SUBUNIT CHARACTERIZATION OF SOME HUMAN AND DEER HEMOGLOBINS

by

Charles F. Plese

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This thesis submitted by Charles F. Plese has been examined and approved by an appointed committee of the faculty of the Graduate Division of the Medical College of Georgia.

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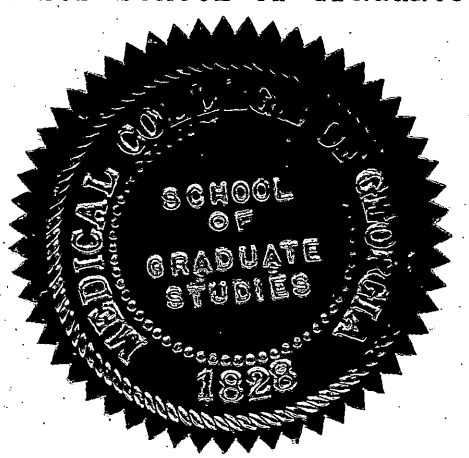

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INTRODUCTION

Mammalian hemoglobins are tetrameric molecules consisting of polypeptide chains which are identical in pairs, each chain possessing a heme group. Sequence analyses of the major adult human hemoglobin component, hemoglobin A, showed one of the chains (designated as the α chain) to be composed of 141 amino acid residues while the nonidentical chain (designated as the β chain) was found to be composed of 146 amino acid residues. The native hemoglobin tetramer is designated as $\alpha_2\beta_2$ (Rhinesmith et al, 1957).

The existence of an equilibrium between tetramers and dimers at neutral pH and low ionic strength has been established by studies which show an increase in the diffusion coefficient (Svedberg and Pedersen, 1940; Lamm and Polson, 1936) and a corresponding decrease in the sedimentation coefficient of hemoglobin at high dilution (Lamm and Polson, 1936; Kegeles and Gutter, 1951; Gutter et al, 1957; Ganguly et al, 1963). In addition Guidotti and Craig (Guidotti and Craig, 1963) found by thin film dialysis a rate of diffusion for hemoglobin which corresponded to a molecular weight slightly larger than one-half that of the hemoglobin tetramer. Guidotti et al (Guidotti et al, 1963) further showed that hemoglobin dissociated into symmetrical dimers ($\alpha_2\beta_2 \rightleftharpoons 2\alpha\beta$) rather than asymmetrical ones ($\alpha_2\beta_2 \rightleftharpoons \alpha_2 + \beta_2$).

Depending upon the dissociation plane in symmetrical dimer formation, two types of $\alpha\beta$ subunits may be formed (Schroeder, 1963). These are designated as $\alpha^1\beta^1$ and $\alpha^1\beta^2$, depending on the

distance between the iron atoms of the individual chains (Cullis et al, 1962). Kawahara et al (Kawahara et al, 1965) dissociated CO-hemoglobin with various reagents and concluded that one of the planes of contact was relatively weak, and that all reagents acted upon it. The other plane of contact was much stronger and was disrupted only when all noncovalent interactions were broken. Rosemeyer and Huehns (Rosemeyer and Huehns, 1967) dissociated several animal and human hemoglobins into dimers by attachment of p-chloromercuribenzoate (p-CMB) to the reactive cysteine, β-93, which earlier had been shown to occur near the α^1-β^2 and α^2-β^1 region of contact (Muirhead and Perutz, 1963; Perutz, 1965). From this they concluded that the initial dissociation was along the α^1-β^2 and α^2-β^1 contacts with formation of $\alpha^1\beta^1$ and $\alpha^2\beta^2$ dimers. Further proof as to the nature of the dimeric species came from detailed X-ray crystallographic studies which showed the α^1-β^2 contact of liganded hemoglobin to be the less extensive of the two contacts (Perutz et al, 1968). This contact is composed of 19 residues contributing about 80 atoms within a distance of 4 Å of each other, while the α^1-β^1 contact is composed of 34 residues with 110 atoms coming within a distance of 4 Å of one another. Thus, the more extensive α^1-β^1 and α^2-β^2 contacts should be more stable, and symmetrical dissociation should favor formation of $\alpha^1\beta^1$ and $\alpha^2\beta^2$ dimers.

X-ray crystallographic comparison of the liganded and non-liganded forms of hemoglobin showed marked differences in their quaternary structures. Upon deoxygenation the α^1-β^2 contacts

undergo the most noticeable change, shifting by 13° relative to each other (Perutz et al, 1968). In the deoxy form there appears to be a small decrease in the number of Van der Waals interactions in the α^1-β^2 contact (Bolton and Perutz, 1970). However, this is apparently more than compensated for by the formation of six additional interchain salt bridges (Perutz, 1970) because both differential sedimentation measurements (Goers and Schumaker, 1970; Kellett, 1971) and light scattering studies (Noren et al, 1971) have shown that deoxyhemoglobin, unlike the liganded derivative, is not appreciably dissociated at low hemoglobin concentration and high concentrations of salt.

Recent subunit dissociation studies on the abnormal human hemoglobins Kansas ($\alpha_2\beta_2^{102\ Thr}$) (Bonaventura and Riggs, 1968) and Richmond ($\alpha_2\beta_2^{102\ Lys}$) (Efremov et al, 1969) have shown the effects of substituting two different amino acid residues at one of the critical α^1-β^2 contact positions. In normal liganded hemoglobin A, an asparaginyl residue in position 102 of the β chain forms a hydrogen bond with an aspartic acid residue at position 94 of the α chain. When compared with hemoglobin A by sedimentation velocity analyses, the liganded forms of the two variants were found to show an increased extent of dimer formation, with hemoglobin Kansas showing a much greater tendency than hemoglobin Richmond to form the dimeric species at neutral pH and low ionic strength.

Three abnormal human hemoglobins, each having a substitution at position 95 of the α chain, have been isolated and

described. The three variants are hemoglobins G Georgia ($\alpha_2^{95\ Leu}\beta_2$) (Huisman et al, 1970; Smith et al, 1972), Rampa ($\alpha_2^{95\ Ser}\beta_2$) (De Jong et al, 1971; Charache et al, 1971; Smith et al, 1972), and St. Luke's ($\alpha_2^{95\ Arg}\beta_2$) (Bannister et al, 1972). Position 95 of the α chain, which in normal hemoglobin A is occupied by a prolyl residue, is part of the invariant α^1-β^2 contact which is ruptured upon dissociation of tetrameric hemoglobin into dimers. The three variant hemoglobins offered an ideal opportunity to establish not only the importance of an individual residue at the α^1-β^2 contact, but also to demonstrate the influence of different substitutions for one and the same amino acid residue in the α^1-β^2 contact region. The purpose of this research was to characterize, by means of sedimentation velocity, diffusion, and viscosity measurements, the effects of the three above-mentioned amino acid substitutions at one critical invariant contact - namely position 95 of the α chain. The results describe the subunit dissociation behavior of the liganded (oxy or cyanferri) and nonliganded (deoxy) forms of hemoglobins A, G Georgia, Rampa, and St. Luke's as a function of salt concentration, pH, and temperature.

MATERIALS AND METHODS

A. Isolation and Purification of Hemoglobin Samples

Hemoglobins A, G Georgia, Rampa, and St. Luke's were obtained already purified from Dr. T. H. J. Huisman. These hemoglobin components had been isolated from freshly prepared red cell hemolysates on columns of DEAE-Sephadex, using procedures similar to those described under deer hemoglobins. The purity of each fraction was checked by starch gel electrophoresis at pH 9.0 (Efremov et al, 1969). The isolated hemoglobin components were dialyzed against distilled water and concentrated by ultrafiltration under reduced pressure at 4°. The samples were stored at 4° as the cyanferrihemoglobin derivative for most ultracentrifugation and viscometry measurements, or in the oxy form when deoxygenation experiments were to be done.

B. Preparation of Samples for Ultracentrifugation Experiments

Preparation of the samples (liganded) for sedimentation velocity experiments as a function of pH consisted of diluting the concentrated stock solutions of hemoglobin to 1.0 ml (to give a hemoglobin concentration of 0.5 g per 100 ml) with one of the following buffers (at 0.10 to 0.15 ionic strength):

Citric acid-Na_2HPO_4	pH range 4.9-5.9
KH_2PO_4-NaOH	pH range 6.9-7.9
NaCl-Glycine-NaOH	pH range 8.9-10.7
Na_2HPO_4-NaOH	pH range 11.2-12.0

The samples were then dialyzed for 24 hours at 4° against 3 changes (600, 700, 700 ml) of the appropriate buffer. Preparation of the samples for sedimentation velocity measurements as a function of NaCl concentration consisted of extensively dialyzing a hemoglobin solution at a protein concentration of approximately 1.0 g per 100 ml against a KH_2PO_4-Na_2HPO_4 buffer pH 7.4 and diluting this solution 1:1 (v/v) with a NaCl solution of twice the molarity needed.

Solutions of oxyhemoglobin were deoxygenated in tonometers which were flushed with high purity, water-saturated nitrogen (Matheson, Morrow, Georgia) by the following procedure:

(1) A solution of oxyhemoglobin (1.5 ml) was placed in the tonometer and a stream of nitrogen passed over the sample for 10 minutes.

(2) The tonometer was then sealed and rotated for 10 minutes.

(3) A stream of nitrogen was passed over the sample for another 5 minutes.

(4) The tonometer was resealed and rotated while the ultracentrifuge cell was flushed with nitrogen for 30 minutes.

(5) The sample was removed from the tonometer with a hypodermic syringe containing a trace of sodium dithionite and transferred to the ultracentrifuge cell.

Effective deoxygenation of the hemoglobin solution was established by recording on a Cary model 14 spectrophotometer the absorption spectra of the solution in the sealed ultracentrifuge cells over the wave length range of 600 to 800 mμ, following the procedure of Benesch et al (Benesch et al, 1962).

C. Ultracentrifugation

A Spinco model E analytical ultracentrifuge equipped with an electronic speed control, Schlieren optics, and Wratten No. 29 red filter was used for the determination of the sedimentation coefficients. Sedimentation velocity experiments were done in an AnD rotor at a speed of 60,000 rpm and at temperatures of 4° and 25°. Hemoglobin concentrations were approximately 0.5 g per 100 ml. Kel F single-sector centerpieces were used because of the high alkalinity or high salt content of several of the samples. Standard, twelve millimeter single-sector cells were used; a wedge window in one of the cells allowed hemoglobin A to be run simultaneously with each of the abnormal hemoglobins.

The light source for the schlieren optical system was a General Electric AH6, high intensity, mercury vapor lamp. The schlieren patterns were recorded at 8 minute intervals on Kodak I-N red sensitive plates. After the plates were developed, the schlieren peak positions were measured on a Nikon Model 6 C Profile Projector.

D. Principle of Measurement

The theoretical principles of sedimentation velocity for

a two component (solute-solvent) system have been developed and extensively discussed by Svedberg and Pedersen (Svedberg and Pedersen, 1940), Schachman (Schachman, 1959), and Tanford (Tanford, 1963), and will not be rigorously derived here, since such an undertaking would be beyond the scope of this thesis. Briefly summarized, this theory states that in the presence of a high centrifugal field, large molecules such as hemoglobin sediment at constant velocity. Opposing this sedimenting or centrifugal force is the frictional force exerted by the medium. The net result is a redistribution of solute molecules in the two component system. This produces a region which contains only solvent molecules, a solvent-solute boundary (interface) at which a concentration gradient of solute molecules exists, and a plateau region in which the solute molecules are uniformly distributed throughout the solvent. The movement of the boundary is described by the equation:

$$S = \frac{1}{\omega^2 X}\frac{dX}{dt} = \frac{1}{\omega^2}\frac{d\ln X}{dt} = \frac{2.303}{\omega^2}\frac{d\log X}{dt}$$

where S is the velocity of migration per unit centrifugal field, or sedimentation coefficient.

ω is the angular velocity in radians/sec. and is equal to $2\pi v$.

v is the velocity in revolutions/sec.

X is the distance in cm of the boundary from the center of rotation.

t is the time in sec.

The concentration gradient, and hence refractive index gradient, at the sedimenting boundary is translated by the

schlieren optical system into the series of peaks seen on the photographic plates (Fig. 1-1). By measuring the distance of the peak maximum ordinate from the axis of rotation for each time interval and then plotting the logarithms of these distances versus the time, a straight line is obtained. The observed sedimentation coefficient (s_{obs}) is calculated from the slope of this line. In order to have a valid comparison of values obtained under different experimental conditions, the observed sedimentation coefficients are converted to a reference solvent having the viscosity and density of water at 20°. The following equation was used: (Svedberg and Pedersen, 1940)

$$s_{20,w} = s_{obs} \left(\frac{\eta_{t^\circ}}{\eta_{t^\circ,w}} \right) \left(\frac{\eta_{t^\circ,w}}{\eta_{20,w}} \right) \left(\frac{1 - \bar{V}_{20,w}\, \rho_{20,w}}{1 - \bar{V}_t\, \rho_t} \right)$$

where

s_{obs} is the observed sedimentation coefficient.

η_{t° is the viscosity of the solvent at the temperature of the run.

$\eta_{t^\circ,w}$ is the viscosity of water at the temperature of the run.

$\eta_{20^\circ,w}$ is the viscosity of water at 20°.

$\bar{V}_{20,w}$ is the partial specific volume of hemoglobin at 20° in water.

$\bar{V}_t$ is the partial specific volume of hemoglobin in the solvent at the temperature of the run.

$\rho_{20,w}$ is the density of water at 20°.

