PHYSICAL CHEMICAL STUDIES OF REACTIONS OF HUMAN HEMOGLOBINS

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William Day Hutchinson

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ABSTRACT

The molecular dissociation of the derivatives, carbonmonoxy-, oxy-, ferro-, and ferri-, of normal adult hemoglobin has been studied at a pH of five. Of these derivatives, ferrohemoglobin is least dissociated while ferrihemoglobin is most extensively dissociated at a given concentration. The apparent dissociation constants have been calculated and related to the acid dissociation of the "Heme-linked" acid groups of these derivatives.

Hemoglobins labelled with Carbon-14 have been prepared. Using C^{14} labels on one hemoglobin, the exchange of molecular subunits has been studied after dissociation of mixtures of normal adult and sickle cell hemoglobins at acid and alkaline pH. Hemoglobin "hybrids", in which one type of subunit is radioactive and the other not, have been formed and isolated. These hybrids have been used in the identification of the polypeptide chain in which the anomaly occurs in sickle cell hemoglobin. This anomaly was found to occur in the β chains. The kinetics of the α chain exchange between the two hemoglobins has been studied at alkaline pH. Presumptive evidence of the mode of dissociation at this pH has been obtained. Preliminary investigations are reported on the isolation of hemoglobin hybrids in which only the β chains are radioactive.

Spectral data for several derivatives of hemoglobin have been related to a recently adopted standard. The use of the ferroversenate ion in forming ferrohemoglobin from oxyhemoglobin is reported.

TABLE OF CONTENTS

PART I

DIFFERENCES IN THE ACID INDUCED MOLECULAR DISSOCIATION BEHAVIOR OF SEVERAL DERIVATIVES OF NORMAL ADULT HEMOGLOBIN

Introduction 1

Procedure 2

Preparation of Materials 3

Experimental Method 5

Results 7

Discussion of Results 18

PART II

CARBON-14 LABELLED HYBRIDS OF NORMAL ADULT AND SICKLE CELL HEMOGLOBINS

A. PREPARATION AND ISOLATION OF CARBON-14 LABELLED HYBRIDS OF NORMAL ADULT AND SICKLE CELL HEMOGLOBINS 22

Introduction 22

Procedure 25

Preparation of Materials 26

Chromatography 30

Results of Hybridization Experiments 32

Significance of Hybridization of Radioactive Hemoglobins . . . 39

B. THE IDENTIFICATION OF THE ABERRANT CHAIN IN SICKLE CELL HEMOGLOBIN 40

Introduction 40

Procedure 40

Preparation of Materials 40

Results 41

Discussion 42

C. THE KINETICS OF SUBUNIT EXCHANGE IN THE ADULT-SICKLE CELL HEMOGLOBIN SYSTEM AND THE MODE OF DISSOCIATION OF HEMOGLOBIN AT pH 11.0 44

Introduction 44

Experimental Procedure 45

Results 46

Discussion of Results 49

Conclusion 60

D. PRELIMINARY INVESTIGATION OF THE ISOLATION OF HEMOGLOBIN HYBRIDS LABELLED IN THE CHAINS 61

Introduction 61

Procedure 61

Experimental Procedure 62

Preparation of Materials 62

Results 63

REFERENCES 68

APPENDICES

I. SPECIFIC EXTINCTION COEFFICIENTS ($E^{1\%}_{1cm.}$) OF HEMOGLOBIN DERIVATIVES 70

II. REDUCTION OF OXYHEMOGLOBIN USING THE FERROVERSENATE ION . . 73

III. PREPARATION OF VARIOUSLY LABELLED RADIOACTIVE SICKLE CELL HEMOGLOBINS 75

IV. CHROMATOGRAPHY PROTOCOL SHEETS 77

PART I

DIFFERENCES IN THE ACID INDUCED MOLECULAR DISSOCIATION BEHAVIOR OF SEVERAL DERIVATIVES OF NORMAL ADULT HEMOGLOBIN

Introduction

In 1955 Field and O'Brien reported a decrease in the sedimentation coefficient of human carbonmonoxyhemoglobin in solutions of pH below five (1). This decrease in sedimentation coefficient was accompanied by an increase in the diffusion coefficient, which suggested a dissociation of the hemoglobin molecule into a new species having half the molecular weight of the original hemoglobin molecule. Field and Ogston (2) concluded from considerations of the rate of boundary spread in the ultracentrifuge that the reaction was reversible and that the dissociation equilibrium was established in less than 200 seconds. It was later demonstrated (3) that the mass action expression obeyed by the reaction was given by:

$$\mathrm{HhHh} + \mathrm{H}^{+} \longrightarrow \mathrm{HhH}^{+} + \mathrm{Hh} \qquad (1)$$

where Hh is a hemoglobin half molecule. From studies of the dependence of the reaction on protein concentration and hydrogen ion concentration, apparent equilibrium constants were calculated. The constants were found to have a scatter which could not be attributed to errors inherent in the method of their evaluation. Since ferrihemoglobin is rapidly formed in carbonmonoxyhemoglobin solutions at acid pH (4) it appeared possible that the scatter in the values found for the equilibrium constants was due to contamination of the carbonmonoxyhemoglobin with ferrihemoglobin. Preliminary work indeed showed that at equal concentrations carbonmonoxyhemoglobin and ferrihemoglobin did dissociate to different extents at pH 5.0.

It has been demonstrated (5) that certain acid groups in various hemoglobin derivatives have acid dissociation constants which depend upon the nature of the bonding state of the iron in the heme, or the nature of the ligand bonded to the heme group. The difference observed in the extents of dissociation of carbonmonoxyhemoglobin and ferrihemoglobin suggested that the molecular dissociation reaction of hemoglobin might also be "heme-linked". A study of the correlations between the reactions of a molecule with a structure as complex as that of hemoglobin can be instructive in elucidating relative positions of reactive groups in the molecule. It is with this view in mind that a study of the dissociation properties of several derivatives of normal adult hemoglobin was undertaken.

Procedure

The method of study has been the determination of the sedimentation coefficients of various derivatives of normal adult hemoglobin as a function of the total protein concentration at a pH of 5.0. The derivatives which have been studied are carbonmonoxy-, oxy-, ferro-, and ferrihemoglobin. These studies were carried out at a pH of five because the concentration range in which dissociation occurs coincides with the optimum range of protein concentration for use of the schlieren optical of the ultracentrifuge.

It has been shown that the measured sedimentation coefficient, S, in a system in dissociation equilibrium is given by (3):

$$S = \sum_i f_i S_i \alpha + (1 - \alpha) S_o \qquad (2)$$

where S_o and S_i are the sedimentation coefficients of the original species and the products of its dissociation respectively and α is the

degree of dissociation. The quantity f_i is the ratio of the molecular weight of the ith species produced in the dissociation to the molecular weight of the original materials. For the case of dissociation into subunits having half the molecular weight of the original material the expression for the sedimentation coefficient becomes:

$$S = (1 - \alpha)S_o + \alpha S_H \qquad (3)$$

where S_H is the sedimentation coefficient of the half molecule.

Preparation of Materials

Hemoglobin stock solutions were prepared from freshly drawn red blood cells from normal adult donors. These cells were washed four times with isotonic saline solution; and the supernatant from each washing was removed using a suction pipette. The packed red blood cells from the last wash were mixed with an equal volume of distilled water and 0.4 volume of toluene. This mixture, red blood cells, water, and toluene was shaken vigorously to rupture cell walls and to effect complete lysis. The lysate was centrifuged for 15-20 minutes at 1800 RPM in a clinical centrifuge after which the toluene and stroma were removed. The remaining hemoglobin solution was then centrifuged for 1-2 hours at 30,000 RPM in the Spinco Model L Ultracentrifuge to remove the last traces of stroma.

To avoid any differences in the behavior of the derivatives of hemoglobin due to the effects of the technique used to prepare ferro-hemoglobin, all of the protein was initially reduced, then treated as necessary to prepare the other derivatives. The method of Adams (6) was first used to prepare ferrohemoglobin; this method was, however, abandoned because of the deleterious effects of the reducing agent

sodium dithionite after partial oxidation (7).

It was found that hemoglobin could be quantitatively reduced by evacuation. The evacuation technique used was as follows:

> A stock solution of the non-crystallized oxyhemoglobin was placed in a stoppered serum bottle. After placing this serum bottle in a water bath held at 37°C., a hypodermic needle connected to a water aspirator was introduced into the bottle through its stopper. The evacuation of oxygen from the bottle was carried out while swirling the contents around to avoid bubbling of the solution. After 15 minutes the evacuation was complete, as indicated by the change in color of the solution from a brilliant red to a deep blue red. Before removal of the aspirator needle, nitrogen was introduced into the bottle to bring the pressure above the solution to a point above atmospheric pressure. The concentration of the ferrohemoglobin solution was determined using the Brice-Pheonix Differential Refractometer. The value of the specific refractive index increment was taken as 0.00181.

An aliquot of the above ferrohemoglobin solution was dissolved in a deoxygenated Acetate-NaCl buffer (0.1 M NaOAc, 0.042 M HOAc, 0.15 M NaCl) of pH 5.0, u = 0.25; this solution was of a concentration appropriate for use in the ultracentrifuge. For sedimentation studies on ferrohemoglobin, a portion of this solution was transferred, using a hypodermic syringe, to an ultracentrifuge cell which had been flushed out with nitrogen. Other portions of this solution were used to prepare the oxy-, carbonmonoxy-, and ferrihemoglobin solutions run in the ultracentrifuge. Oxyhemoglobin and carbonmonoxyhemoglobin solutions were prepared by blowing oxygen and carbon monoxide, respectively, over portions. Ferrihemoglobin solutions were prepared by allowing a portion of the ferrohemoglobin solution of pH 5.0 to stand for 24 hours under oxygen at room temperature. After 24 hours under these conditions the conversion to ferrihemoglobin is 95% complete.

Experimental Method

All sedimentation coefficient determinations were made using the Spinco Model E Ultracentrifuge, operating at speeds of 52,640 or 56,100 RPM. At these speeds of rotation the protein in all cases moved through at least half the length of the cell in three hours running time. In all experiments two cells were used, one of which was fitted with a 1° positive wedge shaped window. The schlieren diagram corresponding to the solution in this cell was displaced upward on the viewing screen of the ultracentrifuge, thus facilitating simultaneous sedimentation experiments on identical or non-identical solutions. It was possible in this way to make a visual comparison of the sedimentation behavior of any two different hemoglobin derivatives.

The sedimentation coefficient, S, is defined as the rate of movement of a dissolved molecule in unit centrifugal field (8):

$$S = \frac{dx/dt}{\omega^2 x} = \frac{d\ \ln x/dt}{\omega^2} \qquad (4)$$

where x is the position of the molecule at time t and ω is the angular velocity of the ultracentrifuge rotor. The rate of movement of a sedimenting molecule in the bulk of a solution is taken to be the rate of movement of the boundary between solution and solvent. Using the second form of the equation for S the logarithm of the boundary position was plotted as a function of the time. The slope of this line divided by the square of the angular velocity gives the sedimentation coefficient uncorrected for the viscosity and bouyancy of the solvent. It is convenient to refer the values of the sedimentation coefficient to a standard solvent, water at 20°C. The sedimentation coefficients were reduced to this standard by the use of the equation:

$$S_{w,20} = \eta_s^T/\eta_w^T \times \eta_w^T/\eta_w^{20} \times \frac{(1 - \bar{V}\rho_w^{20})}{(1 - \bar{V}\rho_s^T)} \tag{5}$$

where:

T = the temperature at which the sedimentation experiment is carried out.

η_s^T/η_w^T = the relative viscosity of the solvent at temperature T.

η_w^T/η_w^{20} = the viscosity of water at temperature T relative to its value at 20°C.

$\bar{V}$ = the partial specific volume of the sedimenting molecule.

ρ_s^T = the density of the solvent at temperature T.

ρ_w^{20} = the density of water at 20°C.

All sedimentation experiments were carried out at 20°C. The results of the sedimentation coefficient determinations correspond therefore to the dissociation equilibria at this temperature.

The schlieren optical system of the ultracentrifuge was fitted with a #29 Wratten filter, and the schlieren patterns were recorded on Eastman 103-F Spectroscopic plates. The boundary positions were taken as the position of the perpendicular bisector of the schlieren peaks; it was not found necessary to apply the method of Goldberg (9) to the determination of the boundary position. The boundary positions were measured on enlargements of the photographic plates such that a measured 1 cm. corresponded to a distance of 0.05 cm. in the ultracentrifuge cell.

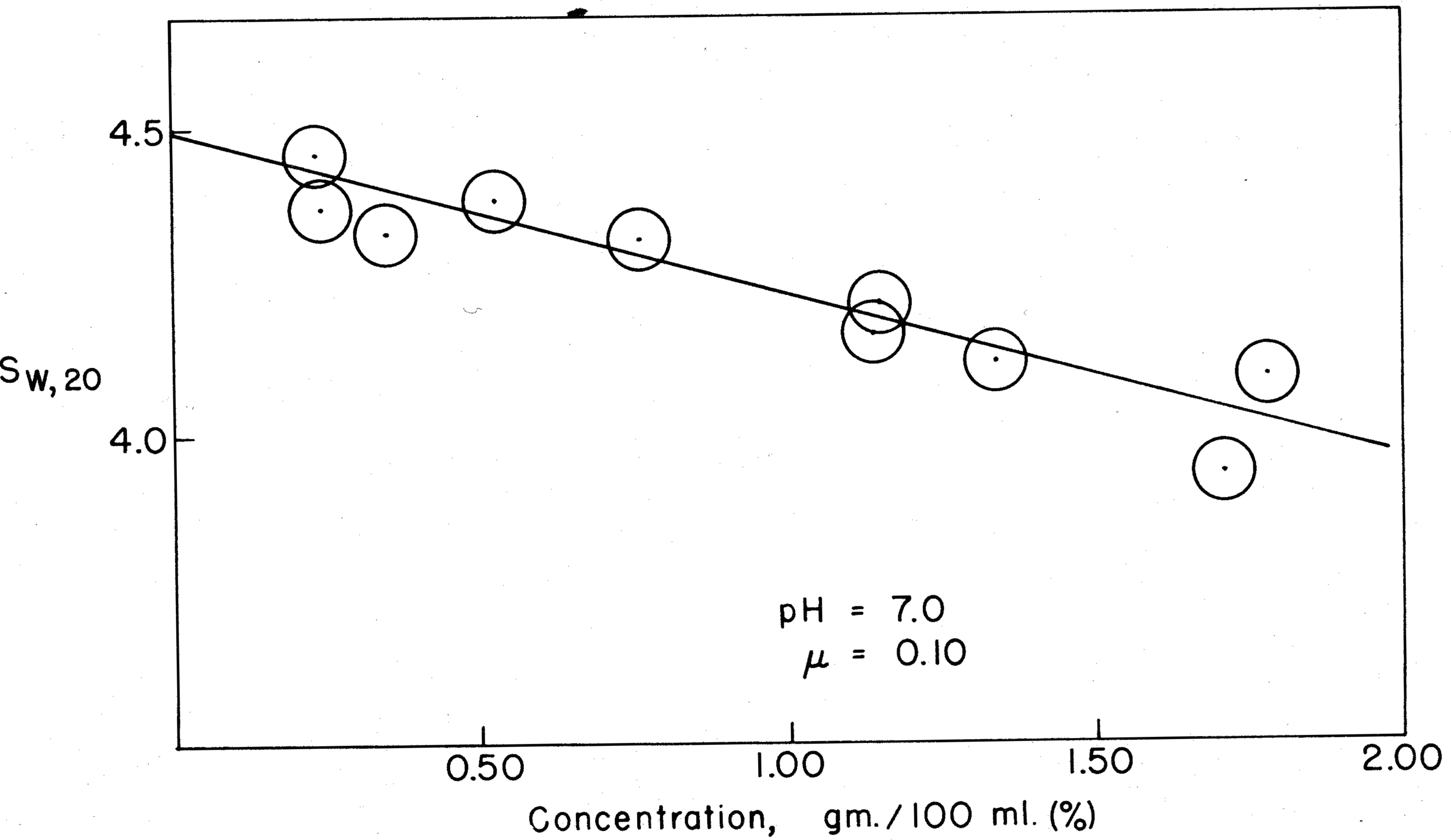
Results

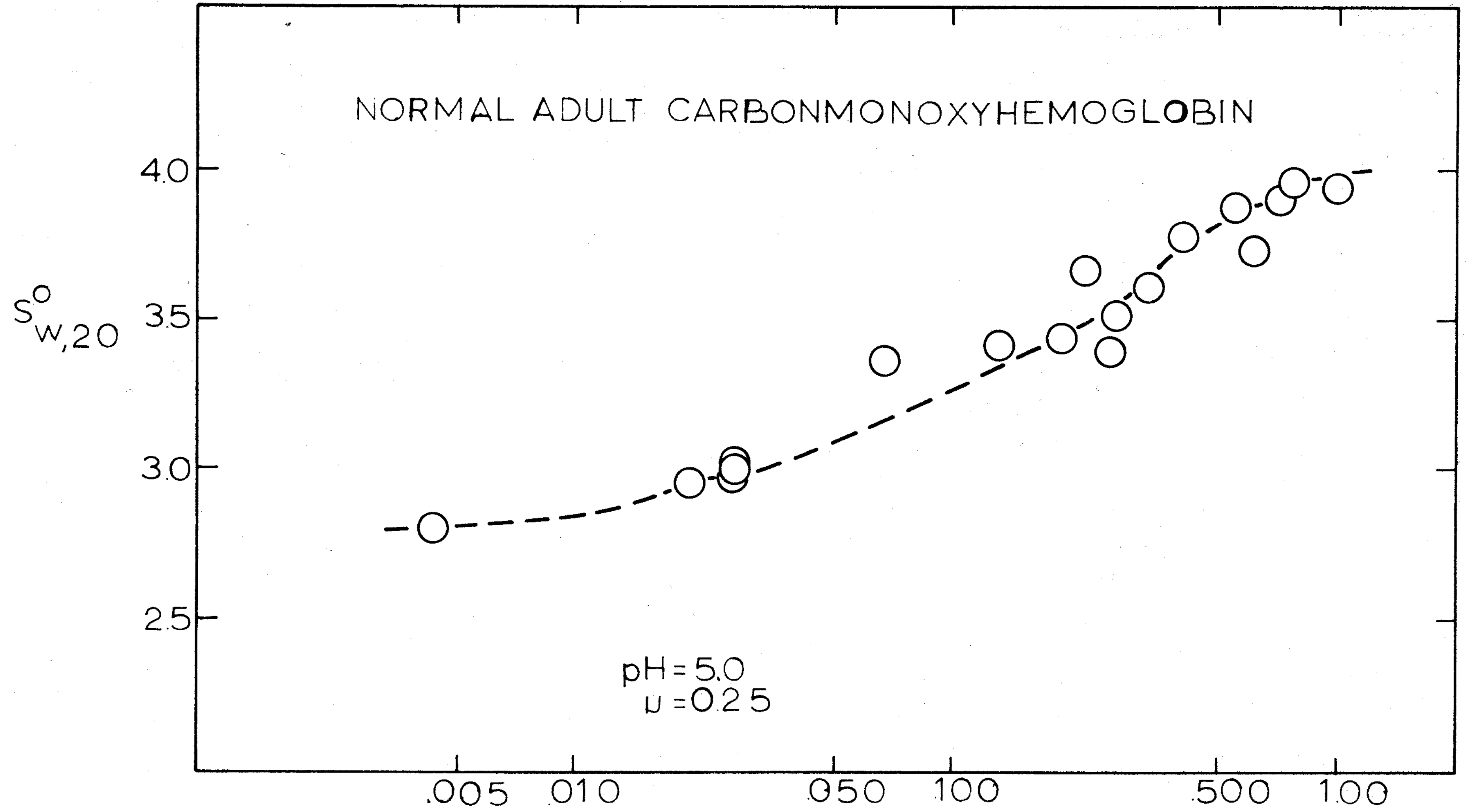
It is seen in equation 3 that the quantities required for the evaluation of the degree of dissociation α from the measured sedimentation coefficient S are the values of the sedimentation coefficients of undissociated hemoglobin and of the hemoglobin half molecules. To determine the value of the sedimentation coefficient of undissociated hemoglobin, sedimentation measurements of carbonmonoxyhemoglobin were performed at several concentrations in a buffer of pH 7.0, μ = 0.1 (0.0286 M K_2HPO_4, 0.0143 M KH_2PO_4). At the concentrations chosen for these measurements the hemoglobin is not appreciably dissociated; these measurements do show the effect of the total protein concentration on the sedimentation coefficient. In figure 1 is seen a plot of the sedimentation coefficient of carbonmonoxyhemoglobin against concentration at neutral pH. At zero concentration the value of the sedimentation coefficient of hemoglobin approaches the value 4.50 Svedbergs; this value is taken to be the sedimentation coefficient of undissociated hemoglobin. The concentration dependence of the acid dissociation of carbonmonoxyhemoglobin has been studied at pH 5.0. In figure 2 the sedimentation coefficient of carbonmonoxyhemoglobin as a function of total concentration is given at pH 5.0. The value approached by the sedimentation coefficient at zero concentration at this pH is 2.70 Svedbergs. This value is taken to be the sedimentation coefficient of the hemoglobin half molecule.

Since the values of the sedimentation coefficients of hemoglobin and the hemoglobin half molecule have been obtained from data extrapolated to zero concentration, it is necessary to correct the measured sedimentation coefficients to the values which would obtain

Figure 1

Sedimentation Coefficient of Normal Adult Carbonmonoxyhemoglobin





Concentration, gm/100 ml.

