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Aggregation of Hemoglobin S and Hemoglobin C_{Harlem} With Nonsickle Hemoglobin in Concentrated Phosphate Buffer

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The effect of HbA and HbF on both the aggregation and deaggregation of deoxy HbS and deoxy HbC_{Harlem} in concentrated phosphate buffers was studied by a turbidimetric method. Although pure deoxy HbA is fully soluble in 2.4 *M* potassium phosphate buffer, the same concentration of deoxy HbAS was insoluble in the same buffer. Since there was little dissolved hemoglobin in the solute of a mixture of HbA and HbS, and since the intensity of turbidity was approximately twice that which would be expected from deoxy HbS alone, deoxy HbA must have coaggregated with deoxy HbS. Similar studies with mixtures of HbS and HbF showed different results. The rates of aggregation of deoxygenated mixtures of HbS and HbF were much slower than those of similar mixtures of HbS and HbA. Measurements of the absorption spectrum of the solute after centrifugation and the electrophoresis of both the aggregates and the solutes showed that a portion of the HbF was coaggregated with HbS, while some of the HbS was still in solution. The solubility of HbS and mixtures of sickle and nonsickle hemoglobins in 2.2 *M* phosphate buffer increased in the order of HbS, $HbAC_{Harlem}$, HbAS, HbC_{Harlem}, HbSF, and $HbFC_{Harlem}$.

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THE SICKLING of red cells containing hemoglobin S (HbS) is caused by the intracellular polymerization of deoxygenated HbS molecules.[1] Although the molecular mechanism of fiber formation is still not totally understood, in the past few years a number of important findings have been made regarding both the kinetic and structural aspects of the polymerization of deoxy HbS.[2-9] In addition, the nature of interactions between unlike hemoglobin molecules have been studied by examining the gelation properties of mixtures of HbS and other hemoglobins. Early studies by Singer and Singer[10] showed that the presence of other hemoglobins with HbS increased the minimum concentration of total hemoglobin required for gelation (minimum gelling concentration, MGC). The interaction of HbS with other hemoglobins has also been studied by measuring the viscosity[11] and solubility of mixtures.[12,13] Results of these studies suggest that the polymerization, viscosity, and solubility of deoxy HbS are affected by other hemoglobins. Among various hemoglobins examined, HbF is believed to inhibit red cell sickling. However, conflicting results have been reported on the mechanism of this effect. Bertles et al.[14] reported that in a centrifugation experiment, HbF was virtually excluded from the condensed gel phase, and Bookchin and Nagel[15] reported that in MGC experiments HbF in the deoxy state appeared not to participate in gelation either with deoxy HbC_{Harlem} (β6 Glu→Val, β73 Asp→Asn) or with deoxy HbS. On the other hand, results of solubility experiments by Goldberg et al.[16] showed that HbF copolymerized with HbS.

Recently we have developed a turbidimetric method for the determination of the kinetics of both the formation and solubilization of hemoglobin aggregates in concentrated phosphate buffer under controlled buffer and temperature conditions.[17-19] This method not only allows us to measure the amounts of deaggregated deoxy HbS and its mixtures with other hemoglobins using a small amount of hemoglobin sample, but also provides us with accurate data on the fraction of HbS and non-HbS components in both aggregates and solutes. In addition, the delay time prior to aggregation of deoxy HbS can be determined in concentrated phosphate buffer (below 2.0 *M*).[19] The purpose of this article is to compare the nature of the aggregation of mixtures of deoxy nonsickle and sickle hemoglobins in concentrated phosphate buffer (over 2.4 *M*) with that obtained in low phosphate buffer.

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MATERIALS AND METHODS

Hemoglobin

Heparinized blood was washed 3 times with 0.9% NaCl. Packed red cells were hemolyzed by adding 5 volumes of 5 m*M* potassium phosphate buffer, pH 7.4, containing 0.5 m*M* EDTA, and the stromata were removed by centrifugation at 27,000 *g* for 10 min.[20] HbS, HbA, and HbF were isolated from sickle cell trait and cord blood on DEAE Sephadex column by gradient elution in 40 m*M* Tris-HCl from pH 8.3 to pH 7.3.[17] HbC_{Harlem} (HbC_H) was isolated from $HbAC_H$ blood on CM Sephadex by gradient elution from 10 m*M* phosphate buffer, pH 6.0, to 20 m*M*, pH 8.0.[21] HbC_H was identified by fingerprints after tryptic and chymotryptic digestions and amino acid analysis.[21] All isolated hemoglobins were confirmed to show a single band on cellulose acetate electrophoresis at pH 8.4. Concentrations of oxyhemoglobin and deoxyhemoglobin were determined spectrophotometrically by using the millimolar coefficient of 60 at 577 nm (mME_{577}) and 50 at 555 nm (mME_{555}).

Turbidimetric Method

Measurements of the kinetics of both the aggregation and solubilization of deoxyhemoglobin were carried out using an anaerobic

photometric cuvette as described in previous reports.[17-19] The mixtures of oxy-forms of sickle hemoglobins were injected into an anaerobic cuvette containing concentrated phosphate buffer and sodium dithionite (10 mg/2 ml). The changes in the turbidity at 700 nm were determined after successive additions of aliquots of distilled water. Turbidity was measured with a Perkin-Elmer 124 double-beam spectrophotometer at 700 nm and recorded on a Perkin-Elmer 56 recorder. Absorption spectra of dissolved supernatant solutions in the cuvette were recorded between 500 and 700 nm after centrifugation of the cuvette at 2500 g for 20 min at room temperature (22°C).