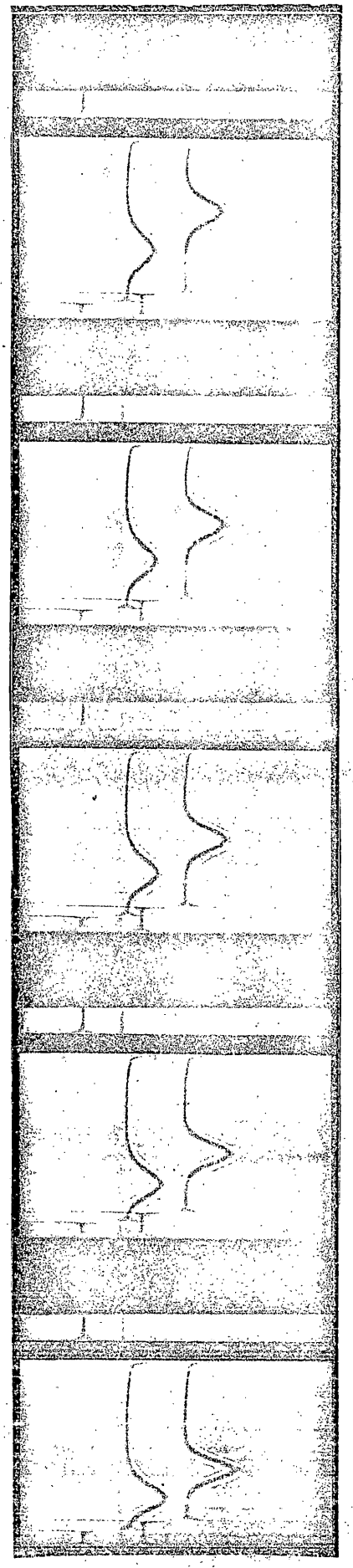
ρ_t is the density of the solvent at t°.

Figure 1-1

Sedimentation velocity pattern of cyanferrihemoglobins A and G Georgia. Sedimentation velocity experiments were done at 60,000 rpm and the schlieren patterns recorded at 8 minute intervals.

Upper pattern: Hemoglobin A, 0.5 g/100 ml in 0.1 M NaCl pH ~ 7.2, 25°.

Lower pattern: Hemoglobin G Georgia, 0.5 g/100 ml in 0.1 M NaCl pH ~ 7.2, 25°.



E. Diffusion

Diffusion coefficients were determined for hemoglobins A, G Georgia, and Rampa in the ultracentrifuge using a double sector capillary-type synthetic boundary centerpiece. The hemoglobin samples, ranging in concentration from 0.7 to 1.0 g per 100 ml, were extensively dialyzed against 0.1 M NaCl (pH 7.2-7.4) and run in an AnJ rotor at 20° at a speed of 12,000 rpm. Schlieren patterns were recorded at 8 minute intervals (Fig. 1-2). The observed diffusion coefficient, D_{obs}, was calculated using an approximation of Lamm's equation for the estimation of diffusion coefficients from sedimentation boundary curves (Lamm and Polson, 1936). The relation derived by Lamm is given by:

$$(A/H)^2 = 4\pi D_{obs} t \ (1 + s\omega^2 t)$$

where A is the area enclosed by the sedimentation boundary curve above its base line (see Fig. 1-2).

H is the maximum height of the sedimentation boundary curve.

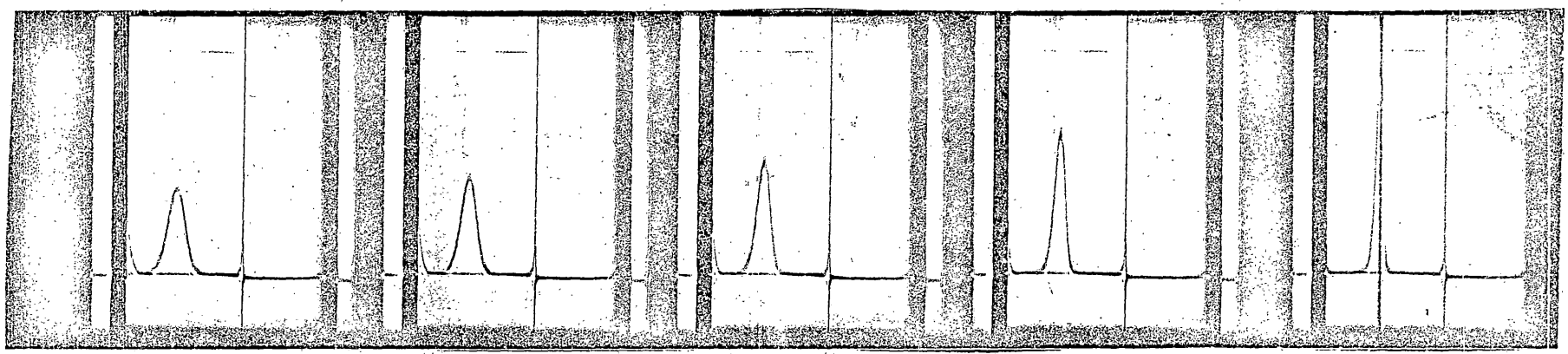
t is the time measured from the start of centrifugation.

ω is the angular velocity of rotation.

When the experiment is done at low speed the term $s\omega^2 t$ becomes negligibly small compared with unity (A is independent of time for the short duration of the run) and one obtains the follow-

Figure 1-2

Sedimentation boundary patterns for the evaluation of the diffusion coefficient of cyanferrihemoglobin A, 1.0 g/100 ml in 0.1 M NaCl, pH ~ 7.2, 20°. Pictures were taken at 8 minute intervals.



ing equation (Kawahara, 1969):

$$(A/H)^2 = 4\pi Dt$$

The desired value of D_{obs} was obtained from the slope of the line of a plot of $(A/H)^2$ versus t (the value of the slope equals $4\pi D$). Correction of D_{obs} to $D_{20,w}$ (reference solvent with viscosity and density of water at 20°) was done using the following equation: (Svedberg and Pederson, 1940)

$$D_{20,w} = D_{obs} \left(\frac{293.2}{T} \right) \left(\frac{\eta_t}{\eta_{20}} \right) \left(\frac{\eta_{sol}}{\eta_t} \right)$$

where T is the temperature of the run in °K.

η_t is the viscosity of water at the temperature of the run.

η_{20} is the viscosity of water at 20°.

η_{sol} is the viscosity of the solvent at the temperature of the run.

Since the runs were done at 20° the above correction reduces to:

$$D_{20,w} = D_{obs} \left(\frac{\eta_{sol}}{\eta_t} \right)$$

F. Viscometry

Viscosity measurements were made at 25° with Cannon - Ubbelohde (State College, Pennsylvania) semimicro dilution viscometers with flow times for distilled water of approximately 225 seconds. Approximately 2 ml of purified sample were extensively dialyzed against 0.1 M NaCl, pH 7.2-7.4, and then equilibrated in the viscometer (25 ± 0.01°) for one hour. The sample was drawn from the reservoir by suction and allowed to wet the capillary before the first flow time measurement was made. Measurements were made with a stopwatch which could be read to one one-hundreth of a second. The flow time for a given hemoglobin concentration was the average of at least three measurements which were in close agreement with one another (± 0.1 sec.). The hemoglobin sample was then diluted and the procedure was repeated, so that data were obtained at three or four different hemoglobin concentrations.

The specific viscosity, η_{sp}, was calculated from the flow times and densities of the sample solutions and solvents as described by Bjork and Tanford (Bjork and Tanford, 1971). The specific viscosity, η_{sp}, was calculated from the relation:

$$\eta_{sp} = \frac{t\rho - t_o\rho_o}{t_o\rho_o}$$

where t_o is the flow time of the solvent.

t is the flow time of the hemoglobin solution.

ρ_o is the density of the solvent.

ρ is the density of the hemoglobin solution.

The density of the hemoglobin solution was computed from the protein concentration, partial specific volume of the protein, and the density of the solvent. The reduced viscosity, η_{sp}/c, was plotted versus the concentration of hemoglobin, c; the intrinsic viscosity, $[\eta]$, was then obtained by extrapolation of the data to zero concentration.

RESULTS

A. Sedimentation Velocity

The differences in sedimentation behavior of the oxy (or cyanferri) and deoxy derivatives of hemoglobins A, G Georgia, Rampa, and St. Luke's in NaCl solutions of increasing molarity are shown in Figures 1-3 and 1-4. The $s_{20,w}$ values in these figures were obtained by correcting the observed sedimentation coefficients to 20° in water without taking account of the possible effect of preferential solvent interactions at high concentrations of NaCl (Kellett, 1971). In unbuffered 0.1 M NaCl near neutral pH the $s_{20,w}$ value of oxyhemoglobin A was 4.5 S, compared to values of 3.0-3.1 S for the liganded derivatives of hemoglobins G Georgia, Rampa, and St. Luke's. In contrast, the corresponding values of the deoxy derivatives of hemoglobin A and the three variant hemoglobins were all in the range of 4.5 to 4.8 S.

At 25° in pH 7.4 buffer which was 0.1 M in NaCl and also 0.1 M in phosphate, or in 0.1 M phosphate buffer alone near neutral pH (6.9 to 7.4), $s_{20,w}$ for oxyhemoglobin St. Luke's was approximately 3.8 S. This value is significantly greater than the value of 3.1 S observed for oxyhemoglobin St. Luke's in 0.1 M NaCl alone, pH ~ 7.2 (Fig. 1-4). The presence of phosphate had a similar, although smaller effect, upon oxyhemoglobin Rampa.

In concentrated solutions of NaCl the liganded and non-liganded derivatives of hemoglobins A, G Georgia, Rampa, and

Figure 1-3

The dependence of sedimentation coefficients ($s_{20,w}$) of the oxygenated and deoxygenated derivatives of hemoglobins A, G Georgia, and Rampa upon NaCl concentration. The analyses were made at pH ~ 7.2 and at 25°. ○ , hemoglobin A; □ , hemoglobin G Georgia; △ , hemoglobin Rampa.

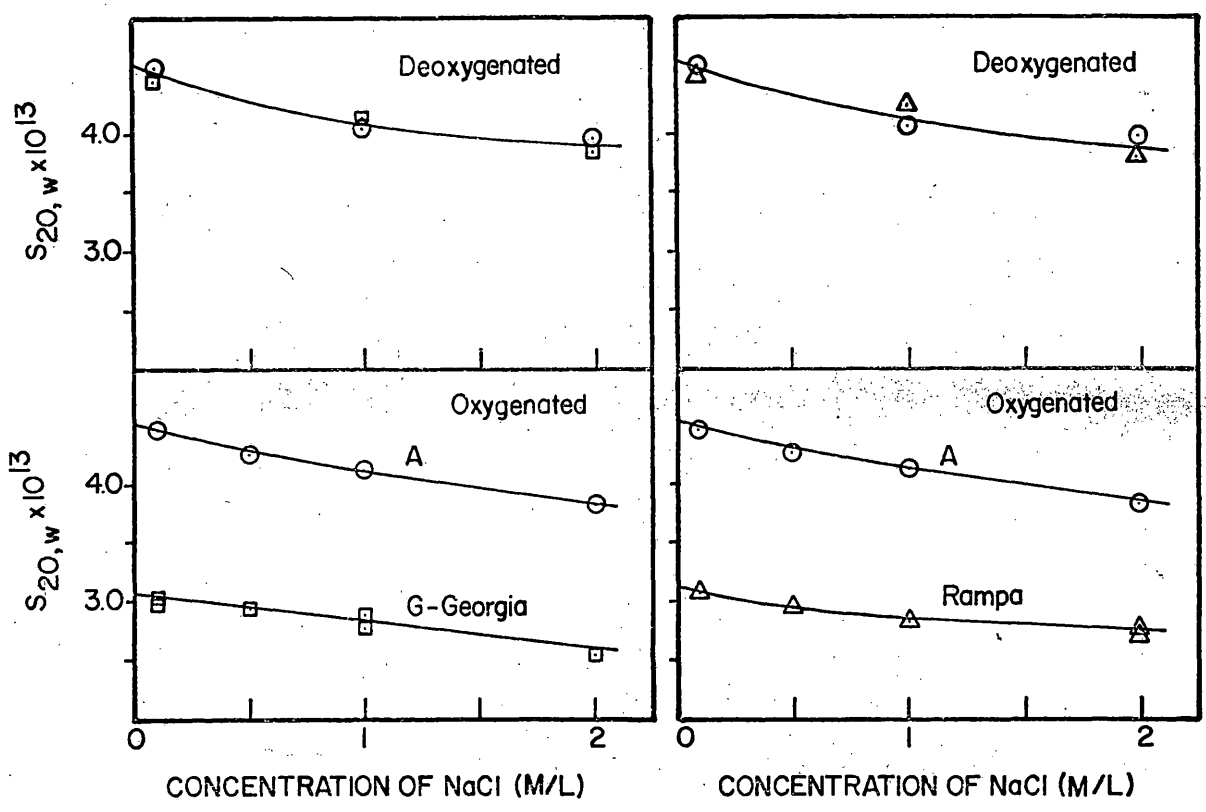
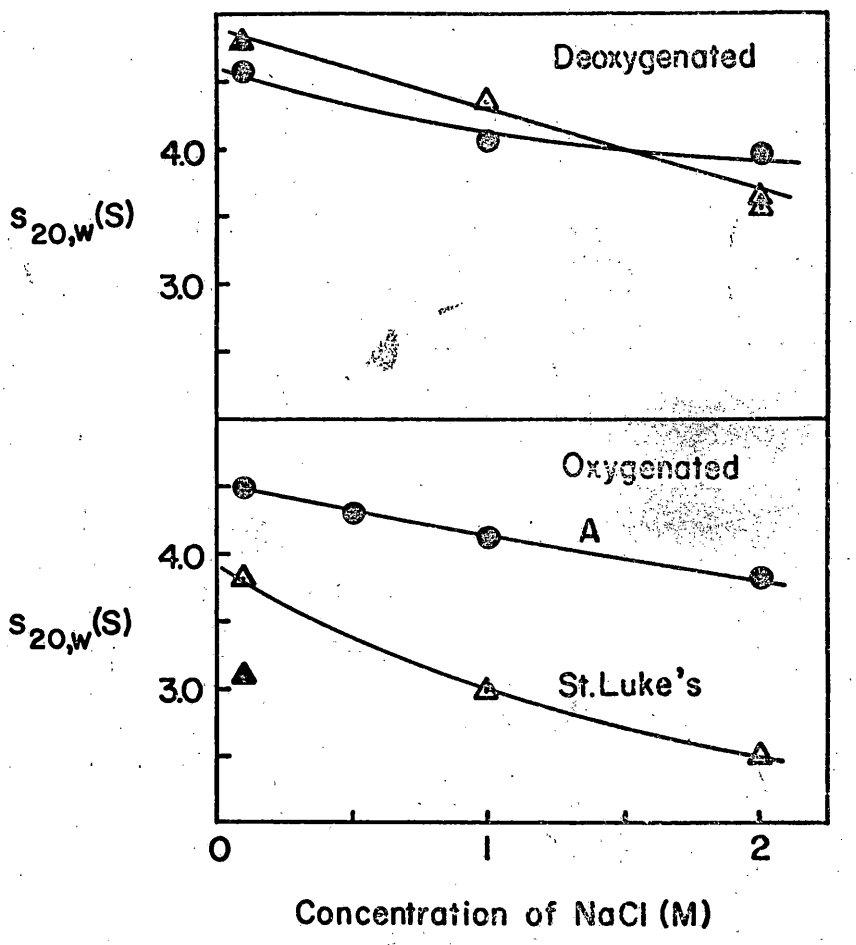

Deoxygenated
Deoxygenated
Oxygenated
Oxygenated
A
A
G-Georgia
Rampa
$S_{20,w} \times 10^{13}$
$S_{20,w} \times 10^{13}$
4.0
3.0
4.0
3.0
0
1
2
0
1
2
CONCENTRATION OF NaCl (M/L)
CONCENTRATION OF NaCl (M/L)

Figure 1-4

The dependence of sedimentation coefficients ($s_{20,w}$) of the oxygenated and deoxygenated derivatives of hemoglobin St. Luke's and hemoglobin A upon NaCl concentration. The analyses were made at pH ~ 7.2 and at 25°. ◍ , hemoglobin A in unbuffered NaCl; ▲ , hemoglobin St. Luke's in unbuffered NaCl; △ , hemoglobin St. Luke's in NaCl + 0.1 M phosphate.