Figure 2

at infinite dilution. It is seen in figure 1 that the sedimentation coefficient of undissociated hemoglobin is 4.25 Svedbergs at a total concentration of 1%; it is assumed that the contribution to the total correction of the measured sedimentation coefficients due to the whole molecule will amount to 0.25 Svedbergs/% total protein. The β lacto-globulin molecule has a frictional coefficient and a molecular weight close to that of the hemoglobin half molecule. The effect of the total protein concentration on the sedimentation coefficient of this material amounts to 0.125 Svedbergs/% total protein (10). This same value has been assumed to hold for the hemoglobin half molecule.

In order to apply these corrections to the measured sedimentation coefficients, an approximate value of the degree of dissociation was calculated using the experimentally determined sedimentation coefficient in equation 3. Substituting the values of the sedimentation coefficients of hemoglobin and the hemoglobin half molecule into this equation and rearranging, this equation becomes:

$$\alpha_1 = \frac{4.50 - S_{w,20}}{4.50 - 2.70} \qquad (6)$$

The values of α_1 obtained from this equation were used in an expression for $S_{w,20}$, the correction to be applied to the measured sedimentation coefficient:

$$S_{w,20} = \mathrm{conc}(\%) \times \{0.125\,\alpha_i + 0.25(1 - \alpha_i)\} \qquad (7)$$

The value of the sedimentation coefficient obtained by adding this increment to the experimentally determined value is the sedimentation coefficient corrected for frictional effects. In table I are given the results of the sedimentation coefficient determinations for hemoglobin derivatives and the values of these quantities after applying the above

Table I

Measured Sedimentation Coefficients ($S_{w,20}$) and Their Corrected Values ($S^{\circ}_{w,20}$) of Derivatives of Normal Human Hemoglobin at pH 5.0

Expt. No.	Derivative	Concentration %	$S_{w,20}$	$S^{\circ}_{w,20}$
238	Carbonmonoxy	0.25	3.60	3.64
238	Ferri	0.25	3.40	3.44
249	Carbonmonoxy	0.54	3.70	3.81
249	Ferri	0.54	3.37	3.46
250	Carbonmonoxy	0.27	3.60	3.65
250	Ferri	0.27	3.26	3.30
255	Ferro	0.64	4.02	4.15
255	Oxy	0.64	3.62	3.74
256	Ferro	0.66	3.94	4.08
256	Oxy	0.66	3.73	3.86
258	Ferro	0.59	3.65	3.76
258	Oxy	0.59	3.51	3.62
261	Ferro	0.78	4.08	4.25
261	Carbonmonoxy	0.78	3.63	3.78
262	Oxy	0.78	3.55	3.70
262	Ferri	0.78	3.47	3.61
270	Oxy	0.56	3.36	3.45
270	Ferro	0.56	3.93	4.05
278	Oxy	0.56	3.69	3.80
278	Ferro	0.56	4.00	4.12
279	Oxy	0.80	3.59	3.74
279	Carbonmonoxy	0.80	3.59	3.74

correction. These corrected sedimentation coefficients are plotted in figures 3 - 6 for these derivatives at several concentrations.

In figure 3 are shown the corrected sedimentation coefficients for oxyhemoglobin at different concentration at pH 5.0. The great scatter of these points is probably due to the extreme lability of oxyhemoglobin to oxidation to ferrihemoglobin in acid solution. In figures 4, 5, and 6, are shown the corrected sedimentation coefficients of ferrihemoglobin, ferrohemoglobin, and carbonmonoxyhemoglobin, respectively, at different concentrations.

Of the hemoglobin derivatives studied, it appears that at a given concentration at pH 5.0 the sedimentation coefficient of ferrohemoglobin is greater than that of the other materials. For example, in experiment 261 which was a simultaneous sedimentation experiment on the derivatives carbonmonoxy- and ferrohemoglobin at 0.78% each, the corrected sedimentation coefficient of carbonmonoxyhemoglobin is 3.78 Svedbergs while that of ferrohemoglobin is 4.25 S. A difference in the values of the sedimentation coefficients of the derivatives may arise from differences in the values of the frictional coefficients of the materials. In table II are shown the results of sedimentation coefficient determinations on the derivatives ferro-, oxy-, and ferrihemoglobin at a pH of 7.0. It is seen that the corrected sedimentation coefficients of these derivatives agree well with the value of the sedimentation coefficient of carbonmonoxy hemoglobin at this pH. Shape factors, therefore, do not account for the differences observed at pH 5.0. In table III are presented the results of molecular weight determinations by the method of Archibald (11) on ferrohemoglobin and oxyhemoglobin at pH 5.0. These molecular weights, though not in

Figure 3

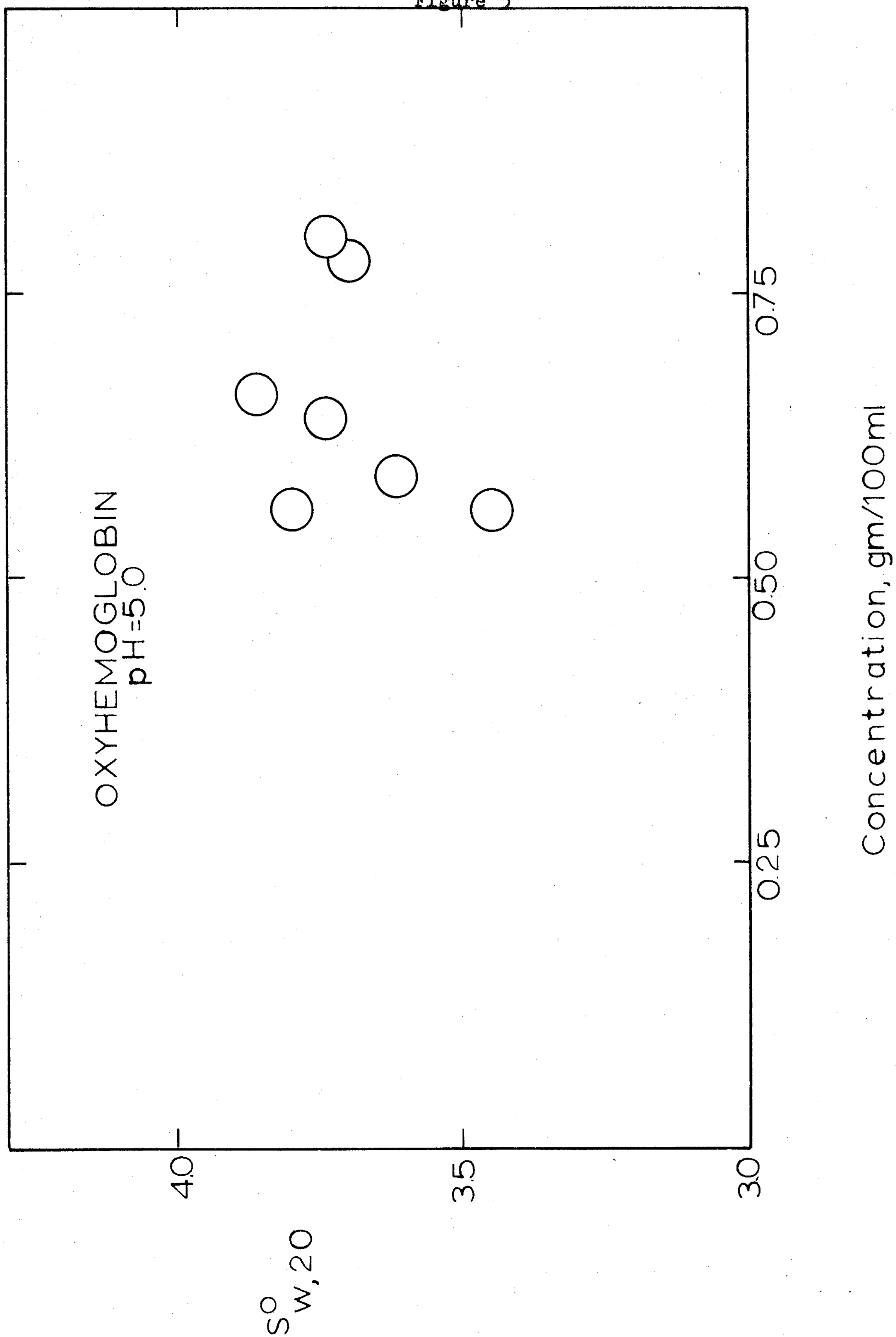


Figure 4

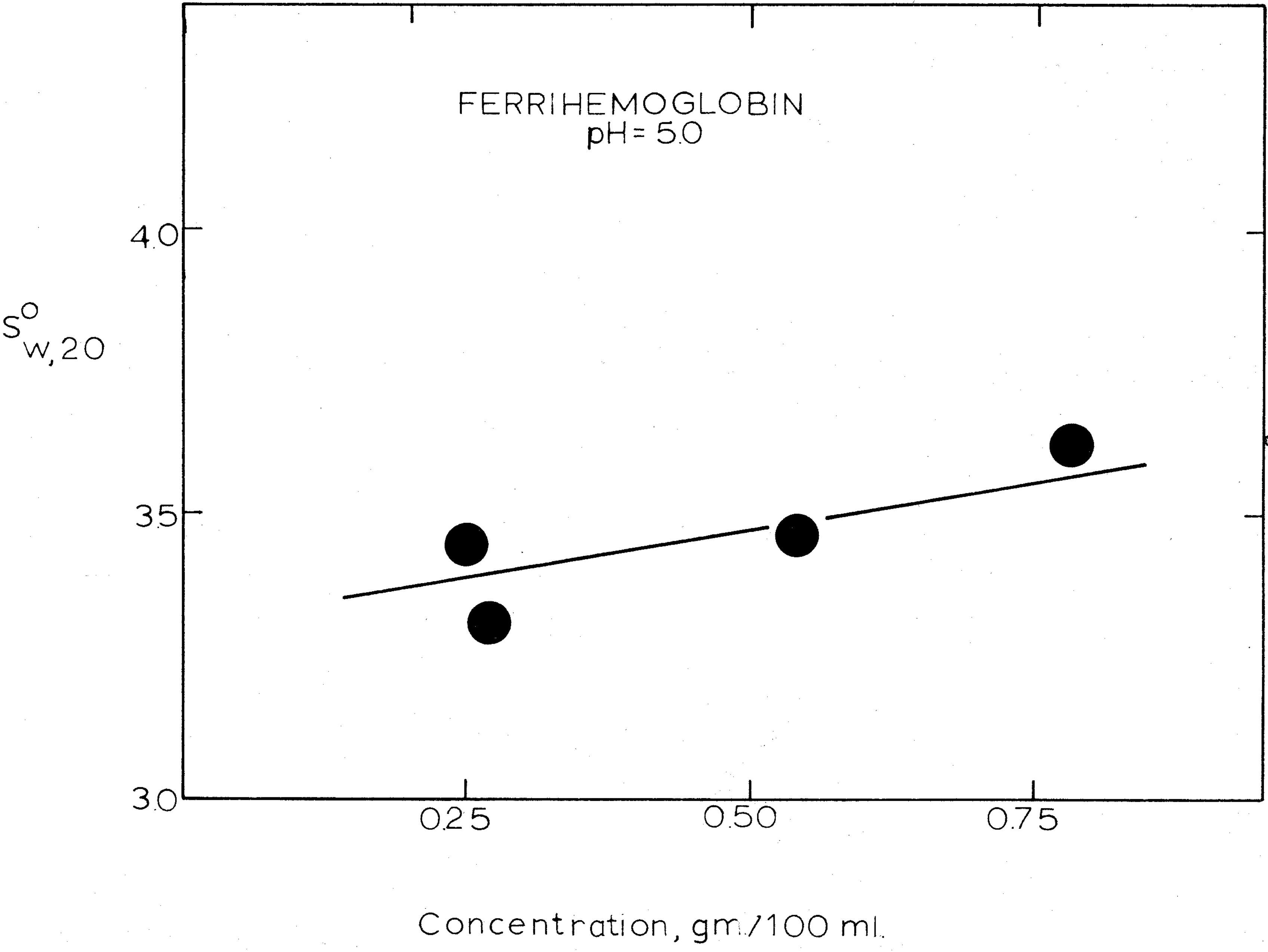


Figure 5

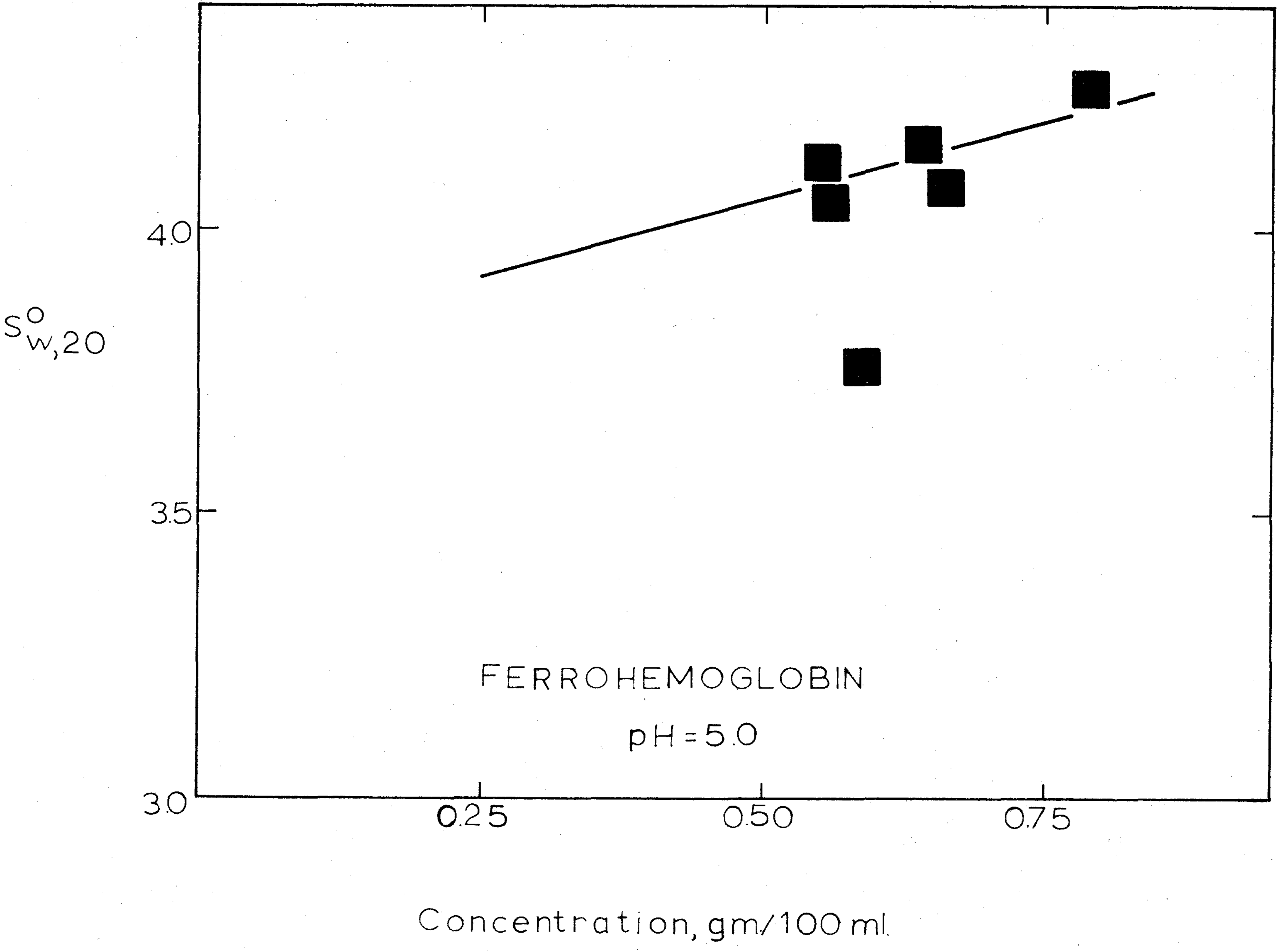
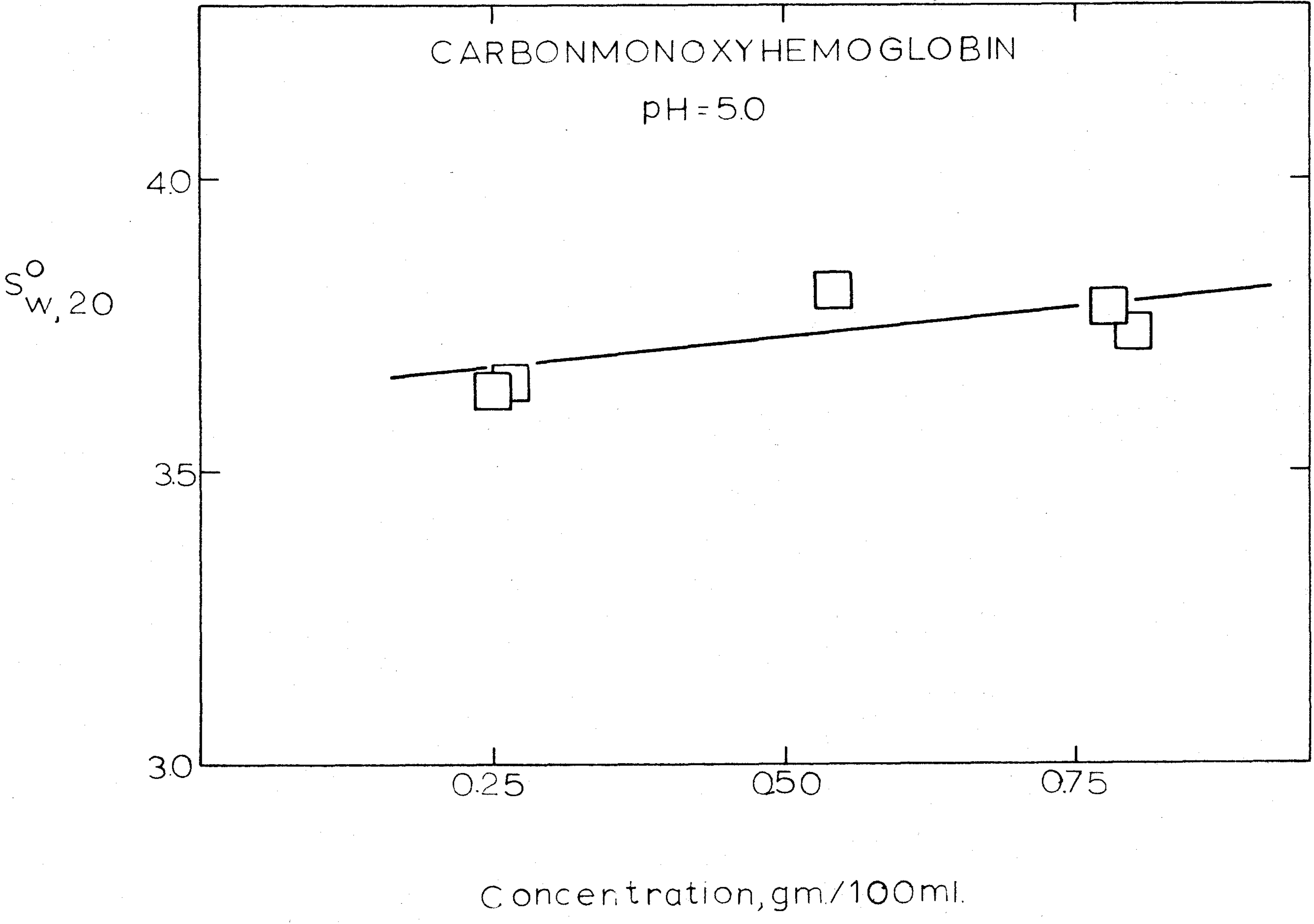


Figure 6



agreement with the sedimentation coefficient data for these materials at pH 5.0 and at the concentration 0.56%, do show the molecular weight of oxyhemoglobin to be significantly less than that of ferrohemoglobin.

Table II

Measured Sedimentation Coefficients ($S_{w,20}$) and Their Corrected Values ($S^{\circ}_{w,20}$) of Derivatives of Normal Human Hemoglobin at pH 7.0

Expt. No.	Derivative	Concentration %	$S_{w,20}$	$S^{\circ}_{w,20}$
266	Ferro	0.86	4.24	4.46
266	Oxy	0.86	4.23	4.45
267	Ferro	0.43	4.37	4.48
267	Oxy	0.43	4.34	4.45
282	Ferri	0.27	4.40	4.47
282	Ferri	0.54	4.26	4.40

Table III

Molecular Weights of Oxyhemoglobin and Ferrohemoglobin at Equal Concentration in Solutions of pH 5.0

Expt. No.	Derivative	Concentration %	Molecular Weight
268	Oxy	0.56	56,700
269	Ferro	0.56	64,600

A suitable parameter for the demonstration of any intrinsic difference in the molecular dissociation behavior of these derivatives is the apparent dissociation constant K_{app}. For the purposes of these calculations, it has been assumed that the dissociation of hemoglobin derivatives in acid solution produces non-identical half molecular species (13). The apparent dissociation constant K_{app} is, in this case:

$$K_{app} = \frac{\text{conc(mg/ml)} \times \alpha_2^2}{MW_{undissoc.} \times (1 - \alpha_2)}$$

In this expression $MW_{undissoc.}$ is the gram molecular weight of human hemoglobin, 66,800. The quantity α_2 is the second approximation of the degree of dissociation calculated using the corrected values of the measured sedimentation coefficients tabulated (table I) in equation 6. In table IV are the values of the apparent dissociation constants for the different derivatives using the data of table I.

