INTERMOLECULAR INTERACTIONS OF OXYGENATED SICKLE HEMOGLOBIN MOLECULES IN CELLS AND CELL-FREE SOLUTIONS

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ABSTRACT We have measured the intermolecular interactions of oxygenated sickle hemoglobin molecules in cells and in cell-free solutions, and have compared the results with similar data for liganded normal adult hemoglobin. The experiments involve the measurement of the spin-lattice relaxation time T_1 of protons of solvent water molecules, as a function of an externally applied static magnetic field. From such data, one can derive a correlation time τ_c, for each sample, which is a measure of the time taken for a hemoglobin molecule to randomize its orientation due to Brownian motion. Thus τ_c is a measure of the freedom of rotational motion, on a molecular or microscopic level, of hemoglobin molecules. Intermolecular interactions will reduce this freedom of motion and lengthen τ_c. We find that oxygenated sickle hemoglobin molecules have an additional intermolecular interaction not found for normal hemoglobin. This extra interaction is increased by the presence of either inorganic phosphate or diphosphoglycerate, and is greater for sickle hemoglobin within cells than in cell-free solutions. By comparing the present results with published data on the viscosity of oxygenated sickle and normal hemoglobin, we conclude that, at concentrations comparable to intracellular values, oxygenated sickle hemoglobin molecules form aggregates several tetramers in size. The possibility exists that these aggregates are the earliest stage of fiber formation itself, the physical basis of the sickling phenomena.

INTRODUCTION

The physiological properties of red blood cells are sensitive to intermolecular interactions of hemoglobin molecules within cells. For example, the flexibility of red cells, and hence the rheological (flow) properties of whole blood, depends very much on the viscosity of the intracellular hemoglobin solution, which in turn depends upon the hemoglobin intermolecular interactions (1, 2). A notable example of the influence of intermolecular interactions on blood rheology is of course sickle cell disease, a result of the aggregation, upon deoxygenation, of molecules of sickle hemoglobin (Hb S) into microtubules with the concomitant formation of characteristic rigid and deformed

(sickled) cells (reviewed in ref. 3). The deformed cells impede normal patterns of blood flow, particularly in smaller capillaries. In addition, the anomolous oxygen-affinity behavior displayed by sickle cell anemia (Hb SS) erythrocytes (4) may well be another expression of the enhanced intermolecular interactions of Hb S.

Intermolecular interactions restrict the freedom of motion of macromolecules, including the continued rotational tumbling motion of the molecules produced by thermal agitation (Brownian motion). We have recently used measurements of magnetic relaxation, a technique related to magnetic resonance (5), to investigate properties of hemoglobin solutions and erythrocyte suspensions (6). The technique is known to give a measure of the orientational relaxation time of protein molecules in solution, i.e. the time required for a molecule to lose memory of its initial orientation because of thermal agitation. It was demonstrated that, for cell suspensions, the technique can be used to measure the relaxation of intracellular hemoglobin, even though one measures the behavior of water molecules external to cells.

In view of the demonstrated importance of interactions of hemoglobin molecules in the pathogenesis of human disease states involving Hb S, we have carried out magnetic relaxation measurements to study the freedom of motion of Hb S molecules in cells and in cell-free solutions. We have previously shown (6) that the mean orientational relaxation time of intracellular normal adult hemoglobin (Hb A) is not demonstrably different from cell-free hemoglobin solutions of similar concentrations. We now report that Hb S molecules, under conditions of complete oxygenation, interact with each other more strongly than do Hb A molecules, and that this characteristic of Hb S molecules is accentuated within cells.

METHODS AND MATERIALS

Sample Preparation

Samples of oxy Hb A and oxy Hb S were prepared from freshly drawn venous blood (AA and SS) with EDTA added (2 mg/ml) to prevent coagulation. Erythrocytes were washed with 0.134 M phosphate buffer, pH 7.2, and lysed by the addition of 0.1 volume of toluene followed by shaking with glass beads. Membrane-free hemolysates were obtained by centrifugation at 90,000 *g* for 30 min and then passed through 50 × 4 cm Sephadex G-25 columns equilibrated with 0.15 M Tris or bis-Tris buffer. The eluted hemoglobins were concentrated by vacuum dialysis and solutions were stored in oxygen filled tubes. KCl, NaCl, 2,3-diphosphoglycerate (DPG), and potassium phosphate from stock solutions were added to aliquots of some concentrated hemoglobin solutions, as indicated in Table I.