Deoxygenated
4.0
3.0
$s_{20,w}(S)$
Oxygenated
A
4.0
$s_{20,w}(S)$
3.0
St.Luke's
0
1
2
Concentration of NaCl (M)

St. Luke's were corrected to standard conditions (a reference solvent with the viscosity and density of water at 20°) by two methods. In the first, the partial specific volume, $\bar{V}$, of hemoglobin at 25° was assumed to be independent of NaCl concentration and taken to be 0.751 ml/g. This method assumes that preferential binding of solvent or salt to the hemoglobin molecule is negligible (Kirschner and Tanford, 1964). In the second method, the values of Kellett (Kellett, 1971) for the apparent specific volume, ϕ, of hemoglobin at 25° in NaCl solutions of increasing molarity were used to correct the observed sedimentation coefficient to standard conditions. The values for ϕ in 0.5, 1.0, and 2.0 M NaCl were 0.764, 0.775, and 0.779 ml/g respectively. In contrast to the first method, the second assumes appreciable preferential interaction of water with the hemoglobin molecule.

The $s_{20,w}$ values of the liganded and nonliganded derivatives of hemoglobins A, G Georgia, Rampa, and St. Luke's, corrected to standard conditions by the procedures described above, are compared in Tables 1-1 and 1-2.

Correction of the observed sedimentation coefficients using Kellett's data for the apparent specific volume of hemoglobin gives $s_{20,w}$ values characteristic of a tetrameric structure for oxyhemoglobin A and for deoxyhemoglobins A, G Georgia, Rampa, and St. Luke's over the NaCl concentration range of 0.1 to 2.0 M. The $s_{20,w}$ values, calculated on the basis of a constant value of 0.751 ml per g for the partial

Table 1-1

Comparison of $s_{20,w}$ values[a] of liganded hemoglobins A, G Georgia, Rampa, and St. Luke's as a function of increasing concentrations of NaCl.[b]

a. Average $s_{20,w}$ values from two or more experiments.
b. A final hemoglobin concentration of 0.5 g/100 ml was used in all experiments.
c. The analyses were made at pH ~ 7.2 and at 25°.
d. The partial specific volume of hemoglobin, $\bar{V}$, was taken as 0.751 ml/g.
e. The values of the apparent specific volume of hemoglobin, ϕ, at increasing concentrations of NaCl are given in the text.
f. Hemoglobin St. Luke's in NaCl + 0.1 M phosphate.

Table 1-1

Concentration[c]	Hemoglobin A			Hemoglobin G Georgia			Hemoglobin Rampa			Hemoglobin St Luke's		
		$s_{20,W}$			$s_{20,W}$			$s_{20,W}$			$s_{20,W}$	
M	s_{obs}	$\bar{v}$[d]	ϕ[e]	s_{obs}	$\bar{v}$[d]	ϕ[e]	s_{obs}	$\bar{v}$[d]	ϕ[e]	s_{obs}	$\bar{v}$[d]	ϕ[e]
0.1	4.95	4.50	4.50	3.32	3.02	3.02	3.38	3.07	3.07	3.40	3.09	3.09
0.5	4.34	4.30	4.55	2.97	2.94	3.11	2.99	2.96	3.13			
1.0	3.71	4.12	4.63	2.51	2.79	3.13	2.54	2.82	3.17	2.69[f]	2.98	3.36
2.0	2.72	3.83	4.57	1.81	2.55	3.04	1.94	2.73	3.26	1.84[f]	2.59	3.08

Table 1-2

Comparison of $s_{20,w}$ values of deoxyhemoglobins A, G Georgia, Rampa, and St. Luke's as a function of increasing concentrations of NaCl.[a]

a. A final hemoglobin concentration of 0.5 g/100 ml was used in all experiments.
b. The analyses were made at pH $\sim$ 7.2 and at 25°.
c. The partial specific volume of hemoglobin, $\bar{V}$, was taken as 0.751 ml/g.
d. The values of the apparent specific volume of hemoglobin, ϕ, at increasing concentrations of NaCl are given in the text.

Table 1-2

Concentration[b]	Hemoglobin A			Hemoglobin G Georgia			Hemoglobin Rampa			Hemoglobin St Luke's		
		$s_{20,W}$			$s_{20,W}$			$s_{20,W}$			$s_{20,W}$	
M	s_{obs}	$\bar{v}^{c}$	ϕ^{d}	s_{obs}	$\bar{v}^{c}$	ϕ^{d}	s_{obs}	$\bar{v}^{c}$	ϕ^{d}	s_{obs}	$\bar{v}^{c}$	ϕ^{d}
0.1	5.03	4.57	4.57	4.90	4.46	4.46	4.95	4.50	4.50	5.28	4.80	4.80
1.0	3.65	4.05	4.56	3.71	4.12	4.63	3.81	4.23	4.76	3.93	4.36	4.91
2.0	2.82	3.97	4.73	2.72	3.83	4.57	2.69	3.79	4.52	2.55	3.60	4.28

specific volume of hemoglobin in concentrated salt solutions at 25°, are somewhat lower. However, regardless of which correction factors are used, it is apparent that the liganded derivatives of hemoglobin G Georgia, Rampa, and St. Luke's dissociate into dimers to a much greater extent than does the liganded derivative of hemoglobin A; whereas the degree of dissociation for the unliganded forms of these mutants is significantly less, and similar to that for unliganded hemoglobin A.

$s_{20,w}$ values of liganded (oxy or cyanferri) hemoglobins A, G Georgia, Rampa, and St. Luke's as a function of pH at 25° are shown in Table 1-3 and also in Figures 1-5 and 1-6. In contrast to normal human hemoglobin A, the variant hemoglobins Rampa and St. Luke's were shown to be pH dependent. Maximum $s_{20,w}$ values of 3.6-3.7 S were observed for hemoglobin Rampa at 25° between pH 5.9 and 6.9. At pH 7.9 and above, the sedimentation coefficients of liganded hemoglobin Rampa at 25° were below 3.0 S. At 25° hemoglobin St. Luke's had $s_{20,w}$ values similar to those of hemoglobin Rampa at pH 5.9 to 6.9; however, maximum $s_{20,w}$ values of 4.3 S were found between pH 8.9 and 9.4. In the pH range 5.9-9.8 hemoglobin A had an average $s_{20,w}$ value of 4.45 $\pm$ 0.06 S, whereas the corresponding average value for hemoglobin G Georgia was 2.88 $\pm$ 0.08 S.

$s_{20,w}$ values for deoxygenated hemoglobins G Georgia and Rampa at pH 9.8 were 3.8 S and 4.1 S, respectively, compared to values of 2.8 S and 2.9 S for the corresponding liganded

Table 1-3

Comparison of $s_{20,w}$ values[a] of liganded hemoglobins A, G Georgia, Rampa, and St. Luke's as a function of pH.[b]

a. Average $s_{20,w}$ value from two or more experiments.

b. A final hemoglobin concentration of 0.5 g/ 100 ml was used in all experiments.

c. The composition of the buffers is given under "Materials and Methods."

d. pH measurements were made at 25°.

Table 1-3

pH[c,d]	Hemoglobin			
	A	G Georgia	Rampa	St Luke's
4.9	4.07	Denat.	2.86	
5.9	4.52	2.93	3.66	3.71
6.9	4.44	2.94	3.63	3.73
7.4	4.48	2.87	3.30	3.84
7.9	4.40	2.87	2.92	4.15
8.9	4.45	2.86	2.77	4.30
9.4	4.48			4.27
9.8	4.40	2.78	2.94	3.78
10.1	4.26		2.88	3.15
10.3	3.96			2.92
10.7	2.86	2.78	2.70	2.93
11.2	2.30	2.50	2.45	2.54
12.0	2.15	2.15	2.04	2.36

Figure 1-5

The dependence of the sedimentation coefficients ($s_{20,w}$) of hemoglobins Rampa (A) and G Georgia (B) upon pH and temperature. △, oxy or cyanferrihemoglobin Rampa, 25°; ▲, oxy or cyanferrihemoglobin Rampa, 4°; □, oxy or cyanferrihemoglobin G Georgia, 25°; ▣, oxy or cyanferrihemoglobin G Georgia, 4°; ○, oxy or cyanferrihemoglobin A, 4° and 25°; the data for hemoglobin A in A and B are identical.

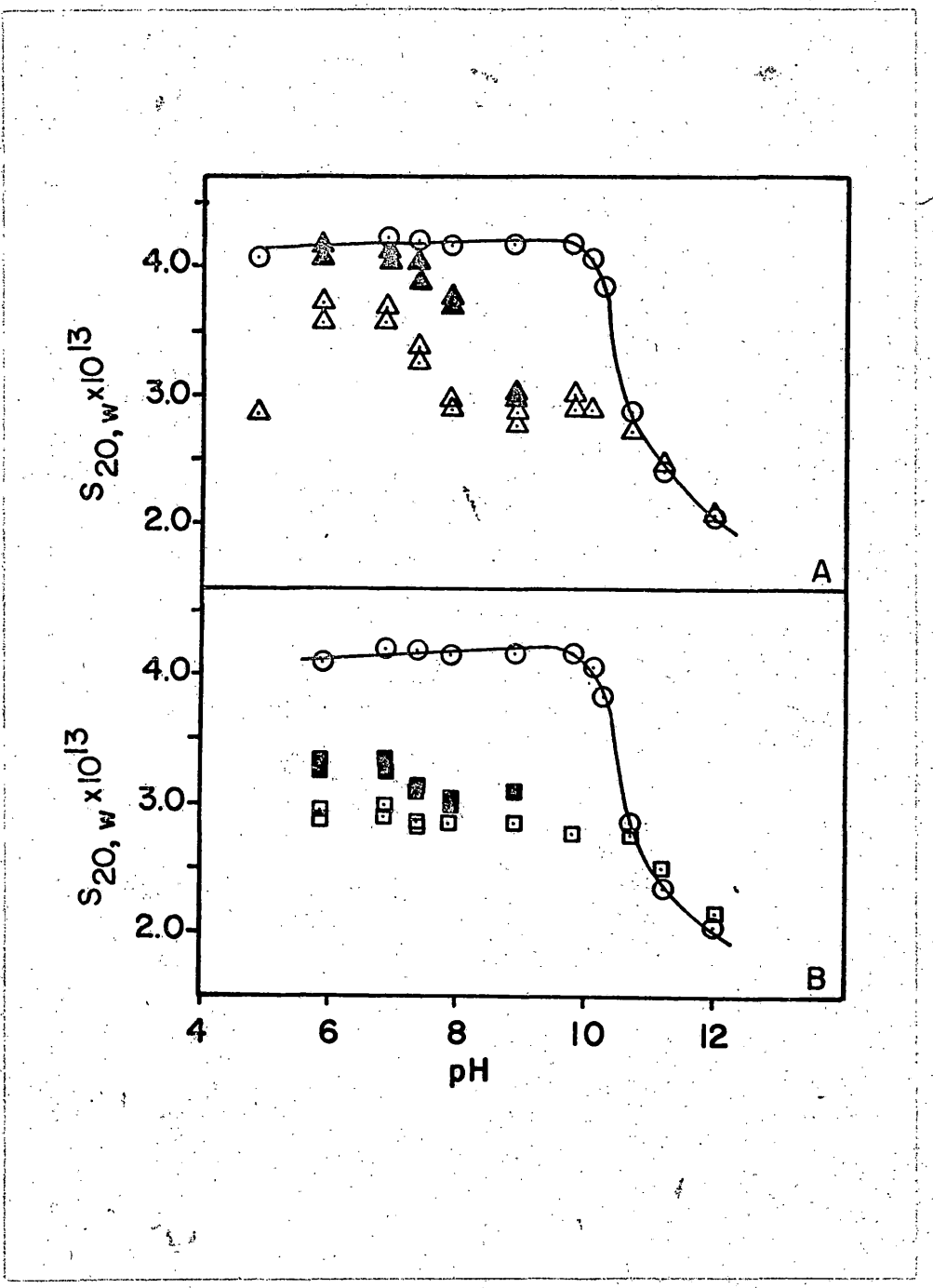
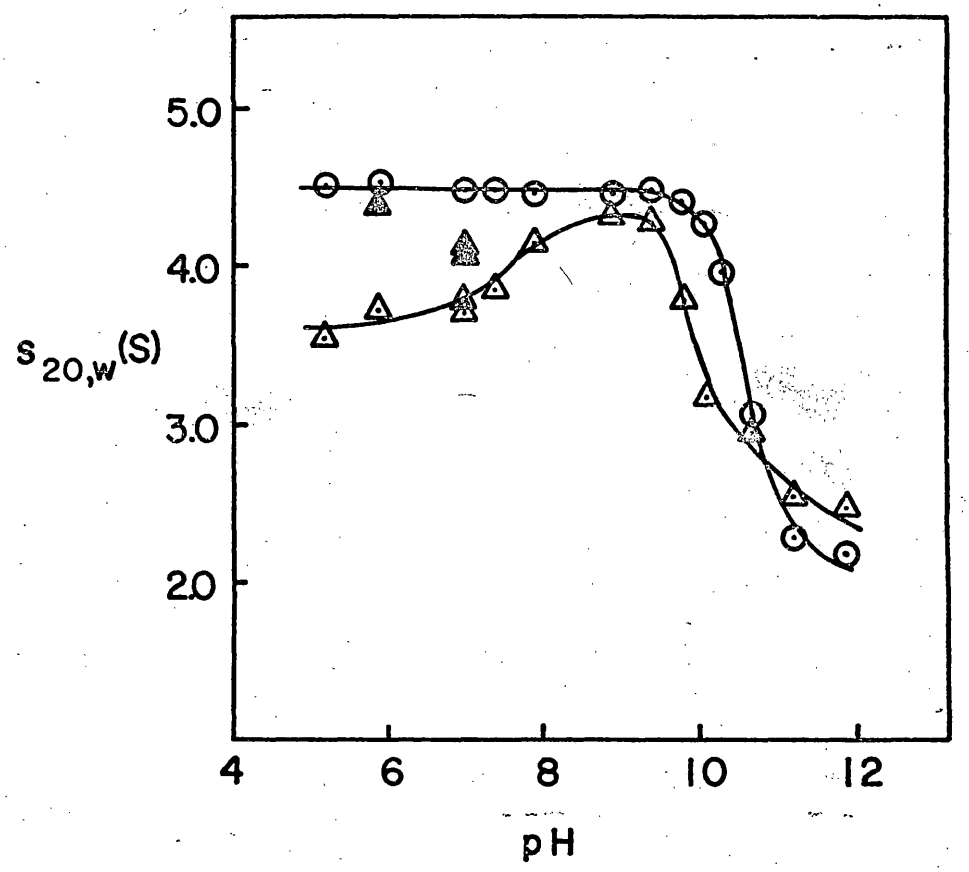