It appears from the apparent dissociation constants in table IV that there is an effect of the nature of a hemoglobin derivative on the extent of dissociation in acid. From the values of these constants it may be seen that ferrihemoglobin dissociates to the greatest extent while at the same concentration ferrohemoglobin is least dissociated. The scatter of the sedimentation coefficients of oxyhemoglobin seen in figure 3 is again manifested in the dissociation constants calculated for this material. Since the values for oxyhemoglobin fall between those of carbonmonoxyhemoglobin and ferrihemoglobin, it appears that the latter is the contaminant in the oxyhemoglobin experiments.

Discussion of Results

The "hemelinked" acid groups of hemoglobin derivatives have been extensively investigated (5). The acid dissociation constants for these groups have been determined and found to vary from one derivative to another. Tabulated in table V are the pK_a values of these groups in the different derivatives. Of interest are the pK_a values of the first hemelinked acid group, for this group would be most affected at pH 5.0 where the molecular dissociation reaction has been

Table IV

Apparent Dissociation Constants of Derivatives of Normal Adult Hemoglobin in Ac-NaCl Buffer pH 5.0, μ = 0.25

Expt. No.	Derivative	Concentration %	$K_{app} \times 10^5$
255	Oxy	0.64	2.9
256	Oxy	0.66	1.9
258	Oxy	0.59	4.2
262	Oxy	0.78	4.0
270	Oxy	0.56	6.8
278	Oxy	0.56	2.1
279	Oxy	0.80	3.7
255	Ferro	0.64	0.42
256	Ferro	0.66	0.68
258	Ferro	0.59	2.5
261	Ferro	0.78	0.27
270	Ferro	0.56	0.70
278	Ferro	0.56	0.48
238	Ferri	0.25	3.2
249	Ferri	0.54	6.6
250	Ferri	0.27	5.4
262	Ferri	0.78	5.5
238	Carbonmonoxy	0.25	1.7
249	Carbonmonoxy	0.54	1.9
250	Carbonmonoxy	0.27	1.7
261	Carbonmonoxy	0.78	3.1
279	Carbonmonoxy	0.80	3.6

shown to be affected by the nature of the hemoglobin derivative. It has been shown that the molecular dissociation reaction involves the uptake of at least one proton for each hemoglobin molecule dissociated (3). If the first hemelinked acid group were the group receiving this proton in the dissociation reaction, it would be expected that the hemoglobin derivatives would dissociate to different extents at pH 5.0. Further, the ferrohemoglobin would be least dissociated since the first hemelinked acid group in this material is, at pH 5.0 only 64% protonated as opposed to 85% protonation of this group in the other derivatives.

Table V

Hemelinked Acid Groups

Hemoglobin Derivative	Group One pK_a	Group Two pK_a	Group Three pK_a
Ferri	5.75	6.68	8.01
Oxy	5.75	6.68	
Carbonmonoxy	(5.75)*	(6.68)*	
Ferro	5.25	7.93	

*No values were found for the pK_a values of carbonmonoxyhemoglobin. Since the iron is bonded covalently through $d^2s\ p^3$ bonds in both oxy and carbonmonoxyhemoglobin it has been assumed that their pK_a values are equal.

In order to reasonably compare the change of the pK_a of this first hemelinked acid group with the change of the apparent dissociation constants of the derivatives, the negative logarithms of these quantities have been calculated. These pK_{app} values are shown in table VI. It is seen that the value of the pK_{app} of ferrohemoglobin, $5.3^5 \pm 0.1^5$, differs most from the corresponding values for the other

derivatives; and also the pK_{app} values for ferri-, oxy-, and carbonmonoxyhemoglobin are equal within the limits of error.

Table VI

Values of the Negative Logarithms of
Apparent Dissociation Constants of
Hemoglobin Derivatives

Hemoglobin Derivative	Average K_{app} ($\times 10^5$)	pK_{app}
Ferri	5.2 ± 1.2	4.3 ± 0.2
Oxy	3.1 ± 0.9	$4.5^{5} \pm 0.1^{5}$
Carbonmonoxy	2.4 ± 0.8	$4.6^{5} \pm 0.1^{5}$
Ferro	$0.5 + 0.2$	$5.3^{5} \pm 0.1^{5}$

The acid constants of the hemelinked acid groups of hemoglobin were determined by titrations of the derivatives (5). There was no consideration made of the molecular dissociation of these derivatives in acid solution at the time these constants were evaluated. It is possible that the titrations reflect not only the protonation of the hemelinked acid groups but also the protonation of acid groups exposed to reaction by the dissociation. The difference between the pK_a values of ferrohemoglobin and the other derivatives is therefore not directly comparable to the differences between the pK_{app} value of ferrohemoglobin and the other materials.

It is significant that, in the tertiary structure published for horse hemoglobin (12), the hemelinked histidine is seen to form the bond between the non-identical chains of this material. Human hemoglobin is known to dissociate into non-identical subunits in acid solution (13), and may be expected to have a structure similar to that of horse hemoglobin.

PART II

CARBON-14 LABELLED HYBRIDS OF NORMAL ADULT AND SICKLE CELL HEMOGLOBINS

A. PREPARATION AND ISOLATION OF CARBON-14 LABELLED HYBRIDS OF NORMAL ADULT AND SICKLE CELL HEMOGLOBIN

Introduction

The disease sickle cell anemia has received much study since it was first reported in the medical literature by Herrick (14) in 1910. Herrick described the clinical manifestations of an individual afflicted with the disease. Other workers have described the peculiar change in shape of the red blood cells of such individuals under conditions of decreased oxygen pressure and have noted the reversibility of this change on returning the pressure to normal. The role of oxygen and other ligands on the heme in effecting this shape change has been studied qualitatively and quantitatively for many years. It was not until 1949, however, that the peculiar properties of these red blood cells were recognized as being due to an abnormality of the oxygen carrying pigment, hemoglobin (15).

In this work electrophoretic studies were done on the hemoglobin from normal individuals and on the hemoglobin from persons who had the disease sickle cell anemia (15). It was found that there existed mobility differences between the two hemoglobins in the region of their isoelectric points. This mobility difference corresponded to a difference in net charge of the hemoglobins. Since this discovery there have been identified more than 15 different human hemoglobins using the electrophoretic technique and others (16).

The main emphasis in the study of these "abnormal" hemoglobins has been in the elucidation of chemical distinctions between the sickle cell type and normal adult hemoglobin. With the electrophoretic difference to account for, the first step taken was a study of the heme groups of the two proteins (15). These studies indicated no differences in the crystal structures of the prosthetic groups of the proteins. Further electrophoretic studies of the globin moieties from these hemoglobins showed that it was in the polypeptide chains that the anomalies were to be found. Titration studies were undertaken in an attempt to identify the amino acid residues responsible for the difference in charge of the proteins. Lack of suitable sensitivity of the titrations obviated the success of this method. Total amino acid analyses (17) failed also to account for the differences. These amino acid analyses did, however, point out the close similarity of amino acid composition of the two hemoglobins. End group analyses using the method of Sanger demonstrated that the two hemoglobins were each composed of two different polypeptide chains, occurring in pairs and having the same N-terminal sequences (17,18). Using combined paper chromatography and paper electrophoresis on hydrolysates of normal adult and sickle cell hemoglobins Ingram (19) was able to obtain characteristic "fingerprints" of the two hemoglobins. Elution of the differing polypeptide spots on the "fingerprints" and analysis of these peptides showed the difference between the hemoglobins to be due to the substitution in sickle cell hemoglobin of a valine residue for the glutamic acid residue found in normal adult hemoglobin. This finding accounted quantitatively for the observed difference in electrophoretic mobility between the two hemoglobins.

In his work, Ingram found only 26 peptide fragments for both normal adult and sickle cell hemoglobin apart from a tryptic hydrolysis resistant "core". This number of peptides was significant in the sense that it demonstrated again the overall similarity of the two hemoglobins. With such outstanding similarities of the two hemoglobins, the possibility existed that the anomaly was confined to only a small region of the sickle cell molecule. It was known that the two polypeptide chains, designated as the α and the β chains, of the two hemoglobins had the same N-terminal sequences. The work of Ingram, however, left unanswered the question of the chemical identity of the polypeptide chains having the same composition in the two hemoglobins.

In Part I of this Thesis, mention has been made of the acid induced dissociation of human hemoglobin. After passing through a minimum in dissociation in the region of neutrality, hemoglobin undergoes another dissociation in alkaline solution; this molecular dissociation is more strongly dependent on the hydrogen ion concentration (20).

Knowing of the acid dissociation of normal adult and sickle cell hemoglobins, Singer and Itano (21) attempted to form "hybrids" of the two hemoglobins by dissociation of mixtures of the proteins followed by dialysis to neutrality. Free boundary electrophoresis was done on these recombined mixtures. In explaining their lack of success in forming new electrophoretic species, these workers suggested that the hemoglobins recombined asymmetrically such that, even in the event of chain exchange, no new electrophoretic species would be formed. It was at this point that the experiments reported in this Thesis were begun. The first attempts made in this investigation at forming "hybrids" consisted in labelling one of the hemoglobins with

iodoacetic acid, iodoacetamide, para-chloromercuribenzoic acid, or dimethylnaphthalene sulfonylchloride. Due either to lack of sufficient time for chain exchange to occur or to unfavorable steric effects of these labels, none of these early experiments clearly demonstrated that chain exchange had taken place. It was then decided to attempt hybridization experiments with radioactively labelled hemoglobins. Indeed, the first evidence of chain exchange between the two hemoglobins in this work was obtained using C^{14} labelled normal hemoglobin and unlabelled sickle cell hemoglobin. Because of the potential uses of suitably active hybrid hemoglobins, a method was developed for producing and isolating highly radioactive hemoglobin hybrids, in which only one type of subunit is radioactive and the other is substantially inactive. In the course of this work chain exchange has been observed after dissociation of hemoglobin mixtures in both acid and alkaline solution.

Procedure

Since hemoglobin dissociates reversibly in both acid and alkaline solution, hybrid hemoglobins have been formed after dissociation under both conditions. Equimolar mixtures of carbonmonoxy sickle cell hemoglobin HbS(CO), and carbonmonoxy adult hemoglobin HbA(CO) were dialysed for 24 hours against either an acid or alkaline buffer at 3°C. The acid buffer was an acetic acid-sodium acetate buffer of pH 5.0 having the composition: 0.042 M HAc, 0.10 M NaAc, 0.15 M NaCl. The alkaline buffer was the buffer of Hasserodt and Vinograd (20) having a pH of 11.0, and u = 0.35 with the composition: 0.033 M Na_2HPO_4, 0.0167 M Ha_3PO_4, 0.15 M NaCl. After dissociation of a mixture of the

hemoglobins, the materials were dialyzed to a pH of 7.22, against the buffer specified as developer no. 1 by Allen, Schroeder, and Balog (22). This developer has the composition: 0.024 M NaH_2PO_4, 0.050 M Na_2HPO_4, 0.01 M KCN. The hemoglobin mixtures were separated chromatographically on IRC-50 columns in equilibrium with this developer.

In these experiments one of the hemoglobins in the mixtures was uniformly labelled with carbon-14 containing amino acids. Upon formation of hybrids radioactivity was transferred to the other initially non-radioactive hemoglobin. In experiments using radioactive sickle cell hemoglobin it was necessary to remove the fast minor components from the HbS before hybridization since these minor components have the same chromatographic properties as HbA (23). This was accomplished by chromatography of from 50 to 100 mg. of the radioactive sickle cell hemoglobin on columns in equilibrium with developer no. 2. Only the major sickle cell hemoglobin component recovered from these columns was used in the subsequent hybridization experiments.

Preparation of Materials

Unlabelled Normal Adult and Sickle Cell Hemoglobins

The unlabelled hemoglobins were prepared from freshly drawn red blood cells from either normal or sickle cell anemic donors. This procedure has been described in Part I of this Thesis.

Uniformly Labelled Adult and Sickle Cell Hemoglobins

It was necessary that the radioactive hemoglobins used in preparing hemoglobin hybrids be uniformly labelled in the α and the β chains. It was also desirable that the amino acid chosen for the label occur with high frequency in the hemoglobin molecule to assure

the highest possible incorporation of radioactivity into the hemoglobin. At the outset of this work nothing was known of the relative compositions of the α and the β chains; it was felt, however, that leucine would fulfill these two requirements. This was a fortunate choice for it has been shown by Hill and Craig (24) that there is an equal number of leucine residues in the two chains. The incorporation of C^{14}-l-leucine thus assures equal specific activities in both chains.

Blood samples containing from 15% to 66% reticulocytes were obtained from donors having acquired hemolytic or sickle cell anemias. After four washings of the reticulocyte-rich red blood cells with cold isotonic saline solution the incorporation of the C^{14}-l-leucine was begun using a modification of the procedure of Borsook, et al (25) for the incorporation of radioactive amino acids into rabbit hemoglobin. This procedure was begun within two hours after the red blood cells were drawn. The complete incubation medium was prepared using the stock solutions described in Table VII. The following amounts of these stock solutions were added to each 10 ml. of packed red blood cells from the last wash: 5.85 ml. of amino acid mixture without leucine, 0.60 ml. of glucose solution, 1.2 ml. glutamine solution, 1.8 ml. of $NaHCO_3$ solution, and 11.7 ml. of ferrous ammonium sulfate solution. To this mixture was added 7 micromoles, 50 uc, of Nuclear-Chicago uniformly labelled C^{14}-l-leucine dissolved in 0.5 ml. NKM saline solution.

The complete incubation mixture was separated into 2 ml. portions in 20 ml. beakers which were then placed in a Dubnoff Shaking Incubator under an atmosphere of 95% CO_2-5%O_2. After incubation for four hours, the red blood cells were washed four times in cold isotonic saline solution. The supernatant solutions resulting from the

Table VII

Solutions Required for Incubations

NKM Saline. 0.13 M NaCl, 0.005 M KCl, 0.0075 M $MgCl_2$. This solution was prepared using redistilled water.

Glucose in NKM. 0.22 M Glucose in NKM saline. This solution was prepared freshly for each incubation.

Ferrous Ammonium Sulfate. 0.01 M $Fe(NH_4)_4(SO_4)_3$ in NKM saline. This solution was prepared freshly for each incubation.

Glutamine in NKM. 0.01 M glutamine in NKM saline. This solution may be prepared in quantity, stored frozen and thawed out as needed for use.

Sodium Bicarbonate. 0.27 M $NaHCO_3$ in redistilled water. This solution was prepared fresh for each incubation.

Total Amino Acid Solution. This solution contained the following amounts of amino acids expressed as mg. per 1000 ml. of NKM saline solution: l-alanine 180, l-arginine 100, l-aspartic acid 380, l-cysteine 50, glycine 400, l-histidine $HCl \cdot H_2O$ 500, l-hydroxyproline 150, l-isoleucine 52, (l-leucine 52), l-lysine HCl 330, l-methionine 50, l-phenylalanine 260, l-proline 160, l-serine 175, l-threonine 200, l-tryptophan 60, l-tyrosine 150, and l-valine 370. This solution was heated to a boil to dissolve all amino acids after which the pH was adjusted to 7.0 with 5 M NaOH. This stock solution was stored frozen in small lots which were thawed out as needed for incubations. For the incubations in which C^{14}-l-leucine was incorporated into hemoglobin this stock solution was made up omitting l-leucine.

centrifugation of each of these washings was consolidated and disposed of with radioactive waste. The further preparation of the radioactive hemoglobins was the same as previously described in this Thesis. The resulting hemoglobin solutions were dialyzed against four 2 liter changes of distilled water to remove the last traces of unincorporated C^{14}-l-leucine. Depending roughly on the percentage of reticulocytes in the red blood cells incubated, the specific activities obtained in the leucine labelled hemoglobins ranged from 1900 to 10,000 cpm/mg.

The specific activities of hemoglobin samples from several of these preparations are given in table VIII.

Table VIII

Specific Activities of Hemoglobins in Several Preparations

Material	% Reticulocytes	Label	Specific Activity cpm/mg.
Hb S	18.9	leucine	1660
Hb A	40.0	leucine	7000
Hb S	34.0	leucine	2700
Hb A	24.4	leucine	2800
Hb A	66.0	leucine	11000
Hb S	18.9	Algal Hyd.	5100
Hb S	18.9	Methionine	112
Hb S	18.9	Tryptophan	135
Hb S	18.9	Fe^{59}	2660

Hemoglobin was prepared using amino acids other than C^{14}-1-leucine. Also one preparation was made using an algal hydrolysate and one using Fe^{59} as the label. The specific activities obtained in these preparations are also given in table VIII. The detailed method of preparation of these labelled hemoglobins is given in Appendix III.

The hemoglobins labelled respectively with methionine and tryptophan were to be used in forming hybrids; tryptic digestion of these hybrids and fingerprinting by the method of Ingram (19) were planned. Radioautograms of the fingerprints would show the distribution of the relatively rare amino acids, methionine and tryptophan,

between the α and the β chains. During the course of this work, a report of the successful separation of the α and the β chains and the amino acid analyses of the individual chains was published (24). The program for solution of the problem using the methionine and tryptophan labelled hybrids was therefore abandoned.

Chromatography

Preparation of Resin for Chromatography

Two pounds of IRC-50 Amberlite resin were transferred in a hood to a jar containing ~16 liters of distilled water. The suspension of resin in water was stirred vigorously for five minutes using an electric stirrer after which the resin was allowed to settle and the supernatant decanted off. This process was repeated a total of four times. The precipitate from the last wash with distilled water was placed on a larger Buchner funnel and washed with 6 liters of 4 N HCl. With the resin now in its acid form it was again washed with distilled water to remove excess HCl.

The resin of the correct size for use in the chromatographic columns was separated and isolated by the hydraulic method of Hamilton (26), as described by Moore and Stein (27). This method consisted in the differential flotation of the resin particles in a Corning 6400 conical separatory funnel. Particles of a size smaller than 250 mesh were removed by flotation at a flow rate of 315 ml./min.; those of a size from 250 to 200 mesh were removed by flotation at a rate of 525 ml./min. The apparatus used in these separations was previously calibrated using resin particles of 200-250 mesh which had been separated by screening.

The resin particles of the correct size were changed into the sodium form by suspension in 2 M NaOH. Excess base was removed by suspending the resin again in 4 x 10 liters of distilled water. After the last of these four suspensions, the supernatant gave only a slightly alkaline reaction with universal litmus paper. The resin was then suspended in the chromatographic developer with which it was to be used.

Preparation of Columns for Chromatography

The chromatographic columns were prepared by the method of Allen, Schroeder, and Balog (22), and were the same dimensions as those of these authors. The columns were equilibrated at 6°C. by allowing approximately one liter of developer no. 1 to flow through the resin bed.

Separation of Mixtures of Normal Adult and Sickle Cell Hemoglobins

Mixtures of the two hemoglobins containing from 20 to 50 mg. total protein in 2 to 3 ml. of developer no. 1 were layered on top of the resin beds of the equilibrated columns. The normal adult hemoglobin was eluted at 6°C. reaching a maximum of concentration at 22 ml. eluate, and tailing off to inappreciable concentrations at 40 through 50 ml. The flow of developer was then stopped, after which the temperature of the jacketed columns was raised to 28°C. Allowing from 15 to 20 minutes for the resin to reach this temperature, the flow of developer was again started. The sickle cell hemoglobin was desorbed at this temperature, reaching a maximum of concentration at about 52 ml. eluate.

The concentrations of the chromatographic fractions were measured spectrometrically with a Beckman Model DU spectrophotometer. The optical densities of the solutions were measured at the wavelengths of

415 mu or 539 mu, corresponding to absorption maxima of carbonmonoxy-hemoglobin; or, in cases where it was believed that the hemoglobin fractions were contaminated with ferrihemoglobin cyanide, the measurements were made at the carbonmonoxy-ferrihemoglobin cyanide isobestic point, 522 mu.