Solutions of carbonmonoxy Hb A were similarly prepared, except that initial lysates were saturated with carbon monoxide, clarified, and concentrated solutions were ultimately dialyzed against 0.2 M phosphate buffer at various values of pH, as shown in Table I.

Suspensions of AA and SS erythrocytes were prepared as follows. Freshly drawn venous blood, oxygenated and adjusted to a packed cell volume of 60%, was centrifuged at 25°C in a Spinco SW 50.1 swinging bucket rotor (Beckman Instruments, Spinco Div., Palo Alto, Calif.) for 1 h at 270,000 *g*. Cells of low mean corpuscular hemoglobin concentration (MCHC) were obtained from the packed cell column immediately below the buffy coat, and high MCHC cells were obtained from the bottom of the packed cell column (7). Cells were resuspended in autologous plasma for determinations of MCHC by routine techniques at 35°C. Cells to be used for

TABLE I

PROPERTIES OF HEMOBLOBIN SOLUTIONS USED FOR RELAXATION MEASUREMENTS, AND VALUES FOR τ_C AT 35°C

Sample	[Hb]	pH	Buffer	τ_C
	mM			*ns*
Hb CO A	1.47	7.25	0.2M PO_4	56
"	1.56	5.9	"	60
"	1.67	8.5	"	49
"	1.70	7.7	"	64
"	2.17	7.2	"	56
"	2.28	5.9	"	66
"	2.92	7.1	"	69
"	3.17	5.9	"	66
"	3.77	7.1	"	89
Hb O_2 A	4.08	7.20	0.14 Tris, 0.02M NaCl, 0.25M PO_4	110
Hb CO A	4.19	5.94	0.2M PO_4	100
Hb O_2 A	4.20	7.52	0.15M Tris, 0.02M NaCl‡	110
Hb O_2 A	4.51	7.52	"	120
Hb CO A	5.52	5.88	0.2M PO_4	190
Hb O_2 A	5.59	6.69	0.14M bis-Tris, 0.009M DPG	240
Hb O_2 A	6.17	6.59	0.15M bis-Tris‡	310
Hb CO A	6.27	7.10	0.2M PO_4	330
Hb O_2 S	2.52	7.18	0.1M PO_4	68
"	4.47	7.11	0.14M Tris, 0.02M NaCl, 0.25M PO_4	180
"	4.65	7.51	0.15M Tris, 0.02M NaCl‡	160
"	4.90	7.14	0.1M PO_4	210
"	5.08	6.00	0.6M PO_4	500
"	5.18	7.28	0.1M PO_4	230
"	5.39	7.12	0.3M PO_4	380
"	5.51	6.70	0.14M bis-Tris, 0.009M DPG	370
"	5.63	7.28	0.1M PO_4	320
"	5.81	7.60	0.35M PO_4	700
"	5.86	7.15	0.1M PO_4, 0.33M KCl	370
"	5.91	7.10	0.1M PO_4	440
"	6.20	6.61	0.15M bis-Tris‡	460

*Hb CO A means carbonmonoxy Hb A, and HbO_2 means oxy Hb.
‡Isotonic preparations.

magnetic relaxation measurements were washed three times and resuspended in either of two buffer solutions: 0.01 M phosphate buffer, pH 7.4, made isotonic by addition of NaCl; or isotonic 0.05 M bis-Tris buffer, pH 7.34, containing 0.118 M NaCl and 0.004 M KCl. Both buffers contained 1.5 mg/ml glucose and autologous plasma at a concentration of 5% to preserve cell shape. Magnetic relaxation measurements and measurements of red cell indices were all made at the same time in order to eliminate effects resulting from changes of cell indices with time. Cell suspensions used for relaxation measurements were stored under oxygen and measurements

were made in sample tubes kept filled with oxygen at all times. In addition, optical spectra were taken to verify the oxygenation and assure that the amount of methemoglobin was negligible.