4.0
3.0
2.0
$S_{20,w} \times 10^{13}$
A
B
4
6
8
10
12
pH

Figure 1-6

The dependence of sedimentation coefficients ($s_{20,w}$) of the oxy (or cyanferri) derivatives of hemoglobin St. Luke's and hemoglobin A upon pH. ○ , hemoglobin A, 25°; △ , hemoglobin St. Luke's, 25°; ▲ , hemoglobin St. Luke's, 4°.


5.0
4.0
3.0
2.0
$s_{20,w}$(S)
4
6
8
10
12
pH

species.

At pH 10.7 and above the sedimentation properties of liganded hemoglobins A, G Georgia, Rampa, and St. Luke's were similar. At low pH, the abnormal hemoglobins appeared to be less stable than hemoglobin A, with hemoglobin G Georgia being almost completely denatured below pH 5.9.

Comparison of the sedimentation properties of the liganded derivatives of hemoglobins A, G Georgia, Rampa, and St. Luke's over the pH range of 5.9 to 8.9 at 4° and 25° are shown in Table 1-4 and in Figures 1-5 and 1-6. In contrast to hemoglobin A, the sedimentation properties of the three variant hemoglobins were temperature dependent. At 4° and pH 6.9 hemoglobins Rampa and St. Luke's were found to have nearly identical $s_{20,w}$ values of 4.1 S, compared to values of 3.6-3.7 S at 25°. Maximum differences in $s_{20,w}$ values at 4° and 25° were seen at pH 7.9 for hemoglobin Rampa, and at pH 5.9 for hemoglobin St. Luke's. Throughout the pH range studied, hemoglobin G Georgia showed a slight temperature dependence, which was most pronounced between pH 5.9 and 6.9.

B. Diffusion Coefficients

Approximate $D_{20,obs}$ and $D_{20,w}$ values of cyanferrihemoglobins A, G Georgia, and Rampa in 0.1 M NaCl are shown in Table 1-5. The $D_{20,w}$ value for hemoglobin A of 7.0×10^{-7} cm^2/sec is similar to the literature value for CO-hemoglobin A (Lamm and Polson, 1936; Field and O'Brien, 1955), while the $D_{20,w}$ values for hemoglobins G Georgia and Rampa are within

Table 1-4

Comparison of $s_{20,w}$ values[a] of liganded hemoglobins A, G Georgia, Rampa, and St. Luke's as a function of pH and temperature.[b]

a. Average $s_{20,w}$ value from two or more experiments.
b. A final hemoglobin concentration of 0.5 g/100 ml was used in all experiments.
c. The composition of buffers is described under "Materials and Methods."
d. pH measurements were made at 25°.

Table 1-4

pH[c,d]	Hemoglobin							
	A		G Georgia		Rampa		St Luke's	
	4°	25°	4°	25°	4°	25°	4°	25°
5.9	4.49	4.52	3.30	2.93	4.10	3.66	4.36	3.71
6.9	4.55	4.44	3.30	2.94	4.08	3.63	4.07	3.73
7.4	4.50	4.48	3.14	2.87	3.93	3.30		3.84
7.9	4.39	4.40	3.04	2.87	3.73	2.92		4.15
8.9	4.46	4.45	3.11	2.86	2.97	2.77		4.30

Table 1-5

Comparison of $D_{20,w}$ values[a] of liganded hemoglobins A, G Georgia, and Rampa in 0.1 M NaCl, pH ~ 7.2.[b]

a. The analyses were made at 20° and 12,000 rpm.

b. pH measurements were made at 25°.

Table 1-5

Hemoglobin	Concentration g/100 ml	$D_{20,obs}$ $X10^7$	$D_{20,W}$ $X10^7$
A	1.0	6.90	6.96
G Georgia	1.0	7.26	7.33
	0.7	7.44	7.51
Rampa	0.85	7.70	7.77

the range of values reported by Kawahara et al (Kawahara et al, 1965) for partially dissociated hemoglobin in 6.4 M urea.

C. Viscosity

The intrinsic viscosity in 0.1 M NaCl of the cyanferri derivatives of hemoglobins A, G Georgia, and Rampa was determined from viscosity measurements at a minimum of three different hemoglobin concentrations. The reduced viscosity, η_{sp}/c, of hemoglobins G Georgia and Rampa was found to show a slightly greater dependence on protein concentration than hemoglobin A (Fig. 1-7). Extrapolation of the data to zero protein concentration gave values which were essentially the same for the intrinsic viscosity, $[\eta]$, of hemoglobins A, G Georgia, and Rampa; values of 3.45 ml/g were found for hemoglobins A and Rampa, and 3.50 ml/g for hemoglobin G Georgia. These values are within the range of 3.3 to 4.0 ml/g, which is considered characteristic of a compact, globular protein (Tanford, 1963).

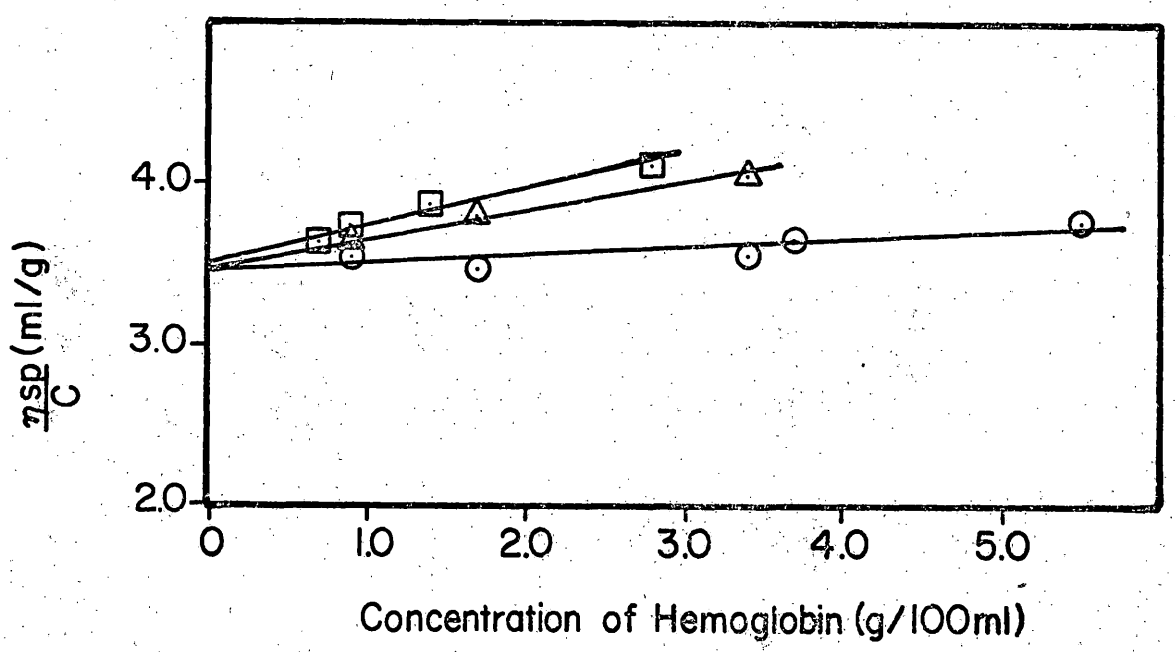
D. Dissociation Constant

An estimate of the dissociation constant, K, of cyanferrihemoglobins A, G Georgia, Rampa, and St. Luke's in 0.1 M NaCl at pH 7.2 to 7.3 and 25° and at a protein concentration 0.5 g per 100 ml was made using the equations (Kirshner and Tanford, 1964):

$$s_{20,w} = (1 - \alpha)s_T + \alpha(s_D) \quad \text{and} \quad K = \frac{4\alpha^2 C_o}{(1 - \alpha)M}$$

Figure 1-7

Intrinsic viscosities of hemoglobins A, G Georgia, and Rampa in 0.1 M NaCl, pH ~7.2, 25°. ○ , cyanferrihemoglobin A; □ , cyanferrihemoglobin G Georgia; △ , cyanferrihemoglobin Rampa.


4.0
3.0
2.0
0
1.0
2.0
3.0
4.0
5.0
ηsp/C (ml/g)
Concentration of Hemoglobin (g/100ml)

where $s_{20,w}$ is the experimental sedimentation coefficient.

α is the weight fraction of hemoglobin in the dissociated form.

s_T is the sedimentation coefficient of tetrameric hemoglobin; 4.50 S (Kirshner and Tanford, 1964).

s_D is the sedimentation coefficient of dimeric hemoglobin; 2.83 S (Kirshner and Tanford, 1964).

C_o is the protein concentration in grams per liter.

M is the molecular weight of undissociated hemoglobin; 64,500 (Kirshner and Tanford, 1964).

On the basis of the viscosity data, changes in the $s_{20,w}$ values were interpreted to be the result of changes in molecular weight; preferential binding was assumed to be the same for both tetramer and dimer, and negligibly small in 0.1 M NaCl (Kirshner and Tanford, 1964). The dissociation constants calculated for hemoglobin A and the three variants are compared in Table 1-6.

E. Molecular Weight

The molecular weights of hemoglobins G Georgia and Rampa in 0.1 M NaCl at neutral pH were calculated by two procedures which are compared in Table 1-6. In the first, the $s_{20,w}$ and $D_{20,w}$ values of the respective hemoglobins were used in the classical Svedberg equation (Svedberg and Pederson, 1940):

$$M = \frac{R\ T\ s_{20,w}}{(1 - \bar{V}\rho)\ D_{20,w}}$$

Table 1-6

Dissociation constants and molecular weight determinations for hemoglobins G Gèorgia, Rampa, and St. Luke's.

Table 1-6

Hemoglobin	$\bar{s}_{20,W} \times 10^{13}$	$K \times 10^5$ M	$D_{20,W} \times 10^7$	[η]	Molecular Weight	
					Svedberg	Sheraga-Mandelkern
G Georgia	2.99	225	7.51	3.50	38,500	36,100
Rampa	3.07	165	7.77	3.45	38,200	37,400
St Luke's	3.09	142				

where R is the gas constant (8.314×10^7 ergs per deg per mole).

T is the absolute temperature.

$\bar{V}$ is the partial specific volume of hemoglobin (0.749 ml/g).

ρ is the density of water at 20 .

The $D_{20,w}$ value of 7.5×10^{-7} cm^2 sec^{-1} was used to determine the molecular weight of hemoglobin G Georgia. This value was obtained at the lower hemoglobin concentration, where the dependence of $D_{20,w}$ upon protein concentration should be less. The molecular weight estimated in this manner was 38,500 for the liganded derivative of hemoglobin G Georgia and 38,200 for that of hemoglobin Rampa (Table 1-6).

In the second procedure, the molecular weights were determined from the Sheraga-Mandelkern equation (Sheraga and Mandelkern, 1953):

$$\beta M^{2/3} = \frac{N s_{20,w} [\eta]^{1/3} \eta}{(100)^{1/3} (1 - \bar{V}\rho)}$$

where β is equal to 2.16×10^6, the value of the parameter generally assumed for globular proteins (Schachman, 1959; Martin, 1959).

N is Avogadro's number (6.02×10^{23}).

η is the viscosity of the solvent.

ρ is the density of the solvent.

$[\eta]$ is the intrinsic viscosity of the solution.

$\bar{V}$ is the partial specific volume of hemoglobin.

The molecular weights determined by this method were 36,100 and 37,400, respectively, for the liganded derivatives of hemoglobins G Georgia and Rampa (Table 1-6).

DISCUSSION

The liganded derivatives of hemoglobins G Georgia, Rampa, and St. Luke's are 80 to 90 % dimeric under conditions where normal hemoglobin is predominantly tetrameric. Values of the dissociation constant, K, for cyanferrihemoglobins A, G Georgia, Rampa, and St. Luke's were 0.6×10^{-5} M, 225×10^{-5} M, 165×10^{-5} M and 142×10^{-5} M, respectively, at protein concentrations of 0.5 g per 100 ml in 0.1 M NaCl near neutral pH at 25°. Extensive dimer formation has been previously observed for the liganded forms of hemoglobins Bibba (α 136 pro) (Smith et al, 1970), Kansas (β 102 thr) (Bonaventura and Riggs, 1968), and Richmond (β 102 lys) (Efremov et al, 1969) under the same, or nearly identical, conditions. The corresponding values of the dissociation constants were 50×10^{-5} M for cyanferrihemoglobin Bibba (Smith et al, 1970), 20×10^{-5} M for oxy or carbonmonoxyhemoglobin Kansas (Bonaventura and Riggs, 1968), and 3×10^{-5} M for cyanferrihemoglobin Richmond (Efremov et al, 1969).