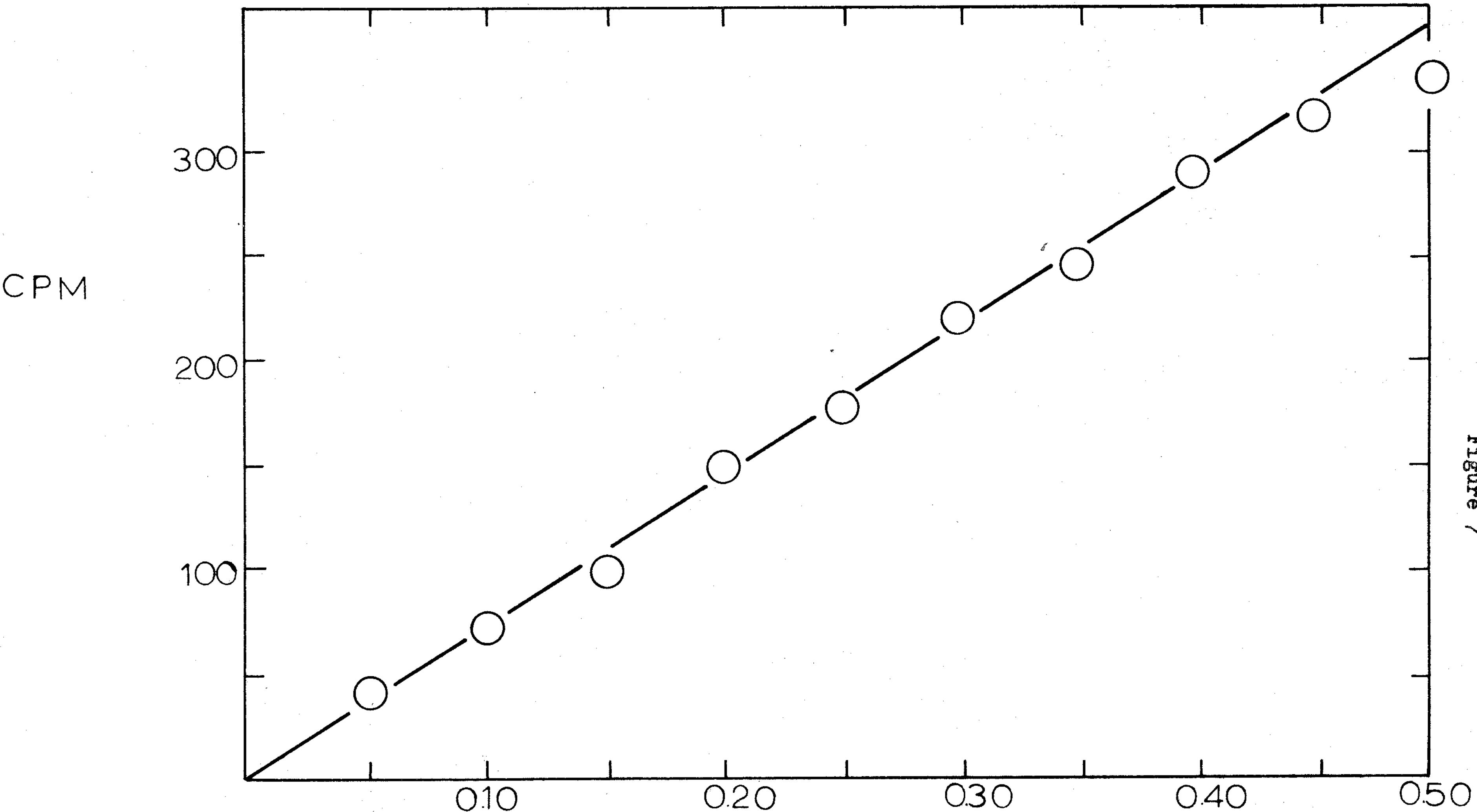
Measurement of Radioactivity in Hemoglobin Samples

The specific activities of hemoglobin samples and chromatographic fractions were measured using a Nuclear-Chicago gas flow Geiger counter, Model D-47, equipped with a "micromil" window. For counting, the hemoglobin samples were placed on planchettes in volumes of 0.5 or 1.0 ml. of developer. The solutions were evaporated to dryness with a heat lamp before counting. Generally, no more than 0.5 mg. of protein was placed on these planchettes. As may be seen from figure 7, no corrections were required for self absorption by the hemoglobin. No corrections were made for absorption by the buffer salts since these were always present in constant amounts.

Results of Hybridization Experiments

Early experiments indicated that hybrids were formed only after dissociation at acid pH. These experiments consisted in allowing mixtures of C^{14}-l-leucine labelled normal adult hemoglobin and inactive sickle cell hemoglobin to stand at pH 4.5 or 11.0 for a time of one hour. After dissociation of the materials at the respective pH values, the solutions were dialyzed against a barbital buffer of pH 8.6, u = .01. The hemoglobins were separated by free boundary electrophoresis in the Spinco Model H, current 8 ma for 5 hours. Good resolution of the hemoglobins was obtained in both the ascending and descending limbs. The hemoglobin samples were removed from the regions

Figure 7



ACTIVITY OF C^{14} LEUCINE LABELLED
NORMAL ADULT HEMOGLOBIN

corresponding to the fast and slow components using the sampling mechanism of the Spinco instrument. Due to the small amount of pure normal adult or sickle cell hemoglobin which could be recovered by this method, it was not possible to determine accurately the amounts of hemoglobins ultimately counted. Attempts were made to count comparable amounts of the two hemoglobins however. The results of experiments of this type are seen in table IX. It may be seen that there is an appreciable transfer of activity to the initially unlabelled sickle cell hemoglobin only after dissociation at pH 4.5.

Separations of the hybridized mixtures of the hemoglobins by free boundary electrophoresis did not allow the isolation of useful amounts of hybrids and were abandoned in favor of chromatographic separations. In the course of this work it was found that by allowing hemoglobin mixtures to remain at pH 11.0 for 24 hours at 3°C. a significant transfer of activity to the previously unlabelled hemoglobin would occur. In these experiments the hemoglobins were labelled as described in this Thesis and were separated chromatographically as described. The results of a typical hybridization experiment after dissociation at pH 11.0 and the chromatographic separation of the hemoglobins in the mixture is seen in figure 8. Also seen in this figure are the results of a chromatographic separation of an undissociated mixture of the same materials. The sickle cell hemoglobin, uniformly labelled with C^{14}-1-leucine, and the unlabelled normal adult hemoglobin used in these experiments were substantially freed chromatographically of minor components prior to these hybridization experiments. In figure 9 are shown the results of chromatographic separations of C^{14}-1-leucine labelled normal adult hemoglobin and unlabelled sickle cell

Table IX

Hybridization of C^{14} Labelled Normal Adult Hemoglobin with Unlabelled Sickle Cell Hemoglobin

Expt. No.	Net Activity Normal Adult (CPM)	Net Activity Sickle Cell (CPM)	Total Activity Expected (CPM)
pH 11.0			
1	7.4 ± 0.5	0.9 ± 0.7	
2	4.9 ± 0.2	-0.2 ± 3.0	
pH 4.5			
1	3.3 ± 0.5	1.5 ± 0.6	6.0 ± 0.6
2	2.7 ± 0.4	1.7 ± 0.4	4.1 ± 0.2

hemoglobin in the absence of hybridization and after dissociation at pH 5.0. In experiments using labelled normal hemoglobin it was not necessary to remove minor components prior to hybridization since these minors do not contaminate the chromatograph of the sickle cell hemoglobin fraction. Inspection of the chromatograms of the hybridized mixtures and comparison with the chromatograms of the unhybridized mixtures show that there are no new chromatographic species formed as the result of chain exchange.* Observed, however, is a transfer of radioactivity into the initially unlabelled hemoglobins as a result of the dissociations.

In table X are summarized the results of several hybridization experiments. Experiments 1 - 7 show the results of chain exchange after alkaline dissociation, while experiments 10 - 13 show the results of subunit exchange after acid dissociation. Experiments 8, 9,

*The chromatography protocol sheets from which the data of these figures were taken are presented in Appendix IV.

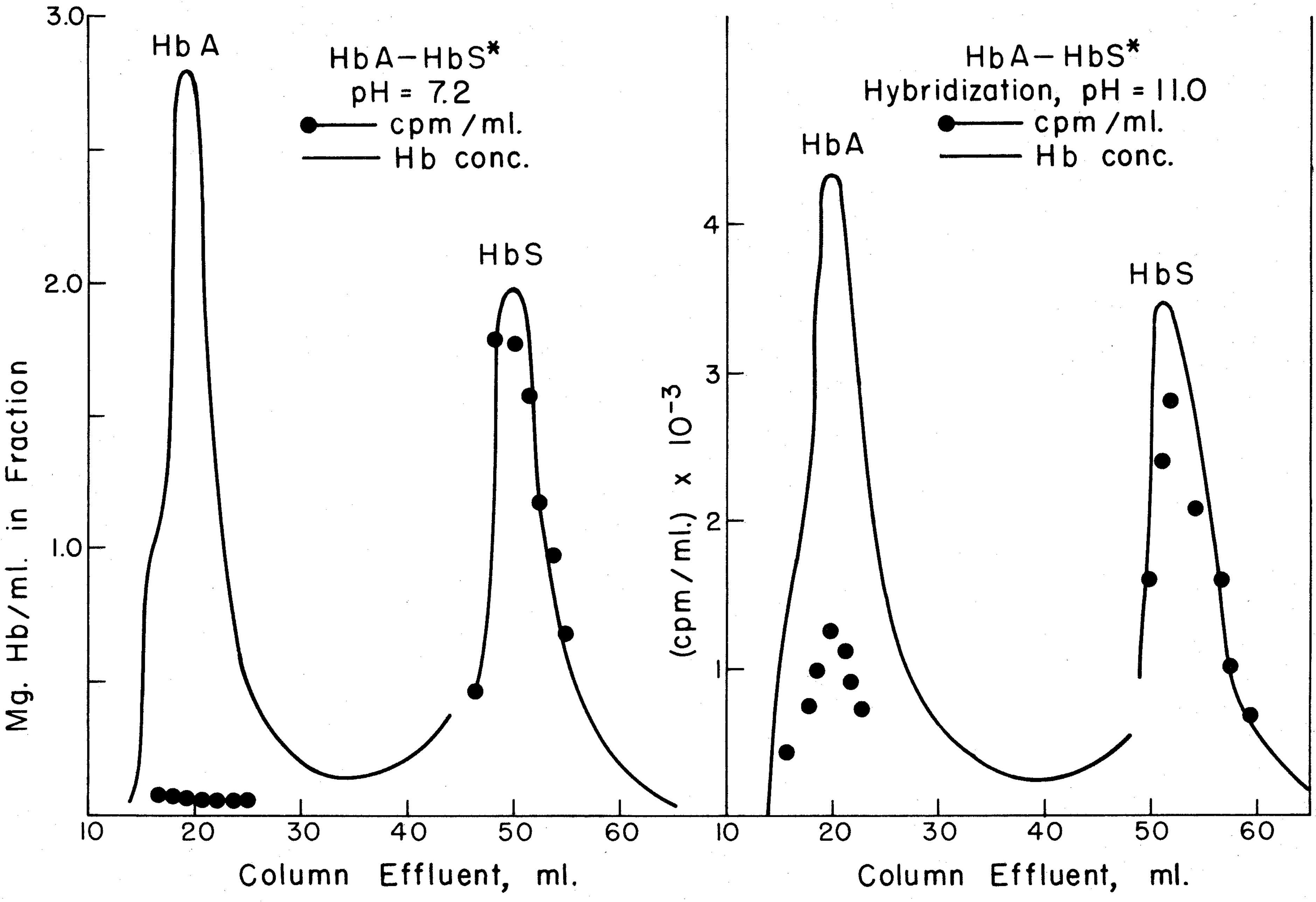


Figure 8

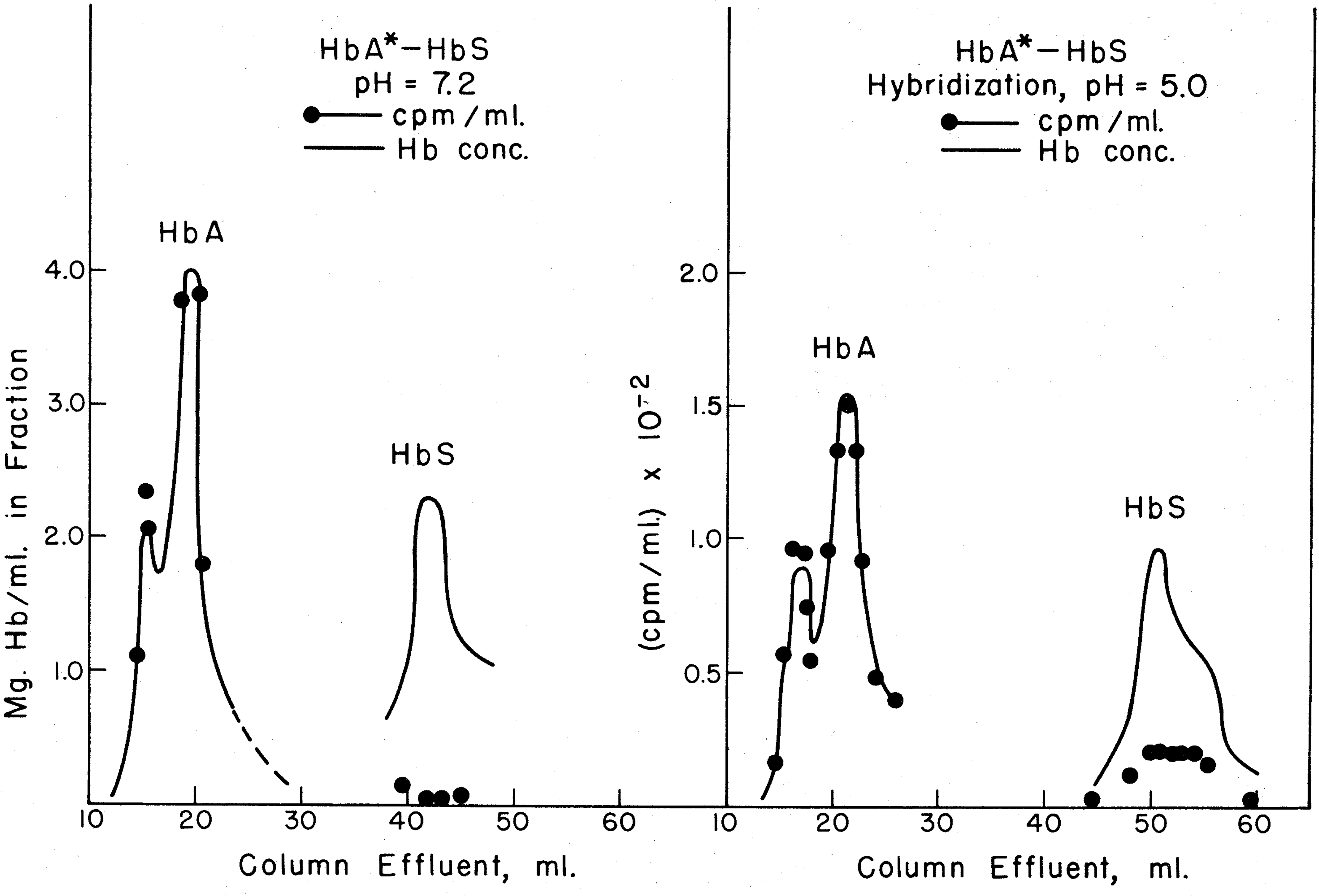


Figure 9

Table X

Results of Hybridization Experiments

Expt. No.	pH	Specific Activity of Reactants		Specific Activity of Products		Percent Hybridization
		HbA	HbS	HbA	HbS	
1*	11.0	3500	0	2700	800	88
2	11.0	0	1900	500 ± 30	1580 ± 200	94.8 ± 13.3
3	11.0	3500	0	2650 ± 140	800 ± 50	91.2 ± 7.4
4	11.0	0	1900	370 ± 50	1570 ± 100	70.8 ± 10.5
5	11.0	6900	0	6200	570 ± 30	27.6 ± 1.5
6**	11.0		0		900 ± 100	39.0
7	11.0	6100	0	4600 ± 500	1000 ± 200	65.1 ± 14.8
8	7.2	3500	0	3200 ± 300	64 ± 15	6.0 ± 1.5
9	7.2	0	1900	90 ± 40	1650 ± 200	16.4 ± 7.6
10	5.0	700	0	500 ± 40	130 ± 20	78.0 ± 13.8
11	5.0	3500	0	2500 ± 400	540 ± 20	64.8 ± 10.7
12	5.0	3000	0	2700 ± 260	460 ± 40	51.1 ± 6.6
13***	5.0	3500	0	2350 ± 170	960 ± 70	120.3 ± 12.5
14	7.2	600	0	550 ± 50	12 ± 4	6.5 ± 2.3

*Only samples of the most concentrated chromatographic fractions of the products were counted.

**Only the resulting HbS hybrid was assayed for radioactivity.

***This hemoglobin mixture was allowed to remain at pH = 5.0 for 5 days.

and 14 demonstrate the results of chromatographic separations of undissociated hemoglobin mixtures. The specific activities given in this table are the averages of specific activities of hemoglobin samples from chromatographic fractions containing more than 0.2 mg. Hb/ml.

Singer and Itano (13) have pointed out that transfer of one quarter of the total activity into the initially inactive hemoglobin is presumptive evidence of the identity of one half of the adult and

sickle cell hemoglobins. The transfer of this amount of activity to the initially unlabelled hemoglobin after dissociation of equimolar mixtures of the two hemoglobins represents complete hybridization. The percentage hybridizations tabulated in table X have been calculated assuming a ratio of specific activities in the products of three to one (21).

Significance of Hybridization of Radioactive Hemoglobins

Hybridization of hemoglobins using C^{14} labelled hemoglobins is an important tool for the identification of identical or compatible subunits in the different hemoglobins. It has been used in the elucidation of the gross structures of fetal hemoglobin (28) and hemoglobin "H" (29). The hemoglobin hybrids themselves are of significant use in many types of experiments. These materials contain one type of subunit which is radioactive while the other is not. In the next section of this Thesis will be described the manner in which use has been made of C^{14}-l-leucine labelled hybrids in the identification of the chain containing the anomaly in sickle cell hemoglobin. It is possible, using a hybrid, to follow the exchange of a specific subunit between other major hemoglobins or minor hemoglobin components. Radioautography of "fingerprints" of an algal hydrolysate labelled hybrid would afford the identification of all peptides emanating from a specific chain. Sequence studies could be done on the individual chains, using this material, without the necessity of separating them.

B. THE IDENTIFICATION OF THE ABERRANT CHAIN IN SICKLE CELL HEMOGLOBIN

Introduction

The formation of hybrids, or the exchange of subunits, in the normal adult-sickle cell hemoglobin system is presumptive evidence of the identity or, at least, the compatibility of subunits in the two hemoglobins. The apportionment of radioactivity between the hemoglobins in a hybridized mixture suggested that the unit transferring had a molecular weight one-half that of the hemoglobin molecule. Hybrid formation alone, though, affords no knowledge of the nature of the subunit transferred. At the time these hybridization experiments were done, the N-terminal sequences of the α and the β chains of both hemoglobins were known (17). The α chains were known to terminate in the sequence val-leu, and the β chains in the sequence val-his-leu.

Procedure

Since the hemoglobins were labelled with leucine, assessment of radioactivity in the terminal peptides of a hybrid hemoglobin, in which only one type of subunit was radioactive, allowed identification of the transferring subunit. The non-transferring subunit therefore contained the anomaly found by Ingram (19).

Preparation of Materials

For the identification of the aberrant subunit in sickle cell hemoglobin two hemoglobin hybrids were prepared. In one case C^{14}-1-

leucine labelled normal adult hemoglobin (7000 cpm/mg.) was hybridized with inactive sickle cell hemoglobin after dissociation at a pH of 5.0, thus forming a sickle cell hemoglobin hybrid. The second hybrid was formed after dissociation of minor free C^{14}-l-leucine labelled sickle cell hemoglobin with inactive normal adult hemoglobin at pH 11.0; this hybrid was a normal hemoglobin hybrid. In both cases only the chromatographic fractions of highest concentration in the respective hemoglobins were consolidated for the end group isolations. The hybrids were concentrated by centrifugation of their solutions for 24 hours in the No. 30 rotor of the Spinco Model L Ultracentrifuge at 27000 RPM. Approximately 100 mg. of the appropriate inactive carrier hemoglobins were added to the resulting concentrated solutions; this was done to facilitate the N-terminal peptide isolations.* The N-terminal peptides were prepared and isolated by the method of Rhinesmith, Schroeder, and Martin (18).

The dinitrophenylated peptides were estimated spectrophotometrically in an acetone solvent. Aliquots of these solutions were layered into planchettes and evaporated to dryness. In the assessment of the activity 50,000 counts were taken on each sample.

Results

Activity measurements were done on the dinitrophenylated terminal peptides from both chains. These materials were DNP-val-leu from the α chains, and DNP-val-his-leu from the β chains. In order to estimate the amount of activity in these peptides which could be attributed

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*I wish to thank Dr. W. A. Schroeder and Miss Joan Balog for carrying out these N-terminal peptide isolations.

to contamination of the peptides, measurements of the radioactivity in two other components, di-DNP-val-his and dinitroaniline, isolated from the DNP-globin hydrolysates were made. These components, which contained no leucine, give an idea of the purity of the leucine containing terminal peptides. In table XI are shown the results of this investigation. It is seen that the dinitrophenylated peptide from the α chains, DNP-val-leu, is significantly radioactive in both hybrids, while that from the β chains, di-DNP-val-his-leu, is substantially inactive.

Table XI

Activity of Components Isolated from Dinitrophenylated Hemoglobin Hybrid Hydrolysates

Material	Hybrid HbS* from HbS and HbA*		Hybrid HbA* from HbS* and HbA	
	cpm/u mole	u moles	cpm/u mole	u moles
DNP-val-leu	47.5	2.21	32.2	2.33
di-DNP-val-his-leu	6.2	0.52	3.4	1.08
di-DNP-val-his	7.2	1.09	1.8	0.56
dinitroaniline	0.6	2.79	0.2	5.36

It therefore appears that exchange of the α chains alone occurs after hybridization at either acid or alkaline pH. The non-transferring β chains must contain the amino acid substitution which confers upon sickle cell hemoglobin its peculiar properties.

Discussion

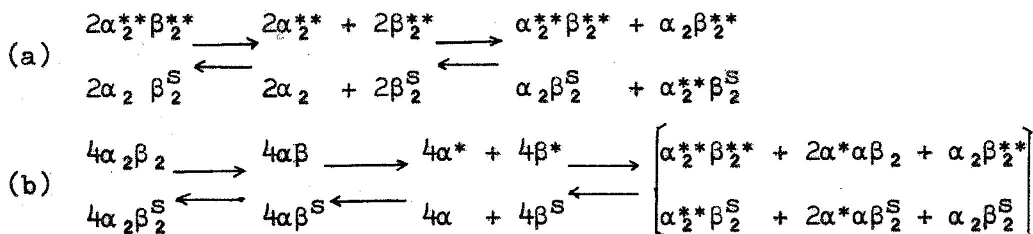
The identification of the aberrant chain in sickle cell anemia hemoglobin suggests a more descriptive system of nomenclature for the abnormal hemoglobins. We may assign the designation $\alpha_2\beta_2$ to normal

adult hemoglobin. From the preceding, it has been shown that the anomaly in sickle cell hemoglobin occurs in the β chains. We may therefore assign the designation of $\alpha_2\beta_2^S$ to this material, the lack of superscripts indicating that a subunit is common to normal adult hemoglobin. This system has been extended to fetal hemoglobin which has α chains which are common to normal adult hemoglobin, and γ chains (30) which have N-terminal peptides different from those of the α and β chains. Fetal hemoglobin is therefore designated as $\alpha_2\gamma_2$ (28). Hemoglobin "H" has been shown to be a tetramer of β chains which are identical to those of normal adult hemoglobin; its designation is therefore β_4 (29). As more subunits of abnormal hemoglobins are identified this system may be extended.