Magnetic Relaxation Measurements

We measure a magnetic property of the solvent water protons, the spin-lattice relaxation time T_1, as a function of the magnitude H_o of a static magnetic field in which the sample is immersed. A description of the apparatus and methods of measurement has been given previously (5). The relaxation measurements were all made on 0.6 ml of sample material in 1 cm outer diameter glass test tubes inserted in a temperature controlled environment within the magnetic field generated by the apparatus. All data presented are for samples at 35°C.

Interpretation of Relaxation Data

The dependence on magnetic field of the relaxation rate ($1/T_1$) of solvent protons in protein solutions has been well documented (5, 6, 8–10). For liganded hemoglobin, and other diamagnetic proteins, $1/T_1$ is greatest at low fields and decreases continuously with increasing field to a constant value for H_o above about 2,500 Oe, corresponding to about 10 MHz. (It has become traditional to express the magnitude of H_o in frequency units, the Larmor precession ("magnetic resonance" or NMR) frequency ν of the proton spins in magnetic field H_o. There are 4,258 Hz per oersted. Since the frequency dependence of a quantity is often called its dispersion, we will refer throughout to the "dispersion curve" of the magnetic relaxation, meaning thereby the full variation of $1/T_1$ with H_o). The dispersion curve inflects at a relaxation rate half way between the low and high field limiting values of $1/T_1$. From this frequency of inflection ν_I, we derive a "correlation time" $\tau_C = 1/2\pi\nu_I$, which is the main quantity of interest in the present experiments, since it is related directly to the freedom of rotational motion of the macromolecules.

For a given τ_C, the amplitude of the low field data is proportional to Hb concentration, though the proportionality depends somewhat on ionic strength and pH (6). The amplitude is, in essence, a measure of the interaction between the protein molecules and the solvent molecules. Though a detailed theoretical understanding of this interaction is not yet at hand (10), it is possible to perform a least squares fit of the data points on a single dispersion curve to a theoretical function, with four adjustable parameters (including ν_I), which is known from previous experience to give an excellent representation of the dispersion data (5, 6). Values derived for τ_C for each sample were obtained using such a fit. It is well established that τ_C is related by Stokes' Law and simple hydrodynamics to the orientational relaxation time by (5, 6, 10)

$$\sqrt{3}\tau_C \simeq \tau_R = 4\pi\eta r^3/kT, \tag{1}$$

where η is the solvent viscosity, T the absolute temperature, r the hydrated radius of a hemoglobin molecule (assumed spherical), k the Boltzmann constant, and τ_R the usual orientational relaxation time of a protein molecule, as obtained, for example, from the inflection frequency of dielectric dispersion data (11).

The effect of the mean lifetime of water molecules within the erythrocyte on the observed value of τ_C has been considered earlier (6). It was indicated that τ_C as measured in samples of cell suspensions is, in principle, not the same quantity as the value of τ_C for the intracellular hemoglobin solution; rather, the observed τ_C is somewhat reduced from its true value. It can readily be estimated from the theory given to be a negligible 3% for the present 35°C data, and the effect is ignored throughout.

RESULTS

Fig. 1 shows representative dispersion data for solutions of oxy Hb A and oxy Hb S to indicate the magnitude of the relaxation effects and the form of the dependence of H_o.