The limited number of sedimentation velocity studies suggest that liganded hemoglobin St. Luke's is considerably more dissociated in dilute, unbuffered NaCl solutions at neutral pH ($s_{20,w} \sim$ 3.1 S) than in phosphate buffers of comparable pH and ionic strength ($s_{20,w} \sim$ 3.8 S). In the case of hemoglobin Rampa, a similar, however smaller, effect was observed ($s_{20,w} \sim$ 3.1 S in 0.1 M NaCl, compared to ~ 3.3 S in 0.1 M phosphate). Although a detailed study of this phenomenon has not been made

in either case, the present data appear to support the investigations of Ruckpaul et al (Ruckpaul et al, 1971), who compared the effects of inorganic phosphate and NaCl on the ESR spectra of methemoglobin and obtained evidence that phosphate ions may exert a specific effect on the structure of the hemoglobin molecule.

$s_{20,w}$ values of deoxyhemoglobins G Georgia, Rampa, and St. Luke's indicate that the nonliganded derivatives are tetramers in dilute salt solution near neutral pH. Also in pH 9.8 buffer at low ionic strength, the deoxy derivatives of hemoglobins G Georgia and Rampa, and presumably St. Luke's, are still predominantly tetrameric.

Extensive dimer formation by liganded hemoglobin molecules in dilute salt solution near neutral pH, in contrast to the tetrameric behavior of the unliganded forms, has been observed for hemoglobins Kansas (Bonaventura and Riggs, 1968) and Bibba (Smith et al, 1970). The liganded form of normal hemoglobin A is also more dissociated than the unliganded one, except at an acid pH in acetate buffer (Kellett, 1971; Benesch et al, 1962; Guidotti, 1967; Antonini et al, 1968; Anderson et al, 1970). However, recent differential sedimentation studies by Goers and Schumaker (Goers and Schumaker, 1970) show that both oxy and deoxyhemoglobin A are tetrameric in structure when analyzed in dilute salt solutions at neutral pH and at the protein concentrations similar to those used in this study. Under these conditions the $s_{20,w}$ value of oxyhemoglobin A is slightly

greater than that of deoxyhemoglobin A, which agrees with X-ray crystallographic measurements indicating that oxygenated hemoglobin A occupies a slightly smaller volume than the deoxygenated molecule (Muirhead and Perutz, 1963).

Previous studies on the dissociation of hemoglobin at high salt concentrations have neglected the effects of preferential solvent interactions, although Kirschner and Tanford (Kirschner and Tanford, 1964) showed that failure to consider such interactions results in sedimentation coefficients which are lower than the true values if water is preferentially bound to the hemoglobin molecule. Thus, when preferential interactions are considered negligible and $s_{20,w}$ values are calculated on the basis of a value of 0.751 ml g^{-1} for the partial specific volume of hemoglobin in concentrated salt solutions at 25°, the fraction of dimers in 2 M NaCl is approximately 30 % for deoxyhemoglobin A, 40 % for oxyhemoglobin A and deoxyhemoglobins G Georgia and Rampa, and 50 % for deoxyhemoglobin St. Luke's. However, when preferential interactions are taken into consideration and the observed sedimentation coefficients are corrected using Kellett's values (Kellett, 1971) for the apparent specific volume of hemoglobin in sodium chloride solutions of increasing molarity, the $s_{20,w}$ values obtained in this manner indicate that oxyhemoglobin A, and deoxyhemoglobins A, G Georgia, Rampa, and St. Lukes all retain a tetrameric structure in 2 M NaCl at the hemoglobin concentrations used in this study.

The evaluation of preferential interactions in the case

of hemoglobin is an unsettled issue. Evidence suggesting that hemoglobin may bind water preferentially has been obtained from both X-ray crystallography measurements (Perutz et al, 1968) and also from measurements of crystal density (Perutz, 1946). On the other hand, the studies by Kirshner and Tanford (Kirshner and Tanford, 1946), and more recently those by Noren et al (Noren et al, 1971), suggest that any preferential binding of NaCl or water by hemoglobin is too small to have an appreciable effect on molecular weight determined by light scattering or ultracentrifugation. Although these questions cannot be resolved at the present time, they do not affect our comparisons between the behavior of normal and mutant hemoglobins.

The greatly enhanced degree of dimer formation observed in the liganded forms of hemoglobins G Georgia, Rampa, and St. Luke's in contrast to the predominantly tetrameric structure of the deoxy derivatives, is the result of the replacement of prolyl residue in position 95 (G2) of the α chains by a leucyl residue, a seryl residue, and an arginyl residue, respectively. This site involves one of the critical, invariant, mainly nonpolar α_1-β_2 contacts which are broken when hemoglobin tetramers dissociate into dimers (Perutz et al, 1968). In both liganded and unliganded hemoglobin A, this prolyl residue forms a nonpolar contact with tryptophan residue in position 37 (C3) of the β chain (Perutz et al, 1968; Muirhead and Greer, 1970; Bolton and Perutz, 1970). Substitution of a leucyl, or a seryl, or an arginyl residue into position α95 (G2) ruptures these contacts in

oxygenated hemoglobin and makes the contact accessible to water. That a single amino acid substitution causes such extensive disruption of the contacts in one form and not in the other is likely due to the conformational differences which exist between the oxy and deoxy structures and to the stabilizing effect of the six additional salt bonds which are present in the deoxygenated molecule (Perutz, 1970; Perutz, 1970).

The occurrence of maximum $s_{20,w}$ values in the pH range of 6 to 7, which was observed both at 4° and 25° for liganded hemoglobin Rampa and to some extent at 4° for liganded G Georgia, is difficult to explain. Also the dependence of $s_{20,w}$ upon temperature, which was observed in this pH range for all three hemoglobin variants (and in the case of hemoglobin Rampa, up to pH 8), is not readily understood. For hemoglobin Rampa, the data suggest the possibility of an intra- or interchain hydrogen bond between the hydroxyl group of serine and a positively charged imidazole group of a histidyl residue. The imidazole groups of the seven titratable histidine residues in the α chains of normal human hemoglobin have an average pK value of 6.7 at 25°, while those of the six titrable histidines in the β chain have values ranging from 5.6 to 8 (Janssen et al, 1970). However, replacement of prolyl residue by seryl residue in position α95 of an oxyhemoglobin model does not suggest the formation of such a bond, according to Perutz. The arginyl residue in position α95 of hemoglobin St. Luke's might possibly be able to participate in a salt bridge with

a side chain carboxyl group, or in hydrogen bond formation with a carboxyl, carbonyl, hydroxy, or phenolic group; however, the leucyl residue in position $\alpha 95$ of hemoglobin G Georgia, which also shows a slight temperature effect, would not be able to form either salt or hydrogen bonds.

The fact that the experiments in this pH range were done in phosphate buffer may be significant. Johnson and Parrella (Johnson and Parrella, 1971) observed a similar affect at 5° and 25° for sheep hemoglobin B in the presence of phosphate at neutral pH and low protein concentration. They found that dissociation was not significantly affected by a temperature increase from 5° to 25° in dilute NaCl solutions, whereas in phosphate solutions of comparable ionic strength, significantly less dissociation occurred at 5°.

In a single experiment, (due to lack of sufficient hemoglobin sample), $s_{20,w}$ values of 3.35 S and 3.37 S were found at 4° and 25°, respectively for liganded Rampa in unbuffered 0.1 M NaCl near neutral pH; corresponding $s_{20,w}$ values of 3.90 S at 4°, and 3.30 S at 25° were observed in a phosphate buffer of pH 7.4. The sedimentation coefficients in unbuffered NaCl could not be considered reliable, however, because the sample of hemoglobin Rampa was denatured during dialysis and the protein concentration was considerably less than 0.5 g per 100 ml.

The increased tendency of liganded hemoglobin St. Luke's to form tetramers over the pH range 8.0 to 9.4 is also not readily apparent. One possible explanation is that in this pH

range small conformational changes occur which permit the arginine in position 95 (G2) of the α chain to form a van der Waals contact - either with tryptophan residue in position β 37 (C3) (which in normal hemoglobin forms an α_1-β_2 contact with position α 95), or else with some other residue in the β chain. Such an explanation appears reasonable in view of the fact that in normal oxyhemoglobin, arginine in position α 92 (FG4) forms three van der Waals type bonds in the critical α_1-β_2 contacts and arginine in position β 40 (C6) participates in four such contacts by means of van der Waals interactions (Perutz et al, 1968); upon deoxygenation, conformational changes occur which disrupt one of these contacts in the α 92 arginine, and two of the contacts in the β 40 arginine (Bolton and Perutz, 1970). A further understanding of the physicochemical properties of hemoglobins G Georgia, Rampa, and St. Luke's must await the X-ray analyses of their oxy and deoxy structures.

PART II DEER HEMOGLOBINS

INTRODUCTION

Sickle cell anemia was demonstrated by Pauling et al (Pauling et al, 1949) and Ingram (Ingram, 1957) to be a molecular abnormality of the hemoglobin molecule. The current postulate concerning the molecular mechanism responsible for the sickled erythrocyte is based on the theory of stacking of hemoglobin S molecules proposed by Murayama (Murayama, 1967). Support for this molecular model has come largely from light and electron microscopy studies which showed tactoid formation in both sickled erythrocytes and cell free hemoglobin solutions (Harris, 1950; White and Heagan, 1970).

In vitro sickling of erythrocytes has been demonstrated in most species of deer (Kitchen et al, 1964; Kitchen et al, 1967). These were first described in 1840 by Gulliver (Gulliver, 1840) as bizarre shaped erythrocytes and were later found to have a morphology which resembled that of erythrocytes associated with sickle cell anemia in man (Herrick, 1910; O'Roke, 1936; Undritz et al, 1960; Pritchard et al, 1963).

The Virginia white-tailed deer (Odocoileus virginianus) has, in addition, been found by starch gel electrophoresis to have an unusual degree of hemoglobin heterogeneity (Weisberger, 1964; Kitchen et al, 1964; Kitchen et al, 1967; Huisman et al, 1968). At least five electrophoretically distinct major adult hemoglobin components and an equal number of minor components are seen on starch gel electrophoresis (Figure 2-1). The major hemoglobin components have electrophoretically identical α chains,

Figure 2-1

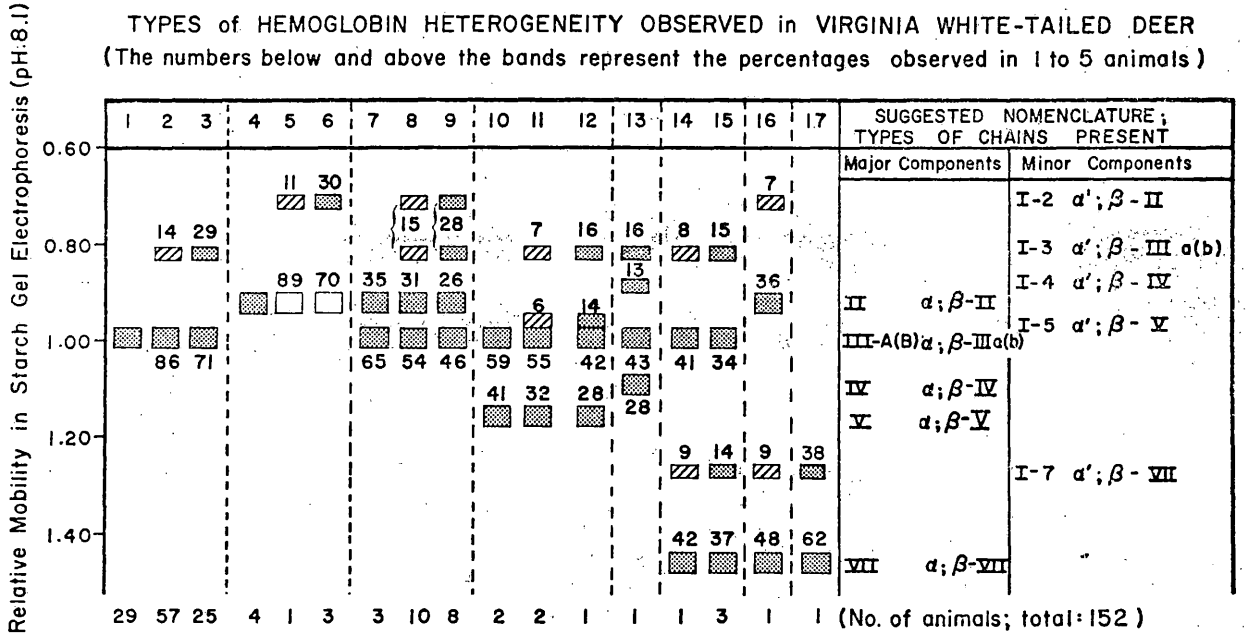
Types of hemoglobin heterogeneity[a] observed in Virginia white-tailed deer.[b] (From Huisman et al, 1968)

a. The α chains are electrophoretically indistinguishable but differ at a minimum in two amino acid residues at positions 5 and 24. See text for details.

b. The numbers above and below the bands represent the percentages observed in 1 to 5 animals.

TYPES of HEMOGLOBIN HETEROGENEITY OBSERVED in VIRGINIA WHITE-TAILED DEER

(The numbers below and above the bands represent the percentages observed in 1 to 5 animals)



SUGGESTED NOMENCLATURE; TYPES OF CHAINS PRESENT		
	Major Components	Minor Components
I-2		α'; β - II
I-3		α'; β - III a(b)
I-4		α'; β - IV
I-5		α'; β - V
I-7		α'; β - VII
II	α; β-II	
III-A(B)	α; β-III a(b)	
IV	α; β-IV	
V	α; β-V	
VII	α; β-VII	

but different β chains, and are catagorized according to their relative mobilities as components II, III, IV, V, and VII. Recently, Harris et al[1] have shown that the α chains of the major hemoglobins can differ, in neutral amino acid residues, at positions 5 and 24; these are positions other that the critical α^1-β^1 or α^1-β^2 contacts (Perutz et al, 1968; Bolton and Perutz, 1970) and not considered any further in this thesis. The minor hemoglobin components likewise differ from one another in their β chains. Each minor component possesses a β chain found in one of the major components but differs from the major component in the type of α chain. The minor components are catagorized according to their relative mobility on starch gel electrophoresis as components I-2, I-3, I-4, I-5, and I-7, with each of the pairs II and I-2, III and I-3, IV and I-4, V and I-5, and VII and I-7 having the same β chain. If the major components are designated as $\alpha_2\beta_2^{II}$, $\alpha_2\beta_2^{III}$, $\alpha_2\beta_2^{IV}$, etc, then their corresponding minor components would be represented by $\alpha'_2\beta_2^{II}$, $\alpha'_2\beta_2^{III}$, $\alpha'_2\beta_2^{IV}$, etc.

