_{II} and D_{II} were chromatographed, only one major zone was observed. By means of introducing a small amount of hemoglobin S containing C-14 labelled amino acids it was possible to demonstrate that hemoglobin D is eluted faster than hemoglobin S_{II}. The difference in chromatographic mobility between S_{II} and D_{II} is apparently not enough to produce two separate zones. The use of the radioactive label for differentiating hemoglobin S and D will be presented and discussed more completely later in this section.

Hemoglobin H.--Extensive chromatographic studies were made on a number of specimens from three different individuals with thalassemia Hemoglobin H disease (39,40,41,42) The most satisfactory separation of components was obtained by chromatography with Developer No. 5: Figure 12 illustrates the results of the chromatography of 100 mg. of hemoglobin from subject P.T. (specimen CH-12a) with Developer No. 5. Five zones or regions were obtained. When separated further with Developer No. 6, the first zone (zone 1, Figure 12) was found to be comprised of at least two hemoglobin components, zones 1a and 1b, Figure 13. Comparison of the optical density values at 280 and 415 mμ revealed the presence of a non-heme protein in addition to the hemoglobin component in zone 1a, Figure 13. Zone 2 of Figure 12 was chromatographically similar to the F_{II} component of umbilical cord blood and to the A_{Ic} component of the normal adult hemoglobin. About 4% of the total hemoglobin from the chromatogram illustrated in Figure 12, was present in the region marked 3; however, no definite color peak was noted in this region. Zone 4 was chromatographically identical with the A_{II} component of the normal adult hemoglobin and zone 5 was chromatographically similar to the A_{III} components described by Clegg and Schroeder (17).

The results of chromatographic studies of each of the specimens obtained from the thalassemia-hemoglobin H disease patients are listed in Table VI. Specimens from subject R.C.

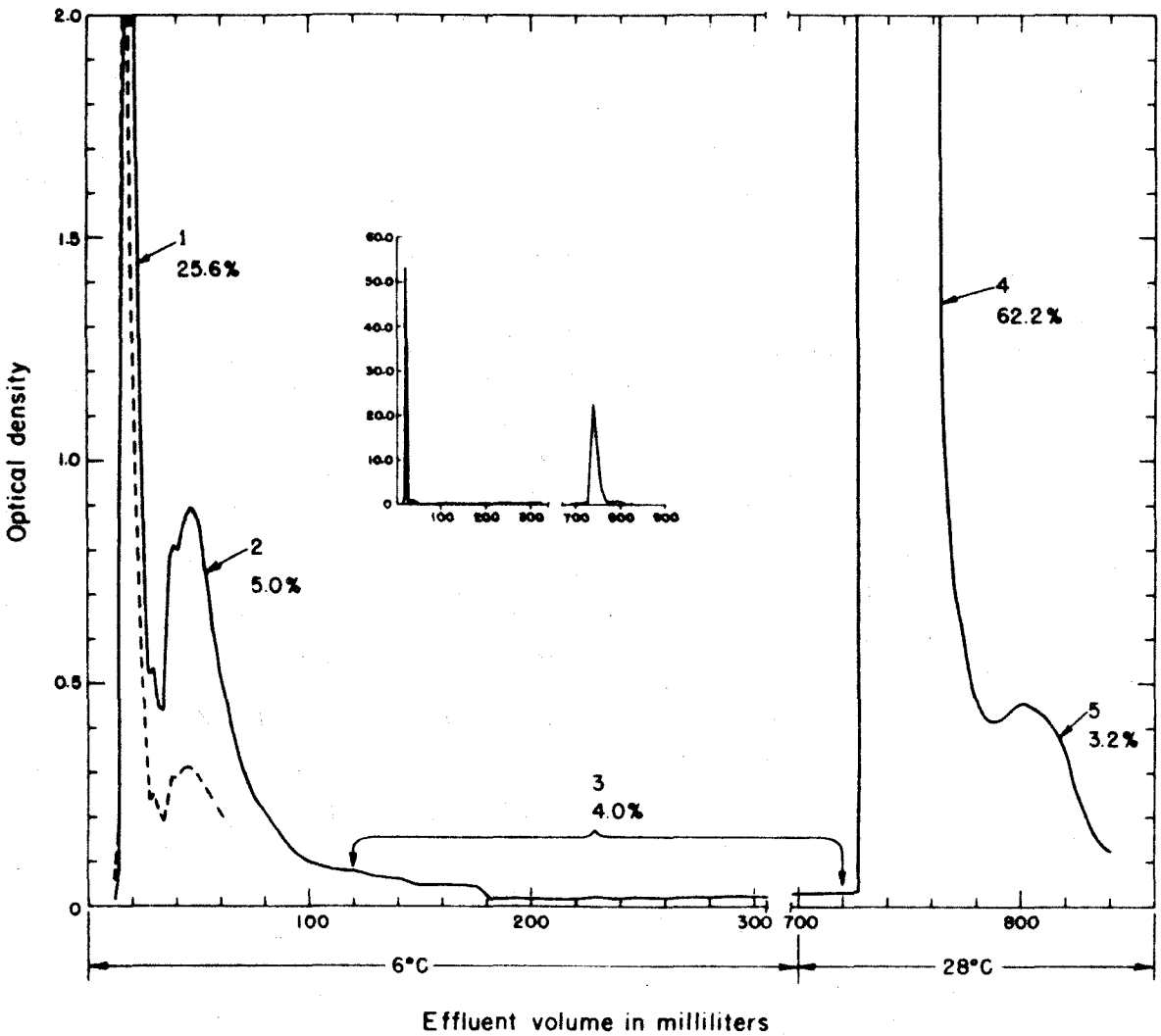


Figure 12

Chromatogram of 100 mg. of hemoglobin from subject P.T. with thalassemia hemoglobin H disease. Developer No. 5 was used for the separation. The optical densities were measured at 280 mμ (dotted line) and 415 mμ (solid line).

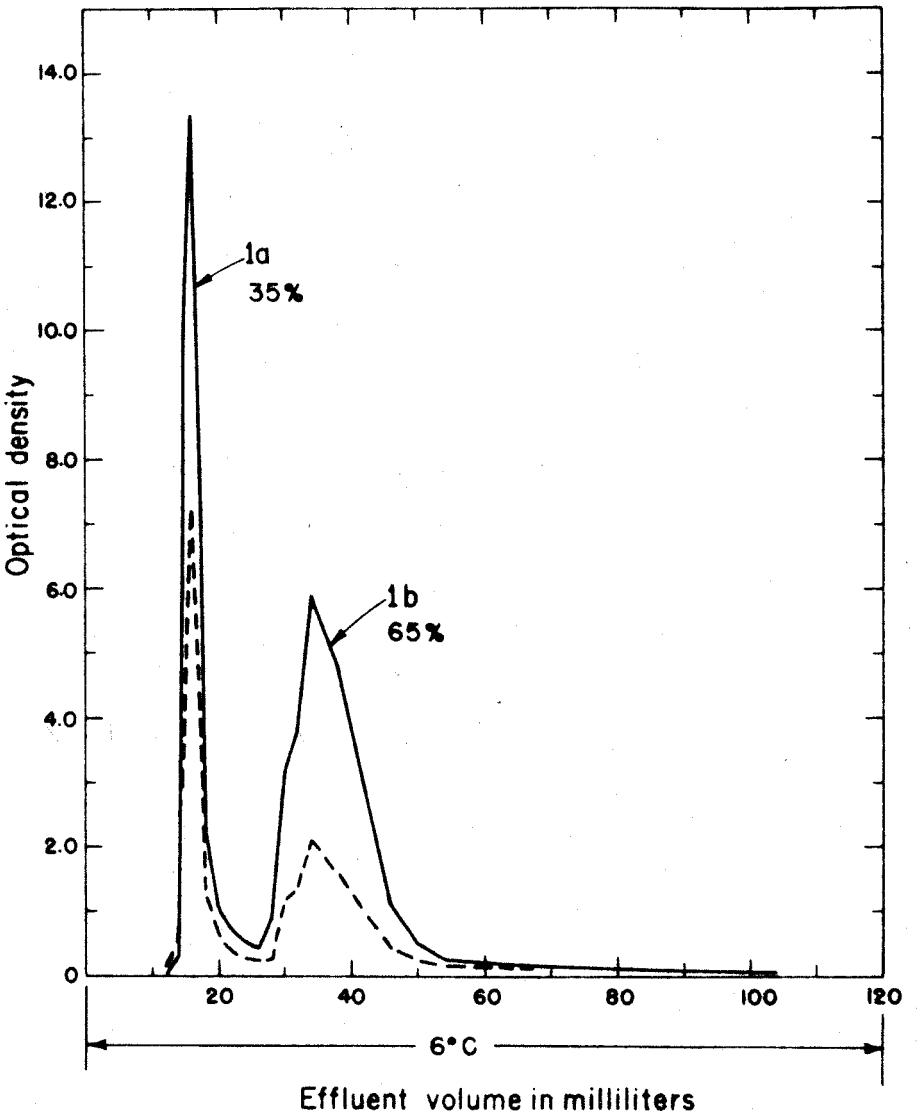


Figure 13

Rechromatography of zone 1 from chromatogram shown in Figure 12. Developer No. 6 was used for the separation. The optical densities were measured at 280 mμ (dotted line) and 415 mμ (solid line).

TABLE VI

Percentage of Components in Hemoglobin from Three Subjects with Thalassemia Hemoglobin H Disease

Subject	Specimen No.	Dates of Specimen	Dates of Chrom.	Percent of zones* 1a	1b	2	3	4	5
R.C.	UO-1a	7/22/1958	8/7/1958	6.3	38.4	3.0	3.0	46.0	3.3
	UO-1b	11/28/1958	12/1/1958	5.2	30.3	3.5	3.7	52.5	4.7
	+UO-1c	1/5/1959	2/20/1959	----44.0----			----56.0----		
H.D.	UO-2	12/3/1958	12/7/1958	6.8	28.6	3.6	3.2	52.4	5.4
P.T.	CH-12a	5/14/1959	5/16/1959	9.0	16.6	5.0	4.0	62.2	3.2
	CH-12b	8/5/1959	8/10/1959	11.5	14.2	5.1	3.8	60.4	5.0
	CH-12c	2/10/1960	11/17/1960	10.8	15.4	3.2	3.3	63.6	3.6
	CH-12d	12/22/1960	1/6/1961	9.6	15.7	3.6	4.0	61.4	5.7

*See Figure 12 for zone numbers.

+This chromatographic separation was with Developer No. 1 only whereas all other separations were first with Developer No. 5 and then Developer No. 6.

were the first to be studied. This subject is No. 7 in the family diagram published by Rigas, Koler, and Osgood (39), and is the brother of the propositus (subject H.D.) in whom the abnormal hemoglobin H was first discovered. The third subject, P.T., and her family have been carefully studied by Sturgeon and co-workers (41,42).

Examination of Table VI reveals that the relative percentage of each component is rather constant for repeated specimens from subjects R.C. and P.T. It is of interest to note that the chromatographic results from specimen CH-12c, which had been stored in Developer No. 5 for 9 months, were very similar to the results from the fresh samples (C-12a, C-12b, and C-12d). This apparent stability of the hemoglobin components associated with hemoglobin H disease is in disagreement with the observations of Rigas et al. (39). The stability of our samples may have been due to our practice of storing the freshly hemolyzed solution in chromatographic developer which is buffered at about pH 7.0 and contains potassium cyanide.

Details of more complete chemical characterization of the components observed in the hemoglobin from these thalassemia hemoglobin H patients will be given below in Section II. In summary, however, zone 1a of subject P.T., but apparently not of subjects R.C. and H.D., is identical to "Bart's" hemoglobin (43,44), and has a molecular formula of γ_4^F. Zone 1b in all subjects is the main abnormal hemoglobin H component

β_4^A. Zone 2 is comprised of a mixture of components A_{Ic} and F_{II} in about a 6:1 ratio. Zone 4 is apparently identical to the main component, A_{II}, from normal adult hemoglobin. Zone 5 appears to be the same as component A_{III}. The heme-component in zones 1a (subject P.T. only), 1b, 2, 4, and 5 (figs. 12 and 13) will be designated as γ_4, β_4, A^H_{Ic}, A^H_{II}, and A^H_{III}, respectively, in the second section of the Thesis.

B. Factors Influencing the Chromatography of Hemoglobins

Schroeder and his co-workers (15,17,18) have observed that the migration of hemoglobins under their chromatographic conditions is particularly sensitive to the temperature of the column, pH and ionic strength of the developers, and the equilibration of the resin. The influence of these factors on the chromatography of hemoglobins was confirmed during the course of this thesis work. Another important factor that influences the migration rates of hemoglobins was found to be the amount of hemoglobin which is chromatographed. Examples of the behavior of two different hemoglobins, will be given to illustrate the magnitude of this load effect.

An influence of the load on the migration rate was suspected when comparisons were made of two chromatograms that were identical except for the amount of hemoglobin applied. When a 50 mg. of sickle-cell hemoglobin was chromatographed with Developer No. 1 the peak of the S_{II} zone emerged at an effluent volume of 94 ml., whereas with

10 mg. the peak emerged at 130 ml. These observations led to an estimation of the migration rate of the S_{II} zone as it moved down the chromatographic column. Figure 14 shows the migration rates of the front of the S_{II} zone during the chromatography of different amounts of sickle-cell hemoglobin under identical conditions.

Similarly, the migration rate of the F_{II} component during chromatography with Developer No. 4 was noted to be dependent upon the load on the column. For example, when chromatographing 12 mg. of the F hemoglobin with Developer No. 4, the peak of the F_{II} zone emerged at about 104 ml. The F_{II} zone for 1.5 mg. of the same hemoglobin F chromatographed on the same column was eluted in a broad zone with a peak at about 240 ml. This influence of the amount of the load on the chromatographic behavior of hemoglobin F was confirmed by Matsuda et al. (32).

It is therefore apparent that the identification of a hemoglobin component by its chromatographic migration is strongly dependent upon the amount of hemoglobin being chromatographed. This dependency and the variation in other factors influencing the migration rate limits the usefulness of the chromatographic procedure in differentiating two similarly migrating hemoglobins unless the two hemoglobins can be compared simultaneously in the same chromatogram.

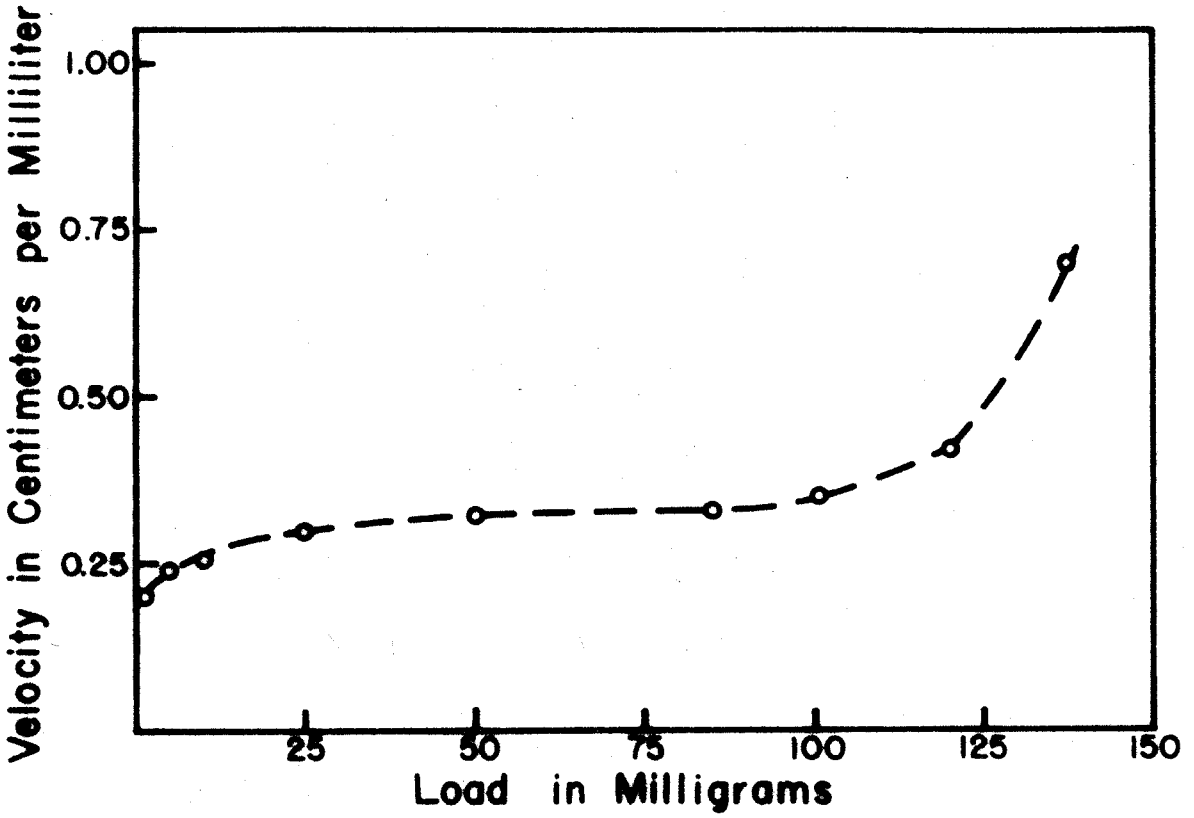


Figure 14

The relation of the chromatographic movement of component S_{II} to the amount of hemoglobin present.

C. Detection of Differences of Chromatographic Behavior with Radioactive Labels

Differences Between Oxyhemoglobins and Ferrihemoglobin Cyanide.--Allen, Schroeder, and Balog (15) introduced the practice of adding cyanide ion to the chromatographic developers in order to convert any ferrihemoglobin present in the sample or formed during the chromatography to ferrihemoglobin cyanide. This conversion of the ferric form of hemoglobin to the cyanide complex seemed desirable to the authors in order to eliminate the formation of slowly moving extraneous zones of ferrihemoglobin during the chromatography of oxyhemoglobins (19). Their rationale was based upon the observation that ferrihemoglobin cyanide and oxyhemoglobin forms of normal adult hemoglobin appeared to have identical chromatographic behavior. Observations of the apparent similarity of the chromatographic movement of the oxyhemoglobin and ferrihemoglobin cyanide forms of hemoglobins A and S were also made as noted previously in this section. However, in the course of studying the chromatographic similarities of different hemoglobin components, it became evident that the chromatographic movement of the ferrihemoglobin cyanide forms of A_{II}, S_{II}, F_{II}, and F_{I} is detectably different from the oxyhemoglobin forms.

Figures 15a and 15b illustrate two chromatograms of fetal hemoglobin using Developer No. 4. Figure 15a is a chromatogram of a mixture of 29 mg. of normal fetal hemo-

Figure 15a. Chromatogram of mixture of 29 mg. non-radioactive whole cord blood oxyhemoglobin and 7 mg. of radioactive fetal ferrihemoglobin cyanide with Developer No. 4. The solid line represents the optical density at 415 mμ. The circles and dashed line represent the specific activity in counts per minute per mg. hemoglobin.

Figure 15b. Chromatogram of mixture of 29 mg. of non-radioactive whole cord blood ferrihemoglobin cyanide and 7 mg. of radioactive fetal ferrihemoglobin cyanide with Developer No. 5. This is a control to Figure 15a. The optical density and specific activity are represented in the same way as in Figure 15a.

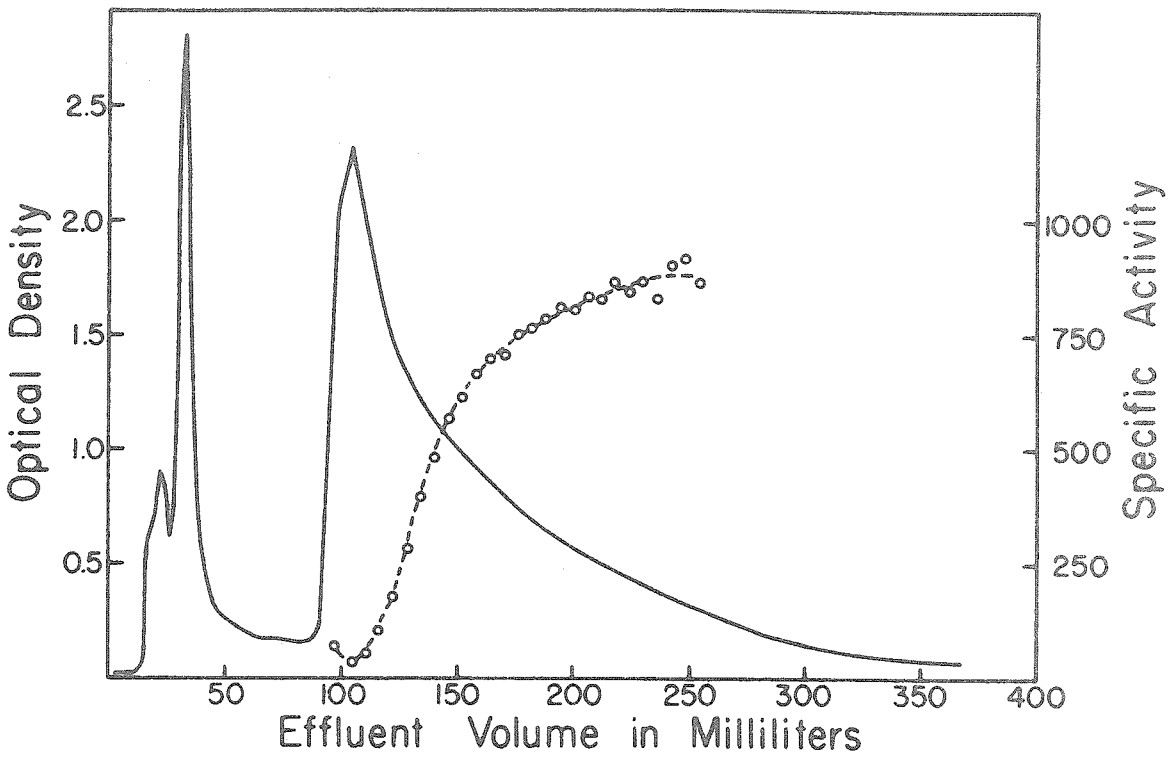


Figure 15a

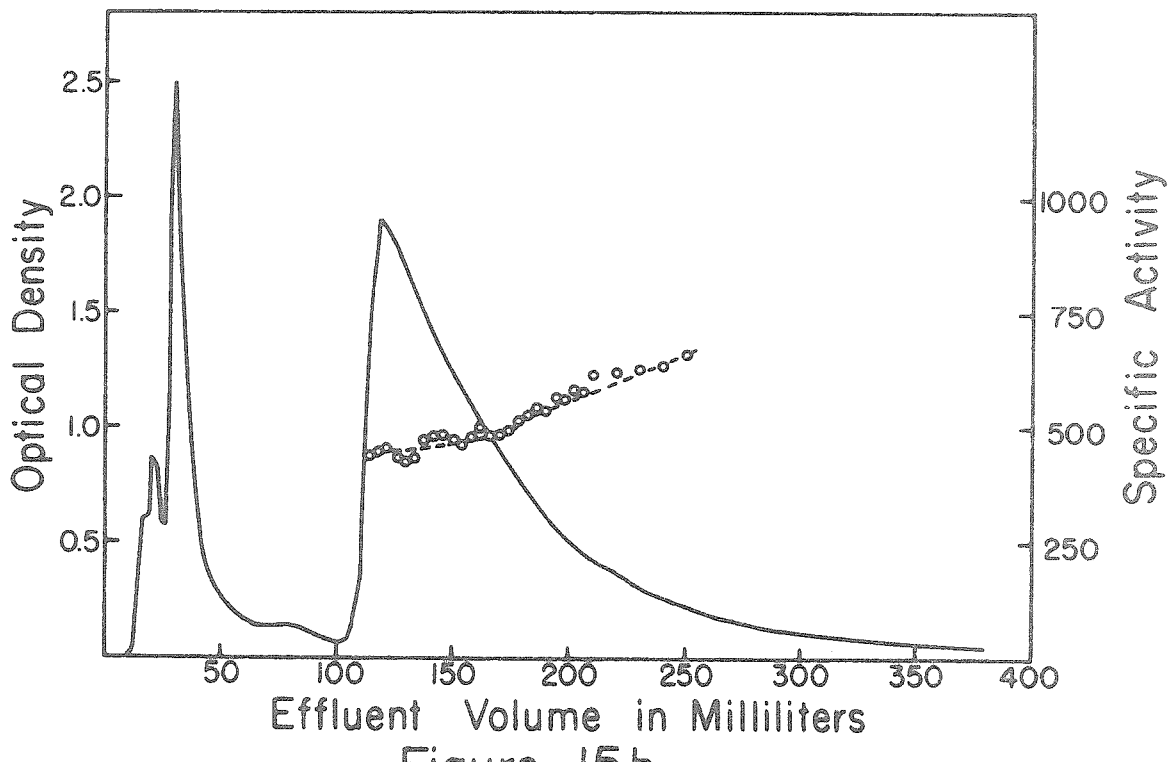


Figure 15b

globin in the oxyhemoglobin form and 7 mg. of fetal hemoglobin containing C-14 labelled L-leucine in the ferrihemoglobin cyanide form. The radioactivity and the hemoglobin concentration of the effluent fractions in the region of the F_{II} zone were determined and the specific activity was calculated in counts per minute per mg. hemoglobin (cpm per mg.). It is readily apparent from an examination of the specific activity curve that the ferrihemoglobin cyanide form of F_{II}, which contained the radioactive label, was not uniformly distributed throughout the whole F_{II} zone. If it were, the specific activity curve should be parallel with the abscissa. Figure 15b represents a control experiment in which a mixture of the same two fetal hemoglobins was first completely oxidized to ferrihemoglobin cyanide before chromatography. The distribution of the radioactivity is essentially uniform throughout the F_{II} zone of this control. Although the migration rates of oxyhemoglobin and ferrihemoglobin cyanide in the experiment shown in Figure 15a may have been influenced by the difference in the relative amounts of the two forms, the experiments do indicate a definite difference in the chromatographic behavior of these two hemoglobins.