C. THE KINETICS OF SUBUNIT EXCHANGE IN THE ADULT-SICKLE CELL HEMOGLOBIN SYSTEM AND THE MODE OF DISSOCIATION OF HEMOGLOBIN AT pH 11.0

Introduction

Hybrid formation using radioactive labels gives no evidence of the mode of dissociation of the hemoglobins. These experiments show only the mode of recombination of whatever subunits are formed in the dissociation reaction. In the preceding section of this Thesis it has been shown that only the α chains are exchanged after dissociation of hemoglobin mixtures at either acid or alkaline pH. This exchange of α chains may occur by either of two mechanisms:



Using radioactive labels in one of the reactants would lead in both cases to the same apportionment of activity in the products.

The exchange of α chains in the adult-sickle cell hemoglobin system is analogous to the isotopic exchange reaction:

where X* is a radioactive isotope label which can exchange with the normal isotope X. The rate law for such an exchange is well known (31) and is given by:

$$2.303 \log x_{oo}/(x_{oo} - x) = (R/ab)(a + b)t$$

where

$a = (AX) + (AX^*)$

$b = (BX) + (BX^*)$

$x = (AX^*)$ at time t, and

$x_{\infty} = (AX^*)$ at time $t = \infty$

The quantity R is the gross rate of exchange of X, whether of like or of different isotopes. A more convenient form of equation 9 for the α chain exchange in the normal adult-sickle cell hemoglobin system is:

$$-\log (1 - \text{Percent Hybridization}/100) = (R/ab)(a + b)t$$

The derivation of the rate law for isotope exchange requires no assumptions of the detailed mechanism by which the exchange occurs. It is possible, however, to use other information with kinetic studies to obtain presumptive evidence of the mode of subunit exchange in the normal adult-sickle cell hemoglobin system.

Experimental Procedure

Normal adult hemoglobin was labelled with 1-leucine as described before in this Thesis. The specific activity found in this hemoglobin preparation was 2800 cpm/mg. Sickle cell hemoglobin was also prepared as described previously. Since labelled normal hemoglobin was used it was not necessary to remove the minor components from either hemoglobin.

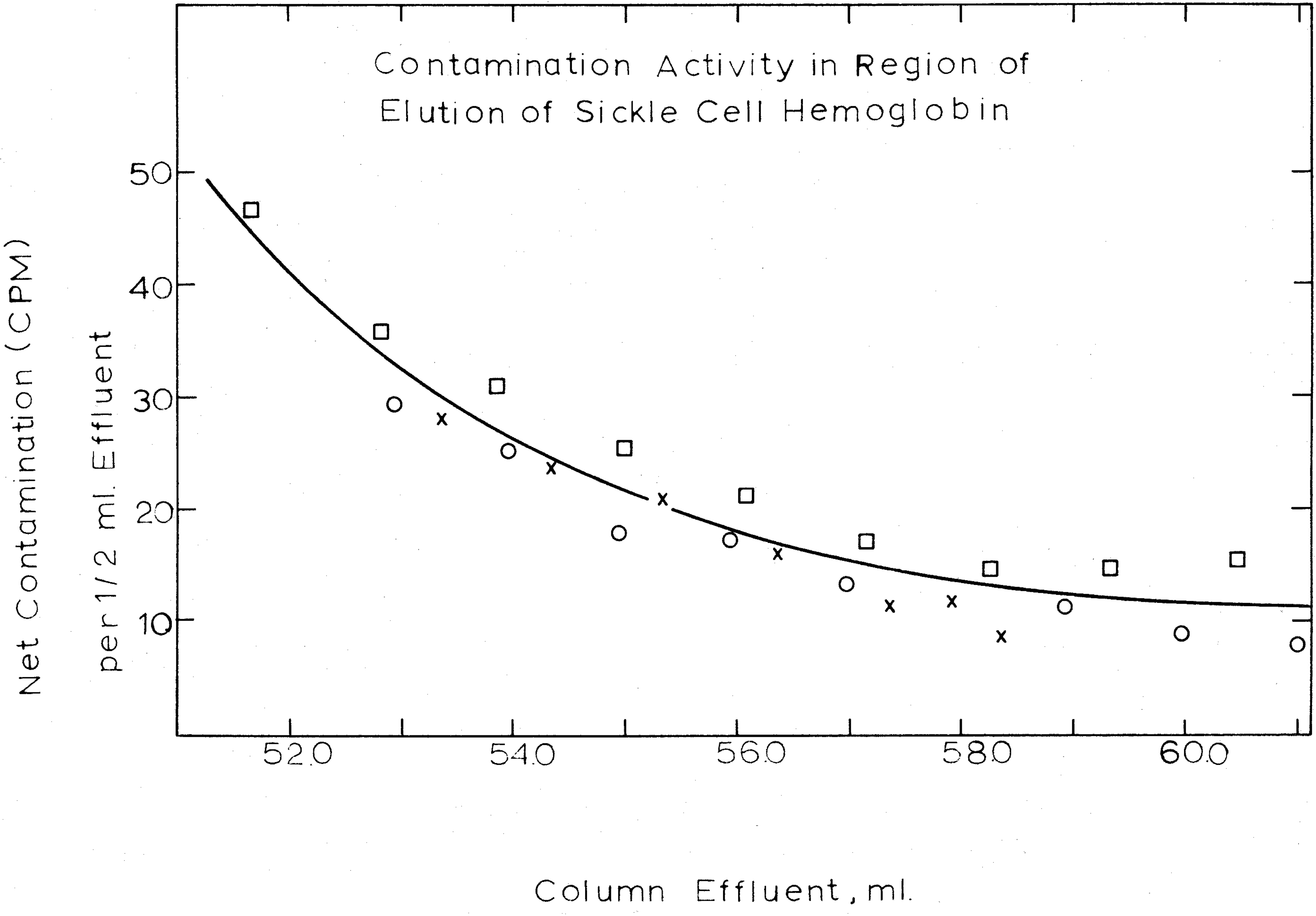
Normal adult hemoglobin labelled uniformly with C^{14}-1-leucine and unlabelled sickle cell hemoglobin in separate dialysis tubes were dialyzed against two changes of the phosphate buffer previously described of pH 11.0. The dialysate was changed twice over a twenty-four hour period. After this dialysis, 15 mg. each of the respective hemoglobins

were mixed and allowed to stand at 5°C. for varying lengths of time. At the end of the allotted reaction time the mixtures were brought to neutrality by quenching with a 2.8 molar phosphate buffer of pH 6.8, this being the buffer of Drabkin (32). These solutions were then dialyzed for twenty-four hours against two changes of the chromatographic developer no. 1. The hemoglobins were separated as before by chromatography. The chromatographic fractions were assayed as before for transfer of radioactivity.

Results

In order to determine the amount of activity actually transferred to the sickle cell hemoglobin in these experiments it was necessary to determine the residual activity from the normal hemoglobin eluted with the sickle cell hemoglobin. For this purpose three types of chromatographic runs were performed, the first on a sample of a radioactive normal adult hemoglobin alone. The second of these experiments was with a mixture of the two hemoglobins which had been carried separately to pH 11.0, mixed and held at this pH for six seconds, neutralized and then dialyzed against developer no. 1. The third of these blank experiments was done on a mixture of the two hemoglobins made after both hemoglobins had been separately at pH 11.0, neutralized, and dialyzed separately against the chromatographic developer. In these three experiments, and the ones done after this in the actual kinetic study, the columns were warmed to 28°C. after exactly 50 ml. of developer had flowed through the columns at 6°C. The pertinent data from these experiments are plotted in figure 10. The specific activities of the sickle cell fractions were calculated

Figure 10



after correction of the gross activities for background activity and the contamination due to adult hemoglobin as deduced from this figure.

In the kinetic experiments specific activities were calculated for each individual sickle cell hemoglobin chromatographic fraction by dividing the net activity by the weight of hemoglobin counted. In each of these experiments, a weight average specific activity of the sickle cell hemoglobin fractions, $\bar{S}_{sc}$, was calculated using the equation:

$$\bar{S}_{sc} = \frac{\sum_i^n w_i S_i}{\sum_i^n w_i} \tag{11}$$

where w_i is the weight of the ith fraction of n fractions and S_i is the measured specific of the hemoglobin in the ith fraction. Weight average probable errors of the weight average specific activities were calculated using the equation:

$$\bar{\Delta} = \frac{\sum_i^n w_i \Delta_i^2}{(n-1)\sum_i^n w_i} \tag{12}$$

where Δ_i is the difference between the weight average specific activity and that of the ith fraction. The percentage hybridization was calculated by dividing the weight average specific activity of the sickle cell hemoglobin by the weight average specific activity of the non hybridized normal adult hemoglobin (2820 $\pm$ 100 cpm/mg.) and multiplying by 400. The probable error in the percentage hybridization was calculated using the equation:

$$\text{p.e.}(\%\ \text{Hyb.}) = \frac{400}{\bar{S}_{na}} \sqrt{\Delta^2_{sc} + \frac{\bar{S}^2_{sc}\ \bar{\Delta}^2_{na}}{\bar{S}^2_{na}}} \tag{13}$$

These values were used in the calculation of the function log(1-percent hybridization/100) as a function of time allowed for

hybridization. The results of these calculations are presented in table XII. In figure 11 the function log(1-percent hybridization/100) resulting from these data is plotted as a function of the time. The vertical bars represent the probable errors of this quantity and the solid line is the least squares line through the points; the slope of this line is 0.032/hour and its intercept at time zero is 0.0365. Due to the nature of the function plotted, the logarithm of differences of small numbers, considerable error is encountered in the regions of near complete hybridization; this is manifested in the large error bars at long times on the plot.

Discussion of Results

By moving boundary electrophoresis of mixtures of adult carbonmonoxy hemoglobin and sickle cell ferrihemoglobin dissociated at pH 4.7 and recombined at pH 6.8, Singer and Itano (13) have shown that the acid dissociation proceeds by mechanism (a). The products formed on recombination of their mixtures indicated that the bond between identical chains remained intact during the dissociation-recombination procedure.

Adult hemoglobin is completely dissociated into half molecules at a pH of 11.0 as shown by Hasserodt and Vinograd (20). Sedimentation coefficient determinations on sickle cell hemoglobin indicate that it too is completely in the form of half molecules at this pH. In table XIII are seen the results of these measurements. If dissociation at pH 11.0 proceeded by mechanism (a) it would be expected that complete hybridization would occur in short time if a mixture of the hemoglobins was dissociated at pH 11 and rapidly neutralized. The kinetic results

Table XII

Evaluation of the Function
log(1 - Percent Hybridization/100) = log(1 - x)

Time	$\bar{S}_{sc}$	Percent Hybridization	x	1 - x	log(1 - x)	Mean
			.929	.071	-1.148	
24 hrs.	619 ± 11	97.6 ± 5.3	.876	.124	-0.907	-0.950
			.823	.177	-0.752	
			.722	.278	-0.950	
18 hrs.	463 ± 13	66.0 ± 6.2	.660	.340	-0.468	-0.477
			.598	.402	-0.397	
			.824	.176	-0.755	
12 hrs.	534 ± 14	76.0 ± 6.4	.760	.240	-0.620	-0.636
			.696	.304	-0.917	
			.622	.478	-0.321	
12 hrs.	339 ± 7	48.7 ± 3.5	.487	.513	-0.289	-0.291
			.452	.548	-0.262	
			.539	.463	-0.226	
9 hrs.	341 ± 9	49.2 ± 4.7	.492	.508	-0.294	-0.295
			.447	.553	-0.296	
			.394	.606	-0.218	
6 hrs.	245 ± 8	34.9 ± 4.5	.349	.651	-0.187	-0.188
			.304	.696	-0.158	
			.340	.660	-0.180	
3 hrs.	213 ± 8	30.5 ± 3.5	.305	.695	-0.158	-0.159
			.270	.730	-0.137	
			.403	.597	-0.296	
3 hrs.	242 ± 9	36.5 ± 9.8	.355	.645	-0.190	-0.207
			.307	.693	-0.158	
			.313	.687	-0.163	
3 hrs.	202 ± 5	29.0 ± 2.3	.290	.710	-0.148	-0.149
			.267	.733	-0.134	

Figure 11

Kinetics of α Chain Exchange between Adult and Sickle Cell Hemoglobins at pH = 11.0

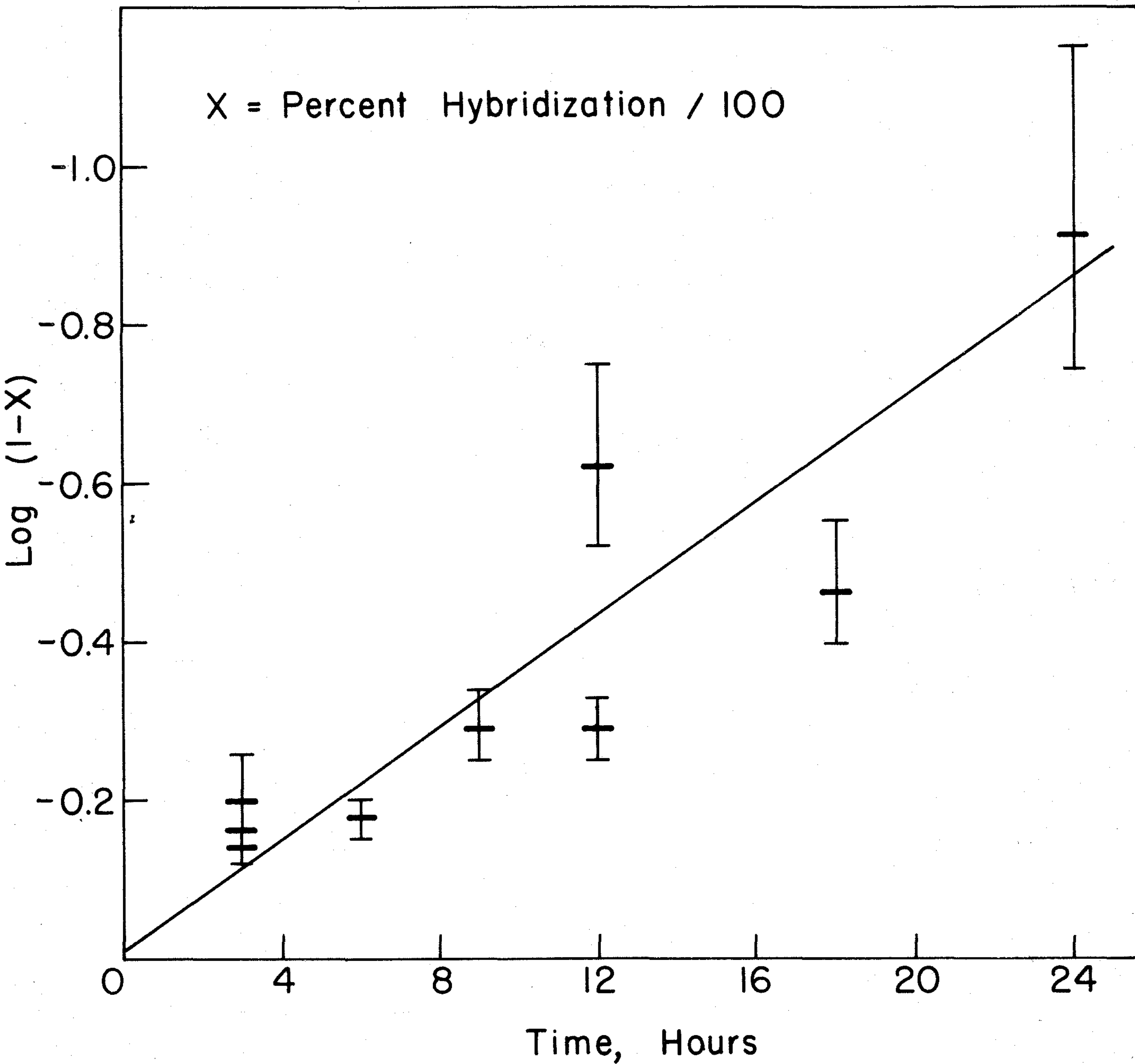


Table XIII

Sedimentation Coefficients of Adult and Sickle Cell Hemoglobins at pH 11.0

Experiment No. C-568

Rotor Speed-56,000 RPM, Solvent-Phosphate Buffer pH 11.0, u 0.35, temperature 20°C.

Material	Concentration	$S_{w,20}$	$S^{\circ}_{w,20}$
Sickle cell	0.55%	2.58	2.65
Adult	0.55%	2.58	2.65

of the chain exchange reaction at this pH obviate this mechanism. In table XIV are shown the results of attempted hybridizations after mixing of the two hemoglobins at acid pH for short times, followed by neutralization. In these experiments substantial hybridization takes place. The ease with which hybrids are formed in acid solution tends to rule out the possibility of changes being required in the tertiary structure of the chains before exchange at either pH. It thus appears that the dissociation of hemoglobins in alkaline solutions first involves the formation of identical subunits, followed by a slow dissociation into the individual chains, i.e., mechanism(b). The slow step may be the dissociation of $\alpha\beta$, $\alpha\beta^{S}$, or both. By mechanism(b) the possibility of forming the hybrid $\alpha_2\beta\beta^{S}$ exists. However, in the preceding section of this Thesis it has been shown that α chain exchange alone occurs. It must be concluded that the hybrid $\alpha_2\beta\beta^{S}$ is not possible because of steric requirements.

Several types of experiments were attempted to confirm the mechanism implied by the kinetic results.

Studies on the Carbonmonoxy-Ferrihemoglobin System

An adapatation of the method of Singer and Itano in elucidating the mechanism of dissociation of hemoglobin at acid pH was

Table XIV

Rapid Mix Experiments at Acid pH

Expt. No.	Materials	Specific Activities of Products		Percent Hybridization
		HbA	HbS	
RM 1	HbA and HbS* held together 20 min., pH 4.7	280	1380	62
RM 2	HbA* and HbS held together 20 min., pH 4.7	4600	1000	65
RM 3	HbA and HbS* held together 10 sec., pH 4.7	300	1485	61
RM 4	HbA* and HbS held together 10 sec., pH 4.7	5700	570	30
RM 5	HbA* and HbS held separately at pH 4.7 for 20 min., neutralized	4700	1270	81
RM 6	HbA and HbS* held together 1 hour at pH 4.7	322	1230	79

attempted at pH 11. This method predicts the formation of two new electrophoretic species in the normal adult carbonmonoxy-sickle cell ferrihemoglobin system if the reaction of chain exchange proceeds by mechanism (a); and if it proceeds by mechanism (b), four new electrophoretic species. For the purpose of demonstrating the mechanism of the alkaline dissociation reaction, a normal adult carbonmonoxy-ferrihemoglobin system was used. This combination predicts the formation of one new electrophoretic species (actually two new molecular species having equal charges) if the reaction proceeds by mechanism (a); and if the α chain exchange proceeds by mechanism (b), three new

electrophoretic species (four new molecular species, two of which have equal charges).

Normal adult ferrihemoglobin was prepared as described by Singer and Itano (13). Equimolar mixtures of normal adult carbonmonoxy- and ferrihemoglobins were dialyzed for 24 hours against the phosphate buffer of pH 11.0 to allow the chain exchange to occur. These mixtures were then dialyzed for 24 hours against the potassium phosphate buffer of Itano and Robinson (33) of pH 6.8, u = 0.01 required for the electrophoretic separation of the materials formed upon chain exchange. In all cases the ferrihemoglobin was selectively precipitated from the solutions. Determinations of the spectrum of the supernatant solutions revealed a 60% loss of the ferrihemoglobin from the solutions.

It was found that normal adult carbonmonoxyhemoglobin and normal adult ferrihemoglobin cyanide could be dialyzed at pH 11.0 for 24 hours, then dialyzed to pH 6.8 against the electrophoresis buffer with no loss of protein. Reaction of ferrihemoglobin with the cyanide ion results in the loss of the charge label required for the electrophoretic separations. It was not feasible to remove the cyanide from the hemoglobin; this procedure was therefore abandoned.

It is interesting to consider the stability conferred upon hemoglobin by compound formation of the iron in the heme. Sedimentation coefficient determinations were made on carbonmonoxyhemoglobin, ferrihemoglobin, and ferrihemoglobin cyanide at equal concentration in solutions of pH 10.4; these results are presented in table XV. Of the hemoglobin derivatives studied it appears that ferrihemoglobin dissociates to the greatest extent. It may be possible that the dissociation of the ferrihemoglobin plays a role in the denaturation of this

material in the alkaline region.