Kitchen et al (Kitchen et al, 1964; Kitchen et al, 1966; Kitchen et al, 1967) showed the morphology of the deer erythrocyte to be characteristic of the type of hemoglobin (Table 2-1). Components II, III, and IV, when present alone or in combination with each other are associated with sickling, while components V or VII, when present alone or in combination with any of the other components preclude the sickling phenomenon.

1. Harris, M. J., Wilson, J. B., and Huisman, T. H. J., Manuscript in preparation.

Table 2-1

Relationship between deer hemoglobin components and red blood cell morphology. (Modified from Kitchen et al, 1967)

a. Identified by starch gel electrophoresis.

Table 2-1

Component[a]	Red Blood Cell Morphology
II, II + III	Matchstick
III	Crescent
V	No Sickling
VII	No Sickling
II + V, II + VII	No Sickling
III + V, III + VII	No Sickling

Numerous experimental techniques have been used in attempts to show similarity between the sickling of deer and human erythrocytes. Sickling of deer erythrocytes is brought about by various agents and conditions, such as the aging of blood at refrigerator temperature, the oxygenation of blood at alkaline pH or exposure of the erythrocytes to high pH. The above mentioned agents either have no effect on sickling of human sickle cell erythrocytes, or else reverse the sickling process (Undritz et al, 1960).

Preliminary polarized light and electron microscopy studies revealed basic similarities between the sickled deer erythrocytes and the sickled erythrocytes of man (Pritchard et al, 1963). More recent electron microscopic investigations of erythrocytes and cell free hemoglobin solutions from the deer have shown the formation of microtubules rather than tactoids, with the transformations characteristic of sickling in deer hemoglobin solutions being more similar, although not identical, to the process of hemoglobin polymerization in gels of normal human hemoglobin (White and Seal, 1971).

The sickling phenomena in deer and human erythrocytes are at least similar, if not identical processes. The ease of preparing sickled erythrocyte and hemoglobin solutions from the deer and the presence of hemoglobin components which are known to both cause and preclude sickling make the deer an extremely valuable animal to use as a model for studying some

of the physical aspects of sickling in man. The possibility of using sedimentation velocity techniques to observe polymer or aggregate formation in the deer hemoglobins which undergo sickling was suggested by ultracentrifugal experiments which have detected polymerization in frogs (Trader et al, 1963; Riggs et al, 1964; Trader and Frieden, 1966), turtles (Riggs et al, 1964; Sullivan and Riggs, 1964; Sullivan and Riggs, 1967), mice (Ranney et al, 1960; Riggs, 1965; Morton, 1966; Bonaventura and Riggs, 1967), and in the abnormal human hemoglobin, Porto Alegre ($\alpha_2\beta_2^{9\ Cys}$) (Bonaventura and Riggs, 1967; Tondo et al, 1963). Normal monomeric lamprey hemoglobin was also found to polymerize but only at low pH in the deoxygenated state, or at high hemoglobin concentration in the oxygenated species. An increase in $s_{20,w}$ from 1.9 (monomer) to 3.7 (weight average trimer) was observed for the polymerized hemoglobin (Briehl, 1963).

The purpose of this research was to characterize the subunit association and dissociation in deer hemoglobin components which are associated with sickling and in those which preclude sickling in order to determine if the sickling components show a greater tendency to polymerize under the conditions known to be favorable to sickling. The results describe comparative sedimentation velocity measurements as a function of pH and salt concentration of the sickling deer hemoglobin components II, III and I-2, and of those which preclude sickling - namely components V, VII and I-7. The sedimentation velocity studies

were supplemented by a limited number of viscometry measurements on hemolysates which contained only the sickling hemoglobin components, and also on hemolysates which contained hemoglobin components known to preclude sickling.

MATERIALS AND METHODS

A. Preparation of Hemolysates

Blood samples of 5 to 10 ml were obtained from the deer with EDTA as anticoagulant and shipped to Dr. T. H. J. Huisman. Whole blood was centrifuged for approximately 10 minutes at 3000 rpm and the plasma removed. The red blood cells were washed at least 3 times with 0.9 % NaCl and the washed erythrocytes lysed by addition of an equal volume of distilled water. One-half volume of CCl_4 was added to remove the stroma. The mixture was then shaken for 15 minutes, centrifuged at 3000 rpm for 30 minutes and the hemoglobin solution removed.

B. Electrophoresis

Starch gel electrophoresis of the hemoglobin in the hemolysates was done by the method of Efremov et al (Efremov et al, 1969). The buffer (pH 9.0) in the electrode vessels contained 20.0 g of Tris, 2.0 g of EDTA, and 1.5 g of boric acid per 1000 ml. The same buffer in 1:4 dilution with distilled water was used for the preparation of the starch gel. Hydrolyzed starch (55 g, Electrostarch Co., Madison, Wisconsin) was dissolved in 500 ml of starch gel buffer and heated until clear. The starch was poured into the starch gel tray and allowed to harden in the cold for 3 hours. One centimeter squares of filter paper were saturated with the hemolysate, inserted into the starch gel, and the sample allowed to diffuse from the paper to the gel. The paper was removed and electro-

phoresis was carried out at room temperature (22-25°) for 16 hours at 6 volts per centimeter and 10 to 12 ma. The gels were cut and stained with o-dianisidine (100 mg 3,3' - Dimethoxybenzidine in 70 ml absolute ethanol, 30 ml of 0.5 M sodium acetate and approximately 5 drops of H_2O_2). Both the hemoglobin types and the purity of the chromatographed samples were determined by this procedure.

C. Chromatography

The different components of the deer were isolated from freshly prepared red cell hemolysates on DEAE-Sephadex (Pharmacia, A-50 medium) columns (2.5 x 50 cm) equilibrated at room temperature with 0.05 M Tris-HCl buffer at pH 8.5. The components were eluted by a stepwise profile with 0.05 M Tris-HCl buffers of pH 8.5-7.8-7.6-7.5 for the slower migrating types, as determined by starch gel electrophoresis, and pH 8.5-7.8-7.3-7.0 for the faster migrating types, as determined by starch gel electrophoresis. The hemoglobin components were concentrated by adjusting the eluted components to pH 8.0 with Tris-HCl buffer (1.0 M, pH 8.5) and repouring on a column of DEAE-Sephadex (pH 8.5, 2.5 x 3 cm). The material was eluted with 0.2 M Tris-HCl, pH 6.5, and dialyzed for several days against distilled water. The purity was checked by starch gel electrophoresis.

D. Sedimentation Velocity Studies

The procedures described in the previous section on materials and methods was used to determine the sedimentation

coefficients of hemoglobin components of the deer. In place of human hemoglobin A, components V and VII were first run against each other and one of these was then run against component II or III. Components I-2 and I-7 were run against one another. Sedimentation properties were studied as a function of NaCl concentration over the salt concentration range of 0.1 to 2.5 M and as a function of hydrogen ion concentration over the pH range of 4.9 to 12.0.

E. Viscometry

Viscosity measurements were done on deer hemoglobin components II, II + I-2, and III + I-3 + VII + I-7 in 0.1 M NaCl, pH ~ 7.3 and on components III + I-3 and III + I-3 + VII + I-7 at pH 10.3. The method was the same as described in the previous section on materials and methods. The intrinsic viscosity was obtained by extrapolation to zero protein concentration, a plot of reduced viscosity, η_{sp}/c, versus hemoglobin concentration.

RESULTS

A. Electrophoretic Studies

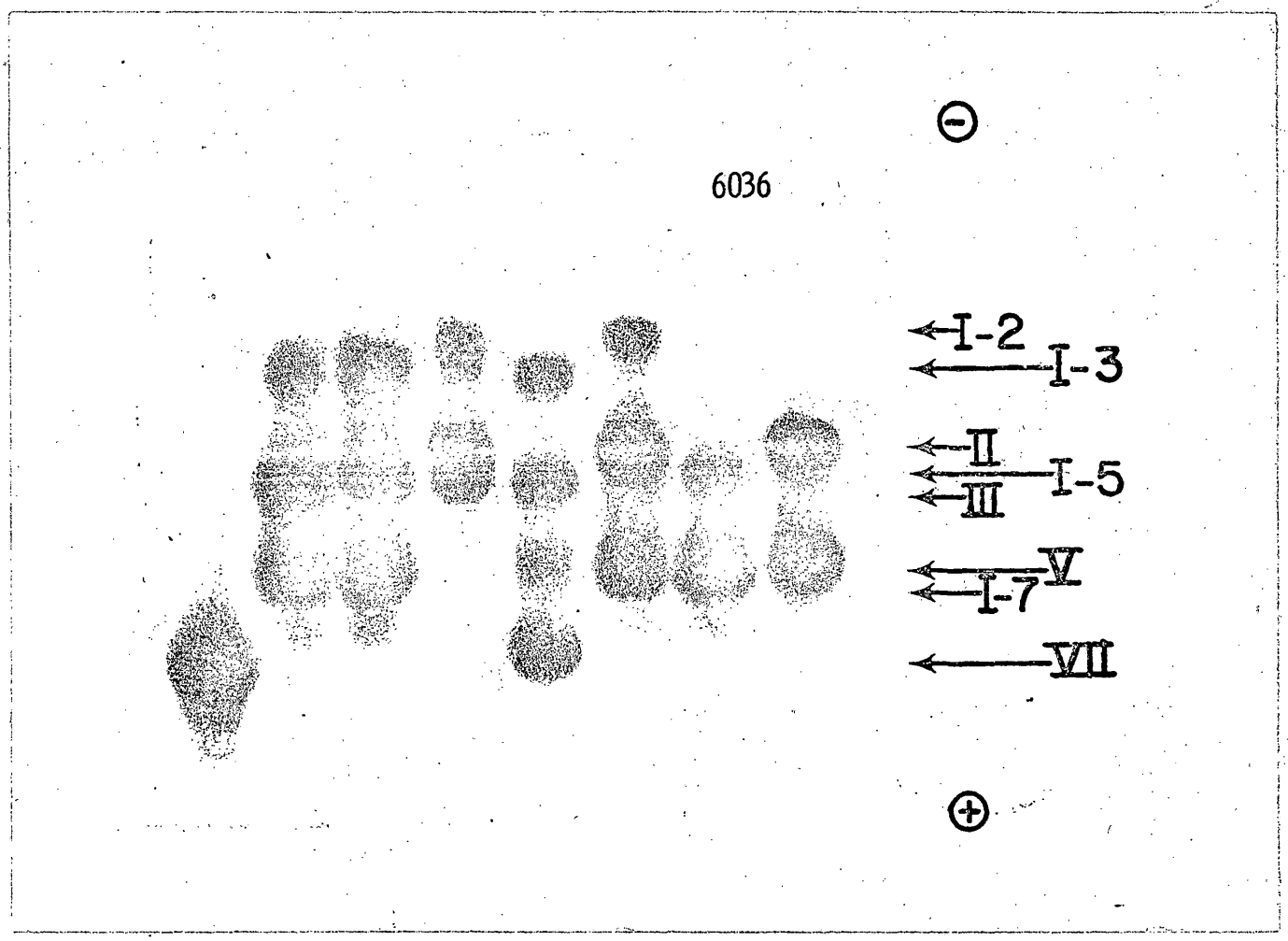
The results of starch gel electrophoresis of hemolysates from a random sampling of Virginia white-tailed deer is shown in Fig. 2-2. Electrophoresis was carried out at room temperature for 16 hours, pH 9.0. The gels were stained with o-dianisidine and the components identified by their relative mobilities.

B. Ultracentrifugal Analysis

Table 2-2 shows the sedimentation velocity data of the deer major hemoglobin components II, III, V, and VII as a function of pH. Component VII was the only one which showed any difference in dissociation properties on comparison with the other major components. The $s_{20,w}$ value for component VII at pH 10.1 was 3.4 S compared to values of approximately 4.0 S for components II, III, and V. At pH 10.3 the $s_{20,w}$ value for component VII was 2.8 S, whereas the corresponding values for components II, III, and V were 3.3 - 3.4 S. (The values for human hemoglobin A at pH 10.1 and 10.3 are about 4.0 S and 3.8 S, respectively). Thus, component VII appears to dissociate into dimers more readily around pH 10 than the other major components which, in turn, are slightly more dissociated than human hemoglobin A in this pH range. The sedimentation coefficients of the deer hemoglobins at pH 10.7 are similar to those observed for human hemoglobin A (Table 1-1) under the same conditions. At pH 11.2 the $s_{20,w}$ values indi-

Figure 2-2

Starch gel electrophoresis of hemoglobin from hemolysates from a random sampling of deer. Identification of hemoglobin components was done as described in the text. The electrophoresis was at pH 9.0, and the gel was stained with o-dianisidine. Roman numerals and roman numerals plus arabic numerals refer to deer hemoglobin components.


⊖
6036
I-2
I-3
II
I-5
III
V
I-7
VII
⊕

cate that dissociation into dimers is complete for all the deer major hemoglobin components. Comparison of the sedimentation data for the deer hemoglobin components in Table 2-2 with that for human hemoglobin A in Table 1-1 shows slightly higher values above pH 11.2 for all of the deer hemoglobins. In citrate-Na_2HPO_4 buffer at pH 4.9 component VII was found to be completely denatured following dialysis and centrifugation. The hemoglobin solutions of the other deer components were brownish in color after 24 hours of dialysis; however, upon centrifugation their schlieren patterns were symmetrical and showed no signs of extensive denaturation. The $s_{20,w}$ values for these components were about 3.5 S, compared to 4.0 S for human hemoglobin A.