Results analogous to those of fetal hemoglobin were obtained for radioactive studies of the relative chromatographic mobilities for oxyhemoglobin and ferrihemoglobin

cyanide A_{II} with Developer No. 2 as well as for oxyhemoglobin and ferrihemoglobin cyanide S_{II} with Developer No. 1. In each of these experiments equal amounts of the two forms of hemoglobin were added. It was concluded that the ferrihemoglobin cyanide forms of probably all hemoglobins are more strongly adsorbed to the resin column than their corresponding oxyhemoglobin forms.

Radioactive Hemoglobins Used as Chromatographic References.--Hemoglobins A, S, and F containing amino acids labelled with C-14 were also employed as reference standards for simultaneous chromatography of mixtures of one of these radioactive hemoglobins and a chromatographically similar, but unknown hemoglobin. In such experiments the specific activity in the region of the peak of unknown hemoglobin should reflect chromatographic similarities or differences between the unknown and the reference hemoglobin containing C-14. If the unknown and the reference hemoglobins are identical in movements, the specific activity curve should be parallel to the abscissa throughout the zone. If the two hemoglobins are different in chromatographic movement, the specific activity curve should be sloped and the sign of the slope should indicate the relative positions of the two hemoglobins. This technique of studying the identity of hemoglobins with similar chromatographic migration was applied to a number of cases. Two

examples, one with radioactive hemoglobin S the other with radioactive hemoglobin A, will be presented as representative cases.

Differences Between Hemoglobin D and Hemoglobin S.-- It has been stated earlier in this section that hemoglobin D appears to be identical to hemoglobin S in its chromatographic movement as judged by the optical density curves of the effluent. This observation is in agreement with the electrophoretic behavior of this hemoglobin (37). Significant differences in the chromatographic mobilities of hemoglobins D and S became apparent when samples containing hemoglobin D were chromatographed with a small amount of radioactive hemoglobin S. Figures 16a, 16b, and 16c illustrate three different chromatograms with Developer No. 1 which bear on this problem. Figure 16a represents the result of chromatographing a mixture containing 2.5 mg. of radioactive hemoglobin S_{II} with 25 mg. of non-radioactive hemoglobin S for a typical sickle-cell anemia subject. Theoretically, the specific activity should be constant throughout the S_{II} zone of this chromatogram; however, the radioactive hemoglobin S was partly in the ferrihemoglobin cyanide form, which accounts for a small slope in the specific activity curve. These experiments had been performed before it was recognized that the ferrihemoglobin cyanide form migrates more slowly than the oxyhemoglobin form. Figure 16b represents a chromatogram of a mixture containing 2.5 mg. of

Figure 16a. Chromatogram of mixture of 25 mg. of hemoglobin S and 2.5 mg. of radioactive hemoglobin S_{II} on IRC-50 with Developer No. 1. The solid line represents the optical density at 542 mμ. The circles and dashed line represent the specific activity in counts per minute per mg. hemoglobin.

Figure 16b. Chromatogram of mixture of 50 mg. of hemoglobin from a subject who is heterozygous for the hemoglobin A and D genes, and 2.5 mg. of radioactive hemoglobin S_{II} on IRC-50 with Developer No. 1. The representation of optical density and specific activity is the same as in Figure 16a.

Figure 16c. Chromatogram of mixture of 25 mg. of hemoglobin from a subject who has sickle-cell hemoglobin D disease, and 2.5 mg. of radioactive hemoglobin S_{II} on IRC-50 with Developer No. 1. The representation of optical density and specific activity is the same as in Figure 16a.

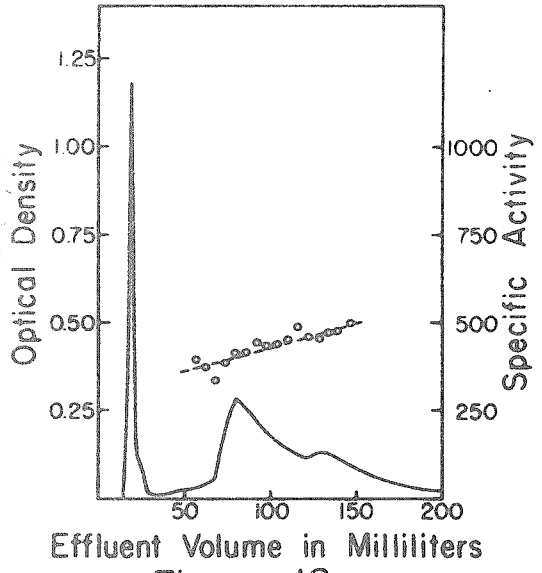


Figure 16a

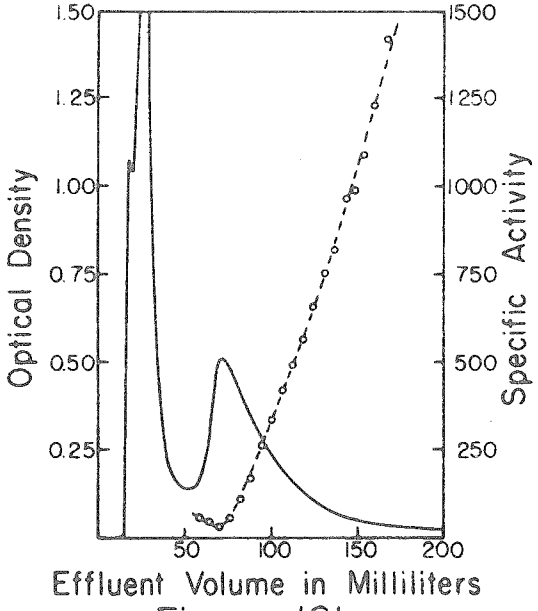


Figure 16b

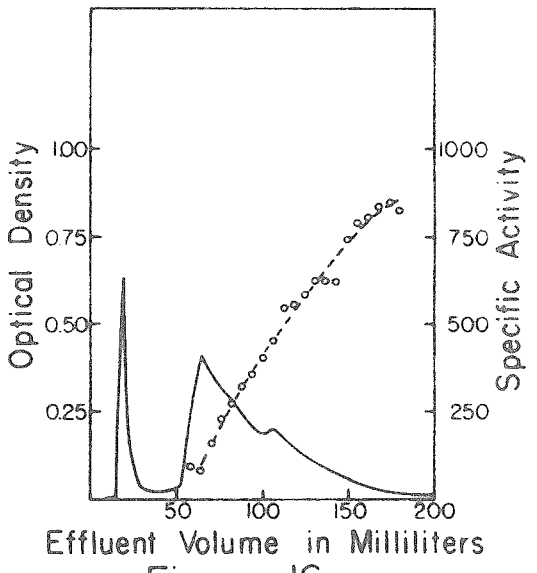


Figure 16c

radioactive S_{II} and 50 mg. of hemoglobin from one of the subjects who is heterozygous for hemoglobin D and hemoglobin A genes (38). Roughly equal amounts of hemoglobins D and A were found in samples from this subject. It is apparent that the slope of the specific activity curve of this chromatogram is very steep and bears no resemblance to the specific activity curve shown in Figure 16a. Figure 16c illustrates the chromatographic results of a mixture of 2.5 mg. of radioactive hemoglobin S_{II} and 25 mg. of hemoglobin from a patient with sickle-cell hemoglobin D disease (38). The slope of the specific activity curve in this chromatogram is intermediate between those shown in Figures 16a and 16b. This intermediate slope suggests that approximately equal amounts of hemoglobin S and hemoglobin D were present in the specimen from this subject with sickle-cell hemoglobin D disease. This conclusion as to the amount of hemoglobin D and S is in agreement with studies by other authors (38).

Hemoglobins Similar to Hemoglobin A.--The set of chromatograms illustrated in Figures 17a, 17b, and 17c represents another example of the use of radioactive hemoglobins for the determination of the relative chromatographic mobilities of hemoglobins. In these experiments all of the samples were converted completely to the ferrihemoglobin cyanide forms in order to avoid having the same hemoglobin in two forms, oxyhemoglobin and ferrihemoglobin cyanide, with different chromatographic mobilities.

Figure 17a. Chromatogram of mixture of 7 mg. of non-radioactive normal adult ferrihemoglobin cyanide and 8 mg. of radioactive ferrihemoglobin cyanide A^{*}_{II} on IRC-50 with Developer No. 2. The solid line represents the optical density at 542 mμ. The circles and dashed line represent the specific activity in counts per minute per mg. hemoglobin.

Figure 17b. Chromatogram of mixture of 7 mg. of non-radioactive ferrihemoglobin cyanide A^{H}_{II} (from subject P.T. with thalassemia hemoglobin H disease, zone 4, fig. 12), and 7 mg. of radioactive ferrihemoglobin cyanide A^{*}_{II} on IRC-50 with Developer No. 2. The representation of optical density and specific activity is the same as in Figure 17a.

Figure 17c. Chromatogram of mixture of 7 mg. of gorilla ferrihemoglobin cyanide (non-radioactive) and 7 mg. of radioactive ferrihemoglobin cyanide A^{*}_{II} on IRC-50 with Developer No. 2. The representation of optical density and specific activity is the same as in Figure 17a.

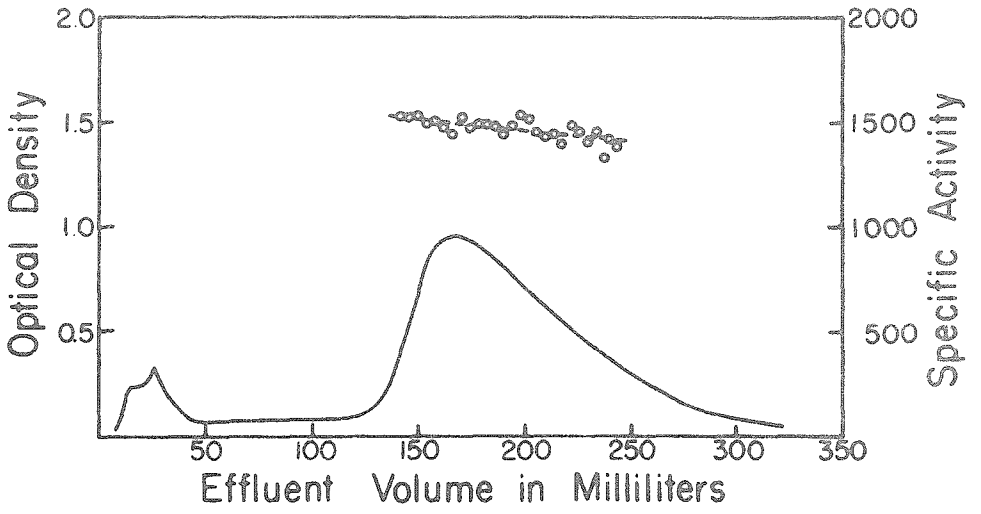


Figure 17a

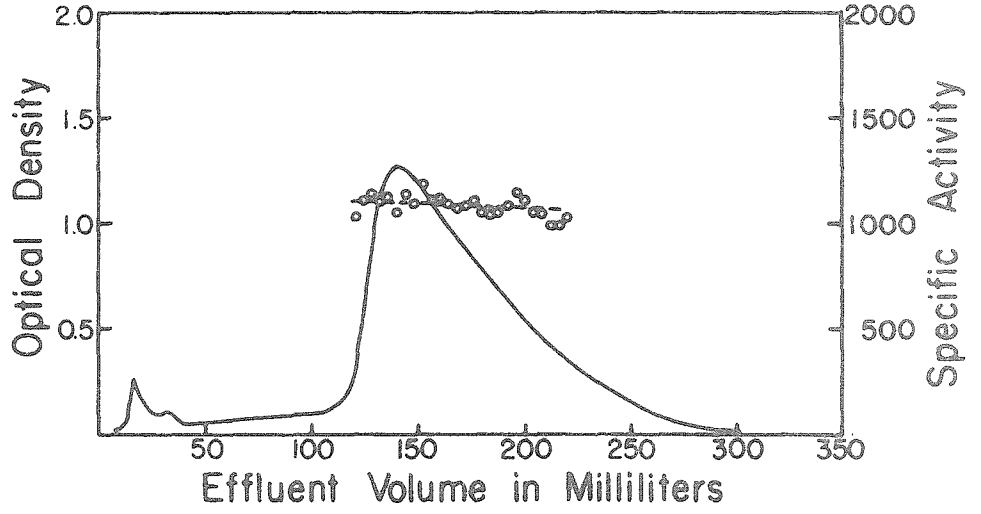


Figure 17b

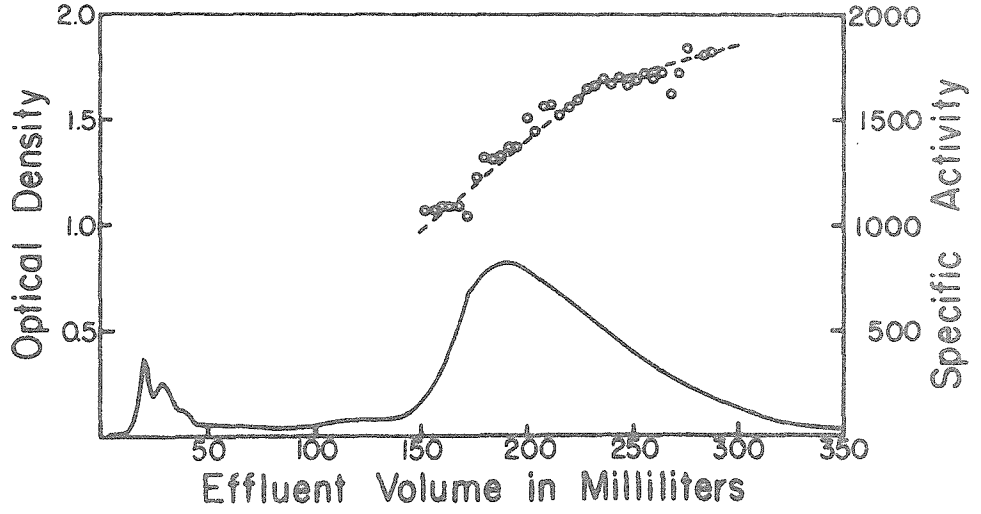


Figure 17c

Figure 17a depicts a chromatogram with Developer No. 2 of a sample containing 7 mg. of normal non-radioactive hemoglobin A and 8 mg. of radioactive hemoglobin A_{II}. It is apparent that the radioactivity is uniformly distributed throughout the A_{II} zone in this control chromatogram. Figure 17b illustrates the chromatography of a mixture containing 7.0 mg. of the "A^{H}_{II}" component from a patient with thalassemia hemoglobin H disease and 7.0 mg. of radioactive hemoglobin A_{II}. The slope of the specific activity curve in this chromatogram is essentially identical with the control shown in Figure 17a. It is concluded that the ferrihemoglobin cyanide form of the A^{H}_{II} component from this patient is chromatographically identical with the normal A_{II} component.

Figure 17c is of a chromatogram of a mixture of 7 mg. of hemoglobin from a lowland gorilla (Gorilla gorilla) (45) and 7 mg. of radioactive hemoglobin A_{II}. The slope of the specific activity curve is significantly different from the horizontal slope of the control, Figure 17a. Since the mixture of gorilla and radioactive A_{II} hemoglobins were both in the ferrihemoglobin cyanide form, it is concluded that the non-uniform distribution of radioactivity in the main zone indicates that the two hemoglobins have slightly different chromatographic mobilities. Because of the positive sign of the slope of the specific activity curve, it appears that the gorilla hemoglobin moves at a faster rate than the normal adult hemoglobin A_{II}. It is interesting to speculate

that this difference in chromatographic behavior may be due to differences in the chemical structure of the normal human hemoglobin and this particular gorilla hemoglobin.

The relative chromatographic mobilities of several other hemoglobin components were also studied by this radioactive label technique. It was possible to demonstrate that the S_{Ie} zone from a typical sickle-cell anemia individual was chromatographically identical with radioactive hemoglobin F_{II} component. The very slow mobility of this S_{Ie} component in the earlier chromatographic studies appears to have been due to the influence of the load and the conversion to the ferrihemoglobin cyanide form. The main fetal component from two different new born sickle-cell anemia infants was demonstrated to be chromatographically identical with the normal hemoglobin F_{II}.

Differences in the chromatographic behavior of the ferrihemoglobin cyanide forms of components A_{Ic} and F_{II} were also demonstrated by this radioactive technique. Although the peaks of the two components had similar mobilities, the distribution of hemoglobin about the peaks of the two components was different. The F_{II} component was eluted as a relatively narrow band as compared to the A_{Ic} component which appeared to migrate as a broad band.

SECTION II. CHEMICAL STUDIES OF SEVERAL HEMOGLOBIN COMPONENTS

INTRODUCTION

Rapid advances in our knowledge of the complete chemical structure of the hemoglobin molecule are currently being made. X-ray analyses of crystalline horse ferrihemoglobin by Perutz and his collaborators (46) have led to a clarification of much of the secondary and tertiary structure of this molecule and may, with further work, yield information about primary structure. The X-ray data indicate that the molecule is comprised of two identical subunits, each containing two separate polypeptide chains, which in turn are folded around single heme groups. Many details of the exact amino acid sequence of each of these chains of human hemoglobins A and F based upon chemical degradative procedures are becoming available at this time (7,8,9,10,11). With this structural information as a frame of reference, the elucidation of the chemical structure of the many hemoglobin components has now become practical. The object of this second section is to present chemical studies which have been useful in determining several features about the chemical structure of a number of hemoglobin components.

Profound structural differences between hemoglobins A and F can be demonstrated by studies of their amino acid composition and sequence (8). The genetic alteration of a single residue, as in the case of hemoglobins S and A,

exemplifies a minimal change in primary structure as a basis of heterogeneity. Structural bases of the minor component heterogeneity may involve these two extremes as well as intermediate differences in the primary structure. However, alteration of primary structure may not be the only cause of minor component heterogeneity. The presence of reactive side groups which can be acylated, oxidized, complexed with metals, or combined with other molecules also may be responsible for heterogeneity of hemoglobins.

A number of approaches which stop short of determining the complete amino acid sequence of each hemoglobin are available for demonstrating structural similarities and differences between many hemoglobin components. These studies include the determination of: amino acid composition, N-terminal amino acid sequence, patterns of peptides from tryptic hydrolysates, ultraviolet spectra, sedimentation coefficients and molecular dissociation, and subunit hybridization. When such data about any hemoglobin are compared with similar data from hemoglobins A and F, any hemoglobin can be stated to resemble either fetal hemoglobin or normal adult hemoglobin (or a genetic variant such as hemoglobin S).

The basic analytical procedure for the study of the primary structure of proteins and peptides is the determination of the amino acid composition. A number of determinations of the amino acid composition of hemoglobins have been made by many groups (8,47,48,49,50,51) by several chromato-

graphic techniques. These results have been reviewed carefully by Schroeder (7,8) and only a few comments concerning the previous amino acid analyses of adult and fetal hemoglobins are necessary for the present study. The amino acid compositions of hemoglobins A and F differ appreciably in a number of amino acids, particularly in isoleucine, which is present in hemoglobin F but absent from hemoglobin A. Also in fetal hemoglobin, as compared to adult hemoglobin, there is more methionine, serine, and threonine but less alanine, histidine, proline, and valine. These and other chemical differences between hemoglobins A and F as well as certain genetic studies were the basis of Itano's earlier conclusion that hemoglobins A and F are quite different chemically and probably under separate genetic control (35). However, as it will be shown later, the α chains of hemoglobins A and F are very similar if not identical; therefore, the difference in amino acids composition between these two hemoglobins must exist entirely in the β and γ chains.

With the development of automatic equipment for the chromatographic determination of amino acids, a study of small amounts of various components has become practical (52). In this way, it is possible to classify the various components into a fetal or an adult group on the basis of their amino acid composition without the requirement of a large amount of protein.

Sanger's dinitrofluorobenzene method for the determination of N-terminal amino acids has frequently been utilized as one of the first procedures in the study of proteins and peptides. Extensive application of this method to the study of hemoglobin A resulted in the clarification of the gross structure of this molecule in terms of the variety and number of polypeptide subunits (53,43,55,56). A similar study of the N-terminal residues of hemoglobin F also indicated the nature of its gross polypeptide structure and served as the first experimental evidence of the possible structural similarity of hemoglobins A and F (12).

Sanger's procedure is based upon the reaction of dinitrofluorobenzene (DNFB) with the free amino groups of the protein to produce a yellow dinitrophenyl protein (DNP-protein). The DNP-protein is then subjected to acid hydrolysis under conditions which will cleave the peptide bonds but not the bond between the DNP-group and the amino nitrogen. Thus the hydrolysate will contain a number of amino acids and, in some cases, peptides all of which are labelled with one or more DNP groups. Through extraction and chromatographic procedures, these DNP-compounds can be isolated and identified. Any amino acid or peptide containing a DNP-group combined with an α amino nitrogen must necessarily have been in a N-terminal position of the protein.

Through careful application of Sanger's method, Rhinesmith, Schroeder, and coworkers (53,54,55) and Braunitzer (56),

independently, concluded that the normal adult hemoglobin has four N-terminal valyl residues per molecular weight of 66,000. From an examination of the α amino DNP-peptides which were isolated under conditions of incomplete hydrolysis, these authors concluded that two of the chains have the N-terminal sequence val-leu and the other two have the sequence val-his-leu. Schroeder and Matsuda found two N-terminal val-leu sequences and two N-terminal glycyl residues per mole of fetal hemoglobin (12).

The first terminology of the polypeptide chains in hemoglobins was based upon this N-terminal sequence work. Thus the val-leu peptide was designated as the α chain, the val-his-leu peptide as the β chain, and the glycyl peptide as the γ chain (7). By combining these symbols with superscripts to designate the type or source of the hemoglobin and subscripts to show the number of chains, gross polypeptide formulas can be written for shorthand purposes. The gross structural formula of hemoglobin A_{II} is $\alpha_2^A\beta_2^A$ and that of hemoglobin F is $\alpha_2^F\gamma_2^F$. Recently the basis of this terminology has been extended to include the entire polypeptide chain and genetic data, not just the N-terminal sequences (see Appendix No. II and ref. 57).

The "fingerprint" or peptide pattern method of Ingram (13,14) is a useful procedure for rapidly obtaining qualitative information about the internal sequences of much of the polypeptide chains of hemoglobins. It was first utilized by

Ingram to demonstrate a difference in the primary structure of hemoglobins A and S and has now become a useful tool for structural studies of many other hemoglobins (58).

The Ingram procedure involves a heat denaturation of the hemoglobin followed by a tryptic hydrolysis at pH 8.0 which converts about two thirds of the protein to soluble peptides. These peptides are separated into a two dimensional array by a combination of paper electrophoresis followed by paper chromatography. The peptides are then reacted with ninhydrin to produce a pattern of colored spots typical of each hemoglobin.

Ingram's fingerprint studies not only showed the difference between hemoglobins A and S, but also indicated that each of these hemoglobins is comprised of identical half molecules. This conclusion is based upon a knowledge that trypsin's hydrolytic action is limited essentially to the splitting of peptide bonds involving carboxyl groups of lysine and arginine. Although there are about 56 of these residues per molecule, only 26 different peptides were observed on the peptide patterns. Thus if the hydrolysis of all of the lysine and arginine residues was fairly complete, only a molecule which was comprised of two identical halves would be expected to yield but one half of the maximum number of peptides. This interpretation is also in agreement with the N-terminal sequences, X-ray data (59) and hybridization results (60,61,62).

The peptide pattern technique of Ingram has been applied in this Thesis work to compare the structure of a number of hemoglobin components in order to separate them roughly into groups which indicate the kinds of polypeptide chains present.

By an examination of the ultraviolet spectra in the region of 290 mμ, an estimate of the content of tryptophan can be made. The spectral curve of normal adult hemoglobin has an inflection at 289 mμ, whereas the curve for fetal hemoglobin has a small maximum at 290 mμ, and a minimum at 289 mμ (63). Both hemoglobins have a high and broad absorption maximum with a peak at 275 mμ that results from the presence of aromatic amino acids: it is on a slope of this peak that the maximum at 290 mμ is superimposed. The difference in the spectral curves of hemoglobins A and F in the region of 290 mμ is considered to be due primarily to the tryptophan absorption, although a decrease in the tyrosine content of fetal hemoglobin may serve to accentuate the contribution of tryptophan at 290 mμ. Again, as in the case of the determination of the amino acid composition and the study of peptide patterns, an examination of the ultraviolet spectra of hemoglobins is useful in classifying hemoglobin components into fetal and adult groups.

Any gross structural formula proposed for a hemoglobin component depends upon at least an approximate knowledge of the molecular weight of the protein. Reliable determinations of the molecular weight of normal adult hemoglobin have been

obtained from ultracentrifugal studies (64). Although subject to errors, an estimation of the molecular weight of different hemoglobins merely by the determination of their sedimentation coefficients has become a useful, rapid method which utilizes relatively small amounts of material. It has been suggested by Field and O'Brien (65) and Hasserodt and Vinograd (66) from examination of sedimentation coefficients of adult hemoglobin in solutions over a pH range of 2.5 to 11.5, that a dissociation of adult hemoglobin into half molecular subunits occurs in acid and alkaline solutions. Therefore, estimation of the sedimentation coefficients of certain hemoglobin components was made for two purposes: first to establish the correct molecular formula and second to determine whether dissociation similar to that of hemoglobin A occurs.