Table XV

Sedimentation Coefficients of Hemoglobin
Derivatives in Alkaline Solution

Buffer: Carbonate
pH 10.4, μ = 0.24

Expt. No.	Material	Concentration	$S_{w,20}$	$S^{\circ}_{w,20}$
298	Carbonmonoxy	0.50	3.96	4.06
303	Oxy	0.50	3.89	3.93
301	Ferricyanide	0.50	3.59	3.69
298	Ferri	0.50	3.41	3.50

Studies on Guanidinated Hemoglobin

It was decided to attempt to produce an effective charge label on the hemoglobin by formation of guanidinium groups from the lysine residues in the hemoglobin molecule. This label becomes effective in the region of pH 11.0. Guanidinated hemoglobin was prepared in the following way (34):

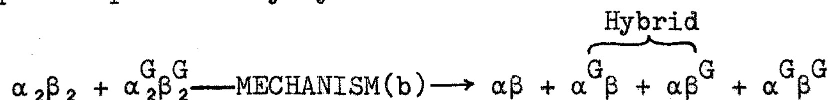
> A solution containing 1 gm. of O-methyl isoruronium sulfate in 15 ml. total volume was titrated to appH of 11.0 with NaOH. To this solution was added 1500 mg. of Normal adult carbonmonoxyhemoglobin in a total volume of 11.4 ml. Before mixing, both solutions were cooled to 0°C. The mixture was allowed to stand for three days at 0°C. After this time the solution was dialyzed against several changes of distilled water to remove excess O-methyl isoruronium sulfate.

The resulting carbonmonoxy guanidinated hemoglobin was crystallizable in the 2.8 molar phosphate buffer of pH 6.8 of Drabkin. The spectrum of guanidinated carbonmonoxyhemoglobin was found to be identical to that of carbonmonoxyhemoglobin. It was found by titration that 25 of the 42 lysines of the hemoglobin were guanidinated; the guanidinated

hemoglobin binding 40 moles of base per molecule and carbonmonoxy hemoglobin binding 65 moles per molecule at pH 11.0. The results of the titrations agree well with the ratios of electrophoretic mobilities of the guanidinated and non-guanidinated hemoglobins in alkaline solution (table XVI). Both hemoglobins give rise to single boundaries in the electrophoretic cell in this region.

Two types of experiments were done with the guanidinated hemoglobin. In one case guanidinated and non-guanidinated carbonmonoxy hemoglobins were dialyzed separately to pH 10.9 in the electrophoresis buffer, mixed and immediately subjected to electrophoresis. In the second type of experiment, the hemoglobins were dialyzed together in this buffer for 24 hours, then subjected to electrophoresis. The results of experiments of this type are presented in table XVI and figure 12. In the figure it is seen that a new component is formed in the mixtures of the hemoglobins dialyzed together. In table XVI it is seen that the mobility of the new component is intermediate between the mobilities of the guanidinated and non-guanidinated hemoglobins. Further, it is seen in the footnote to figure 12 that the area of the middle peak corresponding to the new component is approximately equal to the sum of the decreases of areas of the guanidinated and non-guanidinated hemoglobins. It therefore appears that the new species has been formed from other materials initially mixed.

These experiments were predicated upon the premise that in the electrophoretic buffer all components were to be in the form of half molecules. The electrophoretic components sought were the half molecular species produced by hybridization:



$$\alpha_2\beta_2 + \alpha_2^G\beta_2^G \xrightarrow{\text{MECHANISM(b)}} \alpha\beta + \overbrace{\alpha^G\beta + \alpha\beta^G}^{\text{Hybrid}} + \alpha^G\beta^G$$

Table XVI

Electrophoretic Mobilities of Guanidinated(HbGN) and Non-Guanidinated(HbA) Carbonmonoxyhemoglobins in Carbonate Buffer, pH 10.9, μ = 0.20

Expt. No.	Materials Run	Electrophoretic Mobilities cm^2/sec.-volt x 10^5	
		Left(descending)Limb	Right(ascending)Limb
1	HbA alone	6.2	6.3
2	HbGN alone	3.3	3.6
3	HbA and HbGN dialyzed together	6.1 5.1 3.9	6.4 5.5 4.5
4	HbA and HbGN mixed immediately prior to electrophoresis	6.8 4.4	6.5 4.4
5	HbA and HbGN dialyzed together	6.1 4.9 3.6	6.4 5.2 4.0

The discovery of an electrophoretic species having a mobility intermediate between the guanidinated and non-guanidinated hemoglobins, however, does not resolve the mechanism of chain exchange between the hemoglobins in favor of mechanism(b). Unlike non-guanidinated hemoglobin at pH 10.9 which is completely dissociated, guanidinated hemoglobin is only partially dissociated into half molecules. This may be seen from the results of sedimentation studies made on the two hemoglobins in the electrophoretic buffer of pH 10.9 (table XVII). These experiments were carried out at low temperature to approximate the temperature conditions of the electrophoresis experiments. The mixture of half and whole molecules in the electrophoretic separation confounds a clear interpretation of the results. If the new species formed is whole molecular, it is the same new electrophoretic component

LEGEND FOR FIGURE 12

ELECTROPHORESIS OF GUANIDINATED (HbGN) AND NON-GUANIDINATED (HbA) CARBONMONOXYHEMOGLOBINS IN ALKALINE SOLUTIONS

Buffer: 0.10 N $NaHCO_3$, 0.096 M NaOH
pH 10.9, μ = 0.20
Current: 20 ma
Voltage: 111 volts
Time: 288.9 min.

Experiment Number	Material	Treatment
1	HbA	Dialyzed only against buffer
2	HbGN	Dialyzed alone against buffer
3	HbA and HbGN	Dialyzed together against buffer
4	HbA and HbGN	Dialyzed separately against buffer and mixed immediately prior to electrophoresis

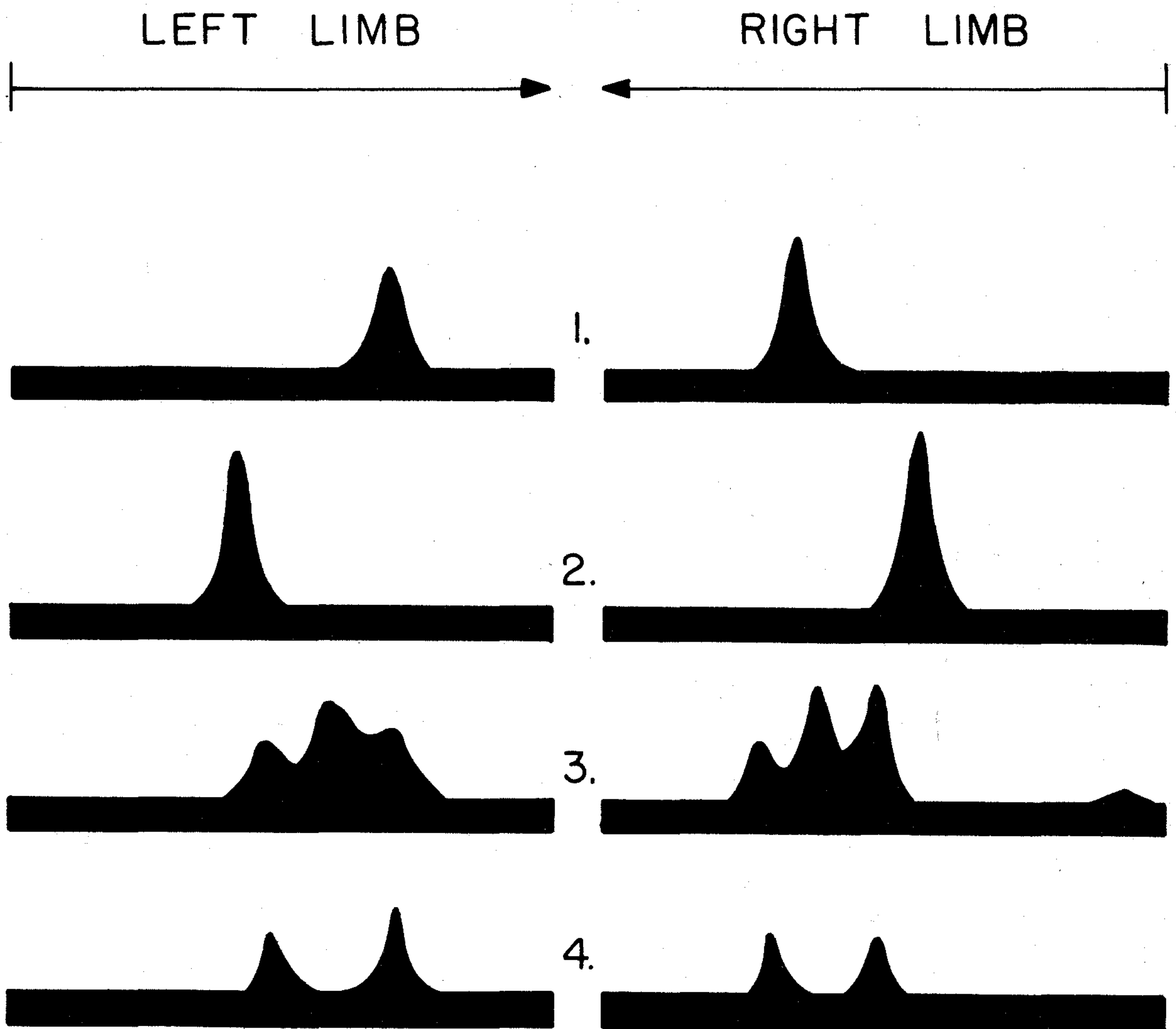
---

Note: The relative areas of the schlieren peaks of experiments 1, 2, and 3 were calculated for the right limbs. These results were:

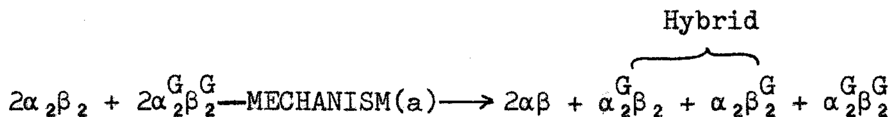
	HbA	Hybrid	HbGN
Expt. 1	161		
Expt. 2			211
Expt. 3	77	154	

Figure 12

Electrophoresis of Guanidinated (HbGN) and Non-Guanidinated (HbA) Carbonmonoxyhemoglobins at pH 10.9



which would result from chain exchange by mechanism (a):



$$2\alpha_2\beta_2 + 2\alpha^G_2\beta^G_2 \text{—MECHANISM(a)} \longrightarrow 2\alpha\beta + \overbrace{\alpha^G_2\beta_2 + \alpha_2\beta^G_2}^{\text{Hybrid}} + \alpha^G_2\beta^G_2$$

Table XVII

Corrected Sedimentation Coefficients of Guanidinated
and Non-Guanidinated Carbonmonoxyhemoglobins in
Carbonate Buffer, pH 10.9, u = 0.20
Hb Conc. = 0.39%

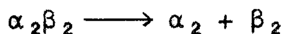
Expt. No.	Material	Temperature °C.	$S_{w,20}$	$S^{\circ}_{w,20}$
C-557	HbGN	9.0	4.03	4.13
C-557	HbA	9.0	2.63	2.68
C-561	HbGN	3.5	4.12	4.22
C-561	HbA	3.5	2.51	2.56

Conclusion

Evidence has been presented which suggests that hemoglobin dissociates in alkaline solutions to produce identical subunits, i.e.,

$$\alpha_2\beta_2 \longrightarrow 2\alpha\beta$$

Singer and Itano have shown that the products formed on acid dissociation are non-identical, i.e.,



$$\alpha_2\beta_2 \longrightarrow \alpha_2 + \beta_2$$

The recently published tertiary structure of horse hemoglobin (12) shows that this hemoglobin consists of four polypeptide chains situated at the corners of an irregular tetrahedron roughly. That human hemoglobin undergoes two types of dissociation is consistent with this model.

D. PRELIMINARY INVESTIGATION OF THE ISOLATION OF HEMOGLOBIN HYBRIDS LABELLED IN THE β CHAINS

Introduction

The hemoglobin hybrids formed on dissociation of mixtures of normal adult and sickle cell hemoglobins are labelled only in the α chains. It would be desirable to have hemoglobin hybrids in which the β chains only are radioactive. Hybrids of this type would be very useful in the identification of abnormal hemoglobins having defects in the α chains. These labelled hybrids could be hybridized with these hemoglobins and the transfer of β chains could be easily determined. For this reason these studies were begun.

Procedure

Acid hybridization of mixtures of normal adult carbonmonoxyhemoglobin and sickle cell ferrihemoglobin produces the species $\alpha_2\beta_2$, $\alpha_2^m\beta_2$, $\alpha_2\beta_2^{sm}$, and $\alpha_2^m\beta_2^{sm}$ having the relative charges 0, 2, 4, and 6, respectively. If uniformly labelled normal adult hemoglobin, prepared as described in this Thesis, were used in such hybridization experiments the species $\alpha_2^m\beta_2$ would be radioactive only in the β chains. If this material were reacted with cyanide ion after separation from the above mixture, it could be used as a tracer in normal adult hemoglobin since ferrihemoglobin cyanide has the same charge as carbonmonoxyhemoglobin.

Experimental Procedure

Attempts were made at separating chromatographically the mixture resulting from the hybridization of normal adult carbonmonoxyhemoglobin and sickle cell ferrihemoglobin. For these separations it was necessary to maintain the integrity of the charge differences between the components of the mixture. A new chromatographic developer, containing no cyanide was used. This developer no. 1 OH had the composition: 0.024 M NaH_2PO_4, 0.050 M Na_2HPO_4, 0.01 M KOH.

Jacketed chromatographic columns were designed having resin beds 70 cm. long. For these separations, IRC 50,200-250 mesh, was again used. The columns were equilibrated at 6°C. by flowing 2 liters of developer no. 1 OH through them over a period of 2 days. The hemoglobin mixtures were layered on the top of the columns in a volume of three ml. of the developer after dialysis against the developer. The normal adult carbonmonosyhemoglobin was eluted in the first 95 ml. of eluate. At 95 ml. the flow of developer through the columns was stopped and the temperature of the jacketed columns raised to 17°C. After allowing 15-20 minutes for the columns to reach this temperature flow of the developer was again begun. The component $\alpha_2^m\beta_2$ was eluted at 17°C. within the next 35 ml. eluate. The flow through the columns was again interrupted and the temperature of the columns raised to 28°C. After allowing sufficient time for the columns to reach this temperature, the remaining protein was eluted.

Preparation of Materials

Normal adult hemoglobin and sickle cell hemoglobin were prepared as described before from the red blood cells of normal and sickle cell

anemic donors, respectively. Sickle cell ferrihemoglobin was prepared by reaction of 100 mg. of sickle cell hemoglobin with a four fold excess of potassium ferricyanide. This reaction was carried out by mixing a 7 to 10% solution of HbS with a $K_3Fe(CN)_6$ solution (4 mg. $K_3Fe(CN)_6$/ml. of 0.01 u K. Phosphate buffer of pH 6.8) at 0°C.

Equimolar mixtures of normal adult carbonmonoxy and sickle cell ferrihemoglobins were dissociated by the methods of Singer and Itano (13). Fifty mg. of total protein in 3.0 ml. of H_2O were mixed with 3.0 ml. of acetate buffer of pH 4.7, u = 0.2. These solutions were allowed to stand at pH 4.7 for 20 minutes at room temperature after which they were dialyzed against cold chromatographic developer no. 1 OH.

Results

Shown in figure 13 are the results of an electrophoretic separation of the recombination products of the normal adult carbonmonoxy-sickle cell ferrihemoglobin dissociations. It is seen that two new electrophoretic species appear as a result of α chain exchange. A charge difference of two between each component of the mixture causes the difference in electrophoretic mobility.

In figures 14 and 15 are shown the results of chromatographic separations of the components in non-dissociated and dissociated mixtures respectively. From the quality of these separations it appears that it is feasible to separate chromatographically these components. Refinement of the chromatographic procedure would perhaps afford a cleaner separation of the components. In these experiments neither of the hemoglobins was radioactive; the use of uniformly labelled normal

Figure 13

ELECTROPHORESIS OF NORMAL ADULT CARBONMONOXY AND SICKLE CELL FERRIHEMOGLOBIN

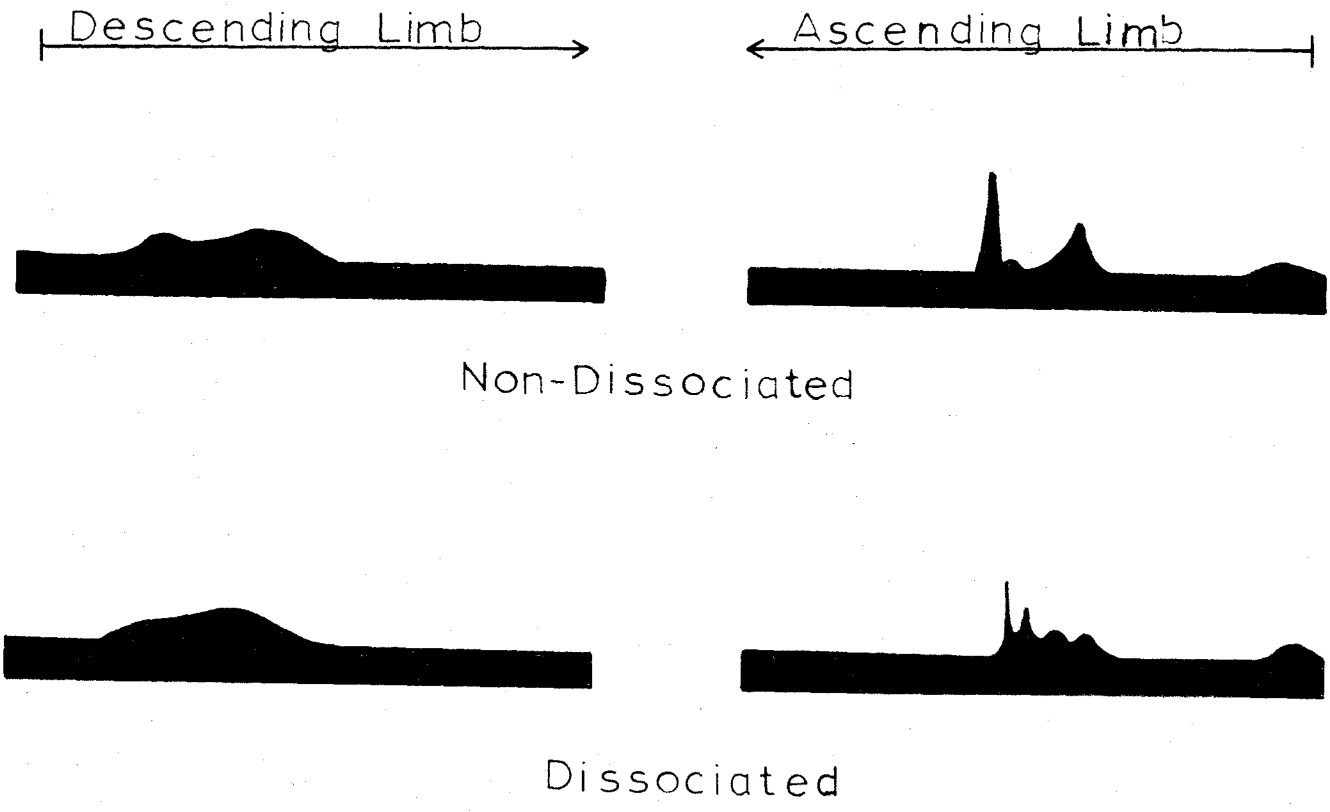
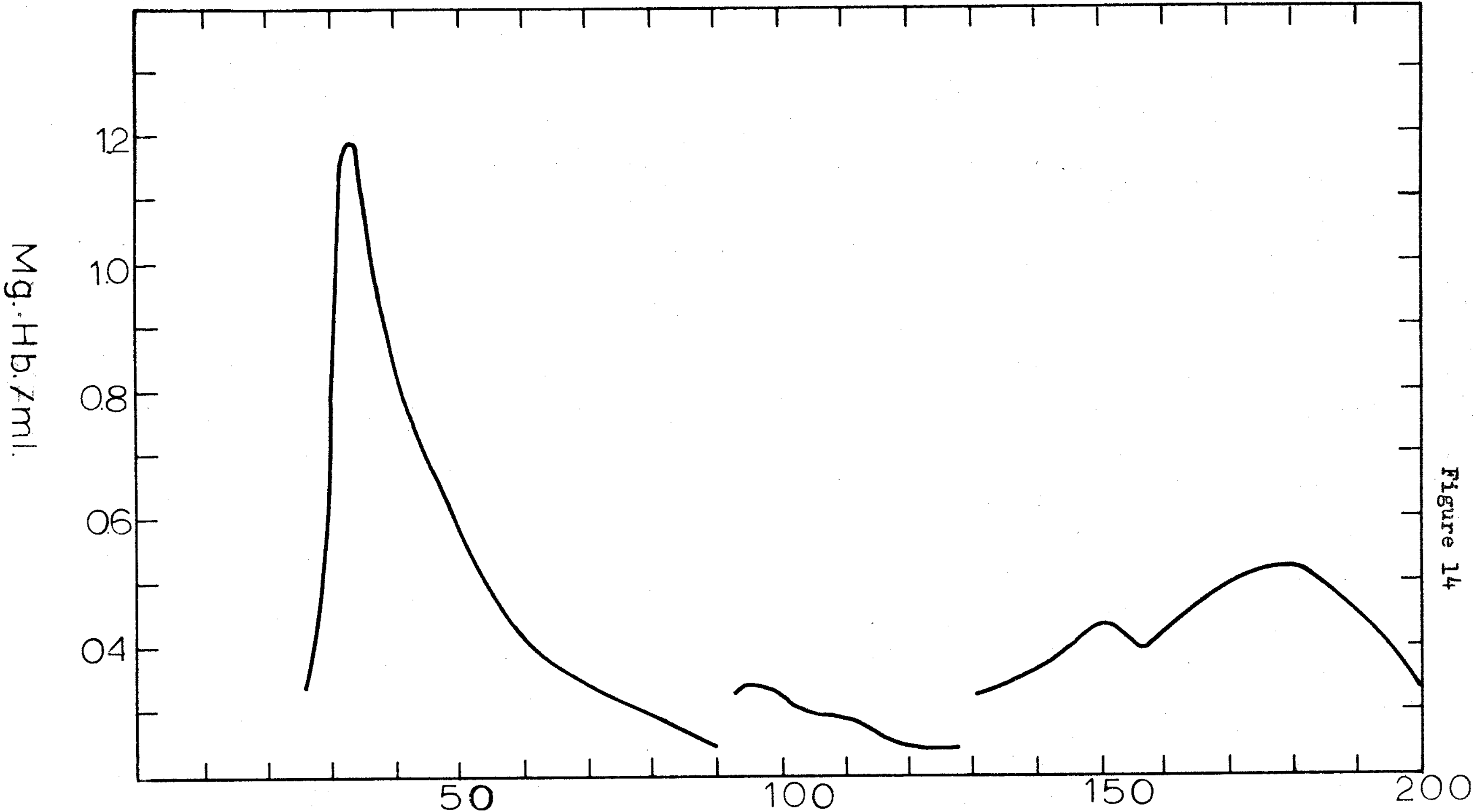