Sedimentation velocity analyses of the major hemoglobin components II, III, V, and VII as a function of increasing NaCl concentration is shown in Table 2-3. The observed sedimentation coefficients were corrected to 20° in water by two methods. In the first, the partial specific volume of hemoglobin at 25° is taken to be 0.751 ml per g at all concentrations of sodium chloride. This first method assumes that preferential binding of solvent or salt to the hemoglobin molecule is negligible. The second method takes preferential solvent interaction into consideration. This was done by correcting the observed sedimentation coefficients using Kellett's values (Kellett, 1971) for the apparent specific volume of hemoglobin in NaCl solutions of increasing molarity. The

Table 2-2

Comparison of $s_{20,w}$ values[a] of liganded deer hemoglobin components II, III, V, and VII as a function of pH.

a. A final hemoglobin concentration of 0.5 g/100 ml was used in all experiments.

b. The composition of the buffers is given under "Materials and Methods;" pH measurements were made at 25°.

Table 2-2

pH[b]	II	III	V	VII
4.9	3.54	3.46	3.50	Denat.
5.9	4.05	4.07	4.15	3.99
6.9	4.03	4.10	4.23	4.12
7.9	4.35	4.50	4.49	4.40
8.9	4.43	4.47	4.47	4.44
9.4	4.34	4.34	4.33	4.38
9.8	4.28	4.12	4.20	4.23
10.1	3.97	4.02	4.05	3.42
10.3	3.40	3.34	3.35	2.77
10.7	2.71	2.72	2.80	2.75
11.2	2.48	2.52	2.60	2.59
12.0	2.42	2.45	2.44	2.40

Table 2-3

Comparison of $s_{20,w}$ values[a] of liganded deer hemoglobin components II, III, V, and VII as a function of increasing molarity of NaCl.

a. A final hemoglobin concentration of 0.5 g/100 ml was used in all experiments.
b. The analyses were made at pH ~7.2 and at 25°.
c. The partial specific volume of hemoglobin, $\bar{V}$, was taken as 0.751 ml/g.
d. The values for the apparent specific volume of hemoglobin, ϕ, at increasing concentrations of NaCl are given in the text.

Table 2-3

Component	II			III			V			VII		
NaCl[b] Conc.		$s_{20,W}$			$s_{20,W}$			$s_{20,W}$			$s_{20,W}$	
	s_{obs}	$\bar{v}^c$	ϕ^d	s_{obs}	$\bar{v}^c$	ϕ^d	s_{obs}	$\bar{v}^c$	ϕ^d	s_{obs}	$\bar{v}^c$	ϕ^d
0.1	4.98	4.53	4.53	4.96	4.51	4.51	4.92	4.48	4.48	4.80	4.37	4.37
0.25	4.64	4.35	4.48	4.71	4.42	4.55	4.74	4.44	4.58	4.69	4.40	4.53
0.5	4.22	4.18	4.79	4.37	4.33	4.58	4.68	4.64	4.90	4.51	4.47	4.73
0.75				4.07	4.26	4.68	4.02	4.20	4.62			
1.0	3.63	4.03	4.54	3.67	4.07	4.58	3.63	4.03	4.54	3.64	4.04	4.55
1.25				3.33	3.90	4.41	3.25	3.81	4.30			
1.5	3.06	3.82	4.29	3.22	4.02	4.51	3.21	4.00	4.50	3.11	3.88	4.36
2.0	2.66	3.75	4.46	2.70	3.80	4.53	2.73	3.85	4.58	2.60	3.66	4.37
2.5				2.42	3.93	4.71	2.39	3.88	4.64			

values used for the apparent specific volume of hemoglobin, ϕ, in 0.1, 0.25, 0.5, 0.75, 1.0, 1.25, 1.5, 2.0, and 2.5 M NaCl were 0.751, 0.764, 0.770, 0.775, 0.775, 0.775, 0.779, and 0.780 ml/g, respectively. A comparison of the values obtained by either of the two methods shows the extent of dissociation of the deer major hemoglobin components to be similar to one another and also to human hemoglobin A over the NaCl concentration range of 0.1 to 2.5 M.

A limited number of sedimentation velocity experiments were done with the deer minor hemoglobin components I-2 and I-7 as a function of both pH and increasing concentrations of NaCl. Table 2-4 shows that the minor components have $s_{20,w}$ values which are similar to those of their major hemoglobin counterparts. For example, comparison of the sedimentation coefficients at pH 10.1 and pH 10.3 shows the minor component I-7 to be dissociated to the same extent as the major component VII, and the minor component I-2 to be dissociated to the same extent as the major component II.

Deoxygenation of deer hemoglobin component III in 0.1 M NaCl showed an $s_{20,w}$ value of 4.3 S. In 0.5 M NaCl deoxyhemoglobin component V showed an $s_{20,w}$ value of 4.4 S when $\bar{V}$ was 0.751 ml/g and 4.7 S when ϕ was taken as 0.764 ml/g and thus revealed no differences in sedimentation properties when compared either to each other or to the corresponding liganded species at the same concentration of NaCl.

Table 2-4

Comparison of $s_{20,w}$ values[a] of liganded deer hemoglobin components I-2 and I-7 as a function of pH and increasing molarity of NaCl.

a. A final hemoglobin concentration of 0.5 g/100 ml was used in all experiments.

b. The composition of the buffers is described under "Materials and Methods."

c. pH measurements were made at 25°.

d. The partial specific volume of hemoglobin, $\bar{V}$, was taken as 0.751 ml/g.

e. The values of the apparent specific volume of hemoglobin, ϕ, at increasing concentrations of NaCl are given in the text.

Table 2-4

Solvent[b]	Concentration M	pH[c]	I-2			I-7		
			s_{obs}	$s_{20,W}$		s_{obs}	$s_{20,W}$	
				$\bar{v}$[d]	ϕ[e]		$\bar{v}$[d]	ϕ[e]
NaCl	0.1	7.3	4.65	4.23	4.23	4.67	4.25	4.25
NaCl	0.5	7.3	3.90	3.87	4.35	4.13	4.09	4.88
NaCl	1.0	7.3	3.52	3.91	4.40	3.60	4.00	4.50
Buffer	0.20	6.9	4.22	3.90		4.12	3.81	
Buffer	0.10	7.9	4.58	4.23		4.51	4.17	
Buffer	0.12	10.1	4.17	3.88		3.56	3.31	
Buffer	0.11	10.3	3.65	3.39		3.11	2.89	
Buffer	0.10	10.7	2.88	2.67		2.86	2.65	

C. Viscosity

Viscosity measurements of the cyanferri derivatives of the deer hemoglobin components II, II + I-2, and III + I-3 + VII + I-7 in 0.1 M NaCl were done at 25° over a protein concentration range of 1.5 to 10 g per 100 ml in order to investigate the possibility of detecting aggregation in components II, and II + I-2, and also to determine the effects (if any) of the presence of component VII. The data shown in Figure 2-3 suggest that the components studied have similar reduced viscosity values, at least up to hemoglobin concentrations of 10 g per 100 ml. The reduced viscosity varied only slightly in a linear manner with hemoglobin concentration. The value of the intrinsic viscosity $[\eta]$ obtained by extrapolation of the data to zero protein concentration appeared to be the same for all deer hemoglobin types studied. In addition, the value for the intrinsic viscosity of 3.38 ml per g is within the range of 3.3 to 4.0 ml per g which is considered characteristic of compact, globular proteins (Tanford, 1961).

Viscometry was also done with deer components III + I-3, and components III + I-3 + VII + I-7 at pH 10.3 (Fig. 2-4). Both hemoglobin types showed a small, linear dependence of reduced viscosity upon hemoglobin concentration. Intrinsic viscosity values were 3.50 ml per g for components III + I-3 and 3.10 ml per g for components III + I-3 + VII + I-7.

Figure 2-3

Intrinsic viscosities of deer cyanferrihemoglobins in 0.1 M NaCl, pH ~ 7.2, 25°. ○, component II; □, component II + I-2; △, components III + I-3 + VII + I-7.

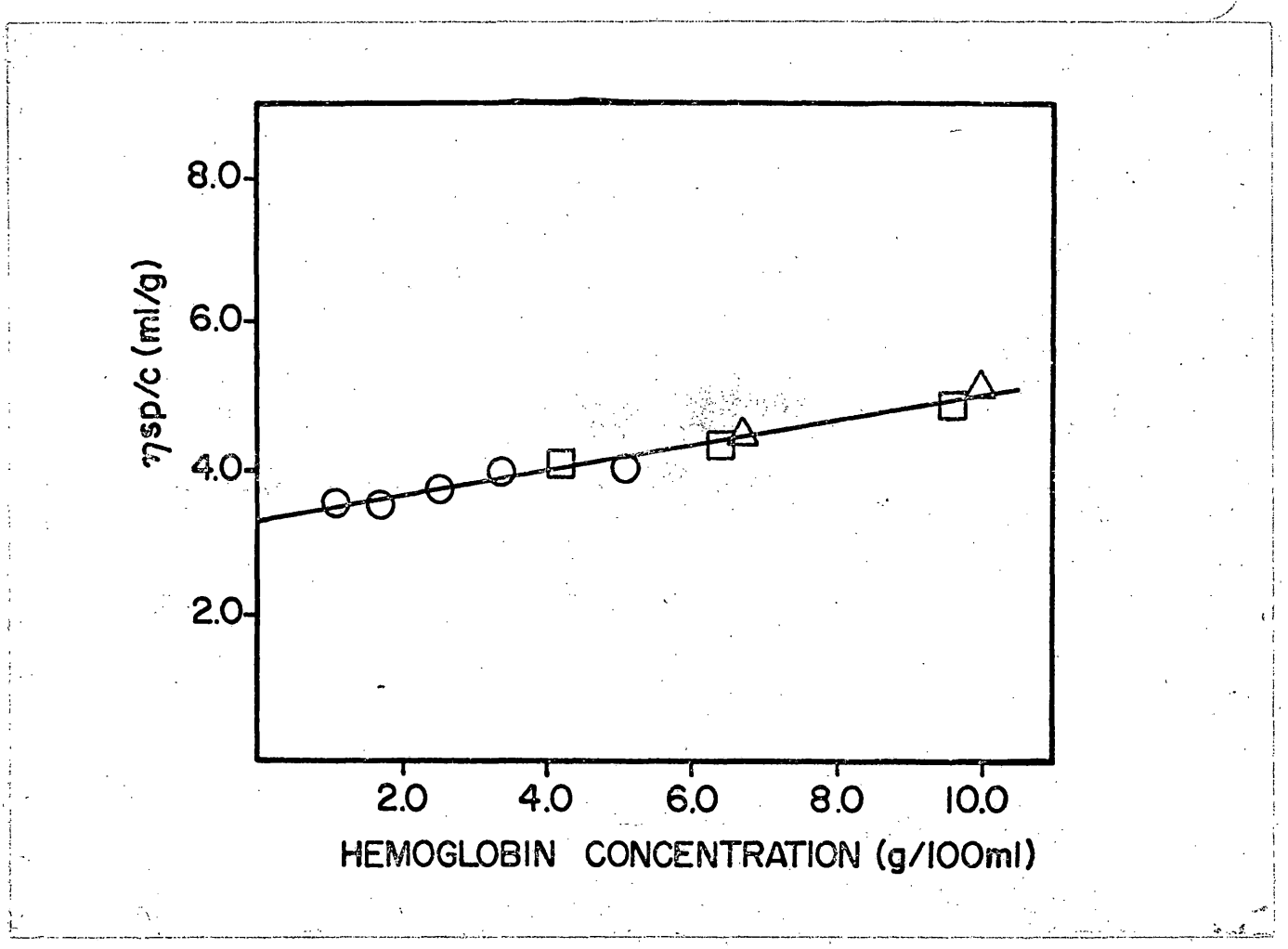
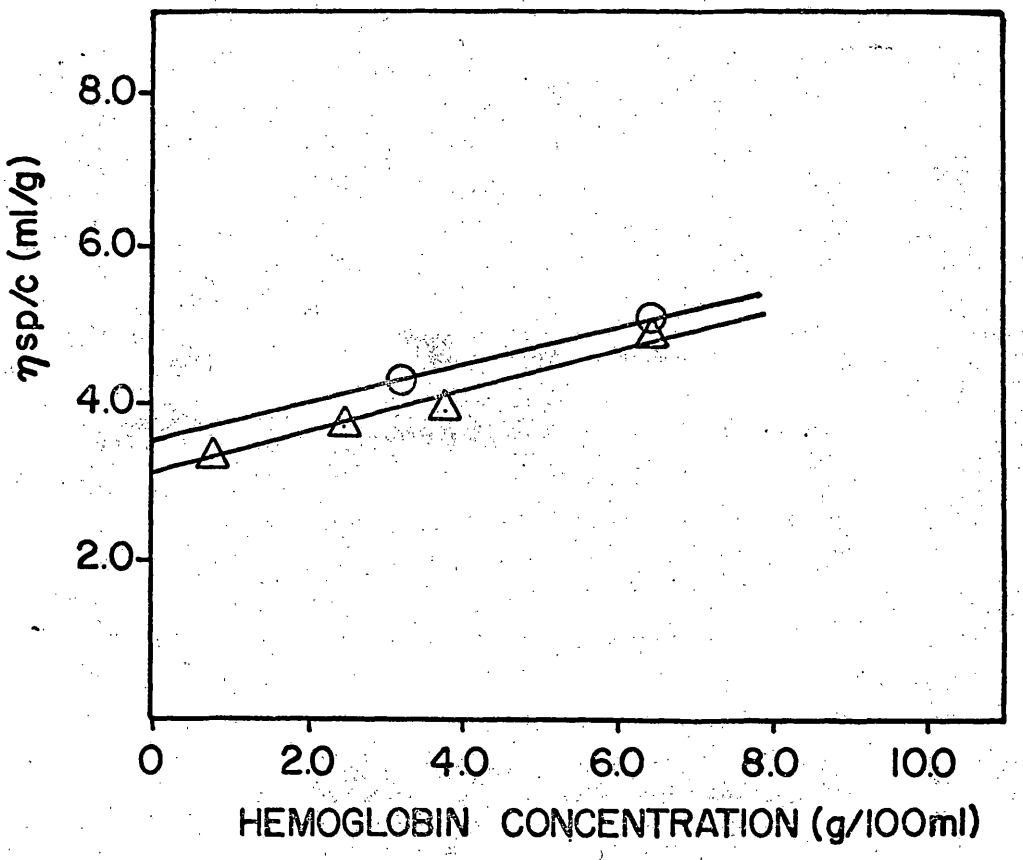

8.0
6.0
4.0
2.0
ηsp/c (ml/g)
2.0
4.0
6.0
8.0
10.0
HEMOGLOBIN CONCENTRATION (g/100ml)

Figure 2-4

Intrinsic viscosities of deer cyanferrihemoglobins in 0.1 M NaCl - Glycine - NaOH, pH 10.3, 25°. ○ , components III + I-3; △ , components III + I-3 + VII + I-7.