A useful technique for studying the polypeptide subunit composition of hemoglobins is the hybridization or chain exchange procedure developed by Singer and Itano (60) and Vinograd and Hutchinson (62). The procedure involves the dissociation of a mixture of two different hemoglobins into subunits in acid or alkaline solution followed by a reassociation and separation of resulting products by electrophoresis or chromatography. An examination of the products reveals whether the original hemoglobins contained subunits which exchanged for one another during the dissociation stage.

Singer and Itano employed various oxidation forms of hemoglobins in their study whereas Vinograd and Hutchinson utilized hemoglobins labelled with C-14 amino acids. As demonstrated by Vinograd, Hutchinson, and Schroeder (61) the latter technique yields information which can be correlated to the N-terminal sequences of the polypeptide chains so that an absolute identification of identical subunits can be made. Their study of hemoglobins A and S led to the conclusion that the β chain of hemoglobin S possesses the amino acid substitution noted earlier by Ingram (13). Several applications of the hybridization procedure have been made in this Thesis work; some of the experiments will be described in detail.

SECTION II. EXPERIMENTAL PROCEDURES

Isolation of Hemoglobin Components.--All of the hemoglobin components studied in this section were isolated and purified by chromatographic procedures which are described in experimental part of Section I. An additional step in the purification of two components (the γ_4 component from hemoglobin H subjects and the A_2 component from normal adults) was made by starch block electrophoresis. These starch block purifications were carried out by Dr. William Bergren and his assistant, Mrs. Helen Fry, at the Children's Hospital in Hollywood, California. The method employed was essentially that described by Schnek and Schroeder (18) using barbital

buffers of 0.05 ionic strength at pH 8.6 and commercial potato starch.

Amino Acid Analysis.--Hemoglobin solutions were dialyzed exhaustively against distilled water at 4° C in order to remove all traces of salts. A portion was then placed in a 13 x 100 mm. pyrex tube, the water was removed at 90° C, and the residue was dried to constant weight in an oven at 110° C. After adding 2 ml. of doubly glass-distilled 6.0 N hydrochloric acid to amounts of 2 to 10 mg. of hemoglobin per tube, the air was evacuated with a water pump and the tubes were sealed and heated at 110 ± 0.5° C for periods of either 22 or 70 hours. Following hydrolysis, the hydrochloric acid was removed by evaporating at 40° C with a stream of air, and the hydrolysate was dissolved in 5.00 or 10.00 ml. of the pH 2.2 buffer solution which is used for applying samples to the ion-exchange columns (52). A small stirring rod was used to remove all traces of material from the sides of the hydrolysis tube and to produce a fine suspension of all insoluble material which resulted from the heme. The sample was centrifuged immediately before removing appropriate aliquots for amino acid analyses.

The amino acid analyses were made with a Spinco Automatic Amino Acid Analyzer, Model 120, following the procedure of Spackman, Stein and Moore (52). In this procedure, the amino acids are separated on columns of IR-120 (a sulfonated polystyrene ion-exchange resin) with sodium citrate

buffers. The effluent from the columns is then automatically measured and recorded for subsequent calculation of the quantity of each amino acid. The precision and accuracy of this procedure is of the order of 100% ± 3% for loads from 0.1 to 3.0 micromoles of each amino acid.

N-Terminal Amino Acid Sequence Analysis.--Hemoglobin components were dinitrophenylated in aqueous solutions with dinitrofluorobenzene (53) at a pH of 8.1 ± 0.1 which was maintained by the addition of dilute sodium hydroxide either manually with a syringe microburet (67) or automatically with a Radiometer Automatic Titrator used as a pH-stat. The DNP-hemoglobin was precipitated at pH 1.5 by adding 6 N hydrochloric acid and the precipitate was washed three times with 0.2 N hydrochloric acid. For removal of the heme in the DNP-hemoglobin, the precipitate was suspended in 20 ml. of an ice-cold solution of 0.05 N hydrochloric acid which was then poured into an ice-cold mixture of 100 ml. of acetone and 2.0 ml. of N hydrochloric acid. This mixture was shaken mechanically for 1 to 3 hours and then the yellow DNP-globin, in the form of a fine precipitate, was allowed to settle overnight in the refrigerator. The settled precipitate was transferred to a fritted glass suction funnel on which it was washed exhaustively with acetone and finally with ether.

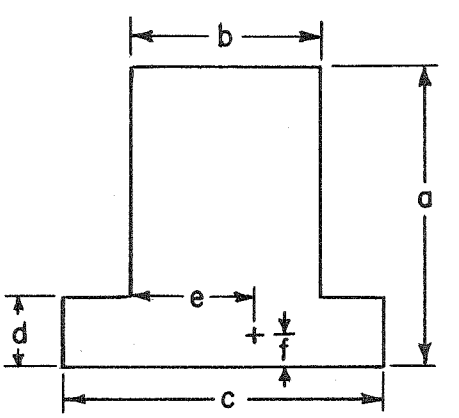
The isolation and determination of the N-terminal residues in the DNP-globin were carried out by Mrs. Joan

Balog Shelton according to the procedures described by Rhinesmith et al. (53,54,55). After the DNP-globin had been hydrolyzed for 15 minutes in refluxing 6 N hydrochloric acid, extraction with ether removed DNP-val-leu, DNP-val, DNP-gly, and dinitroaniline (DNA) from the hydrolysate and extraction with ethyl acetate removed DNP-val-his-leu and DNP-val-his. The DNP-derivatives in the extracts were then separated and characterized chromatographically with silicic acid columns by methods described by Green and Kay (68) as extended by Schroeder and co-workers (55,69). Quantitative measurements were made spectrophotometrically. Complete hydrolyses of the DNP-peptides were carried out for 22 hours in some cases to be certain of their amino acid composition.

Peptide Pattern Analysis.--These analyses were carried out essentially as described by Ingram (13,14) with only minor modifications during the hydrolytic and chromatographic procedures. Hemoglobin solutions were dialyzed against distilled water and then diluted to a volume of about 4 to 5 ml. so that the final concentration ranged from 2 to 25 mg. per ml. After adjusting the pH to 8.0 $\pm$ 0.2 with dilute sodium hydroxide, the hemoglobin was denatured at 90° C for 4 minutes. The denatured hemoglobin was hydrolyzed at 40° C by adding 0.1 ml. of a 0.5% solution of trypsin (2 times crystallized, salt-free, Worthington) in 0.001 N hydrochloric acid while maintaining the pH at 8.0 $\pm$ 0.1 by adding dilute sodium hydroxide either

automatically with a pH-stat (Radiometer) or manually from a syringe microburet (67). One tenth of 1 ml. of 0.4 M $CaCl_2$ was added to the reaction mixture just before the trypsin as suggested by Professor Niemann (69). The hydrolyses were generally carried out for 90 minutes, but additional trypsin was added at 60 minutes to be sure that inactivation of the original enzyme had not occurred. Following the hydrolysis, the mixture was adjusted to pH 6.5 with dilute hydrochloric acid and the insoluble residue (designated as the core by Ingram) was removed by centrifugation. The supernatant, containing the soluble tryptic peptides, was dried over concentrated sulfuric acid in an evacuated desiccator, or stored at -20° C in a freezer before drying.

Two different procedures were used for the separation of the peptides on sheets of Whatman 3MM paper cut into a T shape as shown in the inserted diagram. In Procedure A, papers with the following dimensions in inches were used: a, 12; b, 11; c, 23; d, $2\frac{1}{2}$; e, 7; f, 1. The more successful Procedure B was made on papers with the following dimensions in inches: a, $19\frac{1}{2}$; b, 15; c, 23; d, 5; e, 10; f, $2\frac{1}{2}$. Pairs of papers were either



dipped into or sprayed with electrophoresis buffer (pyridine 50 parts: water 450 parts: glacial acetic acid 2 parts, adjusted to pH 6.40 ± 0.02) and then placed on a one-quarter-inch glass plate which was 1 inch larger than the "a" and "b" dimensions of the papers. Excess buffer was blotted from the papers and the peptides from 2.2 mg. of hemoglobin were applied in 10 microliters of water to the cross mark shown in the diagram after first heavily blotting a surrounding area of about 1 square inch. The papers were then covered with a second glass plate and fifteen minutes of equilibration were allowed before applying an electrical field along the "c" dimension of the papers. In Procedure A, a field of 600 volts was applied for $3\frac{1}{2}$ hours whereas for Procedure B, a field of 1200 volts was applied for $2\frac{1}{2}$ hours. After the electrophoresis, the papers were dried for at least 2 hours in the air before chromatography with one of two solvent systems. Ascending chromatography using the original solvent described by Ingram (13) (n-butanol 3 parts: glacial acetic acid 1 part: water 1 part by volume) was used for Procedure A. Procedure B used descending chromatography with a developer consisting of 7 parts iso-amyl alcohol: 7 parts pyridine: 6 parts water (71,72). The second solvent system was found to be superior to the first system. A modification of the second solvent (7 parts iso-amyl alcohol: 8 parts pyridine: 6 parts water: 0.1 parts formic acid) was useful for chromatographing the more strongly adsorbed peptides.

Detection of the peptides was generally made by dipping the dried papers into a 0.5% solution of ninhydrin in acetone and allowing the color to develop for at least 2 hours at room temperature before heating in an oven at 60 to 80° C for 3 to 5 minutes. Several special reactions were also employed (73). For the detection of tryptophyl peptides, the papers (either treated or untreated with ninhydrin) were sprayed with a solution of 0.4% 4-dimethylaminocinnamaldehyde in ethanol containing 10% 6 N hydrochloric acid (by volume). Arginyl peptides were detected by first dipping in a solution of 0.1% 8-hydroxyquinoline in acetone, drying and then spraying carefully with a solution of 0.2 ml. bromine in 100 ml. of 0.5 M sodium hydroxide in water. Histidyl peptides were localized by spraying with a fresh mixture of equal parts of 0.7% sodium nitrite and 1.0% sulfanilic acid containing 8% concentrated hydrochloric acid, drying until the paper was moist, and then spraying with 10% sodium carbonate. The histidine reaction could be performed after the ninhydrin reaction had been made.

Ultraviolet Spectroscopy.--The ultraviolet spectrum in the region of 250 to 320 mμ of hemoglobin solutions in water or chromatographic developers was examined with a Cary Recording Spectrophotometer, Model 11M or 14. The optical density for an optical path length of 1 cm. was recorded.

Determination of Sedimentation Coefficients.--The measurement of sedimentation coefficients was carried out in a Spinco Analytical Ultracentrifuge, Model E, by Mrs. Janet Morris under the supervision of Dr. J. R. Vinograd. Hemoglobin solutions at a concentration of 0.5 to 0.8% were run in a double-cell rotor so that one of the cells could be filled with normal adult hemoglobin which had been treated in the same manner as the sample under study (66). The composition and pH of the buffer solutions which were tested for the sedimentation experiments are listed in Table VII.

TABLE VII

Buffer Solution for Sedimentation Studies

pH at 25°	Composition in grams per liter of solution
4.70	27.2 g. $NaC_2H_3O_2$ adjusted to pH 4.7 with $HC_2H_3O_2$
7.07	6.54 g. $K_2HPO_4 \cdot 3H_2O$; 1.94 g. KH_2PO_4
9.7	3.0 g. NaOH; 10.2 g. NaCl; 7.7 g. glycine
10.25	3.0 g. NaOH; 10.2 g. NaCl; 7.2 g. glycine
10.50	3.0 g. NaOH; 10.2 g. NaCl; 6.5 g. glycine
10.70	3.0 g. NaOH; 10.2 g. NaCl; 6.0 g. glycine
10.91	3.0 g. NaOH; 10.2 g. NaCl; 5.8 g. glycine
11.61	6.35 g. $Na_3PO_4 \cdot 12H_2O$; 4.68 Na_2HPO_4; 8.78 g. NaCl

Procedures for Subunit Hybridization.--Hybridization experiments were carried out at 4° at either pH 4.60 to 4.70 or pH 11.0 to 11.2. For the hybridizations at low pH, solutions either 0.1 or 0.2 M in sodium acetate adjusted with glacial acetic acid to the proper pH were used. Hybridization experiments at high pH were carried out in solutions containing 6.35 g. trisodium phosphate dodecahydrate, 4.68 g. anhydrous disodium phosphate and 8.78 g. sodium chloride per liter. Hemoglobin mixtures in concentrations ranging from 1 to 50 mg. per ml. were dialyzed against two changes of buffer over a period of 12 to 48 hours and then dialyzed back against at least 2 changes of an appropriate chromatographic developer. These hemoglobin solutions from the hybridization procedure were often concentrated by centrifugation before the chromatographic separations were made. Any precipitates which were formed during the hybridization procedure were centrifuged off before the chromatographic separation was carried out.

SECTION II. RESULTS AND DISCUSSIONS

A. Amino Acid Analyses

Amino acid analyses of normal hemoglobin components A_{Ia}, A_{Ib}, A_{Ic}, A_{II}, and A_{IIIb} are listed in Table VIII. An analysis of the fetal component F_{II} has also been listed for reference purposes (8). The determinations were made on samples which had been hydrolyzed with 6 N hydrochloric acid for either 22 or 70 hours (in one case for 72 hours, see Table VIII). The value of each amino acid is recorded in grams of that amino acid per 100 grams of protein dry weight. The percentage of total recovery for each analysis was calculated by determining the sum of the composition in terms of grams amino acid residues (molecular weight of amino acid minus weight of one mole of water) per 100 grams of protein.

Because the percentage of total recovery varied from 79 to 93%, a comparison of the absolute values of any amino acid between two different experiments can be misleading. The low and varied recoveries were probably due to several factors including: total loss of tryptophan and partial loss of serine, threonine, and tyrosine; incomplete hydrolysis of some valyl bonds; weight of heme; presence of traces of salts and water that were not removed completely; and the precision of the weighing and the analytical methods.

TABLE VIII

Amino Acid Composition of Hydrolysates of Normal Human Hemoglobin Components

Values are given in g. of amino acid per 100 g. protein

	A_{Ia}	A_{Ib}	A_{Ic}			A_{II}			A_{IIIb}	F_{II} Schroeder, et al (8)	
	22 hrs.	22 hrs.	22 hrs.	22 hrs.*	70 hrs.*	22 hrs.+	22 hrs.+	72 hrs.+	22 hrs.	22 hrs.±	70 hrs.±
Ala	9.72	7.86	9.25	10.13	9.94	8.93	8.56	8.68	8.64	9.09	8.78
Arg	3.45	3.31	2.96	3.10	2.99	2.94	2.90	3.78	3.27	3.25	3.12
Asp	10.66	9.93	9.84	10.48	10.14	9.67	9.59	9.06	9.63	10.48	10.10
Cys/2	1.07	0.93	1.03	1.35	1.24	0.62	0.55	0.77	0.78	0.70	0.53
Glu	8.00	7.91	7.23	7.51	7.26	6.83	6.57	6.50	6.83	7.99	7.68
Gly	4.73	4.49	4.56	4.77	4.63	4.35	4.12	4.11	4.05	4.59	4.46
His	8.55	7.33	8.23	8.49	8.35	7.87	8.20	7.95	7.07	8.13	7.99
Ileu	0.42	1.08	0.04	0.03	0.03	<0.02	<0.02	<0.02	<0.02	1.54	1.52
Leu	14.58	12.80	13.79	14.60	14.27	13.62	12.90	12.66	12.62	14.36	13.94
Lys	9.64	8.97	8.79	9.01	8.95	8.27	8.89	8.53	8.37	10.15	10.04
Met	1.52	1.23	1.33	1.36	1.33	1.28	1.21	0.84	1.53	1.84	1.87
Phe	7.58	6.95	7.21	7.68	7.45	7.22	7.05	6.67	6.74	7.74	7.46
Pro	5.28	4.96	4.76	4.97	4.82	4.77	4.54	4.45	4.11	4.06	4.02
Ser	4.67	4.15	4.08	4.35	3.41	4.17	4.12	3.06	4.13	6.19	4.57
Thr	5.59	4.91	5.21	5.66	5.00	5.22	4.93	4.48	4.24	6.71	5.77
Tyr	2.96	3.30	2.80	3.11	2.81	2.19	2.87	2.07	2.45	2.57	2.28
Val	10.59	8.88	10.13	10.94	11.08	9.96	9.56	9.71	8.99	9.45	9.31
Recovery	93%	85%	87%	92%	89%	84%	82%	79%	81%	93%	89%

*Same sample and weight of A_{Ic} for both analyses.

+Same sample and weight of A_{II} for both analyses.

±Same sample and weight of F_{II} for both analyses.

Although the absolute values of the amount of many amino acids appear to differ significantly from one analysis to another, relative values that take the percentage of total recovery into account, are generally very similar. Differences between the amino acid composition of hemoglobin A and F have been noted in the Introduction of Section II and a comparison of these with the differences of the analyses of A_{II} and F_{II} in Table VIII indicates the reliability of these determinations. In general, except for isoleucine, methionine, proline, serine, threonine, and possibly histidine, differences between the analyses of these two hemoglobins do not appear to be significantly greater than the variations of the analyses of the same component. However, the detection of one or more residues of isoleucine for molecular weight of 66,000 (greater than 0.2 grams per 100 grams protein) is a reliable and significant amino acid estimate, and it may be used in comparing several different components.

In component A_{Ia} isoleucine is present to the extent of about two residues per 66,000 molecular weight. Whether this amino acid is actually present in component A_{Ia} is uncertain because it may have had its origin in non-heme proteins which were incompletely removed by the chromatographic isolation. Allen, Schroeder, and Balog (15) have found that this non-heme protein has a high content of isoleucine.

Component A_{Ib} shows an even greater content of isoleucine. About six residues per 66,000 molecular weight are present. In this instance it is less reasonable to postulate a contamination of component A_{Ib} with non-heme protein because as far as is known this component separates readily from the non-heme proteins. The presence of about six residues of isoleucine in A_{Ib} suggests that this component may differ in its amino acid sequence both from component A_{II} and F_{II}. Further determinations of the amino acid composition of both components A_{Ia} and A_{Ib} will be necessary before the significance of the presence of isoleucine can be judged with confidence.

Only traces of isoleucine were detected in component A_{Ic}. This is in contradiction to the results of Allen, Schroeder, and Balog (15) who reported the presence of substantial amounts of isoleucine in this component. The trace amounts of isoleucine in the present analysis could be reduced even further by first denaturing the A_{Ic} with alkali and precipitating it with ammonium sulfate before hydrolyzing it with hydrochloric acid. This suggests that the A_{Ic} normally contains some F_{II} which is not denatured by alkali and precipitated by ammonium sulfate, which contains isoleucine, and which by this procedure is then separated from the A_{Ic}. It is possible that the isoleucine observed by Allen et al. was due to the presence of a small amount of component F_{II} which is chromatographically similar

to component A_{Ic} under the conditions used for isolation (see Section I). Varying amounts of hemoglobin F have been reported in alkali denaturation studies of hemoglobin from normal adults (2).

Comparisons of the other amino acids found in the analyses of components A_{Ia}, A_{Ib}, and A_{Ic} with the values of the amino acids in hemoglobin A_{II} and F_{II} indicate that these minor components are more similar in composition to A_{II} than to F_{II}. More analyses will be necessary before the significance of any differences can be stated with confidence.

As in the case of amino acid analyses of component A_{Ic}, the amino acid composition of component A_{IIIb} appears to be very similar to the composition of component A_{II}. Failure to detect isoleucine in component A_{IIIb} might appear to be a contradiction of the recent amino acid analysis of hemoglobin A_2 by Rossi-Fanelli et al. (74). According to their analysis, a considerable difference exists in the amino acid composition of hemoglobin A_2 and hemoglobin A, especially in the isoleucine content. However, Schnek and Schroeder (18) found as did Kunkel et al. (75) that component A_2, which corresponds to component A_{IIIb}, is often contaminated by appreciable amounts of a non-heme component and suggested that the analysis of A_2 by Rossi-Fanelli et al. was in reality the analysis of a mixture of A_2 and non-heme protein. The validity of Schnek and Schroeder's conclusion is apparent

from a series of amino acid analyses first of the "crude" hemoglobin A_2 prepared by starch block electrophoresis and then of the non-heme protein and the A_{IIIb} component isolated by chromatography from this sample of A_2. These results and the average of four determinations of A_2 by Rossi-Fanelli et al. (74) are shown in Table IX. The presence of this non-heme protein not only will give rise to incorrect values for isoleucine but also will result in incorrect estimates of alanine, arginine, glutamic acid, histidine, leucine, lysine, phenylalanine, tyrosine, and valine.

The amino acid composition of the components isolated from a patient with thalassemia hemoglobin H disease (see figs. 12 and 13) is listed in Table X.

The analysis of the heme component isolated by starch block electrophoresis from zone 1a (fig. 13) is more like the composition of F_{II} than A_{II} but not identical with either. The results are in agreement with other chemical studies that indicate that this component is a tetramer of γ chains. This conclusion will be discussed more completely below.

The presence of a small amount of isoleucine in zone 1b (less than 0.5 residue per 66,000 molecular weight) was probably due to the incomplete chromatographic separation of this component from zone 1a or to the presence of a small amount of non-heme protein. Although Huisman has reported that

TABLE IX

Amino Acid Composition of Hydrolysates of Components A_2, A_{IIIb} and Non-Heme Protein from A_2

Values are given as g. of amino acid per 100 g. protein

Amino Acid	A_2 Rossi Fanelli, et al. (74)	Crude A_2 from starch electrophoresis	Non-Heme Protein from Chromatograms of A_2	A_{IIIb}, Heme-Component from Chromatograms of A_2
Ala	7.584	8.80	5.37	8.64
Arg	3.499	3.58	4.75	3.27
Asp	10.885	10.21	10.83	9.63
Glu	7.615	7.61	10.32	6.83
Gly	4.178	4.28	4.16	4.05
His	5.761	7.38	3.81	7.07
Ileu	1.114	0.51	3.63	0.02
Leu	11.736	12.76	8.06	12.62
Lys	8.595	8.83	6.24	8.37
Met	1.383	1.59	1.71	1.53
Phe	6.596	6.82	5.53	6.74
Pro	4.049	4.62	5.28	4.11
Ser	6.026	4.39	5.10	4.13
Thr	4.225	4.68	4.13	4.24
Tyr	2.818	2.70	5.53	2.45
Val	7.374	9.14	6.00	8.99
Recovery		85%	77%	80%

TABLE X

Amino Acid Composition of Hydrolysates of Components from Thalassemia Hemoglobin H Disease

Values are given in g. of amino acid per 100 g. protein

	γ_4 (Zone 1a, Fig. 13)			β_4 (Zone 16, Fig. 13)			A^H_{Ic} (Zone 2, Fig. 12)	A^H_{II} (Zone 4, Fig. 12)		A^H_{III} (Zone 5, Fig. 12)
	22 hrs.	22 hrs.[a]	70 hrs.[a]	22 hrs.[b]	22 hrs.[b]	70 hrs.[b]	22 hrs.	22 hrs.[c]	70 hrs.[c]	22 hrs.
Ala	6.10	6.34	6.32	7.68	8.13	7.74	8.90	9.45	9.47	9.29
Arg	3.67	3.38	3.32	3.37	3.20	3.20	3.21	3.02	3.47	3.07
Asp	10.34	10.43	10.31	9.97	10.36	10.13	9.51	9.94	9.98	9.61
Cys/2	1.27	0.81	0.72	1.19	1.51	1.22	1.07	0.58	0.76	0.78
Glu	10.45	10.84	10.59	9.42	9.97	9.89	6.91	7.22	7.20	7.15
Gly	5.48	5.61	5.56	5.62	5.82	5.63	4.36	4.52	4.48	4.47
His	6.24	6.11	6.19	8.03	7.88	7.44	8.11	8.28	8.40	7.96
Ileu	3.01	2.89	3.01	0.12	0.15	0.32	0.26	<0.02	<0.02	<0.02
Leu	12.76	13.04	13.05	13.41	14.08	13.73	13.54	14.05	13.89	13.86
Lys	10.05	9.51	9.60	9.07	9.18	8.99	8.64	9.13	9.18	8.73
Met	1.70	1.70	1.68	0.82	0.94	0.93	1.27	1.33	1.31	1.29
Phe	7.60	7.66	7.93	7.65	7.86	7.76	7.10	7.40	7.33	7.28
Pro	3.16	3.16	3.12	4.64	4.94	4.77	4.64	5.09	4.84	4.83
Ser	5.08	5.63	4.40	2.67	2.84	2.29	3.79	4.36	3.29	4.22
Thr	6.27	6.53	5.89	4.56	4.80	4.27	5.12	5.36	4.77	5.17
Tyr	2.29	2.10	2.12	2.40	2.63	2.58	1.96	3.13	2.87	2.52
Val	8.84	8.84	9.12	11.51	11.83	12.01	9.98	10.46	10.93	10.18
Recovery	89%	90%	88%	87%	91%	88%	84%	88%	88%	86%

[a] Same sample and weight of γ_4 for both analyses.

[b] All three analyses of β_4 were of different samples and different weights.