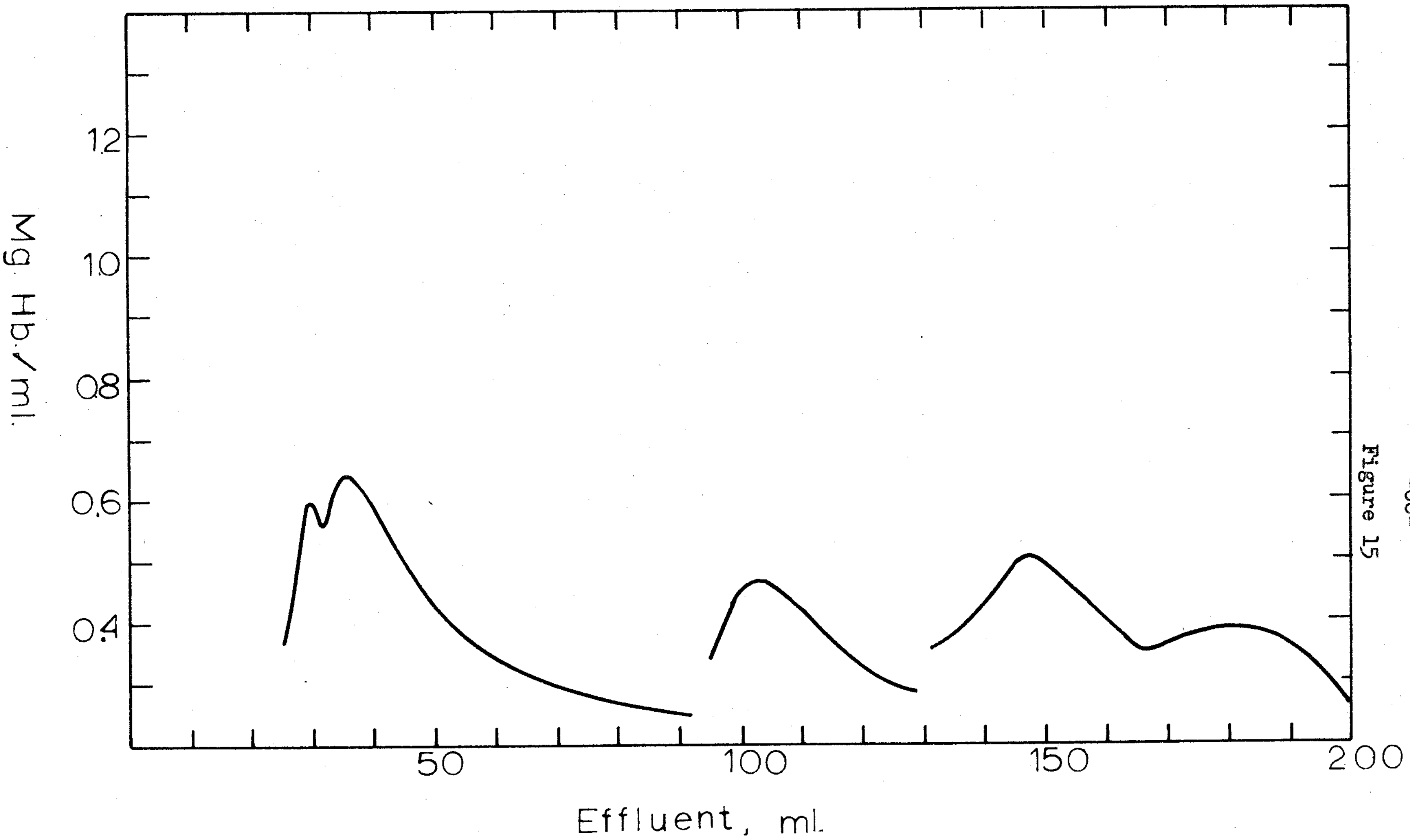
Figure 14



NORMAL ADULT CARBONMONOXYHEMOGLOBIN AND SICKLE CELL FERRIHEMOGLOBIN

HELD SEPARATELY AT pH = 4.7 FOR ONE HOUR

Figure 15



NORMAL ADULT CARBONMONOXYHEMOGLOBIN AND SICKLE CELL FERRIHEMOGLOBIN

MIXTURE AT pH = 4.7 FOR ONE HOUR

adult carbonmonoxy hemoglobin in the dissociation mixture would result in formation of the β chain labelled hybrid.

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APPENDIX I

SPECIFIC EXTINCTION COEFFICIENTS ($E^{1\%}_{1cm.}$)

OF HEMOGLOBIN DERIVATIVES

SPECIFIC EXTINCTION COEFFICIENTS ($E^{1\%}_{1cm.}$) OF HEMOGLOBIN DERIVATIVES

Introduction

There has always existed much confusion in the literature regarding the spectrophotometric determination of hemoglobin concentrations. Recently, however, the National Academy of Sciences-National Research Council has adopted the value 11.5 for the extinction coefficient at 540 mu for a solution of ferrihemoglobin cyanide containing 1 milligram atom of iron per liter.* Calculation of the specific extinction coefficient of ferrihemoglobin cyanide from this standard gives a value for this quantity of 6.96. Spectrophotometric measurements have been done on several derivatives of hemoglobin to relate their extinction coefficients to the adopted standard.

Preparation of Solutions

A stock solution of oxyhemoglobin containing approximately 2 mg. hemoglobin/ml. was prepared. Exactly 20 ml. of this solution was placed into each of five 25 ml. volumetric flasks which had been fitted with serum bottle caps.

Ferrohemoglobin was prepared from the solution in one of these flasks, which had been flushed of oxygen with nitrogen gas. To the contents of this flask were added 5.0 ml. 0.01 M. sodium dithionite in Na-Phos. buffer, pH 8.6.

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*Science, 127, 1376-1378 (1958).

Ferrihemoglobin was prepared by adding to the solution in another flask 1 ml. $K_3Fe(CN)_6$ solution (3 mg. $K_3Fe(CN)_6$ per ml. of 0.2 M phosphate buffer of pH 6.8). To this flask was added, further, 4 ml. of this same buffer.

Ferrihemoglobin cyanide was prepared by adding to the third flask 1 ml. of the previously described $K_3Fe(CN)_6$ solution and 4 ml. of developer no. 1.

Oxyhemoglobin was prepared by blowing into a flask, to which had been added 5.0 ml. of pH 6.8 phosphate buffer, enough oxygen to produce a positive pressure of oxygen.

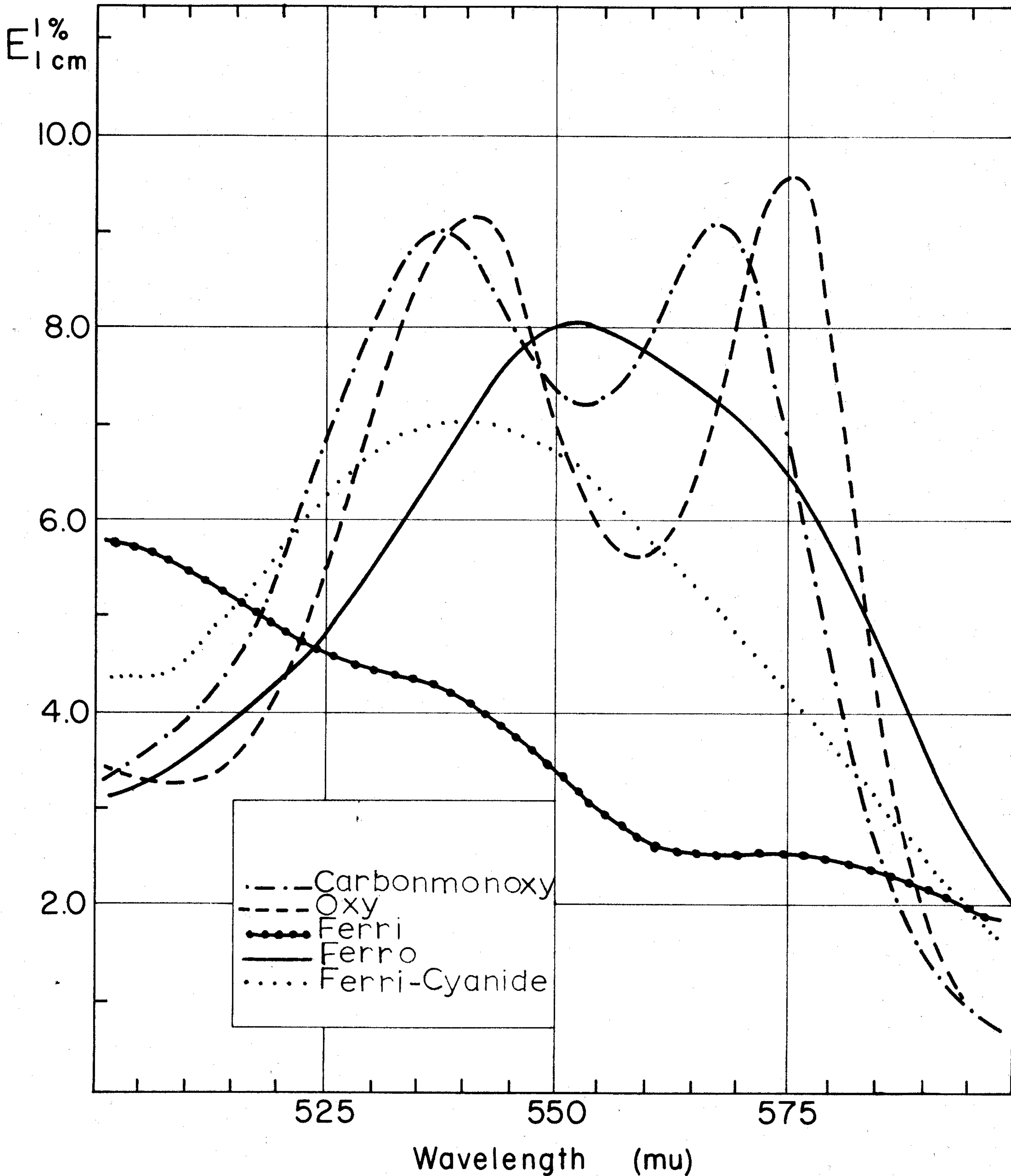
Carbonmonoxyhemoglobin was prepared in the same way as oxyhemoglobin except for the substitution of carbon monoxide for oxygen.

Portions of the above solutions were transferred, using hypodermic syringes, to stoppered Cary spectrophotometric cells.

The results of these measurements are seen in figure 16. The ordinate is given as the specific extinction coefficient ($E^{1\%}_{1cm.}$), the optical density given by a 1 cm. path through a 1% solution.

Figure 16

SPECIFIC EXTINCTION COEFFICIENTS ($E^{1\%}_{1cm.}$)
OF HEMOGLOBIN DERIVATIVES



APPENDIX II

REDUCTION OF OXYHEMOGLOBIN USING THE FERROVERSENATE ION

REDUCTION OF OXYHEMOGLOBIN USING THE FERROVERSENATE ION

The well known methods of formation of ferrohemoglobin from oxyhemoglobin, evacuation of oxygen, equilibration with inert gases, or addition of a chemical reducing agent, may cause a loss of protein from solution because of denaturation or other alterations of the hemoglobin molecule. The first two methods, evacuation and equilibration with inert gases are generally accompanied by extensive denaturation due to the formation of foams. With the chemical reducing agent usually used, sodium dithionite, in forming ferrohemoglobin from oxyhemoglobin, the products of decomposition have deleterious effects on the protein.*

A complex salt such as sodium ferroversenate** would be expected to be free from the harmful effects accompanying sodium dithionite because of the structure of this material and that of its oxidation product. The pH range, 7-9, for use of this reagent would not be harmful to the protein.

Preparation of Ferrohemoglobin Solutions

The reducing agent was prepared in the form of two solutions which were mixed at the time of use.

1. In one liter of deoxygenated phosphate buffer of pH 7.0, u 0.10, was dissolved 0.20 moles of the tetrasodium salt of ethylenediamine-tetraacetic acid. The pH of the solution was brought back to 7.0 by titration with 6 N HCl.

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*Dalziel, K., and O'Brien, J. R. P., Biochem. J., 49, XLVII-XLVIII (1951).

**I am grateful to Professor Normal Davidson for suggesting the use of this material as a reducing agent for oxyhemoglobin.

2. In another liter of the same deoxygenated phosphate buffer 0.001 moles of $FeCl_2$ was dissolved. A precipitate was observed to form.

Equal volumes of solutions 1 and 2 were mixed at room temperature. The precipitate from solution 2 dissolved. Oxyhemoglobin was added to this solution to form a one percent hemoglobin solution. The reaction of the ferroversenate ion with the oxyhemoglobin to form ferrohemoglobin required about five minutes for completion.

Results

The spectrum of the hemoglobin solutions resulting from the treatment of oxyhemoglobin with this reagent was identical to that of ferrohemoglobin.

Measurement of the sedimentation coefficient of ferrohemoglobin produced by this method indicated that no alterations had occurred in the shape of the molecule.

APPENDIX III

PREPARATION OF VARIOUSLY LABELLED RADIOACTIVE SICKLE CELL HEMOGLOBINS

PREPARATION OF VARIOUSLY LABELLED RADIOACTIVE SICKLE CELL HEMOGLOBINS

A stock amino acid solution was prepared according to the data of table VII of this Thesis excluding the amino acids tryptophan and methionine but including leucine. This stock solution was broken up into four 250 ml. portions which were used, respectively, in the preparation of hemoglobins labelled with tryptophan, methionine, Fe^{59}, and, using an algal hydrolysate, with all amino acids.

Total Amino Acid Solution for Preparation of l-Tryptophan Labelled Hemoglobin

To a 250 ml. portion of the above tryptophan-methionine free amino acid solution was added 12.5 mg. of l-methionine. Radioactive d,l-tryptophan (50 uc, 2.2 mg. from Nuclear-Chicago) was dissolved in 1 ml. of NKM saline solution. To 1.29 ml. of the amino acid mixture without tryptophan was added 0.50 ml. of this solution.

Total Amino Acid Solution for Preparation of l-Methionine Labelled Hemoglobin

To another 250 ml. portion of the l-tryptophan-l-methionine free amino acid stock solution was added 15.0 mg. of l-tryptophan. To 1.29 ml. of this solution was added 0.50 ml. of 17.05 uc/ml. (4.52 uc/m mole) C^{14}-methionine.

Total Amino Acid Mixture for Preparation of Fe^{59} Labelled Hemoglobin

To 250 ml. of the tryptophan-methionine free amino acid mixture was added 15.0 mg. l-tryptophan and 12.5 mg. of l-methionine. To

1.29 ml. of this total amino acid mixture was added 0.50 ml. of a $Fe^{59}Cl_3$ solution.

<u>Total Amino Acid Mixture for Preparation of Algal Hydrolysate Labelled Hemoglobin</u>

To the last of the 250 ml. portions of the tryptophan-methionine free amino acid stock solution was added 15.0 mg. tryptophan and 12.5 mg. methionine. The contents of two tubes of Nuclear-Chicago C^{14} algal hydrolysate (2 x 0.51 mg.) were dissolved in 1.29 ml. of the total amino acid mixture. To the resulting solution were added 0.2 ml. of a C^{14}l-leucine (50 uc, 0.75 mg. in 1 ml. NKM saline solution) solution, 0.2 ml. of a C^{14}l-valine solution (0.10 uc Nuclear-Chicago C^{14}l-valine), and 0.3 mg. of l-phenylalanine.

Preparation of Complete Incubation Media

With one exception, to each of the above complete amino acid mixtures were added the following amounts of the solutions listed below and described in table VII of this Thesis. In the solution prepared for the incorporation of Fe^{59} the addition of the ferrous ammonium sulfate was omitted.

Glucose solution	0.13 ml.
Glutamine solution	0.26 ml.
$NaHCO_3$ solution	0.39 ml.
FeAmSul solution	0.63 ml.
Packed Sickle Cell Red Blood Cells (18.9% reticulocytes)	2.00 ml.

The resulting four solutions were incubated as previously described. The variously labelled sickle cell hemoglobins were prepared from these cells also as previously described.

APPENDIX IV

CHROMATOGRAPHY PROTOCOL SHEETS

CHROMATOGRAPHY PROTOCOL SHEETS

HbA-HbS*, pH 7.2

Material Chromatographed: 25 mg. Normal Adult, 25 mg. Sickle Cell-C^{14}

Tube No.	Volume of Fraction	Total Volume	Dilution Factor	mu	O. D.	Mg/ml Diluted Fraction	Mg/ml Original Fraction	Mg. Counted	Net CPM for amt. Counted	Specific Activity
3	1.00 ml.	15.25	6.00	539	.075	.086		.086	10.8	126
4		16.25		539	.168	.193	1.158			
5		17.25		539	.176	.202	1.202	.202	24.8	123
6		18.25		539	.303	.348	2.088	.348	20.5	59
7		19.25		539	.412	.473	2.838	.473	22.6	48
8		20.25		539	.363	.416	2.496	.416	23.8	57
9		21.25		539	.264	.303	1.818	.303	20.0	66
10		22.25		539	.187	.214	1.289	.218	21.0	98
11		23.25		539	.136	.156	.936		22.4	144
12		24.25		539/415	.093/.835	.107/.109	.042/.654			
16		28.25		415	.351	.046	.276	.046		
20		32.25		415	.229	.030	.180	.030	18.2	607
24		36.25		415	.217	.028	.168			

Tube No.	Volume of Fraction	Total Volume	Dilution Factor	mu	O. D.	Mg/ml Diluted Fraction	Mg/ml Original Fraction	Mg. Counted	Net CPM for amt. Counted	Specific Activity
28		40.25		415	.342	.045	.270	.045	55.5	1234
32		44.25		415	.518	.068	.408			
33		45.25		415	.568	.074	.444	.074	104.3	
34		46.25		415	.589	.077	.462	.077	112.2	1457
35	1.30 ml.	47.38	4.85	539/415	.098/.833	.112/.109	.543/.529	.110	167.6	1510
36		48.68	4.85	539	.353	.405	1.964	.405	594.2	1467
37		49.98	4.85	539	.330	.379	1.838	.379	395.5	1571
38		51.28	4.85	539	.280	.321	1.557	.321	538.2	1677
39		52.58	4.85	539	.203	.233	1.130	.233	414.3	1778
40		53.88	4.85	539	.158	.181	.879	.181	350.2	1935
41		55.18	4.85	539	.108	.124	.601	.124	246.1	1985
42		56.48								
43		57.78								
44		59.08	4.85	539	.037	.042	.204			

HbA-HbS*, Hybridization pH 11.0

Material Chromatographed: 25 mg. Normal Adult, 25 mg. Sickle Cell-C^{14}; Developer No. 1

Tube No.	Volume of Fraction	Total Volume	Dilution Factor	mu	O. D.	Mg/ml Diluted Fraction	Mg/ml Original Fraction	Mg. Counted	Net CPM for amt. Counted	Specific Activity
		14.00		415						
2	1.01	15.01	1:11	415	.083	.011	.119	.011	39.0	3606
3	1.01	16.02	1:11	415	.517	.068	.733	.068	33.8	529
4	1.01	17.03	1:11	415	.752	.098	1.082	.098	52.0	529
5	1.01	18.04	1:11	415	.950	.124	1.37	.124	62.7	505
6	1.01	19.05	1:11	415	1.43	.187	2.06	.187	86.5	463
7	1.01	20.06	1:11	415	1.675	.219	2.41	.219	103.4	472
8	1.01	21.07	1:11	415	1.535	.208	2.29	.208	95.1	457
9	1.01	22.08	1:11	415	1.230	.161	1.77	.161	77.1	479
10	1.01	23.09	1:11	415	.990	.130	1.43	.130	65.3	503
11	1.01	24.10	1:11	415	.760	.099	1.09	.099		
12	1.01	25.11	1:11	415	.382	.076	.837			
16	1.01	29.15	1:11	415	.251	.033	.363			
20	1.01	33.19	1:11	415	.162	.021	.231			
25	1.01	37.23	1:11	415	.110	.014	.154			
28	1.01	41.27	1:11	415	.111	.014	.154			
32	1.01	44.31	1:11	415	.149	.020	.220			