8.0
6.0
4.0
2.0
ηsp/c (ml/g)
0
2.0
4.0
6.0
8.0
10.0
HEMOGLOBIN CONCENTRATION (g/100ml)

DISCUSSION

The investigation of the deer hemoglobin components described in this part of the thesis was done primarily in an attempt to gain some insight into the nature of the forces responsible for hemoglobin polymer formation associated with the sickling of erythrocytes. As a secondary objective, comparative association-dissociation studies of the deer hemoglobin components were done to see the effect of amino acid variations in general positions on the subunit behavior of the hemoglobin molecule. The investigations consisted of (1) sedimentation velocity experiments over a pH range of 4.9 - 12.0, and over a NaCl concentration range of 0.05 to 2.5 M at neutral pH, and (2) viscosity measurements in 0.1 M NaCl at neutral pH, and in a NaCl-glycine-NaOH buffer, pH 10.3.

Sedimentation velocity analyses of the deer hemoglobin components associated with sickling showed no indication of hemoglobin polymerization at the protein concentrations used in these studies (0.5 g per 100 ml). $s_{20,w}$ values above a maximum of 4.5 S (weight average for the tetramer) were not observed; nor was there any suggestion of the heterogeneity characteristic of aggregation phenomena in the schlieren patterns. In fact, the schlieren peaks were found to be symmetrical, except at pH 10.1 to pH 10.3 where the equilibrium concentrations of dimers and tetramers were both appreciable (at pH 10.3 component VII was completely dimeric and therefore, the peak was symmetrical). At hemoglobin concentrations below the critical concentration for gel formation the same forces of attraction which

cause polymerization must still exist, although quantatively to a lesser degree. The detection of potential small differences in $s_{20,w}$ values between the deer hemoglobin components associated with sickling and those known to preclude sickling were not possible using the techniques described in this study.

In an attempt to detect hemoglobin polymer formation and/or conformational differences at protein concentrations above 0.5 g per 100 ml, viscosity measurements were done with mixtures of deer cyanferrihemoglobin components in 0.1 M NaCl at neutral pH and in a NaCl-glycine-NaOH buffer, pH 10.3. The results of the viscosity data under both conditions showed a linear relationship and only a slight dependence of relative viscosity on hemoglobin concentration (up to 10 g per 100 ml); thus, in the concentration range investigated, hemoglobin polymer formation could not be detected. Viscosity measurements were also attempted at higher hemoglobin concentrations (25 g per 100 ml) but resulted in precipitation of hemoglobin during the viscosity measurements. Precipitation was observed in both the sickling and nonsickling components and was possibly due to denaturation of the hemoglobin caused by the high shear stress of the capillary-type viscometers.

The sedimentation properties of deer hemoglobin components II, III, and V were found to be similar to one another in the pH range 4.9 - 12.0. At pH 10.1 to pH 10.3, the deer components II, III, and V dissociate into dimers to a greater extent than human hemoglobin A (Tables 2-2 and 1-3), while deer hemoglobin

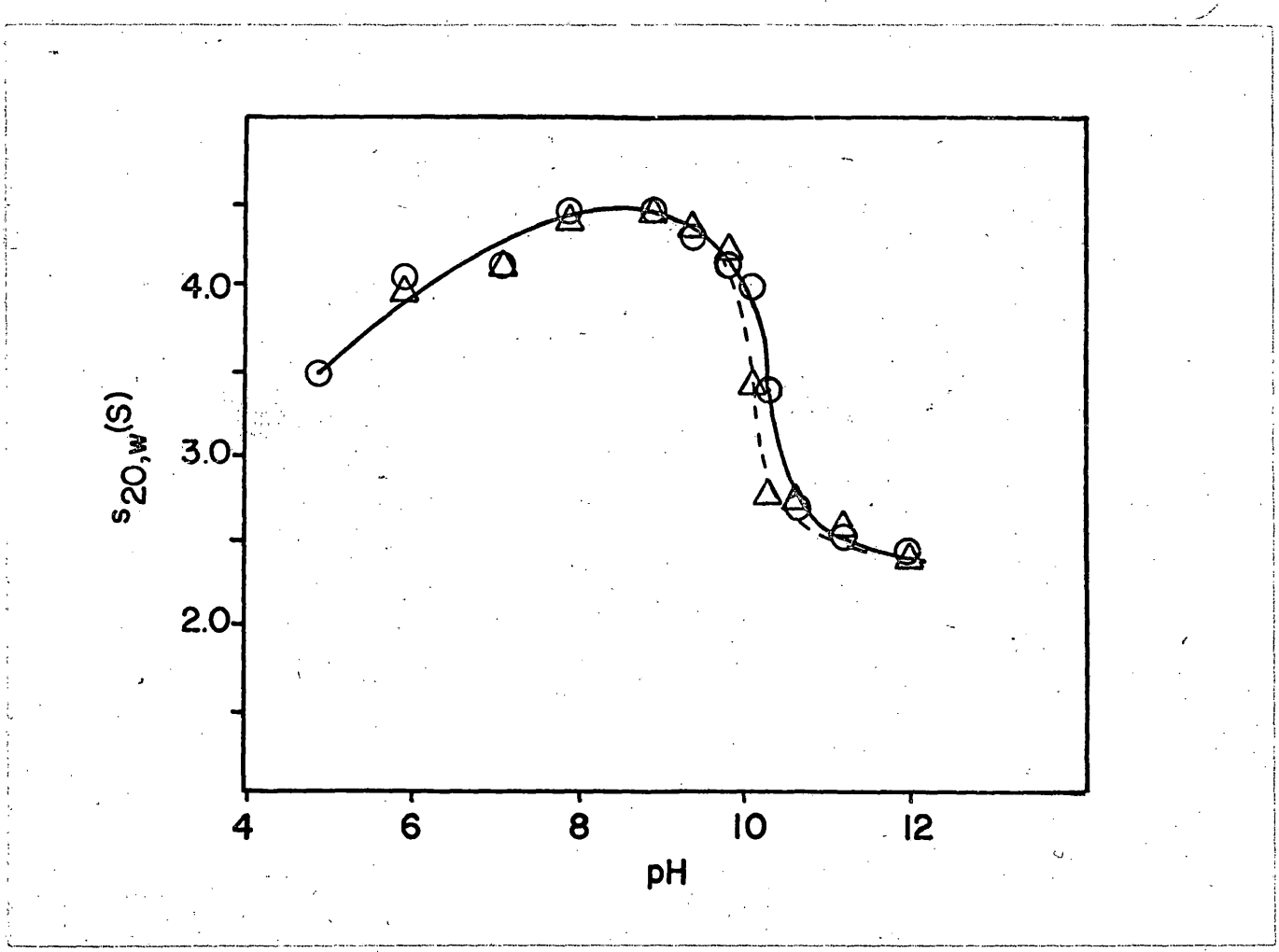
component VII has an even greater tendency to form the dimeric species in this pH range (Fig. 2-5). An increase in the degree of dimer formation similar to that seen in deer component VII has previously been observed in other animal hemoglobins (Gottlieb, 1967; Hanlon, 1971).

A possible explanation of the increased tendency to dissociate around pH 10 may be that charge differences on the various deer hemoglobin molecules cause small changes in the pK values of the tyrosyl residues, $\alpha 42$ and $\alpha 140$, which participate in the α^1-β^2 contact in both liganded and unliganded hemoglobin (Perutz et al, 1968; Bolton and Perutz, 1970). Such charge differences may additionally cause slight conformational changes which make the α^1-β^2 contact either more or less accessible to water and small ions, thereby increasing or decreasing the tendency to dissociate. Although amino acid sequence data for the deer hemoglobins are not yet available and positive identification of the tyrosyl residues in positions $\alpha 42$ and $\alpha 140$ has not been made, the residues which participate in the α^1-β^2 contact have been found to be invariant in all mammalian hemoglobins sequenced to date.

Above pH 11.2 the deer hemoglobin components appear to have less tendency to form monomers than human hemoglobin A, although this was not rigorously established by comparing each deer component against human hemoglobin A. Below pH 5.9 the deer components dissociated to a greater extent into dimers (component VII was completely denatured at pH 4.9) than did human hemo-

Figure 2-5

The dependence of the sedimentation coefficients of the deer hemoglobin components upon pH. ○, deer hemoglobin components II, I-2, III, and V; △, deer hemoglobin components VII, I-7.


4.0
3.0
2.0
$s_{20,w}$(S)
4
6
8
10
12
pH

globin A. Similar results have also been reported for bovine hemoglobins above pH 11.2 and below pH 5.9[2] (Hanlon, 1971; Shreffler, 1962). The amount of ferrihemoglobin present in the deer components was not determined and could explain the increased degree of dissociation below pH 5.9, since ferrihemoglobin is very susceptible to acid denaturation. Until completion of the amino acid analyses, the apparent increased stability of the deer hemoglobin components towards alkali can only be interpreted as an increase in strength of the forces comprising the α^1-β^1 contact.

$s_{20,w}$ values of deer hemoglobin components I-2 and I-7 were studied over a limited pH range and found to be similar to the corresponding $s_{20,w}$ values of components II and VII, respectively. Components II and I-2 and components VII and I-7 were shown by starch gel electrophoresis to have different α chains but the same β chains (Kitchen et al, 1966); consequently, the sedimentation properties of components VII and I-7 appear to be characteristic of the type of β chain.

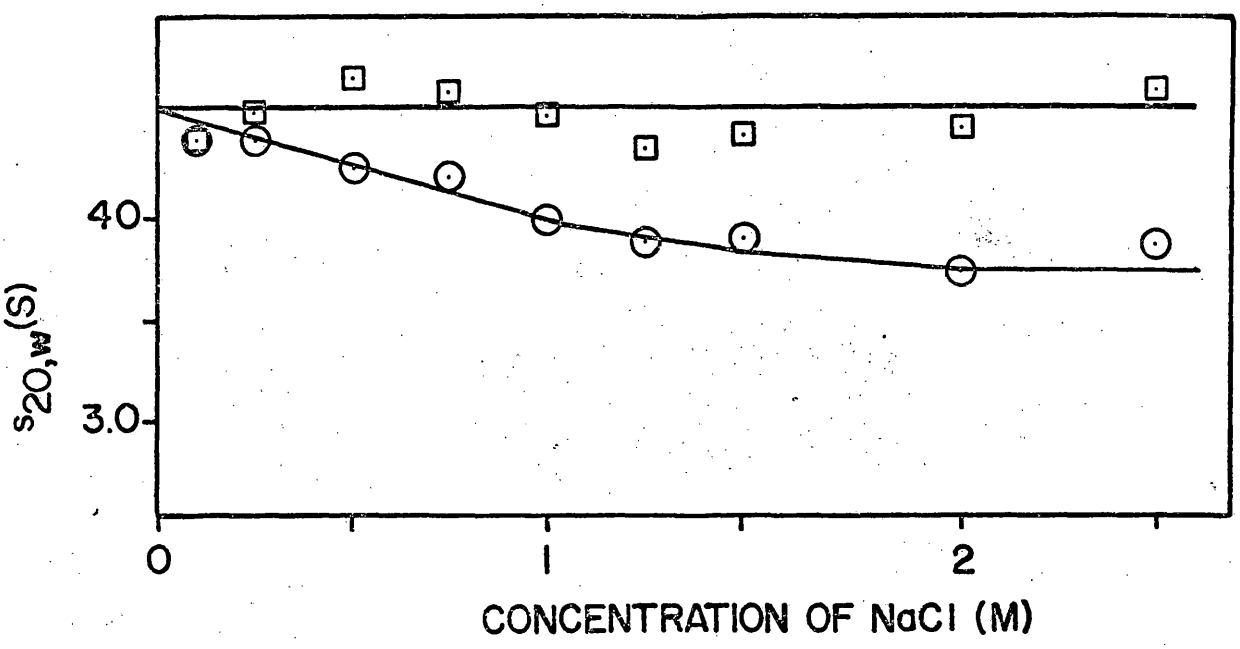
The sedimentation properties of all the deer hemoglobin components were found to be similar in NaCl solutions of increasing molarity. The observed sedimentation coefficients of the deer hemoglobin components were corrected to standard conditions using the values of Kellett (Kellett, 1971) to correct for the preferential binding of water at high salt concentrations and in addition, corrected to standard conditions by assuming

2. Smith, L. L., Private communication.

a constant partial specific volume for the hemoglobin molecule. When the values of Kellett were used, neither the liganded nor the unliganded deer hemoglobin components were found to be dissociated by high concentrations of NaCl (Fig. 2-6). However, when a constant partial specific volume was assumed, the molecules were found to form increased concentrations of dimers with increasing concentrations of NaCl. These findings are the same as those seen for human hemoglobin A (see part 1, "Discussion"). Nonetheless, for the comparative purposes of this research, this discrepancy does not affect the interpretation of results. The extent of dimer formation is directly related to the amino acid residues making up the α^1-β^2 contact (Guidotti et al, 1963) and indirectly to amino acid substitutions which cause conformational changes or charge differences in the α^1-β^2 contact region (Smith et al, 1970). The lack of effect of NaCl on the comparative association-dissociation study of the deer hemoglobin components suggest that the α^1-β^2 contact is similar in the deer hemoglobin components and also similar to the α^1-β^2 contact in human hemoglobin A, with the increase in dimer formation seen in components VII and I-7 at pH 10.1 to pH 10.3 due to charge differences, and/or conformational differences, on the molecule.

Figure 2-6

The dependence of the sedimentation coefficients of the deer hemoglobin components II, III, V, VII, I-2, and I-7 upon NaCl concentration. The data in NaCl were obtained at pH ~ 7.2. □ , the observed sedimentation coefficients were corrected to standard conditions using the values of Kellett (Kellett, 1971) for the apparent specific volume of hemoglobin, ϕ , as described in the text. ○ , the observed sedimentation coefficients were corrected to standard conditions assuming a constant partial specific volume for hemoglobin, $\bar{V}$, of 0.751 ml/g.


40
3.0
$s_{20,w}$(S)
0
1
2
CONCENTRATION OF NaCl (M)

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