[c] Same sample and weight of A^H_{II} for both analyses.

significant amounts of isoleucine are present in hemoglobin H, his preparations were probably not pure β_4 component (36). Disregarding the isoleucine, the analyses of this component in zone 1b (fig. 13) are similar but not identical to component A_{II}. As will be shown by other studies, this component is a tetramer of normal β chains.

Zone 2 from Figure 12 appears to be comprised mainly of A_{Ic} as judged by its amino acid content. However, the amount of isoleucine detected indicates that about one sixth of this zone may have been component F_{II}. This observation is not surprising because the patient was producing γ chains even though she was past the age at which little or no hemoglobin with γ chains (F) is observed in the normal human (2).

No significant differences in the amino acid composition of zone 4 (A^H_{II}) and zone 5 (A^H_{III}) from Figure 12 could be detected with certainty when compared to the normal components A_{II} and A_{IIIb}, respectively.

The amino acid composition of the α, β, and γ chains may be calculated from the amino acid analyses of hemoglobins F_{II} and A_{II} and the main component of hemoglobin H (β_4) because the α chains of hemoglobins A and F are identical (see Appendix IV). Such a calculation has been made and the results are listed in Table XI in terms of amino acid residues per molecular weight; 66,000 for components F_{II} and A_{II} and 16,500 for single chains. The first three columns in

TABLE XI

Amino Acid Composition of Hydrolysates of Components F_{II} and A_{II} and the α, β, and γ Chains

Values are in residues per molecular weight

Amino Acid	Experimentally Determined Compositions of Hemoglobin Components						Estimates of Composition of Chains from Literature		
	F_{II} [a]	A_{II} [b]	$1/4\beta_4$ [c]	α [e]	γ [e]	$1/4\,\gamma_4$ [f]	γ [g]	α [h]	β [i]
Ala	64.2	70.2	15.1	20.0	12.1	11.6	11	21	16
Arg	11.8	12.3	3.0	3.1	2.8	3.3	3	3	3
Asp	50.0	49.4	12.9	11.9	13.2	12.9	13	12	13
Cys/2	3.9	3.8	2.1	0	2.0	1.1	1	1	2
Glu	34.3	32.4	11.2	5.0	12.1	11.9	12	5	11
Gly	38.8	39.6	12.8	7.0	12.4	12.2	12	7	13
His	34.0	35.5	8.4	9.4	7.6	6.6	7	10	9
Ileu	7.6	-	-	-	3.8	3.7	4	0	0
Leu	69.8	70.4	17.8	17.5	17.5	16.3	17-18	18	19
Lys	45.8	41.4	10.4	10.3	12.6	11.0	12	11	11
Met	7.9	5.9	1.0	1.9	2.1	1.9	2	2	1
Phe	29.6	29.5	7.9	6.9	7.9	7.7	8	7	8
Pro	22.8	28.3	7.1	7.1	4.3	4.5	4	7(8)	8
Ser	43.6	31.1	4.9	10.7	11.2	9.9	9-11	11	5
Thr	39.6	31.3	7.0	8.7	12.2	9.5	9-11	9	7
Tyr	10.2	11.9	2.4	3.5	1.6	2.0	2	3	3
Val	51.3	60.3	16.7	13.5	12.2	12.6	13	13	19

a. Analyses from Schroeder, et al. (8).

b. Average of 22 and 70 hrs. hydrolyses of A_{II}^{H}, except for serine and threonine which are extrapolated values.

c. Analysis of 22 hr. hydrolysis of β_4 from Table X in residues per 16,500 molecular weight.

d. $1/2(A_{II}-2\beta)$ = α chain in residues per 16,500 molecular weight.

e. $1/2\,[F_{II}-(A_{II}-2\beta)]$ = γ chains in residues per 16,500 molecular weight.

f. Average of 22 and 70 hr. hydrolysis of γ_4 from Table X in residues per 16,500 molecular weight.

g. Best current estimate of the amino acid composition of γ chain by Schroeder, et al. (8).

h. & i. Estimated from Braunitzer, et al. (11). and (10) and Hill and Konigsberg (9).

Table XI are for F_{II}, A_{II}, and $\frac{1}{4}\beta_4$, respectively. The fourth column represents one half the difference between the compositions of 2β chains and A_{II}, and should correspond to the composition of the α chain. Column five is one half the difference between F_{II} and 2 α chains and is therefore an estimate of the composition of the γ chain. The analysis of the γ_4 component (heme component of zone 1a, fig. 13) from Table X has been listed in a column six on the basis of amino acid residues per single chain for comparison with column five. Finally, the best current estimates of the amino acid composition of the α chain (9,10) β chain (11), and γ chains (8) are listed in the last three columns of Table XI for the purpose of comparison and reference. It is apparent from this table that, in general, the present analyses are slightly low; however, the agreement between these experimental results and the results obtained by others and derived from different materials is good. Hence, the calculations indicate that the analyses of the F_{II}, A_{II}, γ_4 and β_4 components are in agreement with the gross formulas which have been suggested for each of these hemoglobin molecules.

B. N-Terminal Amino Acid Sequences

The results of the determination of N-terminal amino acid sequence of several components are listed in Table XII. Ths characterization and estimation of the DNP-compounds were made by Mrs. Joan Balog Shelton. In addition to the analyses

TABLE XII

Sequences of N-Terminal Amino Acids of Several Hemoglobin Components by the DNP-Method

Values in micromoles of DNP-compound per 0.1 g. of DNP-protein and percentage of yield based on 2.28 micromoles per 0.1 g. DNP-globin

		DNP-compounds From 15-minute and 1-hour hydrolysis					From 22 hours hydrolysis	Percentages of each chain		
Component	Footnotes	val-leu	val	gly	val-his-leu	val-his	val	α	β	γ
A	1	2.00	0.10		1.10	0.40		93	66	
F_{II}	2	1.42	0.30	1.80				98		101
F_{I}	3	1.59	0.24	0.37				80		16
γ_4	4	0.14		1.24	0.12	0.13				54*
	5	0.08		2.24			0.17			98*
β_4	6	0.13	0.16				3.72		170*	
	6	0.15	0.15		1.88	1.11	0.61		164*	
	7	0.15	0.19		1.67	0.96	0.96		158*	
A^{H}_{II}	8	1.79	0.19		1.50	0.72		87	98	
A_{Ic}	9	1.78			0.34	0.26		79	37	
	10	2.11	0.29		0.34	0.32	0.25	105	39	
S_{Ie}	11	2.08	0.10	2.06			0.44	96		91
S_{III}	12	1.67	0.14		1.29	0.72	0.42	80	107	

1. From Rhinesmith, Schroeder, and Martin (55).
2. From Schroeder and Matsuda (12).
3. Unpublished studies by Schroeder and co-workers (76).
4. Crude γ_4 (zone 1a, fig. 13) before starch block purification.
5. γ_4 after purification by electrophoresis on starch block.
6. β_4 corresponds to zone 1b, figure 13 but from subject R.C.
7. β_4 (zone 1b, figure 13) from subject P.T.
8. Corresponds to zone 4, figure 12 from subject P.T.
9. Normal A_{Ic} component.
10. A^{*}_{Ic} from hybridization of A_{Ic} and S^{*}_{II} (see Tables XV & XVI).
11. S_{Ie} from sickle-cell hemoglobin.
12. S_{III} from sickle-cell hemoglobin.

*If four of the same kind of chains are present, theoretical yield should be 200% rather than 100%.

of components γ_4, β_4, A^H_{II}, A_{Ic}, S_{Ie}, and S_{III}, the table includes typical values for hemoglobin A, F_{II}, and F_I. The data have been presented as micro moles of DNP-derivatives per 100 mg. of DNP-globin in the left side of the table. The percentage of yield of each kind of chain has been calculated on the basis of the theoretical yield of 2.28 micro moles of each chain per 0.1 grams of DNP-globin and by assuming that DNP-val-leu and DNP-val found after the short hydrolysis (15 minutes to 1 hour) arise from the α chain; that DNP-val-his-leu and DNP-val-his, found after the short hydrolysis and DNP-val found after the 22-hour hydrolysis arise from the β chain; and that DNP-glycine arises from the γ chain. These percentages are listed in the right side of Table XII and should be 100% for any chain which is present in the ratio of 2 moles chain to 1 mole of hemoglobin (66,000 molecular weight).

Two different analyses of the γ_4 component from patient P.T. who has thalassemia hemoglobin H disease (corresponding to zone 1a, fig. 13) were made. The first analysis was with material purified only by chromatography whereas the second was with material purified by both chromatography and starch block electrophoresis. Only DNP-glycine was obtained in significant yields; however, the amounts were 27 and 49% of the theoretical yield assuming 4.56 micro moles of chain per 100 mg. DNP-protein (54 and

98% based upon 2.28 micro moles). Relatively low yields of DNP-glycine have also been noted for analysis of γ chains isolated from component F_{II} by Schroeder and co-workers (76). The reason for these poor yields is unknown.

Three analyses have been made of the β_4 component (zone 1b, fig. 13) from two different patients with thalassemia hemoglobin H disease (from both subjects R.C. and P.T.). DNP-val-his-leu and DNP-val-his were the principle compounds to be isolated from this hemoglobin component. The yields of these DNP-peptides and the absence of other N-terminal DNP-compounds, indicate that this hemoglobin is composed only of β chains.

The N-terminal amino acid sequences of the main component, A^{H}_{II} (zone 4, fig. 12), were studied and compared with the N-terminal sequences of the normal adult major component, A_{II}. Except for high yields of N-terminal peptides of the β chain, the results appear to be identical to those with normal adult hemoglobin.

Two different analyses of the DNP-N-terminal peptides of component A_{Ic} revealed good yields of α chain peptides but low yields of β chain peptides. An explanation for these low yields is not yet clear, but may be analagous to the reason for low yields of DNP-glycine from the minor fetal component, F_I. Difficulty in obtaining the DNP-derivative of the N-terminal amino acids indicates the possibility

of either an unreactive or unavailable α-amino group. An acyl derivative of the N-terminal amino acid could account for these results.

A single analysis of component S_{Ie} revealed both DNP-val-leu and DNP-glycine in high yields but no DNP-val-his-leu peptide. This result is another indication that component S_{Ie} is identical to component F_{II}.

The slow chromatographic component S_{III} appears to contain the N-terminal sequences of the α and β chains. The relatively high yield of the β chain peptides is unexplained but this component probably is not significantly different in N-terminal sequence from normal adult hemoglobin.

C. Peptide Patterns of Tryptic Hydrolysates

The peptide spots which comprise the peptide pattern or "finger print" of the tryptic hydrolysate of normal hemoglobin have been numbered arbitrarily by Ingram (13). Representative peptide patterns of tryptic hydrolysates of hemoglobin A using two different chromatographic solvent systems are shown in Figures 18a and 18b. Figure 18c represents a peptide pattern of a tryptic hydrolysis of hemoglobin F_{II}. The peptide spots in each of these figures have been outlined and are numbered as completely as possible according to Ingram's system (13,71). A new numbering

Figure 18a. Peptide pattern of tryptic hydrolysate of Hemoglobin A_{II} by combined paper electrophoresis and paper chromatography. The chromatography was ascending and with a solvent composed of 3 parts n-butanol, 1 part glacial acetic acid, and 1 part water by volume. See text and Tables XIIIa and XIIIb for description of Spot Nos.

Figure 18b. Peptide pattern of tryptic hydrolysate of whole Hemoglobin A by combined paper electrophoresis and paper chromatography. The chromatography was descending and with a solvent composed of 7 parts iso-amyl alcohol, 7 parts pyridine and 6 parts water by volume. See text and Tables XIIIa and XIIIb for description of Spot Nos.

Figure 18c. Peptide pattern of tryptic hydrolysate of Hemoglobin F_{II} by combined paper electrophoresis and paper chromatography. Conditions were the same as for Figure 18b. See text and Tables XIIIa and XIIIc for description of Spot Nos.

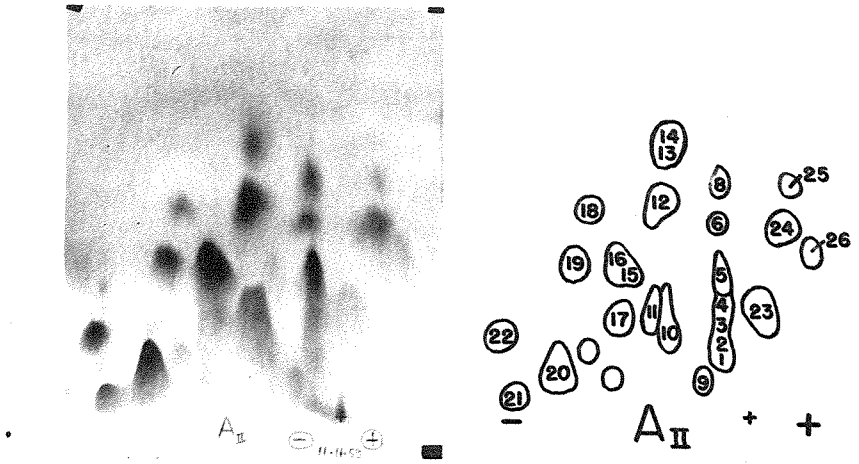


Figure 18a

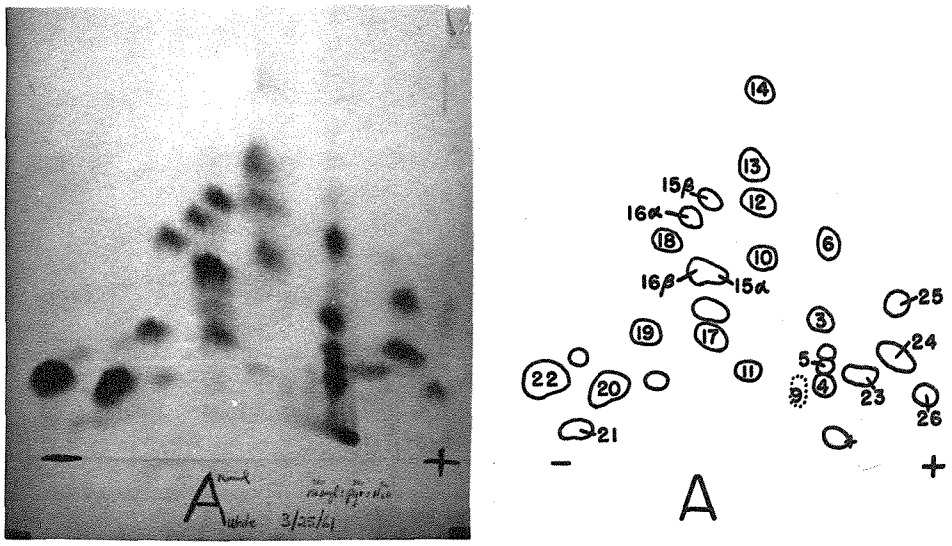


Figure 18b

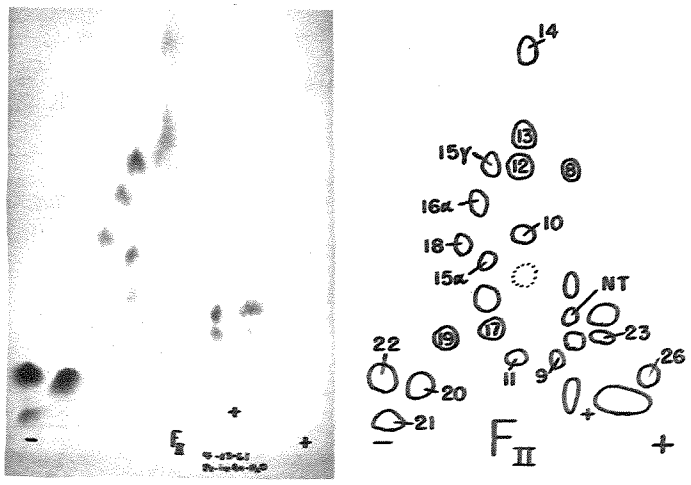


Figure 18c

system for the tryptic peptides of hemoglobin was proposed at the Hemoglobin Structure Workshop held at the M.I.T. Endicott House on December 14-16, 1960 (57). The latter system is based on a number of structural studies that indicate the probable location of all of the arginine and lysine residues in the α and β chains (8,9,10,11) (see Appendix No. II). Tables XIIIa, XIIIb, XIIIc summarize the correlation of the peptide numbers and the amino acid composition or sequence of most of the tryptic peptides and the insoluble residue of the α and β chain. These compilations are based upon studies of Schroeder and co-workers (8), Braunitzer et al. (10,11), Hill and Konings-berg (9), and recently published studies of Baglioni (71).

Peptide patterns of components A_{Ic}, S_{Ic}, S_{Ie}, S_{If}, S_{III}, γ_4, β_4, A^H_{Ic}, A^H_{II}, A^H_{III}, α chains of A_{II}, F_{II}, and S_{II}, β chain of A_{II}, and γ chain of F_{II} were made. Although detailed examination of the peptide patterns of tryptic hydrolysates of various hemoglobin components might indicate small changes in the primary structure, gross examination is often sufficient to determine whether α, β, or γ chains are present in each component. Only the general features of these studies will be presented.

The peptide pattern of component γ_4 (zone 1a, fig. 13) was identical with that of γ chains prepared from hemoglobin F_{II} by the urea gradient method of Wilson and Smith (77).

TABLE XIIIa

Numbering Systems, Sequences, and Compositions of Tryptic Peptides from the α Chain*

"Fingerprint" Numbers according to Ingram (13)	Chain Sequence Numbers (see Appendix II)	Amino Acid Sequence or Composition
3	αT-9	val-ala-asp-ala-leu-thr-aspNH_2-ala-(his,asp,asp,asp, asp,ser,ser,pro,ala,ala,ala,val,val,met,leu,leu,leu)-his-ala-his-lys
NB+	αT-1	val-leu-ser-pro-ala-asp-lys
9	αT-8,9	lys-val-ala-asp-ala-leu-thr-aspNH_2-ala-(his,asp,asp,asp, asp,ser,ser,pro,ala,ala,ala,val,val,met,leu,leu,leu)-his-ala-his-lys
10	αT-6	thr-tyr-phe-pro-his-phe-asp-leu-ser-ser-gly-ala-gluNH_2-his-val-lys
11	αT-1,2	val-leu-ser-pro-ala-asp-lys-thr-aspNH_2-val-lys
13	αT-5	met-phe-leu-ser-phe-pro-thr-thr-lys
15a	αT-3	ala-ala-try-gly-lys
16a	αT-14	tyr-arg
17a	αT-2	thr-aspNH_2-val-lys
18	αT-10	leu-arg
20a	αT-7	gly-his-gly-lys
21a	αT-7,8	gly-his-gly-lys-lys
22a	αT-8	lysine
23a	αT-4	val-gly-ala-his-ala-gly-glu-tyr-gly-ala-glu-ala-leu-glu-arg
α core	αT-11	val-asp-pro-val-asp-phe-lys
	αT-12	leu-leu-(his,his,his,thr,thr,ser,glu,pro,pro,ala,ala,ala, ala,cys,val,val,leu,leu,leu,phe)-ala,ser-leu-asp-lys
	αT-13	phe-(thr,ser,ser,ala,val,val,leu,leu)-thr-ser-lys

*According to Braunitzer, et al. (10), Hill and Konigsberg (9), and Schroeder, et al. (8).

+N.B. indicates a neutral band peptide.

TABLE XIIIb

Numbering Systems, Sequences, and Compositions of Tryptic Peptides from the β Chain*

"Fingerprint" Numbers according to Ingram (13)	Chain Sequence Numbers (see Appendix II)	Amino Acid Sequence or Composition
4	βT-1	val-his-leu-thr-pro-glu-glu-lys
5	βT-13	glu-phe-thr-pro-pro-val-glu-ala-ala-tyr-glu-lys
6	βT-9	val-leu-gly-ala-phe-ser-asp-gly-leu-ala-his-leu-asp-asp-leu-lys
12	βT-2	ser-ala-val-thr-ala-leu-try-gly-lys
14	βT-4	leu-leu-(thr,glu,pro,val,val,try,tyr)-arg
15β	βT-15	tyr-his
16β	βT-14	val-val-ala-gly-val-ala-aspNH_2-ala-leu-ala-his-lys
17β	βT-14,15	val-val-val-ala-ala-gly-ala-asp-leu-ala-his-lys-tyr-his
19	βT-6	val-lys
20β	βT-7	ala-his-gly-lys
21β	βT-7,8	ala-his-gly-lys-lys
22β	βT-8	lysine
24β	βT-5 oxidized	phe-phe-glu-ser-phe-gly-asp-leu-ser-thr-pro-asp-ala-val-metSO_2-gly-asp-pro-lys
25	βT-5	phe-phe-glu-ser-phe-gly-asp-leu-ser-thr-pro-asp-ala-val-met-gly-asp-pro-lys
26	βT-3	val-aspNH_2-val-asp-glu-val-gly-gly-glu-ala-leu-gly-arg
β core	βT-10,11	gly-thr-phe-(lys,his,his,cys,asp,asp,asp,thr,ser,glu,glu,pro,pro,ala,ala,val,val,leu,leu,leu,leu)-phe-arg
	βT-12	leu-(asp,gly,val,leu,leu)-(his,his,cys,gly,ala,val,val,leu,phe)-lys
	βT-13	glu-phe-thr-pro-pro-val-glu-ala-ala-tyr-glu-lys

*According to Braunitzer, et al. (11) and Schroeder, et al. (8).

TABLE XIIIc

Numbering Systems, Sequences, and Compositions of Tryptic Peptides from the γ Chain*

"Fingerprint" Number	Zone Number according to Schroeder, et al. (8)	Amino Acid Sequence or Composition
26	1	val-aspNH_2-val-glu-asp-ala-gly-gly-glu-thr-leu-gly-arg
8	3	val-leu-thr-ser-leu-gly-asp-ala-ileu-lys
22	4	lysine
12-13	6	met-val-thr-gly-val-(ala,ser,ala,leu,ser,ser)-arg
19	9	val-lys
N.B.+	10	his-leu-asp-asp-leu-lys
15	12	tyr-his
12	13	ala-thr-(i)leu-thr-ser-(i)leu-try-gly-lys
20	16	ala-his-gly-lys
14	18	leu(leu,glu,pro,thr,try,tyr,val)-arg
N.T.±	19	gly-his-phe-thr-glu-glu-asp-lys-ala-thr-(i)leu-thr-ser-(i)leu-try-gly-lys
21	21	ala-his-gly-lys-lys

*According to Schroeder, et al. (8).

+N.B. indicates neutral band peptide.

±N.T. indicates N-terminal peptide of γ chain.

No α chain peptides could be detected. Similarly, the peptide patterns of component β_4 (zone 1b, fig. 13) and β chains prepared from hemoglobin A were identical.

No differences in the "fingerprints" of component A^H_{Ic} from one of the subjects with thalassemia hemoglobin H disease and the normal A_{Ic} component could be detected. These patterns as well as those for A^H_{II} (zone 4, fig. 12) were essentially indistinguishable from the peptide pattern of the normal major adult component A_{II}. However, in the case of A^H_{II}, an additional peptide, which contained histidine and was electrophoretically like the number 23 peptide, was detected in low yield when peptide chromatography was made with a developer composed of 7 parts iso-amyl alcohol, 8 parts pyridine, 6 parts water, and 0.1 part formic acid. This extra peptide could have resulted from the presence of a small amount of abnormal α chain in the major component A^H_{II}. The presence of such an abnormal α chain is consistent with the hypothesis that γ_4 and β_4 components result secondarily from a deficiency of α chain formation. The exact nature of this extra peptide is not clear but further study is warranted because this may be the first example of an alteration of a hemoglobin which does not involve a change in the net charge of the molecule.

Although the peptide pattern of the A^H_{III} component (zone 5, fig. 12) was similar to the pattern of A_{II}, it may

have been more like the fingerprints described by Muller and Jonxis for hemoglobin A_2 (78).

Tryptic peptide patterns of components S_{Ic} and S_{Ie} were similar to those of F_{II} whereas the patterns of components S_{If} and S_{III} were similar to those of the major sickle-cell hemoglobin component, S_{II}, as judged by the presence of the No. 4 peptide of hemoglobin S (13,71).

The α chains isolated by the urea gradient procedure (77) from components A_{II}, F_{II}, and S_{II} were found to be identical as judged by their peptide patterns. This identity is in agreement with other chemical studies (Appendix IV and refs. 14 and 61).

D. Ultraviolet Spectra

H. M. Jope apparently was the first to note the difference in the ultraviolet absorption spectra of the adult and fetal hemoglobins of humans (79). The difference has been attributed to the presence of more tryptophan in fetal hemoglobin than in adult hemoglobin. Although precise spectrophotometric methods in the ultraviolet region have been developed for detection of the relative amounts of hemoglobin A and F in mixtures of the two (63), gross examination of the spectra in the region of 250 to 300 mμ reveals rather striking results as shown in Figures 19a, 19b, 19c, and 19d. These figures are representative of the

Figure 19a. Ultraviolet spectrum of Hemoglobin Component A_{II} given as the optical density of a 1 cm. path through a 1% solution.

Figure 19b. Ultraviolet spectrum of Hemoglobin Component F_{II} given as the optical density of a 1 cm. path through a 1% solution.

Figure 19c. Ultraviolet spectrum of Hemoglobin Component β_4 (zone 1b, fig. 13) given as the optical density of a 1 cm. path through a 1% solution.

Figure 19d. Ultraviolet spectrum of Hemoglobin Component γ_4 (zone 1a, fig. 13) given as the optical density of a 1 cm. path through a 1% solution.