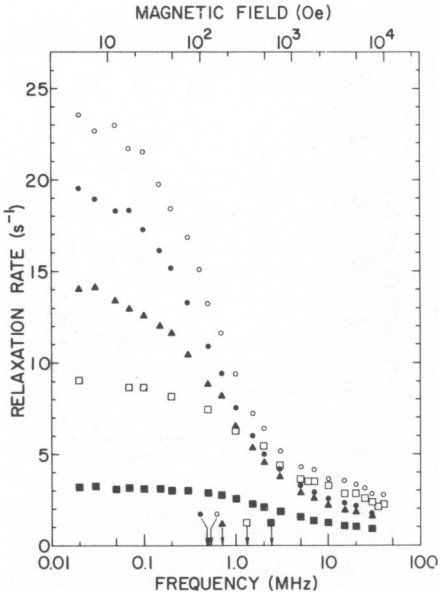

FIGURE 1

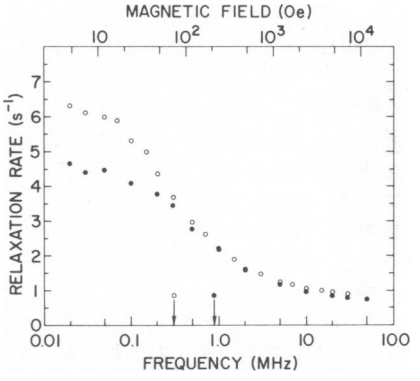

FIGURE 2

FIGURE 1 Magnetic field dependence of the solvent proton magnetic relaxation rate (relaxation dispersion) for representative solutions of oxygenated normal adult hemoglobin (oxy Hb A) and sickle hemoglobin (oxy Hb S) at 35°C. The magnitude of the field is indicated both in oersteds and in units of the Larmor spin precession (NMR) frequency of the protons in that field. ○, 6.17 mM Hb A, pH 6.59; ●, 5.63 mM Hb S, pH 7.28; ▲, 5.18 mM Hb S, pH 7.28; □, 4.51 mM Hb A, pH 7.52; ■, 2.52 mM Hb S, pH 7.2. The buffer compositions of the samples are in Table I. The inflection frequency ν_I of each dispersion curve is indicated by arrows along the frequency axis.

FIGURE 2 Relaxation dispersion data (cf. Fig. 1) for representative suspensions of washed and oxygenated AA and SS erythrocytes at 35°C. The cells are from fractions prepared by ultracentrifugation to restrict the range of mean corpuscular hemoglobin content (MCHC). ○, SS cells, MCHC = 5.8 mM, pH 6.95; ●, AA cells, MCHC = 5.9 mM, pH 7.07. The buffer compositions of the samples are in Table II. The inflection frequency ν_I of each dispersion curve is indicated by arrows along the frequency axis.

Data for numerous preparations, including carbonmonoxy Hb A, for a wide range of pH, concentration, and solvent compositions, are listed in Table I, together with values derived for τ_C for each sample. Fig. 2 shows similar data for suspensions of washed AA and SS cells. Data and τ_C values for these and other cell preparations are in Table II.

Fig. 3 summarizes the variation of τ_C with hemoglobin concentration both for Hb A solutions and AA erythrocyte suspensions. Note that τ_C depends only on the protein concentration, is unaffected by encapsulation of the hemoglobin by cell membrane, and is little affected by variations of pH, salt concentration, and buffer type and whether the iron ligand is CO or O_2. However, τ_C becomes longer as concentration increases, and is particularly sensitive to protein concentration at levels in the intracellular range. Thus, though the fractional variation of MCHC is only about 15%, the change of τ_C is over 50%, allowing for a reasonably sensitive intercomparison of τ_C

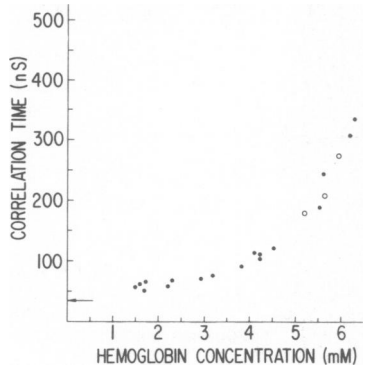

FIGURE 3

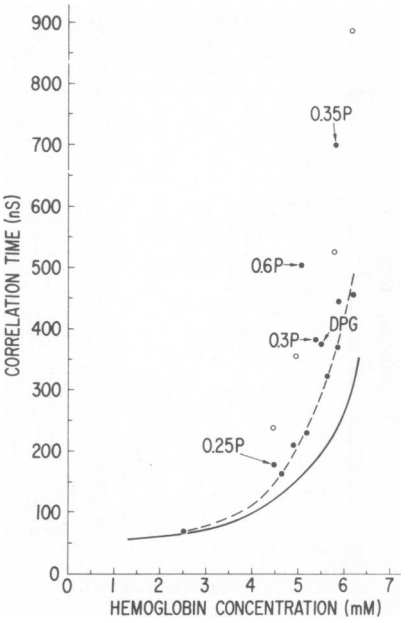

FIGURE 4

FIGURE 3 Variation with hemoglobin concentration of the correlation time (τ_C) for solutions of liganded Hb A (•) and suspensions of oxygenated AA erythrocytes (○) at 35°C. Each value of τ_C is obtained from the inflection frequency ν_I of a complete dispersion curve (cf. Figs. 1 and 2) using the relation $\tau_C = 1/2\pi\nu_I$. The points represent samples with differing values of pH and buffer composition as indicated in Tables I and II. The arrow at the lower left indicates the theoretical value for τ_C in the dilute limit, calculated using Stokes' Law for the orientational relaxation time of a particle the size of a hemoglobin molecule.