Tube No.	Volume of Fraction	Total Volume	Dilution Factor	mu	O. D.	Mg/ml Diluted Fraction	Mg/ml Original Fraction	Mg. Counted	Net CPM for amt. Counted	Specific Activity
36	1.01	48.35	1:11	415	.217	.028	.308			
37	1.01	49.36	1:11	415	.742	.097	1.67	.097	129.2	1332
38	1.42	50.78	1:11	415	1.325	.173	1.905	.173	203.6	1169
39	1.42	52.20	1:11	415	1.325	.173	1.905	.173	236.5	1364
40	1.42	53.62	1:11	415	1.130	.148	1.627	.146	220.4	1491
41	1.42	55.04	1:11	415	.840	.110	1.205	.110	179.8	1636
42	1.42	56.46	1:11	415	.600	.079	.864	.079	128.7	1640
43	1.42	57.88	1:11	415	.403	.053	.579	.053	91.8	1742
44	1.42	59.30	1:11	415	.278	.036	.402	.036	60.0	1651
45	1.42	60.72	1:11	415	.173	.023	.249			
48	1.42	64.98	1:11	415	.084	.011	.121			

HbA*-HbS, pH 7.2

Material Chromatographed: 25 mg. Normal Adult-C^{14}, 25 mg. Sickle Cell, Developer No. 1

Tube No.	Volume of Fraction	Total Volume	Dilution Factor	mu	O. D.	Mg/ml Diluted Fraction	Mg/ml Original Fraction	Mg. Counted	Net CPM for amt. Counted	Specific Activity
1-30				539						
32	0.36 ml.	14.00	1:10		0.052	0.060	0.60			
33	0.36 ml.	14.36			0.095	0.109	1.09	.109	55.8	512
34	0.36 ml.	14.72			0.136	0.156	1.56			
35	0.36 ml.	15.08			0.159	0.183	1.83			
36	0.36 ml.	15.44			0.182	0.209	2.09	.209	121.2	580
37	0.36 ml.	15.80			0.182	0.209	2.09	.209	93.2	449
38	0.36 ml.	16.16			0.163	0.187	1.87			
39	0.36 ml.	16.52			0.156	0.179	1.79			
40	0.36 ml.	16.88			0.188	0.216	2.16			
41	0.36 ml.	17.24			0.225	0.258	2.58			
42	0.36 ml.	17.60			0.283	0.325	3.25			

Tube No.	Volume of Fraction	Total Volume	Dilution Factor	mu	O. D.	Mg/ml Diluted Fraction	Mg/ml Original Fraction	Mg. Counted	Net CPM for amt. Counted	Specific Activity
43	0.36 ml.	17.96			0.342	0.392	3.92	.392	176.1	449
44	0.36 ml.	18.32			0.343	0.393	3.93	.393	183.9	468
45	0.36 ml.	19.04	1:10	539	0.346	3.46				
46	0.36 ml.	19.40			0.294	0.337	3.37			
47	0.36 ml.	19.76			0.262	0.300	3.00			
48	0.36 ml.	20.12			0.218	0.252	2.52			
49	0.36 ml.	20.48			0.206	0.237	2.37			
50	0.36 ml.	20.64			0.158	0.181	1.81	.181	95.8	525
51	0.36 ml.	21.20			0.153	0.175	1.75			
52	0.36 ml.	21.56			0.125	0.143	1.43			
53	0.36 ml.	21.92			0.121	0.139	1.39			
54	0.36 ml.	22.28			0.100	0.114	1.14			
55	0.36 ml.	22.64			0.081	0.093	.93			
3'	0.43 ml.	38.00	1:10		0.042	0.055	.55			
7'	0.43 ml.	38.43			0.056	0.064	.64	.64	7.9	
8'	0.43 ml.	38.86			0.098	0.111	1.11			
9'	0.43 ml.	39.29			0.134	0.154	1.54			
10'	0.43 ml.	39.72			0.173	0.199	1.99			

Tube No.	Volume of Fraction	Total Volume	Dilution Factor	mu	O. D.	Mg/ml Diluted Fraction	Mg/ml Original Fraction	Mg. Counted	Net CPM for amt. Counted	Specific Activity
11'	0.43 ml.	40.15			0.203	0.233	2.33			
12'	0.43 ml.	40.58			0.202	0.232	2.32	.232	0.4	2
13'	0.43 ml.	41.01			0.204	0.233	2.33	.233	-0.6	-2
14'	0.43 ml.	41.44			0.197	0.227	2.27			
15'	0.43 ml.	41.87			0.183	0.210	2.10			
16'	0.43 ml.	42.30			0.167	0.192	1.92	.192	2.5	7.5
17'	0.43 ml.	42.73			0.151	0.173	1.73			
18'	0.43 ml.	43.16			0.131	0.150	1.50			
19'	43.69				0.122					
20'	44.02				0.106					
30'					0.097				1.8	16.1

HbA-HbS, Hybridization pH 5.0

Material Chromatographed: 25 mg. Normal Adult-C^{14}, 25 mg. Sickle Cell, Developer No. 1

Tube No.	Volume of Fraction	Total Volume	Dilution Factor	mu	O. D.	Mg/ml Diluted Fraction	Mg/ml Original Fraction	Mg. Counted	Net CPM for amt. Counted	Specific Activity
132	0.435	14.11	1:10	539	0.007	0.008	0.080			
133	0.435	14.54	1:10	539	0.018	0.021	.210			
134	0.435	14.97	1:10	539	0.040	0.046	.460	.046	17.0	370
135	0.435	15.40	1:10	539	0.072	0.083	.830			
136	0.435	15.83	1:10	539	0.110	0.126	1.260	.126	57.8	459
137	0.435	16.26	1:10	539	0.134	0.154	1.540			
138	0.435	16.69	1:10	539	0.162	0.186	1.860	.186	98.9	533
139	0.435	17.12	1:10	539	0.162	0.186	1.860	.186	96.4	519
140	0.435	17.55	1:10	539	0.148	0.170	1.700			
141	0.435	17.98	1:10	539	0.128	0.147	1.470	.147	71.9	490
142	0.435	18.41	1:10	539	0.117	0.134	1.340	.134	56.6	423
143	0.435	18.84	1:10	539	0.129	0.148	1.480			
144	0.435	19.27	1:10	539	0.153	0.175	1.750	.175	93.6	53.5
145	0.435	19.70	1:10	539	0.197	0.226	2.260			
146	0.435	20.13	1:10	539	0.244	0.280	2.80			

Tube No.	Volume of Fraction	Total Volume	Dilution Factor	mu	O. D.	Mg/ml Diluted Fraction	Mg/ml Original Fraction	Mg. Counted	Net CPM for amt. Counted	Specific Activity
147	0.435	20.56	1:10	539	0.250	0.287	2.87	.287	136.0	457
148	0.435	20.90	1:10	539	0.275	0.315	3.15	.315	151.7	482
149	0,435	21.42	1:10	539	0.263	0.301	3.01	.301	131.7	437
150	0.435	21.85	1:10	539	0.221	0.253	2.43			
151	0.435	22.28	1:10	539	0.208	0.238	2.381			
152	0.435	22.71	1:10	539	0.183	0.210	2.10			
153	0.435	23.14	1:10	539	0.162	0.186	1.86	.186	99.3	536
154	0.435	23.57	1:10	539	0.133	0.152	1.52			
155	0.435	24.00	1:10	539	0.119	0.136	1.36			
156	0.435	24.43	1:10	539	0.107	0.123	1.23	.123	61.9	503
157	0.435	24.86	1:10	539						
158	0.435	25.29	1:10		0.081	0.093	0.931			
159	0.435	25.72								
160	0.435	26.15	1:10	539	0.065	0.074	0.74	.074	41.0	555
103'										
104'								.023	1.7	72
105'										
106'										
107'	47.45		1:10	539	0.038	0.044	0.44			

Tube No.	Volume of Fraction	Total Volume	Dilution Factor	mu	O. D.	Mg/ml Diluted Fraction	Mg/ml Original Fraction	Mg. Counted	Net CPM for amt. Counted	Specific Activity
108'										
109'			1:10	539	0.075	0.086	0.86	.086		142
110'										
111'			1:10	539	0.150	0.172	1.72	.172		141
112'			1:10	539	0.168					
113'			1:10	539	0.182	0.209	2.09	.209		106
115'	0.567	52.01	1:10	539	0.172	0.197	1.97	.197	20.0	
116'	0.567		1:10							
117'	0.567	53.15	1:10	539	0.153	0.175	1.25	.175	22.4	128
118'	0.567	53.72	1:10	539	0.131	0.156	1.5	.150	20.1	134
119'	0.567	54.29	1:10	539	0.118	0.135	1.35			
120'	0.567	54.86	1:10	539	0.106	0.121	1.21			
121'	0.567	55.43	1:10	539	0.101	0.116	1.16	.116	18.8	162
122'	0.567									
123	0.567	56.57	1:10	539	0.083	0.095	.95			
124	0.567									
125	0.567	57.14	1:10	539	0.057	0.065	.65			

Tube No.	Volume of Fraction	Total Volume	Dilution Factor	mu	O. D.	Mg/ml Diluted Fraction	Mg/ml Original Fraction	Mg. Counted	Net CPM for amt. Counted	Specific Activity
126	0.567									
127	0.567	38.28	1:10	539	0.042	0.048	.48			
128	0.567									
129	0.567	58.85	1:10	539	0.034	0.039	.39			
130'	0.567	59.42	1:10	539	0.034	0.039	.39	.039	8.9	154

PROPOSITIONS

PROPOSITIONS

1. Human hemoglobin "H" has been shown to be a tetramer of β chains (1). No naturally occurring hemoglobin consisting of four α chains has been identified. A method is proposed for producing a native hemoglobin which is composed entirely of α chains.

As Singer and Itano (2) have shown, normal adult carbonmonoxy hemoglobin may be hybridized with sickle cell ferrihemoglobin by dissociation of equimolar mixtures of the hemoglobins at pH 4.7 followed by reassociation at neutral pH. The hemoglobin species resulting from this treatment are $\alpha_2\beta_2$, $\alpha_2^m\beta_2$, $\alpha_2\beta_2^{sm}$, and $\alpha_2^m\beta_2^{sm}$ having the relative charges 0, 2, 4, and 6 respectively. As shown in this Thesis the species may be separated chromatographically.

Isolation of the species $\alpha_2\beta_2^{sm}$ would provide a hemoglobin which is half carbonmonoxyhemoglobin and half ferrihemoglobin. The rates of denaturation of carbonmonoxy- and ferrihemoglobins are different in both acid and alkaline solutions. A suitable choice of conditions may be made such that the ferrihemoglobin moieties of the hybrid are preferentially denatured leaving in solution only the α hemoglobin chains originating from the normal adult carbonmonoxyhemoglobin. This differential denaturation may possibly be carried out by allowing the hybrid to stand for 20 minutes at 0°C. in a formate buffer of pH 3.6, $u = 0.02$ (3).

Physical chemical studies could be carried out on the material resulting from this treatment. It could thus be determined if an abnormal hemoglobin, or a minor hemoglobin component is to be expected

containing only α chains.

2. From kinetic studies, presumptive evidence has been presented in this Thesis of the mode of dissociation of human hemoglobin in alkaline solution. Attempts to confirm the mechanism implied by the kinetics using the "heme-labelling" method of Singer and Itano (2) were unsuccessful due to the selective denaturation of ferrihemoglobin after dissociation at pH 11.0. A modification of the "heme-labelling" technique which avoids this difficulty is proposed.

Carbonmonoxyhemoglobin and ferrihemoglobin cyanide may be successfully carried to pH 11.0 without serious loss of protein from the solutions. Sickle cell carbonmonoxyhemoglobin and normal adult ferrihemoglobin cyanide may be hybridized as described in this Thesis. Treatment of the resulting mixture, after removal of all excess cyanide ion, would result in the oxidation of only those hemoglobin subunits which were in the ferroheme-carbonmonoxide complex. Different electrophoretic species would be found in the hybridized mixture depending on the mode of subunit recombination.

If the designation α^{mCN} or β^{mCN} is assigned to the α and β ferrihemoglobin cyanide chains and the designation α^{m} and β^{sm} to the ferrihemoglobin chains from the sickle cell hemoglobin, the electrophoretic species expected would be:

Asymmetric dissociation-mechanism (a).

$\alpha_2^{mCN}\beta_2^{mCN}$, $\alpha_2^{m}\beta_2^{mCN}$, $\alpha_2^{mCN}\beta_2^{sm}$, and $\alpha_2^{m}\beta_2^{sm}$ having the relative charges 0, 2, 4, and 6 respectively.

Symmetric dissociation-mechanism (b).

$\alpha_2^{mCN}\beta_2^{mCN}$, $\alpha^{m}\alpha^{mCN}\beta_2^{mCN}$, $\alpha_2^{m}\beta_2^{mCN}$, $\alpha_2^{mCN}\beta_2^{sm}$, $\alpha^{m}\alpha^{mCN}\beta_2^{sm}$, and $\alpha_2^{m}\beta_2^{sm}$

having the relative charges 0, 1, 2, 4, 5, and 6 respectively. Electrophoretic analysis of the hybridized mixture would therefore allow a distinction to be made between the two schemes of α chain exchange.

3. The antimalarial drug Primaquine has been shown to produce a hemolytic anemia of varying severity in approximately 10% of Negro subjects on which it has been tested (4). After administration of the drug, sickling tests were performed on the red blood cells of those individuals who developed the anemia; no evidence of sickling was found.

The carbonic anhydrase inibitor Diamox has been shown to inhibit sickling of red blood cells from sicklemic individuals (5) if the cells are drawn after administration of the drug. The possibility exists that the sickle cell trait was not evident in the individuals subjected to Primaquine tests for similar reasons. It is therefore proposed that these tests be repeated using the more reliable electrophoretic tests for abnormal hemoglobins.

If the effect of this antimalarial drug can be related to the presence of the sickle cell trait in the affected individuals, further studies might lead to an understanding of the mechanism of the resistance to malaria of sickle cell trait carriers.

4. After blocking of from 2 to 3 sulfhydryl groups in human hemoglobin with mercurials Riggs (6) has observed an increase in the oxygen affinity of hemoglobin. He has interpreted this increase in oxygen affinity and an accompanying loss of heme-heme interactions in terms of the implication of the sulfhydryl in the oxygenation process. It has been found in preliminary sedimentation studies that the molecular weight of hemoglobin, in which the -SH groups are bound to PCMB, is

decreased from its normal value (7). If this is also the case with the mercurial compounds studied by Riggs, his data may be subject to another interpretation.

If blocking of the SH groups with mercurial compounds increases the molecular dissociation of hemoglobin, the hemoglobin half molecules may present less hindrance to the entry of oxygen molecules upon dissociation resulting in an increase in oxygen affinity and a loss of heme-heme interactions. In this case it is not necessary to invoke a direct involvement of the sulfhydryl groups in the oxygenation process.

It is therefore proposed that molecular weight determinations be made on the hemoglobin-mercurial compounds of Riggs.

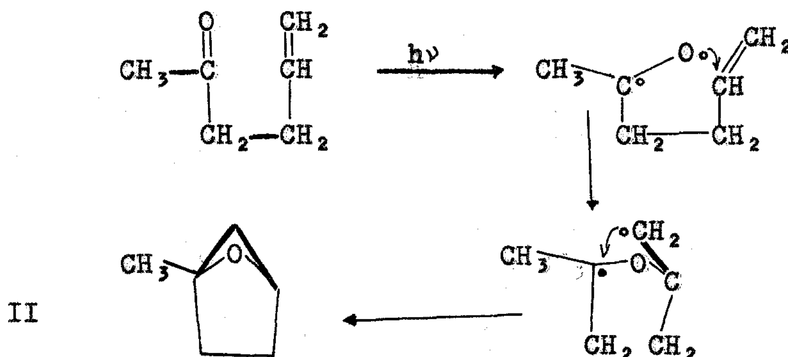
5. Steinhardt and Beychok (8) have reported an increase in acid binding capacity of horse ferrihemoglobin which is brought about by denaturation. They have interpreted this effect in terms of an "unmasking" of groups which are not labile in the native protein. Tanford (9) has found it possible to account for this increase in acid binding capacity on the basis solely of inferred changes in the electrostatic interaction factor w. An experimental procedure is proposed which would allow a distinction to be made between these two interpretations of the results.

If there is an unfolding of the ferrihemoglobin molecule upon denaturation such that there are more groups exposed for reaction, it should be possible to use a specific chemical agent to react with the groups newly exposed. One agent which is specific for the ϵ-amino groups of lysine is O-methyl isouronium sulfate. In the unmasking of groups in the molecule upon denaturation it may be expected that some new lysine residues would become available for reaction in the denatured

protein. Using C^{14} labelled O-methyl isouronium sulfate in the guanidination of native and denatured hemoglobin and measurement of uptake of this material in the two forms of the protein would allow an estimate to be made of the group "unmasked" upon denaturation.

6. The photochemistry of 5-hexene-2-one has been studied in the gas phase by Srinivasan (10) using light of a wavelength of 3130 Å over the temperature range from 27 to 139° C. The most important product of the photochemical reaction, an isomer of the original material, has been assigned the structure:

Studies with molecular models indicate that another isomer of 5-hexene-2-one may be formed after excitation of the carbonyl group:



The structure assignment of the isomer as compound I was made on the basis of infrared and NMR data. These two methods, in themselves, would not allow a clear distinction to be made between structures I and II. Both structures would have, possibly, similar NMR spectra (11). A distinction may be made between the isomers on a chemical basis. Opening of the propylene oxide rings in the two compounds would lead to known compounds.

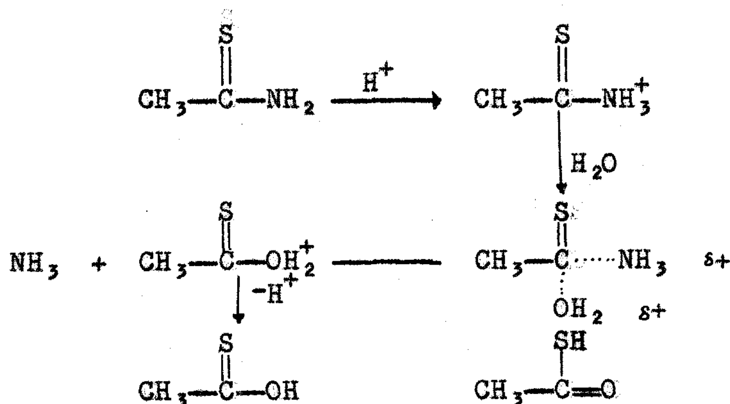
Considerations of strain in the two structures would favor

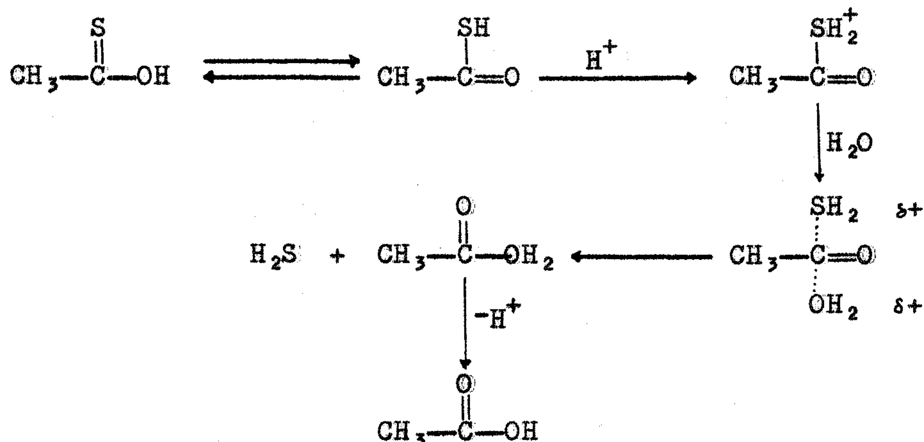
structure II over I. The non-planar cyclopentance ring in structure II is bridged across the 1 and 4 positions by an ether oxygen allowing the propylene oxide ring to assume a dihedral configuration. In structure I both the propylene oxide ring and the cyclobutane ring are planar.

7. The stoichiometric equation for the hydrolysis of thioacetamide is:

In studies of the acid catalyzed hydrolysis of thioacetaminde Swift and Bütler (12) observed a decrease in the hydrogen ion concentration as the reaction proceeded. To account for this change in hydrogen ion concentration it was postulated that acetamide was formed as an intermediate in the reaction and the protonation of this material caused the increase in pH. In other studies of the acid catalyzed hydrolysis of thioacetamide, Rosenthal and Taylor (13) assumed that acetamide was an intermediate in the reaction though they were able to isolate thioacetic acid from the reaction mixture.

It is possible to account for both the decrease in hydrogen ion concentration observed by Swift and Butler and the presence of thioacetic acid in the reaction mixture without assuming acetamide to be an intermediate:

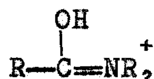




In acid solution, the NH_3 formed in the reaction would take a proton from the free acid in solution to form the ammonium ion rather than from the acetic acid to form the acetate ion and ammonium ion. The net result would be a decrease in the hydrogen ion concentration as the reaction proceeds.

Rosenthal and Taylor assumed the involvement of two monoprotonated tautomers of thioacetamide in the reaction sequence:

By their mechanism, the tautomer (a) leads to the formation of thioacetic acid and (b) leads to thioacetamide. It is interesting that neither (a) nor (b) is the sulfur analogue of the most abundant monoprotonated amide as determined by NMR (14,15):



Extension of these NMR studies to thiamides would afford knowledge of the relative basicities of nitrogen and sulfur in these compounds.

8. Harris (14) has found that when stroma free solutions (15-25 mg/ml) of whole sickle cell anemia hemoglobin are deoxygenated birefringent spindle shaped bodies varying in length from 1 to 15 u are

formed. These bodies are liquid crystals of the nematic type. The role of the minor components of sickle cell anemia hemoglobin in the formation of these tactoids has never been demonstrated. With chromatographic methods now available for the removal of these minor components from whole sickle cell hemoglobin preparations, it is now possible to determine what role, if any, the minor components have in the sickling process. It is therefore proposed that the studies of Harris be repeated on minor free sickle cell hemoglobin preparations.

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