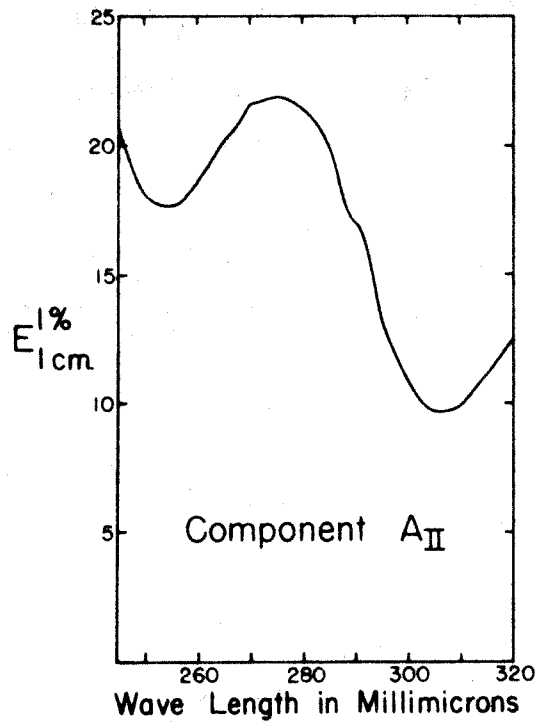


Figure 19a

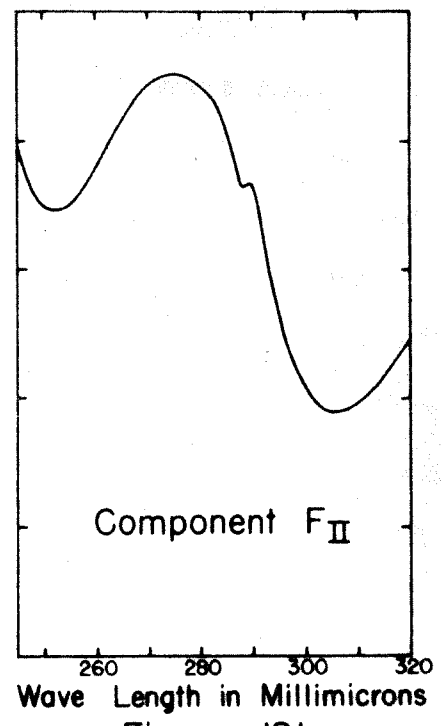


Figure 19b

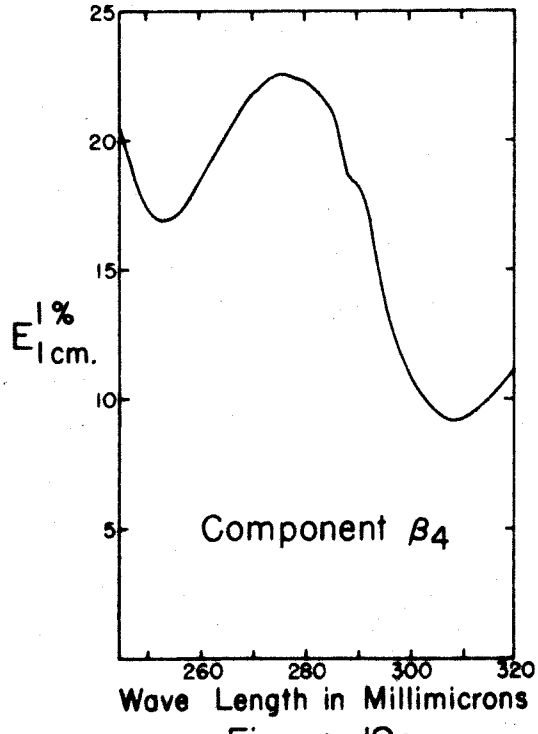


Figure 19c

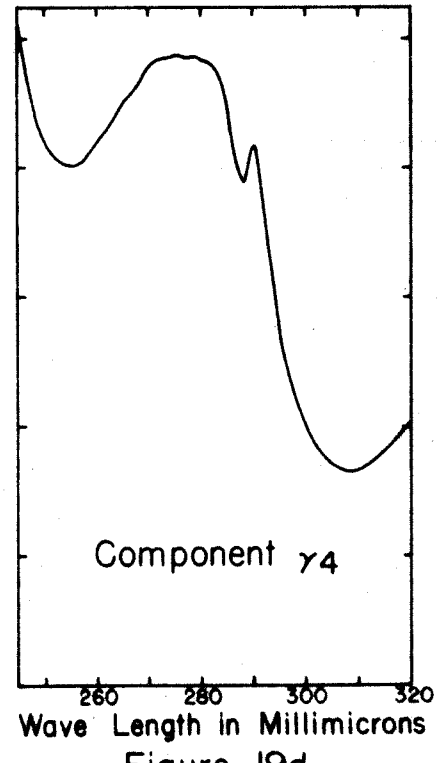


Figure 19d

ultraviolet spectra of hemoglobin components A_{II}, F_{II}, β_4 (zone 1b, fig. 13) and γ_4 (zone 1a, fig. 13). Only inflections in the region of 290 mμ were noted for components A_{II} and β_4, whereas maxima in this region were found for components F_{II} and γ_4. The striking maximum value of 290 mμ in the case of the γ_4 component has been noted on three different preparations from subject P.T. but was not present when the zone 1a from two other thalassemia hemoglobin H patients was studied (subjects R.C. and H.D.).

The observed $E^{1\%}_{1cm.}$ value of 24.4 at 275 mμ for γ_4 compares well with the calculated value of 24.8 for γ_4 which is twice the sum of $E^{1\%}_{1cm.}$ for F_{II} and $\frac{1}{2}\beta_4$ minus the $E^{1\%}_{1cm.}$ for A_{II}, i.e., $2(\alpha_2\gamma_2 + \frac{1}{2}\beta_4 - \alpha_2\beta_2) = \gamma_4$. However, this agreement may be only coincidental because the accuracy of the measurements is probably about $\pm$ 5%.

Ultraviolet spectral studies of a number of other components were found to fall into two groups, those resembling component F_{II} and those similar to component A_{II}. Those components which were like F_{II} included F_I, S_{Ic}, and S_{Ie} whereas those resembling A_{II} included A_{Ia+b} (i.e., mixture of A_{Ia} and A_{Ib}), A_{Ic}, A_{III}, A^H_{Ic}, A^H_{II}, A^H_{III}, S_{Ib}, S_{If}, S_{II} and S_{III}.

E. Sedimentation Coefficients

The sedimentation coefficients of hemoglobin components β_4 (zone 1b, fig. 13) and γ_4 (zone 1a, fig. 13) were compared with the coefficients of the normal component A_{II} and are listed in Table XIV. The $S_{w,20}$ values for the two abnormal components were each 3 to 5% higher than the values of their A_{II} controls. The observation for component β_4 is in agreement with results reported earlier by Rigas et al. (39) who noted that hemoglobin H has a slightly higher sedimentation constant than hemoglobin A. The results for the γ_4 component have recently been confirmed by Kekwick and Lehmann (80). The sedimentation coefficients of components β_4 and γ_4 indicate that their molecular weights are of the order of 60,000 to 70,000 assuming that the frictional coefficients of these components are similar to that of hemoglobin A. The present molecular weight estimates for components β_4 and γ_4 are the basis for concluding that these molecules are tetramers.

An estimation of the dissociation of hemoglobin component β_4 in alkaline solutions was made in order to decide whether subunit hybridization experiments might be practical. The sedimentation coefficients of component β_4 and controls, A, were studied at pH 9.7, 10.0, and 10.5 and are listed in the middle portion of Table XIV. Apparently the β_4 component

TABLE XIV

Sedimentation Coefficients ($S_{w,20}$) * of Hemoglobins γ_4, β_4 and F_{II}

Experiment No.	Hemoglobin Components Experimental	Control	pH	$S_{w,20}$ Experimental	Control	$\Delta S_{w,20}$
1832	γ_4	A_{II}	6.74	4.97	4.86	0.11
1702	β_4	A_{II}	7.07	4.63	4.39	0.24
1710	β_4	A	9.70	3.93	4.09	-0.16
1704	β_4	A	10.0	3.96	4.07	-0.11
1681	β_4	A	10.5	2.81	3.77	-0.96
1709	β_4		7.07	4.51		
	β_4		10.5	2.81		
1736	F_{II}	S_{II}	4.7	3.39	2.92	0.47
1690	F_{II}	A_{II}	10.25	4.12	4.10	0.02
1688	F_{II}	A_{II}	10.50	3.82	3.85	-0.03
1686	F_{II}	A_{II}	10.70	2.81	3.07	-0.26
1689	F_{II}	A_{II}	10.91	2.70	2.63	0.07
1696	F_{II}	A_{II}	11.65	2.42	2.39	0.03

*Sedimentation Coefficients are expressed in Svedberg units. (10^{-13} sec.).

is dissociated into half molecules at pH 10.5 at which hemoglobin A is only partially dissociated (66). Reversible dissociation of component β_4 was demonstrated by comparing the sedimentation coefficients of two samples of this component. The first sample was dialyzed against pH 10.5 glycine-NaCl buffer for 16 hours and then against pH 7.07 phosphate buffer for 16 hours more. The second sample of component β_4 was dialized in the reverse order, first against pH 7.07 buffer then against pH 10.5 buffer. The sedimentation coefficients for these two samples are also listed in Table XIV and indicate that reassociation of the subunits of component β_4 does occur.

Initial attempts to hybridize hemoglobin F_{II} with radioactive hemoglobin S resulted in poor exchange of label (see Subunit Hybridizations below). Therefore, comparison of the sedimentation coefficients of hemoglobin F and either hemoglobin A_{II} or S_{II} were made in acid and alkaline solutions in order to detect possible differences in dissociation. The results are listed in the bottom part of Table XIV and indicate a slight difference in the sedimentation coefficients of hemoglobins F_{II} and A_{II} in the region of pH 10.7. This difference was confirmed by a duplicate determination. A single comparison of components F_{II} and S_{II} which was made at pH 4.7 indicated a substantial difference between the sedimentation coefficients

of these two hemoglobins. Further studies in the acid range are necessary to evaluate the significance of this single observation.

Thus, the dissociation of hemoglobin F_{II} does appear to occur in acid and alkaline solutions; however, the dependency of this dissociation on pH may be different from that of hemoglobin A_{II} and S_{II}.

F. Subunit Hybridization

Two types of information were sought in making a number of subunit hybridization experiments. The first and primary information concerned the kind and relative number of exchangable chains in hemoglobin components A_{Ia+b} (a mixture of A_{Ia} and A_{Ib}), A_{Ic}, S_I (a mixture of rapidly moving S_I minor components), S_{Ie}, C_{II}, D_{II}, F_I, F_{II}, and the two abnormal components of hemoglobin H, γ_4 and β_4. The second type of information was related to the mechanism of subunit hybridization of complementary subunits and included studies with components γ_4, β_4, F_{II}, and A_{II}. A hypothesis of non-random recombination is developed from the latter data which indicates that differences exist in the relative tendency of recombination of some of the complementary subunits in hybridizations of hemoglobin mixtures containing α, β, and γ chains. This non-random recombination may be due to a greater affinity of the α chain-units for the β chain-units than for the γ chain-

units; differences in the relative stability or rate of formation of certain tetramers; or a combination of these two.

Two different approaches were used for the determination of the kind and number of exchangable subunits in a hemoglobin component. The first approach was analogous to the experiment of Vinograd, Hutchinson, and Schroeder (61) in which they identified the aberrant chain in hemoglobin S by detecting C-14 labelled leucine in the N-terminal peptides of the radioactive hybrids of hemoglobin A and S (see Introduction of Section II above and Identification of the Common Chain in Radioactive Hybridizations, below). The second method was to form new hemoglobin products which differed in chromatographic behavior from the hemoglobin reactants. The second approach was unique at the time it was first employed and two communications that include a description of the procedure were published in 1959 (see Appendices III and IV).

For the sake of the present discussion, the terms α chain(s), β chain(s), and γ chain(s) will often be used when referring to the subunits of the hemoglobins that undergo exchange or recombination in the hybridization procedure. Although the exact nature of the hybridizing subunits is not yet clear (31), these subunits probably are either dimers or single chains.

Detection of the Transfer of Identical Chains with Radioactive Hemoglobins.--A typical example of the use of hemoglobins containing C-14 labelled leucine for the detection of a transfer of identical subunits was an experiment in which 20 mg. of component A_{Ic} (non-radioactive) was hybridized with 20 mg. of component S^*_{II} (* indicates presence of C-14 L-leucine) at pH 4.7 for 12 hours at 3°. After hybridization and chromatographic separation, the A_{Ic} component had an average specific activity of about 700 cpm/mg. (counts per minute per milligram) and the S_{II} component had an average specific activity of about 2300 cpm/mg. If Equation No. 1 is assumed to represent the balanced reaction for the hybridization of component A_{Ic} with component S^*_{II}, then the ratio of specific activity of the product A^*_{Ic} to the product S^*_{II} should be 1:3 for complete hybridization (31). The observed ratio of 1 : 3.3 suggests that the experimental hybridization was about 91% of that theoretically possible.

$$2\alpha_2^{A_{Ic}}\beta_2^{A_{Ic}} + 2\alpha_2^{A^*}\beta_2^{S^*} \rightarrow \underbrace{\alpha_2^{A_{Ic}}\beta_2^{A_{Ic}} + \alpha_2^{A^*}\beta_2^{A_{Ic}}}_{A^*_{Ic}} + \underbrace{\alpha_2^{A_{Ic}}\beta_2^{S^*} + \alpha_2^{A^*}\beta_2^{S^*}}_{S^*_{II}} \qquad (1)$$

Similarly, when components A_{Ic} and S^*_{II} were hybridized at pH 11.2 for 24 hours, the transfer of the common subunit was essentially complete as judged by Equation No. 1.

The pertinent experimental data for the above hybridizations and similar data for D_{II}, S_{Ie}, and F_{II} are listed in Table XV. Although the hybridizations of A_{Ic} with S^*_{II}

TABLE XV

Hybridization Studies Based on Transfer of

Common Radioactive Chains

Hybridization Mixture[a]	pH	Duration in hrs.[b]	Specific Activity After Hybridization in cpm/mg		% Theoretical Hybridization[c]
$A_{Ic} \times S^{*}_{II}$	4.7	12	A_{Ic}-700	S_{II}-2300	91%
$A_{Ic} \times S^{*}_{II}$	11.2	24	A_{Ic}-250	S_{II}-750	100%
$D_{II} \times A^{*}$	4.7	24	D_{II}-100	A_{II}-300	100%
$D_{II} \times F^{*}_{II}$	4.7	24	D_{II}-1100	F_{II}-4500	73%
$S_{Ie} \times A^{*}_{II}$	4.7	24	S_{Ie}-60	A_{II}-250	72%
$A_{II} \times F^{*}_{II}$	4.7	12	A_{II}-1000	F_{II}-5500	54%
$F_{II} \times S^{*}_{II}$	11.2	48	F_{II}-200	S_{II}-1250	45%
$F_{II} \times S^{*}_{II}$	11.0	168	F_{II}-35	S_{II}-190	55%

a. For any experiment the two hemoglobin components were present in the same amount. The * indicates the presence of C-14-labelled leucine in the hemoglobin.

b. This is the time during which the mixture is dialyzed against the hybridizing solution.

c. For equal amounts of reactants the ratio of the specific activity of the products after hybridization should be 1:3 provided the exchange of the common subunits has been complete. The % of theoretical hybridization is calculated by dividing the observed ratio of specific activity of the products by 0.0033.

and D_{II} with A^{*}_{II} appeared to go to completion under the conditions employed, none of the studies involving F_{II} went to completion. The extent of hybridization of F_{II} with other components appeared to be greater at pH 4.7 than at pH 11.2 and increased with duration of hybridization at these two pH's.

The result of the hybridization of S_{Ie} with A^{*}_{II} was 72% of the theoretical hybridization based on an equation similar to Equation No. 1, and was essentially identical with the result of the hybridization of F^{*}_{II} with D_{II} (73% of theoretical hybridization). This observation is in agreement with earlier chromatographic studies, N-terminal amino acid determinations, and peptide pattern analyses, all of which indicate that S_{Ie} and F_{II} are identical in chemical properties.

Identification of the Common Chain in Radioactive Hybridizations.--Although the hybridization data in Table XV indicate that each pair of hemoglobins in every experiment has an equivalent chain in common, such studies do not identify the common chain unless combined with other information. The direct way to determine the common chain of hemoglobins that have hybridized is to study that hemoglobin into which radioactivity has been introduced. This determination is possible because of the following facts. First, in all of the hybridizations the

radioactive hemoglobin was labelled with C-14 L leucine. Second, it is known that the second residue from the N-terminus of the α chain is leucine and that the third residue from the N-terminus of the β chain is leucine. Third, it is possible to isolate DNP-peptides from partial hydrolysates of DNP-hemoglobin that contain the leucyl residue mentioned above. Thus the DNP-peptide that is radioactive must stem from the chain that is common and that was exchanged during hybridization. Exchanges of common chains followed by isolation of the N-terminal DNP-compounds were made on components A_{Ic}, C_{II}, D_{II}, F_I and F_{II} and the pertinent data are presented in Table XVI. Apparently all of these components have the α chain of the normal A_{II} component in common. In the cases of F_I and F_{II}, this result is not surprising because the N-terminal amino acid studies indicate that neither of these hemoglobins contains β chains.

Formation of New Hemoglobins by Hybridization.--
Hybridization experiments with hemoglobin H (β_4 component) demonstrate how the formation of new hemoglobin products can yield information about the kinds of chains present in different hemoglobins. When roughly equal amounts (about 22 mg.) of hemoglobin H (zone 1b, fig. 13) and radioactive hemoglobin S were hybridized at pH 11.0 at 3° for 24 hours, the chromatogram shown in Figure 20 was obtained (also see

TABLE XVI

Specific Activity of N-Terminal Peptides

Following Hybridization

Hybridization Mixture[1]	Specific activity[2] in counts per minutes per micromole DNP-val-leu	DNP-val-his-leu	DNP-val-his	DNP-val	DNA
$A_{Ic} \times S^*_{II}$	75.9	5.6	12.6	0.3	0.8
$C^3_{II} \times A^*_{II}$	22.4	1.4	1.8	-	-
$D_{II} \times A^*_{II}$	16.8	4.3	1.4	-	-
$F^*_I \times A_{II}$	219.3	4.6	18.1	1.4	0.9
$F^4_{II} \times S^*_{II}$	53.7	8.6	3.6	-	-

1. The * indicates the presence of C-14-labelled leucine in the hemoglobin.

2. Specific activity of the N-terminal DNP-compounds from the hemoglobin component which was originally non-radioactive.

3. This hemoglobin was from Dr. H. Lehmann, and although an unknown to him, it was shown to be identical to hemoglobin C by chromatography and peptide pattern analysis.

4. Non-radioactive DNP-val-his-leu and DNP-val-his were added during the hydrolysis procedure to act as carrier for any radioactive DNP-val-his-leu which might be present. DNP-glycine was also isolated and had specific activity of 0.05 cpm/umole.

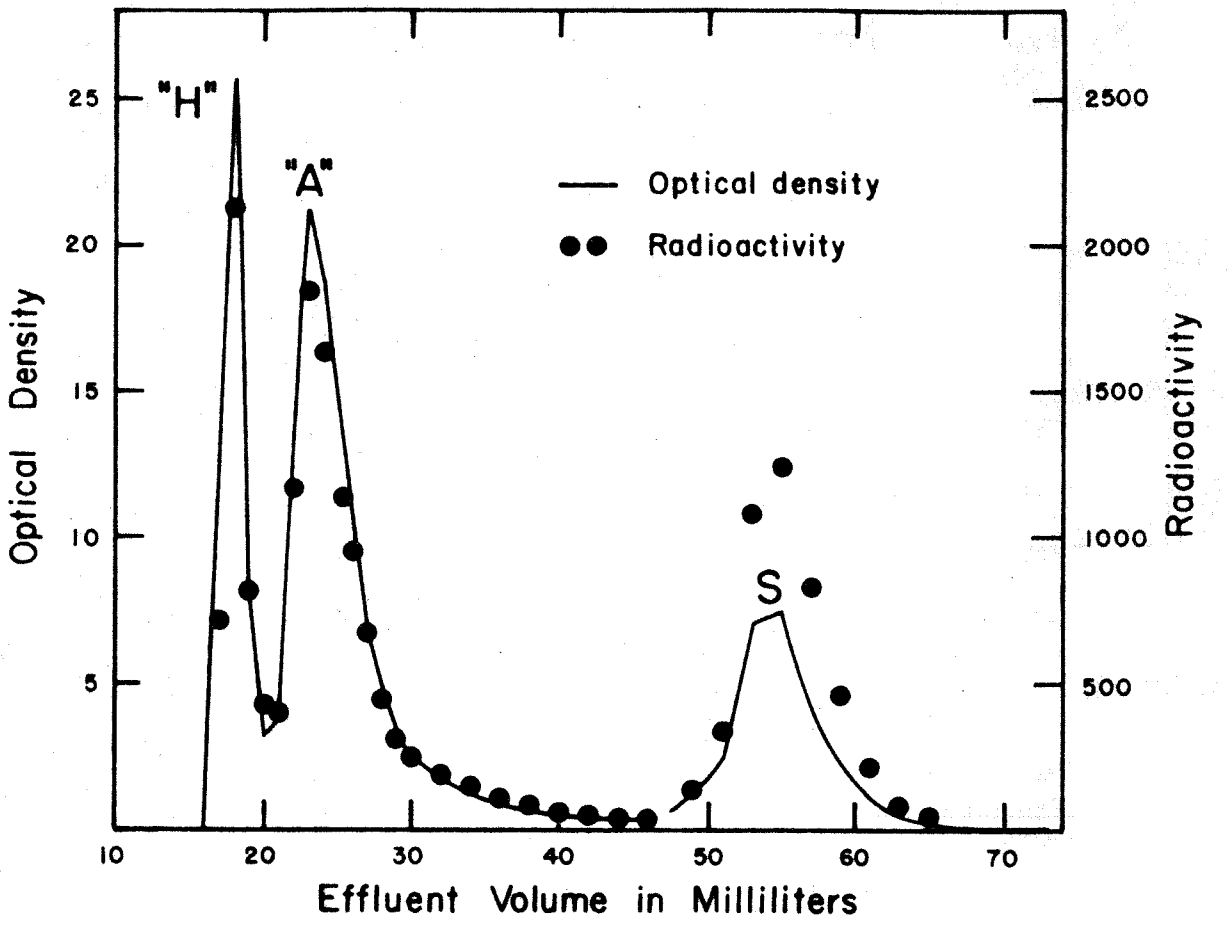


Figure 20

Chromatogram of products from hybridization of components β_4 and S^*_{II}, on IRC-50 with Developer No. 1. The solid line represents the optical density at 415 mμ. The solid circles represent the radioactivity in counts per minute per milliliter of effluent. The "H" zone was separated into two components by rechromatography with Developer No. 4 (not shown). The "A" zone was identical to component A_I by chromatography, electrophoresis on starch block, and sedimentation studies. The S zone was identical to component S_{II}.

Appendix III for further experimental details). Hemoglobin A_{II} as well as hemoglobin S and a fast moving hemoglobin zone were present in this chromatogram. Because the radioactive label, which was originally present only in the S^{*}_{II} reactant, was found by N-terminal peptide studies to be present only in the α chain of the A^{*}_{II} product, the β chain of this A^{*}_{II} product must have arisen from the non-radioactive hemoglobin H. The front zone from Figure 20 was separated into two components by further chromatography with Developer 4. One of these components was identical with the original hemoglobin H (β_4 component), whereas the second component was composed of only the β chains of hemoglobin S as concluded from its chromatographic behavior and specific activity. If one assumes that there was random recombination of the β^A and β^S chains with the available α chains, then the presence of approximately twice as much hemoglobin A_{II} as hemoglobin S_{II} indicates that hemoglobin H is comprised only of β^A chains. This conclusion necessarily assumes that the ratio of β^A to β^S chains which precipitated during the hybridization experiment was the same as the ratio of β^A to β^S chains that were available for combination with α chains. Otherwise, a decrease in the number of β^S chains relative to β^A chains could "drive" the hybridization past a point of the theoretically uniform distribution of the α chains between the β^A and β^S chains.

Ideally the hybridization of equal amounts of hemoglobins β_4 and S^*_{II} can be represented by Equation No. 2.

$$3\ \beta_4^{H=A} + 3\ \alpha_2^*\beta_2^{S*} \longrightarrow 2\ \beta_4^{H=A} + \beta_4^{S*} + 2\ \alpha_2^*\beta_2^{H=A} + \alpha_2^*\beta_2^{S*} \qquad (2)$$

This equation assumes that none of the components is lost, that the affinity of the α chain is the same for the $\beta^{H=A}$ and β^S chains, and that no other recombinants form to any significant extent. It should also be noted that the tetramer form of β_4^{S*} has been assumed by analogy to β_4^A; however, as yet there is no experimental evidence to confirm the latter assumption. The aforementioned hybridization experiment appears to satisfy Equation No. 2 at least so far as the stable products A^*_{II} and S^*_{II} are concerned.