FIGURE 4 Variation with hemoglobin concentration of the correlation time (τ_C) for solutions of oxy Hb S (•) and suspensions of oxygenated SS erythrocytes (○) at 35°C. The solid line represents analogous data for hemoglobin A, Fig. 3. The points represent samples with differing values of pH and buffer composition, as indicated in Tables I and II. The dashed line is fit to data points for solutions containing either zero or 0.1 M phosphate, and no 2,3-diphosphoglycerate (DPG). Points representing solutions with greater phosphate content are indicated (P means potassium phosphate).

for intracellular hemoglobin solutions and cell-free hemoglobin solutions. The arrow at the lower left indicates the value of τ_C calculated using Eq. 1, and is the theoretically predicted value of τ_C in the limit of infinite dilution. The agreement between the value calculated for τ_C and the extrapolation of the experimental data to infinite dilution is one validation of our methods and their interpretation.

Fig. 4 shows τ_C values plotted against hemoglobin concentration for oxygenated Hb S solutions and SS cell suspensions. The solid line represents analogous results for Hb A taken from Fig. 3. The dashed line is drawn through data points for samples containing either 0 or 0.1M phosphate. There are three points to be noted: τ_C for any Hb S solution is, at high concentration, consistently longer than τ_C for Hb A solutions; τ_C increases with progressively increasing amounts of phosphate, and with DPG addi-

TABLE II

PROPERTIES OF ERYTHROCYTE SUSPENSIONS USED FOR RELAXATION MEASUREMENTS, AND VALUES FOR τ_C AT 35°C

	MCHC*	[Hb]	pH	τ_C
	mM	*mM*		*ns*
Top fraction AA cells	5.2	1.56	7.07	180
Unfractionated AA cells	5.6	1.91	7.06	210
Bottom fraction AA cells	5.9	2.02	7.16	290
Top fraction SS cells	4.5	1.27	6.95	240
"	5.0	1.36	6.95	360
Bottom fraction SS cells	5.8	1.68	6.95	530
"	6.2	1.94	6.95	890

*MCHC means mean corpuscular hemoglobin concentration. Cells were fractionated according to MCHC by ultracentrifugation. See text for details.

tion; and τ_C for suspensions of oxygenated SS cells is generally longer than for oxy Hb S solutions.

DISCUSSION

Figs. 1 and 2 show the behavior of the dispersion of the relaxation rate of solvent water protons for a variety of sample compositions. As indicated previously, it is possible to characterize the entire dispersion curve with four adjustable parameters. These are the values of $1/T_1$ in the high and low field limits, the inflection frequency ν_I, and a parameter that describes how steeply the curve drops in the intermediate field region (6). The first two parameters depend explicitly upon protein concentration, and the last gives an indication of polydispersity of the macromolecules in solution: a distribution of states of aggregation of the proteins would broaden the curve (5, 6). However, for the present purposes, it is only ν_I and the value of τ_C derived from it that is relevant.

The correlation time τ_C is a measure of the time it takes for a hemoglobin molecule to randomize its orientation by Brownian rotational diffusion. Direct aggregation and both electrostatic and hydrodynamic long range intermolecular interactions will increase τ_C from its Stokes' Law value, i.e., its value in the limit of infinite dilution, Eq. 1. These effects cause a dependence of τ_C on hemoglobin concentration. For homogeneous systems, the resulting variation is much like the concentration dependence of the macroscopic viscosity (measured as bulk viscosity in standard viscometers). However, characteristics of a heterogeneous system are different. For example, it has been shown (6) that in a gelled sample of deoxy Hb S near the temperature of the gelation transition, the majority of the protein molecules are quite free to rotate. The same is true for Hb A entrained in a gelatin gel (6). As the temperature is increased above the gelation transition of Hb S solutions, progressively more hemoglobin molecules become incorporated into microtubules, and the motion of those remaining in the