As by Equation No. 2, the hybridization of one part of hemoglobin H with 2 parts hemoglobin S^*_{II} can be represented by Equation No. 3.

$$2\ \beta_4^A + 4\ \alpha_2^*\beta_2^{S*} \longrightarrow \beta_4^A + \beta_4^{S*} + 2\ \alpha_2^*\beta_2^A + 2\ \alpha_2^*\beta_2^{S*} \qquad (3)$$

The latter equation was tested experimentally by hybridizing 25 mg. of hemoglobin β_4 with 50 mg. of radioactive hemoglobin S^*_{II} at pH 4.65 for 24 hours. Although a substantial amount of hemoglobin was lost as a precipitate, each of the products in Equation No. 3 was detected. The ratio of the amount of A^*_{II} formed to S^*_{II} remaining was 0.82 indicating that either the hybridization had been incomplete or that

more β^A chains than β^{S*} chains were precipitated during hybridization. Of course, it is also possible that the α chains had a greater affinity for the β^{S*} chains than for the β^A chains.

Hybridization of a Mixture of S_I Components.--A second example of the formation of new hemoglobin products is the hybridization of component A_{II} with an equal amount of radioactive S^*_I minor components (includes all of the rapidly moving minor components of hemoglobin S). This study was made at pH 11.2 for 60 hours and resulted in the formation of 18% S^*_{II} in addition to 53% S^*_I and 28% A^*_{II}. Because the β^S chain is responsible for the difference in the chromatographic movement between hemoglobin A_{II} and S_{II}, production of S^*_{II} in the above hybridization indicates that β^{S*} chains are present in one or more of these minor components of sickle-cell hemoglobin. In addition, the fact that the A^*_{II} component, which was originally non-radioactive, became radioactive indicates the presence of normal α chains in these minors. This experiment suggests that one might be able to form the S_{II} components by simply hybridizing the S_I minor components with themselves. The presence of β^S chains in some of the S_I minor components indicates that these chains are combined with modified α chains, with other chains as yet undetected, or as tetramers of single chains. The last possibility is of special interest in view of Huisman's report

of the presence of a hemoglobin which is probably β_4^S in two infants with the sickle-cell gene (81).

Hybridization of a Mixture of A_{Ia} and A_{Ib} Components.--When a mixture of components A_{Ia} and A_{Ib} (designated as A_{Ia+b} hereafter) was hybridized with an equal amount of radioactive S^*_{II} at pH 4.7 for 24 hours, 7% A^*_{II} was formed in addition to 48% A^*_{Ia+b} and 45% S^*_{II}. The production of A^*_{II} indicates the presence of normal β^A chains in either or both A_I components. However, the presence of radioactivity in the A^*_I minors after the hybridization does not necessarily prove the presence of exchangable α chains because this radioactivity could have been due to the β^{S*} chains which were replaced by the β^A chains or to the formation of "altered" S^*_{II} component which has been observed to move near the A_I components in control experiments.

Hybridization of the S_{III} Component.---Component S_{III}, the slowly moving minor component of sickle-cell hemoglobin, was examined by hybridization with equal amounts of hemoglobin β_4 in order to determine whether products similar to the slowly moving A_{III} components could be formed. Two experiments were performed, the first at pH 11.0 with non-radioactive hemoglobins and the second at pH 4.7 with radioactive S^*_{III} and non-radioactive β_4. In each study a component that resembled A_{II} by chromatography and starch electrophoresis was formed in addition to a rapidly moving zone

and a slowly moving zone which was more like S_{II} than S_{III}. The ratios of the amount of A_{II} product to this sickle component were 2.73 and 3.05, respectively. From an equation similar to No. 2, one would predict that these ratios should have been 2.0.

Although the A component which was observed might have been A_{IIIa} rather than A_{II}, it could not have been A_{IIIb} because its electrophoretic mobility was that of A_{II} and A_{IIIa} rather than A_{IIIb} (18). Assuming this A component was A_{II}, then its formation indicates the presence of normal α chains in S_{III}.

The explanation for the high ratios of this A_{II} product to the S product, which were approximately 3, rather than the theoretical 2.0, is not certain. At least three explanations are: that $\beta^{S^*_{III}}$ chains were present in the S^*_{III} reactant and were replaced by the β^A chains to form a component that was identical to A_{II} in electrophoretic and chromatographic behavior; that the affinity of the α chains was greater for β^A chains than for $\beta^{S_{III}}$ chains; or that more $\beta^{S_{III}}$ chains than β^A chains were lost in the precipitate which formed during the hybridization procedure. The reason for the apparent change in chromatographic mobility of the S product from that of the S_{III} component before the experiment to that of the S_{II} component after the experiment is not clear. It is possible that the S_{III} component is in fact a modification of the S_{II} component which is converted back to the typical S_{II} form during the hybrid-

ization experiment. A reversibly altered β^S chain that is more susceptible to denaturation could be proposed because this would account for both the high ratio of the A product to the S product and the apparent conversion of the S_{III} reactant to the S_{II} product. Further investigation of component S_{III} will be necessary before a complete explanation of these hybridization studies can be reached.

Special Hybridization Studies of Components γ_4, β_4, F_{II}, and A_{II}.--From the data presented in Table XV, it was concluded that hybridizations of component F_{II} with components A^*_{II}, S^*_{II} and D_{II} were incomplete. Thus, further hybridization studies of components F_{II}, β_4, γ_4, and A_{II} were made in order to investigate factors related to subunit recombinations involving only the normal α^A, β^A, and γ^F chains. The data of these studies are summarized in Table XVII.

Although hybridization of approximately equal amounts of components F_{II} (8.2 mg.) and β_4 (7.6 mg.) at pH 11.1 for 14 hours resulted in the formation of only 22% of the theoretical amount of A_{II} components, essentially complete hybridization (97% of theoretical) of the same mixture resulted in the same time at pH 4.7. Equation No. 4 is proposed to describe the over-all reaction taking place in the latter type of hybridization.

$$3\ \beta_4 + 3\ \alpha_2\gamma_2 \longrightarrow 2\ \beta_4 + \gamma_4 + 2\ \alpha_2\beta_2 + \alpha_2\gamma_2 \qquad (4)$$

TABLE XVII

Hybridization Studies of Hemoglobins

γ_4, β_4, F_{II}, and A_{II}

Experiment	Hybridization Mixture[a]	Amount of Reactants in mg.[b]		pH	Duration in hrs.[c]	Percentage of Products[d] Front zones	F_{II}	A_{II}	A_{III}	Ratio of Products F_{II} to A_{II} Exper.	Theor.	% of Theoretical Hybridization
A	β_4 x F_{II}	7.6	8.2	4.7	14	37.5	23.4	39.1		0.6	0.54	97
B	β_4 x F_{II}	7.6	8.2	11.1	14	31.4	58.9	9.7		6.1	0.54	22
C	γ_4 x A^*_{II}	13	26	4.7	24	28	5	67		0.08	1.0	8
D	γ_4 x A^*_{II}	13	26	11.2	24	27	8	65		0.12	1.0	12
E	γ_4 x A^*_{II}	6.6	13.2	4.7	24	27	16	57		0.28	1.0	28
F	β_4 x F^*_{II}	14.5	29	4.6	24	20	25	53.4	1.6	0.47	1.0	214
G	γ_4 x F^*_{II}	5.0	10.0	4.7	24					.83	0.75	68

Footnotes to Table XVII:

a. The molecular formulas of components F_{II} and A_{II} are $\alpha_2\gamma_2$ and $\alpha_2\beta_2$, respectively. The "x" indicates a hybridization procedure. The "*" indicates the presence of C-14 L leucine in the hemoglobin.

b. The amount of each reactant is listed in the same order as given in the preceding column.

c. This is the time during which the mixture is dialyzed against the hybridizing solution.

d. The components are isolated by chromatography with Developer 5. The percentages are relative to the total hemoglobin from the chromatogram. The front zone is comprised of β and γ chains, presumably only as β_4 and γ_4.

e. Percentage of hybridization is based upon the ratio of the relative amounts of the F_{II} and A_{II} products except in Experiment G in which the ratio of the specific activities of the F^*_{II} produced by hybridization to the F^*_{II} of the control was used. See text for derivation of the theoretical ratios of F_{II} to A_{II} for complete hybridizations.

The percentages of theoretical hybridization assuming equal affinity of α chains for β and γ chains and equal stability of the tetramers of γ and β chains were calculated from a graph (not shown) based upon an equation similar to No. 4 but which took into account the unequal amounts of reactants.

When the reverse of the F_{II} x β_4 hybridization was attempted with components γ_4 and A^*_{II} (Experiments C, D, and E, Table XVII) at both pH 4.7 and 11.2, incomplete hybridization resulted. Because twice as much A^*_{II} as γ_4 was employed in these studies, Equation No. 5 was used to calculate the percentage of theoretical hybridization.

$$2\,\gamma_4 + 4\,\alpha^*_2\beta^*_2 \longrightarrow \beta^*_4 + \gamma_4 + 2\,\alpha^*_2\gamma_2 + 2\,\alpha^*_2\beta^*_2 \qquad (5)$$

In the most successful of these experiments (Experiment E), the hybridization was of the order of only 28% of that theoretically possible, even though the duration of hybridization was 24 hours rather than the 14 hours used for the hybridization of F_{II} and β_4. The incompleteness of hybridization of γ_4 with $2A^*_{II}$ might indicate that the overall rate of subunit exchange is relatively slow, especially from the γ_4 plus A_{II} side of the reaction, or that there is non-random recombination of some of the complementary subunits which are responsible for the overall hybridization reaction. As will be shown, both of these factors appear to operate in some of the hybridizations.

The probability of non-random recombination of the complementary subunits that are involved in hybridizations in which the sum of the γ and β chains is greater than the total α chains became apparent when the hybridization of components F^*_{II} and β_4 was repeated at pH 4.6 for 24 hours (Experiment F, Table XVII). In this experiment one part of component β_4 was hybridized with two parts of component F^*_{II}; however, the ratio of the amount of A^*_{II} formed to the amount of F^*_{II} remaining was of the order of 2.14 rather than 1.0 which would be predicted from Equation No. 6, below.

$$2\,\beta_4 + 4\,\alpha^*_2\gamma^*_2 \longrightarrow \gamma^*_4 + \beta_4 + 2\,\alpha^*_2\gamma^*_2 + 2\,\alpha^*_2\beta_2 \qquad (6)$$

This equation takes into account the 1:2 ratio of the reactants and was derived from Equation No. 4, which was concluded to describe Experiment A, Table XVII in which equal amounts of F_{II} and β_4 were hybridized. Because the reactants (β_4 and $2F^*_{II}$) were added so that equal amounts of γ and β chains would be present, the formation of twice as much A^*_{II} as F^*_{II} instead of equal amounts supports a conclusion of non-random recombination of the hybridizing subunits.

Although a precipitate formed during Experiment F, its amino acid composition indicated that it contained approximately equal amounts of γ and β chains (ratios of $\alpha : \beta ; \gamma :: 1.0 : 4.7 : 5.8$). Therefore, differential

loss of γ chains relative to β chains which could have resulted in the formation of more component A_{II} than F_{II} could not have been entirely responsible for the results.

Also, on the basis of Equation No. 6, the specific activity of the rapidly moving zone (γ_4^* plus β_4) would be expected to be about one half of that of the F_{II}^* component. Actually the specific activity of this zone (about 2,000 cpm/mg.) was almost as high as the F_{II}^* component (2,100 cpm/mg.). Although this observation is subject to error, it indicates that the rapidly moving zone probably contained substantially more of the γ_4^* component than the β_4 component. An excess of γ_4^* product in solution after the hybridization relative to the β_4 product also supports the conclusion of non-random recombination. This conclusion of non-random recombination of complementary subunits will be discussed more completely later.

The possibility of slow overall reaction rates as reflected by incomplete hybridization becomes probable when results of the hybridization of β_4 with F_{II} at pH 4.7 for 14 hours (Experiment A) are compared with results of the preceding hybridization, Experiment F, which was made for 24 hours. Similarly, re-examination of the results of the hybridization of γ_4 with 2 A_{II}^* (Experiments C and E) in view of the results of Experiment F (β_4 with $2F_{II}^*$) also raises the question of incomplete hybridization due

at least partly to slow rates of reaction. If the hybridization process is reversible and no slow steps were present under the conditions employed, then the ratios of the amount of product F_{II} to product A_{II} should be the same for both the hybridization of γ_4 with A_{II} and β_4 with F_{II}, and dependent only on the ratio of reactants and the relative affinity of the various hybridizing-subunits for one another. However, the experimentally derived ratios were not the same for these two kinds of hybridizations (examples are Experiments E and F, Table XVII).

In order to obtain direct evidence of the probability of a slow reaction step in these hybridizations, a subunit exchange experiment with components γ_4 and F^*_{II} was made (Experiment G, Table XVII). Five mg. of component γ_4 and 10 mg. of radioactive component F^*_{II} were hybridized at pH 4.7 for 24 hours. A control containing only component F^*_{II} was treated in an identical way. Equation No. 7 predicts that the ratio of the specific activities of the F^*_{II} hybridization product to the F^*_{II} control which is equivalent to the F^*_{II} reactant, should be 0.75, providing that the α and γ chains of the original F^*_{II} component had the same specific activity and assuming that non-radioactive γ chains were not lost in excess relative to radioactive γ^* chains.

$$\gamma_4 + 2\,\alpha^*_2\,\gamma^*_2 \longrightarrow \gamma_2\,\gamma^*_2 + \alpha^*_2\,\gamma^*_2 + \alpha^*_2\,\gamma_2 \qquad (7)$$

The experimental ratio of the two F^{*}_{II} components was 0.84, which indicates that the extent of hybridization was only 68% of that theoretically possible. This percentage of hybridization is similar to those percentages observed for hybridization of F^{*}_{II} with D_{II} and S_{Ie} with A^{*}_{II} (see Table XV). Thus, if one assumes the non-radioactive γ chains to be chemically equivalent to the radioactive γ chains, then the incomplete hybridization of γ_4 with F^{*}_{II} would support a conclusion that the overall hybridization reaction is slow under the conditions employed. Although the radioactive γ chain may not be exactly identical chemically to the non-radioactive γ chain because of the presence of C-14 L leucine, such non-equivalence is probably small and could not account for the incompleteness of hybridization in Experiment G, above. Of course, this question could be answered by comparing the present experiment with the hybridization of radioactive γ_4 with non-radioactive F_{II}.

Thus, both slow rates of reaction and unequal affinities or non-random recombination of the hybridizing subunits appear to occur in hybridizations involving the F_{II} component. Slow reactions were probably important in all of the hybridizations of F_{II}, whereas the unequal affinity of the hybridizing subunits was apparent only in those experiments in which there was competition for the available α chains. Because the γ_4 and β_4 tetramers were not

detected in hybridizations of hemoglobin F_{II} with normal α chain-containing hemoglobins (hemoglobins A, S, and D), competition for α chains was probably important only when the sum of the γ chains plus β chains was greater than the total α chains. The total number of α chains was less than the sum of γ and β chains only in the hybridizations of γ_4 with A_{II} and β_4 with F_{II}.

If it is assumed that the overall reaction of the hybridization of hemoglobins γ_4, β_4, F_{II}, and A_{II} with one another is reversible and that this reaction can be represented as the sum of the separate dissociation equilibria of each of these hemoglobins, then the following equilibrium reaction may be written:

$$2\,\alpha_2\gamma_2 + \beta_4 \overset{K_T}{\rightleftharpoons} 2\,\alpha_2\beta_2 + \gamma_4$$

where K_T is the equilibrium constant. The equilibrium equation for this reaction (eqn. No. 12, below) can be derived from the separate dissociation equilibria of hemoglobins γ_4, β_4, F_{II}, and A_{II} and their respective dissociation constants (eqns. Nos. 8, 9, 10, 11) assuming, for the sake of discussion, that the dissociation products and hybridizing subunits are dimers of identical chains.

$$\gamma_4 \rightleftharpoons 2\,\gamma_2 \qquad \frac{[\gamma_2]^2}{[\gamma_4]} = K_B \tag{8}$$

$$\beta_4 \rightleftharpoons 2\,\beta_2 \qquad \frac{[\beta_2]^2}{[\beta_4]} = K_H \tag{9}$$

$$\alpha_2\,\gamma_2 \rightleftharpoons \alpha_2 + \gamma_2 \qquad \frac{[\alpha_2]\,[\gamma_2]}{[\alpha_2\,\gamma_2]} = K_F \tag{10}$$

$$\alpha_2\beta_2 \rightleftharpoons \alpha_2 + \beta_2 \qquad \frac{[\alpha_2]\,[\beta_2]}{[\alpha_2\beta_2]} = K_A \tag{11}$$

$$2\alpha_2\,\gamma_2 + \beta_4 \rightleftharpoons 2\alpha_2 + 2\,\gamma_2 + 2\beta_2 \rightleftharpoons 2\alpha_2\beta_2 + \gamma_4$$

$$\frac{[\alpha_2\beta_2]^2\,[\gamma_4]}{[\alpha_2\,\gamma_2]^2\,[\beta_4]} = \frac{2\,K_F\,K_H}{2\,K_A\,K_B} = K_T \tag{12}$$

Analogous equations can be derived for subunits that are not dimers of identical chains. Although the dissociation constants would be defined differently, they would still describe the dissociation of each hemoglobin into its hybridizing subunits and the overall equilibrium reaction would have the same form, provided no other association products were stable relative to these four hemoglobins.

Thus, if Equation No. 12 can be used to approximate the overall mechanism of the hybridizations involving hemoglobins γ_4, β_4, F_{II}, and A_{II}, then the final state of the equilibrium depends upon the various dissociation constants

and the relative proportions of the starting reactants. The value of K_T will be other than unity only if the relative stabilities of the γ_4 and β_4 tetramers are not the same, if the relative affinity of the α chain for the β chain is not the same as that for the γ chain, or if a combination of both of these factors occurs. If one compares the results of Experiment E (γ_4 with $2A^*_{II}$) and Experiment F (β_4 with $2F^*_{II}$) with this equilibrium expression, then it would appear that the equilibrium lies on the right side of the reaction, that is, K_T is greater than unity. Whether this is due to a difference in the relative stability of the γ_4 and β_4 tetramers, differences in the affinity of the α chain for the β and γ chain, or a combination, cannot be decided from these experiments.

In addition to revealing factors which may be important in the hybridization mechanism, these studies with γ_4, β_4, F_{II}, and A_{II} yield further proof of the structural formula of the two abnormal components from the patients with thalassemia hemoglobin H disease and are basic to the conclusion that the α chains of hemoglobins A and F are identical (see Appendix IV).

CONCLUSIONS

Although specific conclusions have been made in the subsections on Results and Discussions, several concluding correlations and comments should be presented.

It is evident from the results that minor component heterogeneity is not only present in normal adult human hemoglobin and umbilical cord blood hemoglobin but also exists in hemoglobin from individuals with genetically determined hemoglobinopathies. Several of the minor components from these different sources appear to be similar in their general chromatographic behavior, whereas other minor components bear little chromatographic resemblance to any other component. A summary of the chromatographic similarities and identities of components from several hemoglobins is illustrated in Table XVIII. In this comparison, the components of hemoglobin A and the fetal components of umbilical cord blood have been used as primary chromatographic references. Chromatographic similarities are indicated by "S's" and identities, as judged by radioactive tracer studies, are indicated by "I's".

Because factors such as temperature, pH and ionic strength of developer, amount of hemoglobin, and oxidation state of the heme group can significantly influence the chromatographic behavior of hemoglobin components, the similarities and differences that are observed by chromatography must be judged with care. Thus, components A_{Ic} and

TABLE XVIII

Chromatographic Movement of Several Hemoglobin Components as Compared to Reference Hemoglobin Components[a]

		A_{Ia}	A_{Ib}	A_{Ic}	A_{Id}	A_{Ie}	A_{II}	A_{IIIa+b}	f_3	f_4	F_I	F_{II}
b	f_3	S										
	f_4	S										
	F_I		S									
	F_{II}			S								
	A_{II}^{CB}						I					
	A_{III}^{CB}							S				
c	Zone 1a, PT	Faster than A_{Ia} and f_3										
	Zone 1a, RC&HD	Faster than A_{Ia} and f_3										
	Zone 1b, RC&PT	S							S			
	Zone 2 (A_{Ic}^{H})			I								S
	Zone 4 (A_{II}^{H})						I					
	Zone 5 (A_{III}^{H})							S				
d	S_{Ia}	S							S	S		
	S_{Ib}									S		
	S_{Ic}										I	
	S_{Id}			S								S
	S_{Ie}	Varies from A_{Ic} to A_{Ie} region										I
	S_{If}					S						
	S_{Ig}					S	S					
	S_{II}	Different from D_{II} by R.T.S.										
	S_{III}	Slower than S_{II}										
e	C_{Ia}	S										
	C_{Ib}		S									
	C_{Ic}			S								
	C_{Id}						S					
	C_{Ie}	Moves between A_{II} and S_{II}										
	C_{If}	Similar to S_{II}										
	C_{Ig}	Similar to S_{III}										
	C_{II}	Slowest component studied										

TABLE XVIII (CONTINUED)

Footnotes

a. Comparisons are made of the chromatographic movement of each component in the first column with one or more of the reference component which are listed or with specific hemoglobins as noted. Similarity in movement is indicated by a "S", whereas identity of movement as judged by radioactive tracer studies (R.T.S.) is indicated by a "I". A number of different samples, chromatograms and developers were used in this comparison.

b. Cord Blood Hemoglobin. The adult-like components from cord blood hemoglobin have been indicated by superscript CB.

c. Hemoglobins from Thalassemia Hemoglobin H Disease. The zone nos. refer to chromatographic separations similar to those shown in Figures 12 and 13. Components were from three different subjects, PT, RC, and HD. Except for zone 1a which was the same for RC and HD but different from that of PT, all other components appeared identical. Superscript "H", has been used to designate some of these components.

d. Sickle-cell Hemoglobin. Components from about 20 sickle-cell anemia subjects were studied.

e. Homozygous Hemoglobin C Disease. Components from subject with homozygous hemoglobin C disease.

F_{II} and components S_{II} and D_{II} are similar to each other under most conditions of chromatography; however, by tracer studies with hemoglobins containing C-14 L leucine they have been found to be different. Contrarily, components S_{Ie} and F_{II} often appeared to have different chromatographic movements from one another but radioactive tracer chromatograms as well as chemical studies indicate that these two components are identical.

In spite of these limitations, the chromatographic procedures are very useful for the demonstration of heterogeneity, for a provisional identification of hemoglobins, and for the isolation and purification of most components.

When the chromatographic studies of hemoglobin components are combined with specific chemical studies, the structural relations of many components become clearer. The conclusions from chemical studies of a number of components have been correlated in summary form in Table XIX. Some conclusions from studies that were made in collaboration with others and that were not presented in the Results and Discussions of the Thesis have been included in Table XIX and are enclosed in parentheses. Conclusions from studies made by others and quoted from the literature are listed in brackets. In addition to the chemical procedures which were employed in the present work, the results from studies of the kinetics of alkali denaturation

TABLE XIX

Summary of Conclusions from Chemical Studies

Components	Amino Acid Analysis	N-Terminal Sequences	Ultraviolet Spectrum	Rate of Alkali Denaturation	Peptide Patterns	Subunit Hybridization	Molecular Formulas	Special Comments
A_{Ia}	A+ileu		A			} α		
A_{Ib}	Not A nor F		A			}		Could be a mixture of A and F
A_{Ic}	A	α X	A		A	α	$\alpha_2 X_2$	two unknown chains, X, postulated
A_{II}	A	[α β]	A	A	A	[α β]	$\alpha_2\beta_2$	Primary reference hemoglobin
A_{IIIb}	A	(α β)	A		A	(α)	$\alpha_2 \delta_2$	Apparently same as A_2 component
F_I	(F)	α Y	F	F	F	α	$\alpha_2 Y_2$	Two unknown chains, Y, postulated
F_{II}	F	[α γ]	F	F	F	α	$\alpha_2 \gamma_2$	See appendix IV
A_{II}^{CB}			A	A	(A)			Probably identical to adult A_{II}
Zone 1a PT	γ	γ	> F	>F<A	γ	γ	γ_4	Identical with Bart's hemoglobin
Zone 1a RC&HD			A	>A				May consist of "altered" β_4
Zone 1b	β	β	A	>A	β	β	β_4	Main abnormal component
Zone 2 (A_{Ic}^{H})	A+$\frac{1}{6}$F		A	A	A		$\alpha_2 X_2$+$\alpha_2 \gamma_2$	Mixture of A_{Ic} and F_{II}
Zone 4 (A_{II}^{H})	A	α β	A	A	A+E.S.		$\alpha_2^? \beta_2$	Extra spot (E.S.) see text

Zone 5 (A^{H}_{III})	A	A		A			Probably identical with A_{III}
S_{Ia}		A					
S_{Ib}		A					
S_{Ic}		F	F	F			Probably identical with F_I
S_{Ie}	$\alpha\gamma$	F	F	F	$\alpha\gamma$	$\alpha_2\gamma_2$	Identical with F_{II}
S_{If}		A		S	$?\beta^S$		Hybridization of S_I suggests β^S
S_{II}	[α β]	A	A	S	[α β^S]	$\alpha_2\beta^S_2$	Primary reference hemoglobin
S_{III}	α β	A		S	α $\beta^{S_{III}}$	$\alpha_2\beta^{S_{III}}_2$	See text for $\beta^{S_{III}}$

Explanation of Symbols. - Letters A, F, and S indicate similarity to hemoglobins A_{II}, F_{II}, and S_{II}, respectively for the given procedure.

- Greek letters α, β, and γ indicate similarity to the α, β, and γ chains respectively for the given procedure. Superscripts have been added only in those cases in which differences from the α and β chain of hemoglobin A_{II} and the γ chain of hemoglobin F_{II} have become apparent. The "?" has been used in the molecular formula of A^{H}_{II} because of the possibility that the α chain of some of this component is abnormal.
- The > indicates a property greater than that of the reference, for example tryptophan "notch" greater for the γ_4 than for the F_{II}, alkali denaturation rate greater than that of F_{II}.
- The < indicates a property less than that of reference.
- Parentheses indicate work in collaboration with others but not cited under Results and Discussions. Parentheses have not been used for studies of rate of alkali denaturation, see text.
- Brackets indicate work of others which is cited for reference purposes.

of a number of components have been included in this table. Most of this work was done in collaboration with Dr. Norman Weliky and Professor Pauling (82). Molecular formulas have been written for components only for cases in which such conclusions seem justified by the available chemical evidence and, in a few cases, by assuming a molecular weight of 66,000. The symbols which are used in Table XIX are described in the legend to the table. Each symbol is intended to indicate that the results from a particular chemical study of a given component are similar or indistinguishable from the results obtained from hemoglobin A_{II}, F_{II}, or S_{II} or the α, β, or γ chains.

Although components A_{Ic} and F_I contain normal α chains, their molecular formulas can be written only by postulating the presence of pairs of two unknown types of chains, X_2 and Y_2, respectively. The N-terminal amino acid sequences of these chains remain unknown but much of the internal sequence of the X chain appears to resemble the normal β chain whereas much of the Y chain resembles the γ chain as indicated by peptide pattern studies.

The formula for component A_{IIIb} (A_2) has been concluded mainly from unpublished work of Schnek, Jones, and Schroeder (83) and observations of Stretton and Ingram (84).

Contrary to Itano's earlier conclusion that hemoglobins A and F are very different both chemically and genetically (35), the results of the more recent chemical

studies of hemoglobin F_{II} indicate that these two hemoglobins share half of their structure in common as an examination of their molecular formulas readily indicates. On the basis of this chemical evidence it is possible to postulate that a portion of the genetic control of these two hemoglobins is identical.

Unique among the structures of the various hemoglobin are the tetramer structures of γ_4 and β_4. Component β_4 is concluded to be the one abnormal hemoglobin that is always associated with thalassemia hemoglobin H disease, whereas component γ_4, which is identical with hemoglobin Barts (43, 44), appears to be detectable only in certain individuals with that disease. With the determination of these tetramer structures, it is possible to predict that δ_4 and α_4 hemoglobins may exist naturally or may be produced experimentally. In fact, a component that resembled A^*_{IIIb} was detected in one of the hybridizations of β_4 with F^*_{II} (Experiment F, Table XVII). Although this observation was not discussed earlier, it can reasonably be postulated that this component may be $a_2^{A*}\delta_2^{A_2}$ and could have arisen from the recombination of α^{A*} chains from the F^*_{II} component and δ chains from a "δ_4 hemoglobin" present as a contaminant in the β_4 component which was used in the hybridization. This postulate does not alter the earlier conclusions which were drawn from Experiment F.

From the presence of what appeared to be an "extra" or "new" peptide in the region of spot No. 23 (αT-4) in the peptide pattern of component A^{H}_{II}, it is possible to conclude that the α chain of some of the component in this zone may be altered in their amino acid sequence. Such an alteration in the α chain could be genetically determined and be a primary factor in the apparently deficient formation of these chains in thalassemia hemoglobin H disease (42, Appendix V). This is in agreement with the observation of hemoglobins Q and H in the same individual (85) and unpublished studies which indicate that the α chain of hemoglobin Q has an abnormal No. 23 peptide (86).

Although the molecular formula of component S_{II} has been established by Vinograd, Hutchinson, and Schroeder (61), the exact nature of the $\beta^{S_{III}}$ chain in the formula of S_{III} is not yet clear, except that it has N-terminal val-his-leu and resembles the β^{S} chain of component S_{II}.

Thus, the chemical basis of minor component heterogeneity appears to be multiple. From the studies of component A_2 (A_{IIIb}) made principally by others (78,84), it is evident that some minor component heterogeneity is due to the alteration of portions of the primary structure of the hemoglobin molecule. However, the identification of S_{Ie} as F_{II} and S_{Ic} as F_{I} indicates that this minor component heterogeneity probably results from a persistence in the

formation of fetal hemoglobin. Finally, the presence of non-reactive N-terminal amino acid groups in the minor components F_I and A_{Ic}, possibly because of acylation of the α-amino group, may indicate that modification of the major components F_{II} and A_{II}, respectively, without alteration of primary structure of these molecules may also be a basis of some of the minor component heterogeneity. The possibilities of other causes for this heterogeneity such as alteration in tertiary structure, modification of reactive side groups, or combinations with other molecules have not been excluded by the present studies. The importance of these possibilities is evident from consideration of the incomplete studies of components S_{If} and S_{III} which indicate that these components possess the alteration in primary structure that is characteristic of the major component S_{II}.

APPENDICES

APPENDIX I

FORMATION OF DOUBLE ZONES OF SINGLE HEMOGLOBIN COMPONENTS

The formation of what appears to be two zones when each of the components A_{Ic}, A_{II}, and S_{II} is chromatographed alone was observed early in the Thesis work. This phenomenon may be similar to the "double zoning" which occurs with simpler compounds but which is not completely explained according to Schroeder (87). The studies of these hemoglobins, however, indicate that the two zones formed during chromatography of hemoglobins are variants of a single type of molecule. Studies which support this conclusion will be presented and briefly discussed below. The examples are mainly those of hemoglobin S_{II}, but the conclusions appear to apply to other hemoglobins.

Although the formation of the two zones of hemoglobin S_{II} may be observed on a 1 by 35 cm. column when 5 to 200 mg. are used, the detection of two peaks in the effluent fractions is generally found only when amounts of 40 to 100 mg. are used (see fig. 7). The more rapidly moving of the two zones proceeds down the column at a constant velocity, whereas the more slowly moving zone proceeds at a continually decreasing velocity. In a few experiments the more rapidly moving zone has appeared to divide into two new zones. Conversely, when small amounts of hemoglobin are chromatographed, the demarca-

tion between the more rapidly and the more slowly moving zones often disappears before the front of the faster zone has been eluted from the column. These observations indicate a possible conversion of the component from one chromatographic form to another.

When fractions of hemoglobin from each peak of the two zones of hemoglobin S_{II} were rechromatographed separately, the formation of two zones was again noted for both fractions. Although this result indicates the probability of a reversible change in solution, the conversion which takes place during chromatography appears to be dependent upon interation of the hemoglobin and the resin and occurs principally during chromatographic development. This conclusion is based upon the observation that when the development of a chromatogram of S_{II} was stopped for several hours and then continued, the behavior of the two zones was similar to that of chromatograms in which the development had been continuous.

Analyses of the N-terminal peptides from each zone of hemoglobin S_{II} by Dr. Schroeder and Mrs. Joan Shelton did not reveal any significant differences in the ratio of α chains to β chains. Thus dissociation into asymmetric half molecules with gross separation of half molecules does not seem to be an explanation for the two-zone phenomenon. Dissociation into asymmetric half molecules without gross separation of the half molecules also does not seem to be involved. This was concluded from an experiment in

which hemoglobin S_{II} was applied to a column and allowed to form two zones. Radioactive hemoglobin A_{II} was then applied in such a way that it formed two zones which passed through the region of the two S_{II} zones and which were then eluted from the bottom before the S_{II} component. Because no transfer of radioactivity to the S hemoglobin was detected, apparently dissociation into subunits which are free to transfer between the two hemoglobins did not occur.

That the formation of ferrihemoglobin cyanide is not the explanation of double zones became obvious from the observation of the double zones during chromatography of hemoglobin S_{II} (also A_{II}) which had been converted entirely to the ferrihemoglobin cyanide form.

Thus, although the explanation for the double zone phenomenon is not yet clear, these studies do indicate that the two zones are probably a single molecular species which undergoes a reversible change from one chromatographic form to another. Interaction of the hemoglobin with the resin is probably important in the conversion during chromatographic development, although this conversion does not appear to be caused by the dissociation into asymmetric half molecules. It is possible that dissociation in symmetrical half molecules, the formation of aggregates of the whole molecule, or reversible changes in the tertiary structure may be responsible for this double zone phenomenon.

APPENDIX II

RECOMMENDATIONS FOR THE NOMENCLATURE OF HEMOGLOBINS (57)

Chain Nomenclature

There is now general agreement on the naming of the peptide chains of the major components of normal human adult and fetal hemoglobins as the α, β and γ chains; e.g. adult hemoglobin is written as $\alpha_2^A\beta_2^A$ and fetal hemoglobin as $\alpha_2^A\gamma_2^F$. The superscripts A and F refer to the fact that the particular chain is the one found in the human adult and fetal hemoglobins. It is recommended that this practice be continued and that the symbols α, β and γ, without superscripts, be reserved for those occasions when reference is being made to, for example, α chains in general.

Information concerning the structure of the chains of hemoglobin A_2 is now sufficient to indicate that one of the chains is identical with the α^A chain, while the second differs in a number of residues from the three foregoing chains. In addition there is evidence (e.g. ref. R. Cepellini, CIBA Symp. Biochem. Genetics, 1959) to indicate that the genetic control of this unique chain is independent of the genes for the α, β and γ chains. It is therefore recommended that this chain be designated as δ^{A_2}; Hb A_2 is then written as $\alpha_2^A\,\delta_2^{A_2}$. Again, one could refer

simply to δ chains in the general case.

The simplest method of naming the tryptic peptides of a chain is to number them in the order in which they occur in the chain, beginning with the N-terminus. The symbol for the chain is included as a part of the designation. The letter T is included to insure that there be no confusion between peptide number and amino acid residue number. For example, the third tryptic peptide of the α chain would be αT-3 in this system. Where a lysyl bond is not attacked under the conditions used, the symbol for the resultant "dipeptide" or "double peptide" would contain the numbers appropriate to both tryptic peptides, e.g. αT-1,2. From the published structure of the α and β chains (10,11) and from the amino acid composition it is evident that the α chain will contain the tryptic peptides αT-1 to αT-14 and the β chain the tryptic peptides βT-1 to βT-15. It so happens that the tryptic peptides αT-8 and βT-8 are lysine. In addition, the present methods of tryptic cleavage do not break the bond separating the expected tryptic peptides αT-12 and αT-13, nor the bond between the expected peptides βT-10 and βT-11. In view of the possibility that these bonds might be split in some experiments at a later date, it is felt that the numbering system should correspond with the theoretical number of tryptic peptides.

Nomenclature of the Abnormal Hemoglobins

An ideal nomenclature system for the abnormal hemoglobins would provide for adequate designation of the chemical structure at each stage of the investigation. The following system is an attempt to meet this requirement.

When only the chain in which the abnormality resides is known, then the hemoglobin may be written as $\alpha_2^A\beta_2^S$, or $\alpha_2^A\beta_2^{D\text{Punjab}}$. When the abnormality has been located in a particular tryptic peptide, as by fingerprinting, then the designation should be, for example, $\alpha_2^A\beta_2^{T\text{-}1}$. When the amino acid composition of the tryptic peptide indicates a particular amino acid substitution, then this will be indicated as $\alpha_2^A\beta_2^{T\text{-}1\ (Glu\rightarrow Val)}$ for HbS. Finally, when the amino acid interchange has been located at a particular residue position in the chain, the fully descriptive formula, as in the case of HbS would be in the form: $\alpha_2^A\beta_2^{6Val}$.

Presumably, for use in formulae describing experiments such as reassociation, it will be necessary to define in a given paper a one letter designation for a particular hemoglobin. For example, the formula $\alpha_2^I\beta_2^S$ could be used, provided that wherever possible the hemoglobin has been defined, as, for example, HbI as $\alpha_2^{16Asp}\ \beta_2^A$ and HbS as $\alpha_2^A\ \beta_2^{6Val}$.

It is strongly urged that no further letters be assigned to abnormal hemoglobins. Newly discovered hemoglobins prior to their chemical identification, should be known by the letter designation of the previously described hemoglobin whose electrophoretic mobility they most nearly resemble. To the letter should be attached a subscript indicating the geographic origin of the new hemoglobin.

APPENDIX III

[Reprinted from the Journal of the American Chemical Society, 81, 3161 (1959).]

GROSS STRUCTURE OF HEMOGLOBIN H

Sir:

Human hemoglobin H has been described in some detail by Rigas, Koler and Osgood.[1] Chemical investigations of chromatographically purified[2] hemoglobin H, here presented, lead to a further understanding of its structure and of its relation to other human hemoglobins.

When DNP-globin H was prepared and examined by methods previously described,[3,4,5] the result was approximately four N-terminal valyl residues per molecule of 66,000 molecular weight[1] but only one kind of N-terminal sequence: val-his-leu. This N-terminal sequence defines β chains[5] and suggests that hemoglobin H may be represented[6] as β_4^H.

"Fingerprints"[10] of tryptic hydrolysates of hemoglobins H and A differed markedly. Peptides numbered[10] 5, 10, 11, 13, 17, 18, 23, and probably several others in regions normally poorly resolved were absent on the fingerprint of H but no new peptides were apparent. The absent peptides were present on fingerprints of isolated α^A chains. The likely conclusion that the sequence in β^H and β^A chains is identical was substantiated by the following hybridization experiment.[8,11]

Following hybridization of carbonmonoxyhemoglobin H and radioactive carbonmonoxyhemoglobin S at *p*H 11.0 at 3° for 24 hr., four hemoglobins were chromatographically isolated. These data are pertinent:

	Reactants		Products			
Zone	...	...	1	2	3	4
Mg.	22	22	5[a]	2[a]	15	7
C.p.m./mg.	0	1200	70	1100	600	1200
Identity of material	Hb-H	Hb-S*	Hb-H	β_4^S*	Hb-A*	Hb-S*
Formula	β_4^A	α_2^A*β_2^S*	β_4^A	β_4^S*	α_2^A*β_2^A	α_2^A*β_2^S*

[a] Precipitation that occurred during hybridization must have consisted of β^A and β^{S*} chains because α chains are conserved.

Identification of the products involved chromatographic studies and determination of radioactivity and for hemoglobin A also the study of sedimentation velocity and examination of N-terminal peptides[3,4,5] to show that only the α chains were radioactive. Thus, hemoglobin A and β_4^S* were formed during hybridization but there was no evidence for $\beta_2^A\beta_2^S$*. On the basis of the radioactive and material balance, it was concluded that the four β chains of hemoglobin H are identical with each other and with β^A chains.

Hemoglobin H is the first observed example of a hemoglobin composed of a single kind of polypeptide chain. Possibly, other abnormal hemoglobins or minor components in normal hemoglobin may be built on the scheme α_4, $\alpha_3\beta$, $\beta_2\gamma_2$, etc. Biologically, it suggests that hemoglobin H disease results from an imbalance in the relative production of α and β chains and hence that α and β chains are under separate biosynthetic and genetic control. This latter suggestion is further supported by experiments now in progress which show that the α^A and α^F chains are identical and that β chains are present in several minor hemoglobin components normally associated with hemoglobin A and S.

These experiments were made possible by the interest and generosity of Dr. D. A. Rigas and Dr. R. D. Koler. This investigation was supported in part by grants H-2258 and H-3394 from the National Institutes of Health, United States Public Health Service.

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(2) Extension of methods of D. W. Allen, W. A. Schroeder and J. Balog, THIS JOURNAL, **80**, 1628 (1958).

(3) H. S. Rhinesmith, W. A. Schroeder and L. Pauling, *ibid.*, **79**, 609 (1957).

(4) *Ibid.*, **79**, 4682 (1957).

(5) H. S. Rhinesmith, W. A. Schroeder and N. Martin, *ibid.*, **80**, 3358 (1958).

(6) The N-terminal sequence[5] defines the chain as α or β, the superscript denotes the hemoglobin that is the source of the chain, and the subscript has the usual chemical significance. The glycyl chains[7] of hemoglobin F are termed γ chains. Thus, hemoglobin A and S are $\alpha_2^A\beta_2^A$ and $\alpha_2^A\beta_2^S$ inasmuch as the α chains are identical.[8,9]

(7) W. A. Schroeder and G. Matsuda, THIS JOURNAL, **80**, 1521 (1958).

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APPENDIX IV

[Reprinted from the Journal of the American Chemical Society, **81**, 4749 (1959).]

IDENTITY OF THE α CHAINS OF HEMOGLOBINS A AND F

Sir:

Possible identity of portions of human fetal and adult hemoglobin was suggested by Schroeder and Matsuda,[1] who determined that fetal, like adult,[2] hemoglobin contained two polypeptide chains N-terminal in the sequence val-leu (α chains).[3] This suggested identity now has been substantiated by our present experiments which show not only that "fingerprints"[5] of the soluble portion of tryptic hydrolysates of α^A and α^F chains are grossly indistinguishable[6] but also that α chains may be transferred by hybridization of hemoglobin F with other hemoglobins.

All hemoglobins were isolated from hemolysates by column chromatography[7] to remove minor components normally present. Zone F_{II},[7] the main component of normal full-term cord blood, was taken as hemoglobin F.

The reversible dissociation of hemoglobin F into molecular sub-units is similar but not identical to hemoglobin A[8,9] at both alkaline and acid *p*H. Thus hybridization[10,11] or recombination[10] with other hemoglobins might be expected. Initial hybridization of hemoglobin F_{II} ($\alpha_2^F\gamma_2^F$) and radioactive (C^{14}-labelled leucine) hemoglobin S ($\alpha_2^{A*}\beta_2^{S*}$) at *p*H 11.0 or 11.2 at 3° for 1 to 6 days showed less transfer of radioactivity to hemoglobin F than would have been expected from a hybridization of the type

$$2\,\alpha_2^{F=A}\gamma_2^F + 2\,\alpha_2^{A*}\beta_2^{S*} \rightarrow \alpha_2^{F=A}\gamma_2^F + \alpha_2^{A*}\gamma_2^F + \alpha_2^{A*}\beta_2^{S*} + \alpha_2^{F=A}\beta_2^{S*} \quad (1)$$

if α^F and α^A are identical. Nevertheless, hemoglobin F_{II} after chromatographic isolation was radioactive in the α chains only, as shown by investigation of the N-terminal peptides.[2,4,12] Likewise, when radioactive hemoglobin F_{II} and hemoglobin A were hybridized at *p*H 4.7 at 3° for 12 hr., transfer of radioactivity to hemoglobin A was 53% of that calculated from an equation like (1). These experiments do not determine the number of identical α chains in hemoglobin F. However, after approximately equal amounts of hemoglobin F_{II} and hemoglobin H (β_4^A)[13] had been hybridized at *p*H 4.7 at 3° for 12 hr., chromatography separated three hemoglobins, an "H-like" hemoglobin, "F_{II}", and "A." From the ratio of F_{II} to A which was very close to 1:2, it was calculated that hybridization according to the equation

$$3\alpha_2^{F=A}\gamma_2^F + 3\beta_4^A \rightarrow 2(2\beta_2^A + \gamma_2^F) + \alpha_2^{F=A}\gamma_2^F + 2\alpha_2^{F=A}\beta_2^A \quad (2)$$

was essentially complete (97%): thus, two identical α chains are present in hemoglobin F. The newly formed hemoglobin A was characterized by its chromatographic and starch electrophoretic behavior. The nature of the "H-like" hemoglobin from the hybridization is unknown at present. At *p*H 11.2, the hybridization of F_{II} and H was about 20% in 12 hr.

From the incompleteness of hybridization under certain conditions, the process clearly is a complex one. As more information becomes available, it should give an insight into the apparently different forces between the chains of the various hemoglobins.

The conclusion that the α^F and α^A chains are identical strongly suggests a related genetic control of hemoglobins A and F and is an extension of the idea derived from the structure of hemoglobin H that the α and β chains of adult hemoglobin are under separate genetic control.[13,14] It is clearly apparent that these ideas would be substantiated by the detection of an abnormal fetal hemoglobin in a newborn child who, in adult life, will produce an abnormal hemoglobin that has an aberration in the α chain.

This investigation was supported in part by grants H-2558 and H-3394 from the National Institutes of Health, United States Public Health Service.

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(3) The two γ chains of fetal hemoglobin terminate in glycine[1] and the two β chains of adult hemoglobin in val-his-leu.[4]

(4) H. S. Rhinesmith, W. A. Schroeder and N. Martin, THIS JOURNAL, **80**, 3158 (1958).

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APPENDIX V

THE CHEMICAL SUBSTRUCTURE OF HEMOGLOBIN H; A UNIQUE TYPE OF HEMOGLOBIN MOLECULE*

The chemical substructure of hemoglobin components present in Hb H disease was determined and compared with Hbs A, S, and F. The techniques employed included ion-exchange chromatography (for the isolation of hemoglobin components), N-terminal amino acid sequence analysis, ultraviolet spectroscopy, tryptic peptide pattern analysis, and molecular subunit hybridization. Recent work indicates that Hbs, A, S, and F have the respective molecular formulas $\alpha_2\beta_2^A$, $\alpha_2\beta_2^S$, and $\alpha_2\ \gamma_2$. These α chains are identical and have the N-terminal sequence val-leu. The β chains of Hbs A and S have N-terminal val-his-leu and differ by only one amino acid residue. The γ chain has N-terminal glycine.

Studies of Hb H revealed two abnormal components. One, apparently present in all Hb H subjects, has a formula β_4^A. The other, observed in only one of three subjects, has a formula γ_4 and is chromatographically identical with Hb Bart's. These two components represent a new, unique type of hemoglobin molecule. We conclude

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*Abstract from Clinical Research 8, 129 (1960). Authors Richard T. Jones and W. A. Schroeder.

that γ and β chains can exist <u>in vivo</u> independently of α chains, furthermore that α, β, and γ chains may be biosynthesized as separate units before combining to form normal hemoglobin. It is proposed that insufficient production of normal α chains is responsible for the formation of γ_4 and β_4^A hemoglobins.

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PROPOSITIONS

I The Dissociation Constants of the Diastereoisomers of β-Hydroxyaspartic Acid

Because the separation of the two diastereoisomers of β-hydroxyaspartic acid by ion exchange chromatography may be due to differences in proton dissociation, it is proposed that the dissociation constants of each diastereoisomer be determined and compared.

Background for Proposition

Recently Kornguth and Sallach (1) have reported the separation of the diastereoisomers of β-hydroxyaspartic acid by ion exchange chromatography on Dowex 1, a quaternary ammonium anion exchange resin (2), and on Amberlite IR-120, a sulfonated polystyrene cation exchange resin (2). The separation on Amberlite IR-120 has been confirmed in these Laboratories by the use of an automatic amino acid analyzer (3, 4).

Displacement and elution chromatography of amino acids on ion exchange resins have been summarized by Greenstein and Winitz (5). These authors note, as did Partridge and Brimley (6) earlier, that the order of the displacement of the amino acids is generally that of the order of their pK values. Thus, according to Partridge and Brimley (6), "a study of the order of displacement of a range of solutes confirms that the main factor in determining the order is the basic and acidic strength of the solutes, and it has become the practice to regard any departures from this order based on pK values as 'anomalous'." The

exceptions, which include the aromatic amino acids, proline, and methionine, may be retarded due either to solubility effects or to short-range adsorptive forces associated with ring structures (5).

An explanation for Partridge and Brimley's conclusion becomes apparent from a consideration of the ion exchange process. Because only cations are adsorbed by the strongly acidic $-SO_3^-$ groups in the case of the sulfonated polystyrene resin, the reaction of amino acids with this resin depends on the availability of the completely protonated form of the amino acid. The relative amount of the protonated form, and therefore the order of adsorption of the amino acids, is related to the order of their dissociation constant for the equilibrium reaction $NH_3^+RCOOH \rightleftharpoons NH_3^+RCOO^- + H^+$. The constant for this reaction is generally designated as K_1 (5, 6), and the reaction is often written as $A^+ \rightleftharpoons A^\pm + H^+$. Although bivalent cations such as lysine and arginine should be retarded on the cation exchange resins, the monoaminodicarboxylic acids, aspartic and glutamic acids, should behave like the monocarboxylic acids. Similarly, the order of displacement of amino acids on an anion exchange resin such as Dowex 1 should be related to the dissociation constant for the reaction $A^\pm \rightleftharpoons H^+ + A^-$. For monoaminomonocarboxylic acids this is the K_2 constant for the dissociation of $-NH_3^+$, i.e., $NH_3^+RCOO^- \rightleftharpoons NH_2RCOO^- + H^+$, whereas for the monoaminodicarboxylic acids it is the K_2 constant for the dissociation of the second carboxyl group, i.e.,

$$HOOCRNH_3^+COO^- \rightleftharpoons {}^-OOCRNH_3^+COO^- + H^+.$$

In displacement chromatography with ion exchange resins such as the sulfonated polystyrenes, the displacement of a weaker base, A_2, will depend mainly upon the partial suppression of the cationic form of A_2 by the presence of a stronger base, A_1 (6). The pH of the ambient solution will be controlled only by the presence of the amino acids themselves. On the other hand, in elution chromatography of amino acids as described by Stein and Moore (7), the formation of elution peaks depends upon the use of developers which contain cations capable of competing with the cationic form of the amino acids. The competing cations are protons in acid developers and Na^+ in sodium citrate developers. The order of emergence of the amino acids from the elution columns is very similar to that in displacement chromatography which, according to Partridge and Brimely (6), is not surprising. Thus the relative order of amino acids in elution chromatography also appears to be related to the order of their dissociation constants (5).

Shulgin et al. (8) have demonstrated that DL threonine can be separated from the diastereoisomeric DL allothreonine by chromatography on Dowex 50, a sulfonated polystyrene cation exchange resin (2). Threonine is eluted more rapidly than allothreonine in their system, which one might have been willing to predict from the pK_1 values at 25° of 2.088 for threonine and 2.108 for allothreonine as determined by Smith, Gorham, and Smith (9). From the data of these authors, it appears reasonable to conclude that the difference in these two pK_1 values is significantly greater than variations due to experimental errors.