solution, as indicated by τ_C, becomes progressively more restricted. At 30°C essentially all the deoxy Hb S molecules are immobilized in microtubules (6). Thus τ_C is a measure of the rotational freedom of the protein molecules in their microscopic environment, and its value may well belie the macroscopic physical state of a heterogeneous sample: a drastic change in sample viscosity (e.g. by gelation) may not be indicated by τ_C. By contrast, alterations of the state of aggregation of the protein molecules in a homogeneous sample will change τ_C but not the sample viscosity. The reason is that macroscopic viscosity depends upon the volume fraction of protein in solution and not on the size of the individual macromolecular aggregates, so long as they remain approximately spherical (1).

Fig. 3 shows that τ_C for cell-free solutions of Hb A at 35°C is found to depend on protein concentration, but to be little influenced by ionic strength, buffer type, pH in the range used, 6–8.5, and by whether the iron ligand is O_2 or CO. Fig. 3 also shows that values for τ_C for Hb A inside AA erythrocytes fall on the curve for the cell-free solutions of Hb A, indicating that the encapsulation of a Hb A solution within a cell does not alter its microscopic hydrodynamic behavior.

We conclude that the orientational relaxation time of Hb A molecules in solution is the same in cell-free solutions and inside erythrocytes, and we expect that the macroscopic viscosity should behave similarly. Though no exact theory exists for the concentration dependences of τ_C and macroscopic viscosity at high concentrations, the expectation is that their behaviors would be similar since the concentration dependence in each case arises from the interference by one protein molecule with the streamline flow of solvent around a neighboring protein molecule. In fact, the solid line, Fig. 3, is very much like the viscosity dependence of Hb A solutions at the same temperature (cf. Fig. 8 in ref. 2), except that the fractional increase of τ_C is only about half the viscosity increase at a given concentration. This expectation, that the viscosities of intracellular and cell-free solutions of Hb A are similar, is demanded by studies of the (non-Newtonian) rheological properties of blood (1, 12) and packed red cells (13).

The behavior of oxy Hb S is quite different. As Hb S concentration increases above about 4 mM, τ_C for cell-free solutions of oxy Hb S becomes progressively greater than for Hb A; for oxy Hb S within SS erythrocytes, the increase is greater still (Fig. 4). Moreover, the effect in solutions appears to be enhanced by the presence of high concentrations of inorganic phosphates, and by DPG (Tables I and II). It is interesting to note that the presence of phosphate, which increases τ_C for the Hb S solutions, also favors the formation of deoxyhemoglobin, and one might infer that the observed increases of τ_C result from the partial formation of deoxy Hb S in our samples. This possibility is remote; the Hb S solutions were prepared under one atmosphere of O_2 to insure full oxygenation. Rather, changes in tertiary conformation brought on by phosphates are known for both liganded Hb A (14) and oxy Hb S (15). We suggest, then, that the addition of phosphates alters the conformation of oxy Hb S, including the state of certain surface residues (15) so that the liganded (oxy) Hb S molecules tend to aggregate, though not to the extent that nonliganded (deoxy) Hb S molecules do.

Our major conclusion is that the abnormally high value of τ_C shown by Hb S solu-

tions results from an intermolecular interaction not present between molecules of either oxy or carbonmonoxy Hb A. There is little other published evidence relating to this. Benesch et al. (16) have found that oxy Hb S is less soluble than oxy Hb A, particularly at high phosphate concentrations. On the other hand, there is no difference between the viscosities of oxy Hb A and oxy Hb S solutions (2). These data taken together indicate a tendency of oxy Hb S molecules to aggregate before reaching the solubility limit. This would increase τ_C, which is proportional to the volume of the tumbling aggregate, Eq. 1. However, as already stated, the dependence on concentration of macroscopic viscosity is on the volume fraction of protein in solution, and not on size or aggregation state of the particles, provided they remain roughly spherical (1). Thus limited aggregation of oxy Hb S would increase τ_C without affecting the viscosity, in agreement with observation.

The fact that τ_C increases no more than two- or threefold at the higher concentrations implies that on the average the oxy Hb S molecules are forming aggregates the size of two or three tetramers. The fact that the dispersion curves of $1/T_1$ for oxy Hb S solutions are not significantly broadened compared with the data for Hb A, Fig. 1, implies (5, 6) that the amount of aggregation is not highly heterogeneous, and that the average behavior is the typical behavior of each aggregate.

The reasons for τ_C being greater in oxygenated SS cells than in the equivalent cell-free solutions, Fig. 4, are unclear at the moment. It is known, for example, that the cellular concentration of DPG decreases with increasing MCHC (4), and we therefore discount this as a factor. Apparently the presence of the cell membrane influences the averaged properties of the hemoglobin within. The viscosity of oxygenated SS cells is greater than AA cells (2, 13) even though the viscosities of comparable cell-free solutions are the same. The increased viscosity of SS blood is known to depend on the presence of irreversibly sickled cells (ISC), which usually have elevated MCHC. The dependence of τ_C on MCHC could be a correlate of the fact that higher MCHC samples contain greater proportions of ISC. Lessin et al. (17) have reported that microaggregates of Hb S form on the membranes of ISC in the absence of oxygen. Perhaps the interaction of these microaggregates, or some other as yet unknown characteristic of ISC membranes, with cytoplasmic oxy Hb S can shift its equilibrium towards more aggregation. Such a propagating long range influence resulting from a Hb S-membrane interaction could explain our observations.

Wilson et al. (18) have investigated, by studying the scattering of laser light, pregelation aggregation of Hb S solutions upon deoxygenation. They have argued that the aggregation of deoxy Hb S molecules occurs by a linear condensation scheme in which aggregation proceeds by combination of two smaller aggregates, as opposed to aggregates growing by the repeated stepwise addition of a single molecule at the end of a chain. Presumably, the interactions of oxy Hb S molecules are sufficiently weak so that the rate of dissociation of a long aggregate (which should be proportional to its length) balances the formation rate, when the aggregate is only a few tetramers in size. This would account for our observations.

Cottam et al. (19) have investigated the gelation of deoxy Hb S by studying the trans-

verse or spin-spin relaxation rate T_2 of water protons in Hb S solutions and in samples of packed cells. The measurements were performed at one value of H_o, corresponding to 24.3 MHz. T_2 is another relaxation parameter which, for the conditions used, is essentially equal to 0.3 times the value of the low field limit of T_1 (10). Thus they have a single point on a dispersion curve, and cannot derive a correlation time which, as has been seen, gives information regarding the rotational motion of the macromolecules. Cottam et al. (19) also observe T_1 at the same H_o, and note that it gives little indication of the aggregation and gelation phenomena. Reference to Fig. 1 will show that 24.3 MHz is far above the magnetic field at which the major dispersion phenomena occur, and one should expect little influence of gelation on T_1 at this high a field. Moreover, the gelation phenomena has been investigated by dispersion measurements (6), and the detection of the gelation transition by T_1 dispersion measurements involves many subtle considerations.

In summary, we find that the orientational relaxation time of oxy Hb S molecules in cell-free solutions at high hemoglobin concentrations is longer than that of oxy or carbonmonoxy Hb A molecules in comparable solutions. Since the viscosities of solutions of oxy Hb S and oxy Hb A at the same concentration have been reported to be the same (2), we infer that the oxy Hb S molecules exist as aggregates about two or three tetramers in size at concentrations comparable to intracellular values. The aggregation is sensitive to the concentration of phosphate, and is greater for intracellular oxy Hb S than for oxy Hb S solutions. Aggregation of oxy Hb S molecules has not been previously reported, though evidence of states of aggregation of deoxy Hb S smaller than the usual microtubular fibers but larger than the present suggestion, has been presented (18–22). It would appear, then, that the intermolecular interactions of Hb S molecules that are responsible for gelation of deoxy Hb S, and ultimately for sickle cell disease, are present whether or not the Hb S molecules are oxygenated. The aggregation resulting from this interaction may precede gelation, but whether the contact sites on the protein molecules are the same as those involved in fiber formation is not known. As the molecules are forced towards the deoxy or T conformation, the interaction increases. A point may be reached where a phase transition becomes possible, if the conditions of concentration and temperature are right. Then a solid microtubular phase of Hb S forms in equilibrium with a solution phase (6, 23).

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