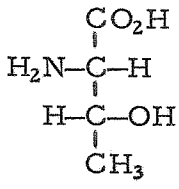
Although the differences in the pK_1's and chromatographic separation are small, their correlation is consistent with Partridge and Brimely's generalization.

Thus, the rather striking separation of the diastereoisomers of β-hydroxyaspartic acid (1, 3) could be due to differences in their relative pK values. Although Chibnall and Cannon (10) determined differences in the pK values of aspartic acid and β-hydroxyaspartic acid of 0.10, 0.29, and 0.82 for the pK_1, pK_2, and pK_3's respectively, the authors do not indicate whether the β-hydroxyaspartic acid which they studied was a single diastereoisomer or a mixture of the two. However, the pK values which they report are consistent with the observation that both diastereoisomers of β-hydroxyaspartic acid are eluted more rapidly than is aspartic acid (1, 3). If the striking difference in the separation of the two diastereoisomers of β-hydroxyaspartic acid by elution chromatography on both anionic and cationic resins is due primarily to dissociation properties of these molecules, then a significant difference in at least the first and second dissociation constants of the two diastereoisomers should exist.

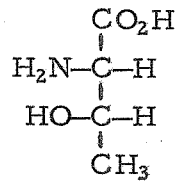
Structure and Nomenclature of the Diastereoisomers of β-Hydroxyaspartic and Aspartic Acids.

Greenstein and Winitz have reviewed the structural configuration and nomenclature of the diastereoisomers of β-hydroxyaspartic acid and threonine (11). The spatial relationship of the substituents surrounding the α- and β-asymmetric centers of L threonine and β-hydroxy-L-aspartic

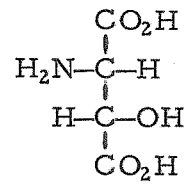
acid and their diastereoisomers is shown below by Fischer projection formulas (L with respect to the α-carbon asymmetry):



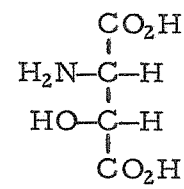
L-threonine



L-allothreonine



threo-β-hydroxy-L-aspartic acid



erythro-β-hydroxy-L-aspartic acid

Execution of the Proposition

Because enantiomers do not differ in dissociation properties (12), the measurement of the dissociation constants could be made on racemic mixtures (mixtures of the D and L enantiomers) of the two diastereomers of β-hydroxyaspartic acid. Although the use of the racemic mixtures would simplify the preparation of the materials, the erythro-hydroxy-aspartic acid racemate can be resolved according to Greenstein and Winitz (13). However, the threo-hydroxyaspartic acid racemate has not yet been resolved (13).

Although the two diastereoisomeric racemates of β-hydroxyaspartic acid have been prepared by several authors (see references 1 and 13), they are probably not yet commercially available. However, according to Kornguth and Sallach (1) the synthesis of the diastereoisomers of β-hydroxyaspartic acid can be effected readily by allowing a mixture of sodium glyoxylate and copper glycinate to react in 1N NaOH at 5° for 12 to 18 hours. The reaction is an aldol condensation and is represented by: $HOOCCHO + (NH_2CH_2COO)_2Cu \longrightarrow \begin{matrix} NH_2CHCOOH \\ CHOHCOOH \end{matrix}$. The copper can

be removed as copper sulfide, and the sodium ion can be adsorbed onto Dowex 50 resin in the hydrogen form without losing the amino acids, provided the pH remains above 6. The diastereoisomers of β-hydroxy-aspartic acid could then be separated and purified chromatographically on either Amberlite IR-120 or Dowex 1 as described by Kornguth and Sallach (1).

Acid-base titrations of each diastereoisomer as done by Cannon (10) might be made initially in order to obtain approximate values of the 3 pK constants for each diastereoisomer (12). Unless a comparison of the respective values of the pK_1 and pK_2 of the two isomers revealed significant differences, more precise measurement of the true thermodynamic dissociation constants as determined from cells without liquid junctions might be necessary (9, 12, 14, 15, 16). The determination of the three pK constants for each diastereoisomer could be carried out in the same way that Batchelder and Schmidt (15) determined the pK's of aspartic acid, in which the electromotive force cell was represented by:

$$H_2(Pt) \mid \text{Amino acid } (m_T),\ NaOH \text{ or } HCl\ (m_B \text{ or } m_A),\ NaCl\ (m_S),\ AgCl \mid Ag.$$

Predictions Based on Proposition

If the separation of the diastereoisomers of β-hydroxyaspartic acid by elution ion exchange chromatography is a reflection of differences in their respective dissociation constants, then one can predict the relative order of their pK_1 and pK_2 constants. Because the threo form precedes the erythro form on elution chromatography on the sulfonated polystyrene cation exchange resins, one would predict that the pK_1 of the threo diastereomer is less than that of the erythro diastereomer. Conversely,

because the erythro diasteriomer precedes the threo diasteriomer on anionic exchange resins (Dowex 1), one would predict that the pK_2 of the erythro form is less than that of the threo form. The pK values determined for threonine and allothreonine (9) are in this relative order, which also supports the prediction.

II A Palliative Treatment for Sickle-Cell Anemia

Because the disease process in sickle-cell anemia is related to the inability to produce normal erythrocytes, it is proposed that a long-term palliative treatment for this disease might result from the establishment of a transplant of erythropoietic tissue from a normal donor whose erythrocytes contain only hemoglobin A. A number of chemical procedures related to the study and detection of human hemoglobins would be required in the execution of this proposition.

Background for Proposition

Wintrobe has reviewed many of the pertinent medical and biological factors related to sickle-cell anemia (17). In discussing the prognosis of sickle-cell disease Wintrobe indicates that it "is a serious malady with an ultimately fatal outcome. Most patients die in the first decade of life and very few survive the third." Harris (18), in discussing the selective pressure against the sickle-cell gene, similarly indicates that the "genetic" survival of the individual who is homozygous for the abnormal gene is very low.

Several authorities (19, 20, 21) conclude that the "sickling" process is a property of the red cell rather than the plasma and that the abnormal

hemoglobin which is present is the primary etiological factor. Thus, according to Itano and Pauling (20), "it is probable that sickle-cell anemia can be described as a molecular disease, resulting from the difference in molecular structure of sickle-cell-anemia hemoglobin and normal adult human hemoglobin, the properties of the abnormal hemoglobin being such that when deoxygenated the molecules combine with one another to form long molecular strings, which, through intermolecular attraction, aggregate into tactoids, which have enough mechanical strength to distort the red cell, changing the viscosity of the blood, and causing the clinical and pathological manifestation of the disease."

One of the most important factors in the pathological physiology of sickle-cell disease is the persistent anemia. The concentration of the circulating red cells in patients with this disease is generally one half or less than that of the normal adult. Although the anemic state is generally attributed to an inability to synthesize erythrocytes at a normal rate (21), a significant decrease in the life span of the erythrocytes from sickle-cell anemia subjects has been observed (22) and may contribute to the lowered steady state concentration of erythrocytes (23). One consequence of any severe anemia is a decrease in the average degree of oxygenation of cells in certain areas of the circulatory system (24), which in the case of sickle-cell disease leads to an increase in the random destruction of the erythrocytes containing the sickle-cell hemoglobin (17). Thus blood from donors with normal erythrocytes often is transfused into the sickle-cell individual in order to relieve his anemia and thereby

decrease the rate of hemolysis of his erythrocytes which contain sickle-cell hemoglobin (25). This is only a temporary measure because of the finite life span of the transfused normal-erythrocytes, which are incapable of reproduction.

Thus the high mortality and morbidity of sickle-cell disease results from the fact that individuals with this disease possess an abnormal erythropoietic tissue, resulting in the production of insufficient erythrocytes, which in turn have a diminished life span because of the presence of the sickle-cell hemoglobin. Although it may be possible at some future time to correct the genetic defect present in these somatic erythropoietic cells which results in the formation of the abnormal ehmoglobin by in vivo conversion or replacement of some of their RNA or DNA molecules, a simpler and more immediate palliative measure would be the establishment of a graft of erythropoietic tissue from a normal donor in the sickle-cell anemia individual.

There are a number of problems and questions which must be considered before any such experimental procedure could be attempted. These include questions concerning: how transplantation of erythropoietic tissue could be accomplished; what type of tissue should be used; whether the recipient should be irradiated; what basis and method should be used in selecting the recipient; what risks to the recipient would be involved; what would justify these risks; what reason would there be for believing that a transplant would be of benefit; and how the therapeutic benefits of the experiment could be evaluated. Although each of these problems can be discussed, only a few comments concerning some of the questions will be presented, for the sake of brevity.

Although bone marrow transplantation is technically possible and has been utilized in satisfactory transplantation of erythropoietic tissue both in experimental animals (26) and humans (27, 28), the use of blood from infants with erythroblastosis fetalis (29) would appear to be the tissue of choice (30) because of its availability and ease of transfusion, the presence of primitive erythropoietic cells, and the higher probability of obtaining a successful transplantation with fetal tissue (31). Irradiation of the recipient has been considered necessary before attempting bone marrow transplantation in the adult human (27); however, this is associated with undesirable side effects and might be avoided if transplants were attempted in infants. Even in the event the transplanted erythropoietic tissue failed to survive in the recipient, it might result in an "active acquired tolerance" (31) towards the donor if the recipient were an infant. If such tolerance did develop, transplantation attempts could be repeated again at an older age. The major risk to the recipient is the development of a graft-versus-host reaction (33). Although this risk is real and could result in death, it is difficult to evaluate accurately in the case of humans. Acceptance of this risk is probably justified in the case of sickle-cell disease because of the high mortality and morbidity associated with this anemia as it is presently treated. In view of this risk and considering the problem of determining whether a transplant has succeeded, only individuals who are homozygous for the sickle-cell gene should be selected as recipients. The clinical diagnosis of sickle-cell anemia at birth is impossible to make, but with the techniques of ion-exchange chromatography a laboratory diagnosis could be made (34). In order to evaluate the

success of the transplant the relative percentages of hemoglobins S and A could be measured at intervals after making the transplant. Serological markers could also be used in this evaluation (26, 30). Finally, whether a transplant, if established, would be of therapeutic value or not would depend upon its ability to produce normal erythrocytes at a rate sufficient to alleviate the anemia and possibly enough to replace the recipient's own sickle-cell erythropoietic tissue. It is possible that a normal erythropoietic graft could produce cells which have a selective advantage over the abnormal sickle-cell erythropoietic tissue. Probably nothing short of attempting such a transplantation will answer this question.

Execution of Proposition

Blood from an exchange transfusion from an infant with severe erythroblastosis fetalis (high nucleated erythrocyte count) would be transfused (by exchanges) into an infant who is homozygous for the sickle-cell anemia gene as judged by family studies and chromatographic evaluation of the infant's hemoglobin. Important serological incompatabilities between the donor and recipient should be avoided in an attempt to minimize the chances of graft-host and host-graft reactions. The recipient should then be followed carefully with respect to the types of hemoglobins and serological markers to be found in circulation. In the event a graft is not established, the recipient should be tested for induced tolerance against the donor's cell so that future transplantations of bone marrow might be considered.

III Incomplete Enzymatic Hydrolysis for the Determination of Primary Structure of Polypeptides

It is proposed that conditions which favor incomplete hydrolysis of proteins and peptides by enzymes such as trypsin and leucine aminopeptidase be investigated for the purpose of extending their utility for the determination of the primary structure of polypeptides.

Background for Proposition

Two different examples of incomplete enzymatic hydrolysis will be presented and their usefulness for the determination of primary structure of polypeptides will be discussed. The first example is the incomplete hydrolysis of proteins and long-chain peptides with trypsin, an endopeptidase. The second example is the incomplete or limited hydrolysis of peptides with leucine aminopeptidase, an exopeptidase. The term incomplete or limited hydrolysis is used to indicate that the enzymatic hydrolysis of susceptible peptide bonds is not complete under the conditions employed.

Many different enzymes have been successfully utilized in the determination of the amino acid residue sequence of polypeptide chains (35). Endopeptidases such as trypsin, chymotrypsin, pepsin, and papain have been employed to effect partial hydrolysis of proteins and peptides. These hydrolyses are partial in that only certain more-or-less specific bonds are hydrolyzed rather than all peptide bonds (36). The hydrolytic specificity of trypsin is for peptide bonds at the carboxyl side of the amino acids lysine and arginine, which, according to Harris and

Ingram (35), is the reason that trypsin has become "the favored tool for the controlled breakdown of proteins."

This hydrolysis at specific bonds of a protein or long-chain polypeptide results in the formation of a number of smaller peptides of which at least n-1 terminate with either lysine or arginine in the case of tryptic hydrolysis. The amino acid residue sequence of each of these smaller peptides can then be determined independently (37). In general, except for the use of papain, conditions of hydrolysis are chosen which result in as complete hydrolysis at each susceptible bond as possible. This maximizes the yield but minimizes the number of peptides which result from each specific enzymatic hydrolysis.

Although the primary structure of the peptides from the partial hydrolysis made with an enzyme such as trypsin can be determined, the complete primary structure of the starting polypeptide(s) of a protein cannot be deduced unless the relative linear order of one peptide to another is known. In general this ordering of peptides has been accomplished by studying another set of peptides that result from a hydrolysis of the original protein with a second enzyme which differs in specificity from the first (35, 37). In this way overlapping segments of the amino acid sequences can be obtained from which the relative linear order of the peptides can be deduced. Although this "double" sequence analysis is and will probably remain one of the most useful approaches to the determination of the complete primary structure of proteins, procedures which result in the incomplete hydrolysis of susceptible peptide bonds would be of real value and more direct.

The practicability of using incomplete hydrolysis of proteins with trypsin for the determination of the relative linear order of the tryptic peptides becomes evident from an examination of the recent studies of the structure of hemoglobins (38). It has become apparent that incomplete tryptic hydrolysis of certain lysyl and arginyl bonds in hemoglobin results under some conditions. Thus, a few peptides which contain a basic amino acid at internal locations of their peptide chain have been obtained, although generally in low yield. Such peptides have been called "ditryptides" (39). The relative order of the two tryptic peptides in the ditryptide can then be deduced by comparing the ditryptide with the peptides which result from a complete tryptic hydrolysis. Although these ditryptides have given very useful sequence information, their production was unexpected because an attempt was made to effect complete hydrolysis of all susceptible bonds.

In a recent experiment which was carried out in part for this proposition, a tryptic hydrolysis of hemoglobin was made and samples were removed after 5, 10, 20, and 90 minutes of hydrolysis (40). The peptide patterns of these samples were compared and found to differ in a number of ways. Several peptide spots were observed in the 5 minute sample but were undetectable in the 90 minute sample and vice versa. Although this preliminary experiment was fairly crude, it does indicate that a more quantitative study by column chromatography would be valuable. Dr. Stanford Moore has indicated that the automatic amino acid analyzer system can be modified and used in the study of peptides in hydrolysates of ribonuclease (4, 41). Similar modifications might be applied in the proposed investigation.

The different relative rates of tryptic hydrolysis of the susceptible bonds in a specific protein might be influenced by altering the pH, ionic strength, temperature, and the state and concentration of the enzyme and protein substrate in such a way that by limiting the duration of hydrolysis a number of di- and "polytryptides" could be obtained. The presence and variation in the production of these "tryptides" could be studied quantitatively by automatic chromatographic procedures.

Exopeptidases such as leucine aminopeptidase (LAP) have been used for the determination of amino acid sequences near the ends of peptides (35, 36). Kinetic studies of the release of amino acids from peptides hydrolyzed with LAP have been used successfully in determining N-terminal sequences (42, 43). However, these studies not only require repeated amino acid analyses but often require the isolation and study of the incompletely hydrolyzed peptides. Because this enzyme is unable to hydrolyze peptide bonds that are associated with the nitrogen of proline at an appreciable rate, partial hydrolysis with LAP can be used to determine the amino acid composition on the N-terminal side of proline in peptides which contain this amino acid (38, 43).

From recent studies of the amino acid sequence of peptides from hemoglobin, it appears that some peptides which contain aspartic acid or glycine, but not proline, are incompletely hydrolyzed by LAP (38). One can calculate that although the amino acids which are N-terminal to one of these two amino acids are hydrolyzed off from the peptide in stoichiometric ratios, the remaining amino acids are released incompletely. Relatively slow rates of hydrolysis of peptide bonds associated

with the carboxyl group of glycine in short peptides have been reported (44); however slow rates of hydrolysis of aspartyl bonds apparently have not been reported. In certain cases the results from an incomplete hydrolysis of peptides due to the presence of either aspartic acid or glycine residues could be useful in locating the amino acids which lie on the two sides of one of these residues, much like the presence of proline is useful. Therefore, an investigation of conditions which would minimize the rate of hydrolysis of bonds associated with either glycine or aspartic acid or the modification of other peptide bonds in order to limit their hydrolysis by LAP could result in an extension of the usefulness of LAP in amino acid sequence analysis of peptides.

Execution of Proposition

In studies of the incomplete hydrolysis of polypeptides with trypsin, a simple protein whose tryptic peptides are known should be chosen. Either ribonuclease or possibly hemoglobin could be selected. Next, procedures for the separation and quantitative detection of the tryptic peptides from this protein should be studied and adapted to automatic chromatographic equipment. Then conditions which might favor incomplete hydrolysis of the reference protein could be examined in order to learn how to obtain di- and polytyptides. The isolation and further chemical characterization of these tryptides could then be made in order to deduce the linear order of the tryptic peptides which might be present.

Results from hydrolysis with LAP under conditions which might favor incomplete hydrolysis of peptide bonds associated with either

aspartic acid or glycine could also be made with automatic chromatographic procedures. It would probably be necessary to study the effect of pH, ionic strength, temperature and concentration of peptide substrate and enzyme on the rates of hydrolysis of these bonds.

IV The Presence of β-Hydroxyglutamic Acid in Human Cerebrospinal Fluid

It is proposed that one of the unknown ninhydrin-positive compounds present in human cerebrospinal fluid may be one of the diastereoisomers of β-hydroxyglutamic acid and that a chemical characterization of this unknown compound be made.

Background for Proposition

During a recent investigation of the amino acid content of human cerebrospinal fluid (3) several unknown, ninhydrin-positive compounds were detected by ion exchange chromatography (4). One of these unknown compounds was present in the analysis of "acidic and neutral" amino acid (4) in relatively high concentration (as much as 1 micromole leucine equivalents per 10 ml fluid). This unknown was eluted just before threonine during analyses at 50° and just after serine during analyses at 30° but always before glutamic acid and after aspartic acid. This was the only compound other than glutamic acid which was observed to change in its order of elution relative to other amino acids with the 20° change in temperature.

Compounds with the chromatographic behavior of this unknown apparently have not yet been reported (45). However, there are several

characteristics of this unknown compound which support the prediction that it may be one of the diastereoisomers of β-hydroxyglutamic acid. First, the ratio of the peaks of the optical density curves at 570 to 440 mμ is similar to that of glutamic acid but different from those of other amino acids. Second, during chromatography on Amberlite IR-120 it is eluted more rapidly than glutamic acid but more slowly than aspartic acid, which could indicate an α-carboxyl group with an acidity between these two amino acids (6). Third, in comparing chromatograms at 30 and 50° the position of this unknown relative to its "neighboring" amino acids is changed significantly and in the same direction as the change for glutamic acid, which, according to Moore (45), is characteristic of dicarboxylic amino acids. Fourth, the chromatographic behavior of the compound is not altered by hydrolysis in 6N HCl for 30 hours, a condition which should cleave any peptide bonds. Fifth, the reaction of this compound with ninhydrin indicates the presence of a "free" amino group. Sixth, the unknown compound cannot be either of the α-hydroxyglutamic acids because both of these diastereoisomers have more rapid chromatographic movements than that of the unknown (3). Although one of the diastereoisomers of β-hydroxyglutamic acids whose absolute conformation was unknown has been studied by chromatography, its chromatographic movement was faster than that of the unknown (3). However, this unknown compound from spinal fluid could be the other diastereoisomer of β-hydroxyglutamic acid which has not yet been studied.

From a consideration of the second, third, fourth, and fifth observations above, one can suggest that the unknown may be a dicarboxylic acid which is neither aspartic nor glutamic acid. In view of this,

the first observation would indicate that the unknown might be a "substituted" glutamic acid. Furthermore, its chromatographic behavior is similar to that which one would predict for a hydroxy-substituted glutamic acid (5, 6). If the unknown compound is a hydroxy-glutamic acid, the sixth observation would indicate that it could be only one of the two diastereoisomers of β-hydroxyglutamic acid.

It is certainly true that the evidence which has been presented to support the prediction is circumstantial. However, there is an aesthetic reason for suggesting this possibility. Historically the presence of β-hydroxyglutamic acid in biological material has been discovered on two separate occasions (46, 47), only to be disproven by a number of later studies (48). Nevertheless the search still goes on (42).

Execution of Proposition

First, the diastereoisomers of β-hydroxyglutamic acid should be obtained from a reliable source or synthesized according to methods outlined by Greenstein and Winitz (49). Then the chromatographic behavior of the diastereoisomers should be compared with that of the unknown from cerebral spinal fluid on columns of Amberlite IR-120 and Dowex 1 resins. If the chromatographic movement of the unknown is identical with one of the diastereoisomers, the unknown should then be isolated in sufficient quantity to be studied by paper chromatographic procedures. If the similarity still persists, further chemical studies (50) might be carried out in order to prove their chemical identity and absolute configuration.

V The Biosynthetic and Genetic Control of Hemoglobin

It is proposed that the synthesis of the various hemoglobin molecules is in terms of the independent formation of separate polypeptide chains or quarter molecules which then combine reversibly to produce the four chain hemoglobin molecules. It is further postulated that there are several types of peptide chains produced in various relative ratios and that the allowed combinations of these chains determine the heterogeneity of human hemoglobin. Finally it is proposed that the genetic control of each type of hemoglobin results from a summation of the separate genetic control of the chain-subunits which are involved.

Background for Proposition

This proposition was presented at the time of my Candidacy Examination in February 1959. Since that time many of its details have been confirmed by observation made in these Laboratories and elsewhere. Therefore, a brief resume of the studies that initiated this proposition and the recent publications that confirm the proposals will be presented.

According to the literature at the time this proposition was formulated, the synthesis of hemoglobin was considered to be in terms of the whole molecule and under some sort of genetic control (21, 51). Many authors believed that fetal hemoglobin and normal adult hemoglobin are synthesized independently of one another and that these syntheses are controlled by completely different genetic factors (21, 51). However,

a number of studies which were in progress or had just been completed in these Laboratories at that time indicated the possibility of chemical similarities between the α chains of hemoglobins A, S, and F and some of the minor hemoglobin components and between the β chains of hemoglobins A and H and other minor components (52, 53, 54, 55, 56). Thus, this proposition was prompted by a consideration of these latter studies in relation to the concepts of biosynthesis and genetic control of hemoglobins which were being propounded in the literature up to 1959.

A number of different chemical and biological observations which would confirm this proposition were suggested at the time it was submitted. These suggested observations included: evidence of several different types of polypeptide chains in various hemoglobins; chemical identity of chains in different hemoglobins; evidence of independent genetic control of chemically different chains in the same hemoglobin but common genetic control of chemically identical chains in two different hemoglobins; chemical and biological evidence of combination of "complementary" chains into different four-chain hemoglobin molecules with the hemoglobins in ratios which might indicate differences of affinity between the complementary chains; and biological existence of molecules composed of single types of polypeptide chains independent of any complementary chains.

Many of the experimental observations which have recently been made and which support the details of this proposition have been reviewed by Ingram (57) and Lehmann and Ager (58).

At least four chemically different polypeptide chains have been found in human hemoglobin (38, 57, 58, 59). These have been designated the α, β, γ, and δ chains. Although these chains are similar in many

portions of their primary structure, they are chemically distinct (38, 57, 58, 60). The α chains which are present in hemoglobins A, S, F_{II}, A_2, and F_I appear to be identical (52, 54, 55) and the β chains of A and H are identical (56).

Proof of the independence of genetic control of chemically different chains has resulted from a variety of studies but is best illustrated by the investigations of the chemical identity of hemoglobins in certain individuals who are heterozygous for two different hemoglobin genes and by genetic studies of their families (61, 62, 63, 64, 65). These studies indicate that the genetic control of the α chain is independent of that of the β chain. Recently Huehns and Shooter have demonstrated the presence of the same abnormal α chain in the main abnormal component G(Ibadan) and the minor component G_2(Ibadan), which they conclude is an abnormal A_2 component (66). In view of their study and the fact that the β chain of hemoglobin A and the δ chain of hemoglobin A_2 are chemically and genetically distinct (60, 67) one can conclude that the α chains of hemoglobins A and A_2 are under a common genetic control.

The studies of individuals with four major hemoglobins (61, 63, 65) and individuals with hemoglobins γ_4, β_4, F_{II}, and A_{II} (34) indicate that the various "complementary" chains probably have different affinities for one another. This in combination with the observation of the existence of β_4 and γ_4 molecules (34, 56) is indicative of independent formation of the polypeptide chain-subunits either as monomers or dimers. Although the fact that no hemoglobins have been found with only one abnormal chain in the four chain structure (for example, $\alpha_2\beta^A\beta^S$) is circumstantial evidence, it is in support of the hypothesis that the chain-subunits are synthesized as monomers.

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