Electrophoresis

The fractions of HbS and non-S hemoglobin in solution and in aggregates were determined electrophoretically by the following procedure. After centrifugation of the anaerobic cuvette, aggregated and dissolved forms of hemoglobin were clearly separated into two layers: the thin layer of hemoglobin aggregates on the top and the transparent solution on the bottom (solute). The dissolved hemoglobin portion in the bottom layer was removed gently with a Hamilton syringe. The aggregates suspended in a small portion of the solute were dissolved in water. After bubbling with carbon monoxide, these solutions were concentrated and dialyzed against cold distilled water. Electrophoresis of the resulting solutions was performed on cellulose acetate at pH 8.4, and the fractions of S and non-S hemoglobin were measured with a densitometer (Helena Laboratories, Beaumont, Texas).

RESULTS

Aggregation of a Mixture of HbS and Non-S Hemoglobin

In 2.4 *M* potassium phosphate buffer, pH 6.95, deoxy HbS aggregates and forms a stable homogeneous emulsion, whereas all the other hemoglobins, including HbA and HbF, are totally soluble.[12] Since dissolved hemoglobin has little absorption at 700 nm, the formation of aggregates can be semiquantitatively determined by the increase in the optical density at 700 nm.[17,18] It is quite interesting that if we mix HbA and HbS at the same concentration, both hemoglobins become insoluble in 2.4 *M* phosphate buffer, pH 6.95; the optical density at 700 nm increased to approximately twice that expected from the concentration of deoxy HbS.[18] Since the solution of a mixture of HbA and HbS in 2.4 *M* phosphate buffer after centrifugation is almost colorless, deoxy HbA must be coaggregated with deoxy HbS. This aggregation of deoxy HbA decreases when the ratio of HbS/HbA is decreased. As shown in Fig. 1A, when deoxy HbA is mixed with the same or greater amounts of deoxy HbS, the deoxy HbA is almost totally aggregated, whereas when HbA is mixed with lesser amounts of deoxy HbS, only a part of deoxy HbA is coaggregated with HbS. For instance, with a 75:25 mixture of HbA and HbS, only 50% of the total hemoglobin aggregates. It appears that one mole of HbA coaggregates with one mole of deoxy HbS. This was further confirmed by measuring the absorption spectrum of the solution after centrifugation (Fig. 1B). The dissolved hemoglobin in the solute exhibits the absorption spectrum of deoxyhemoglobin, and the amount of the soluble form of hemoglobin increases as the fraction of HbS is decreased.

Similar studies with mixtures of HbS and HbF provided us with somewhat different results. First, the

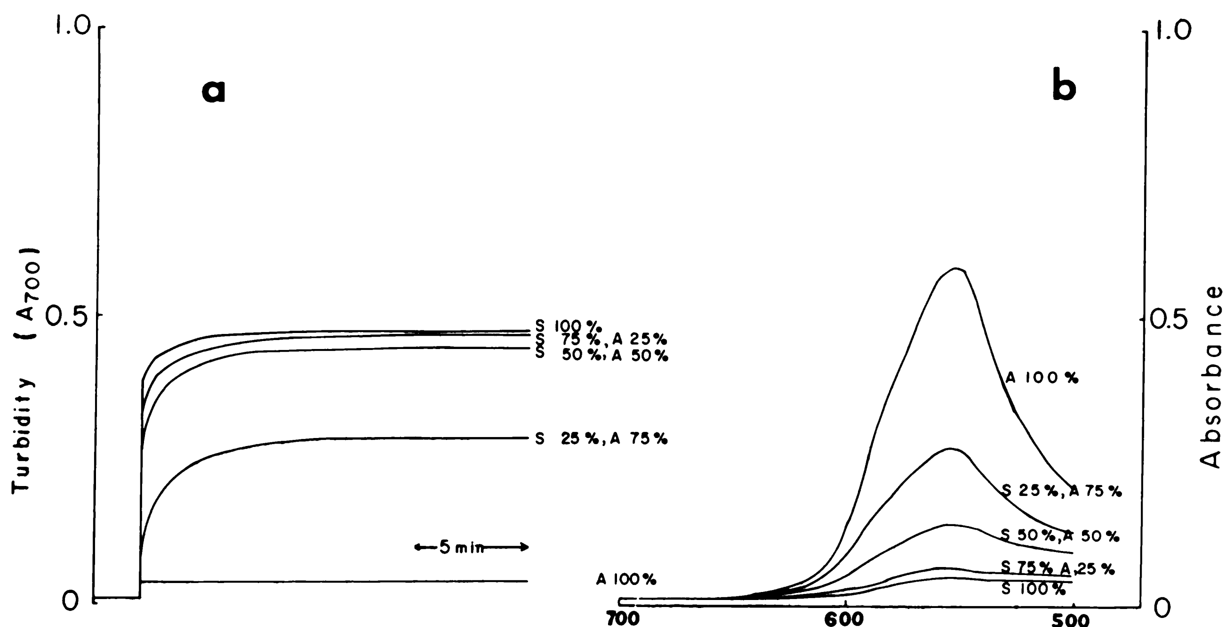

Fig. 1. Aggregation of mixtures of different rations of deoxy HbS and deoxy HbA in 2.4 *M* potassium phosphate buffer, pH 6.95, at ratios 22°C. The total amount of hemoglobin in each solution is 70 mg/dl. Absorption spectra of the solute after centrifugation are shown on the right side of the figure.

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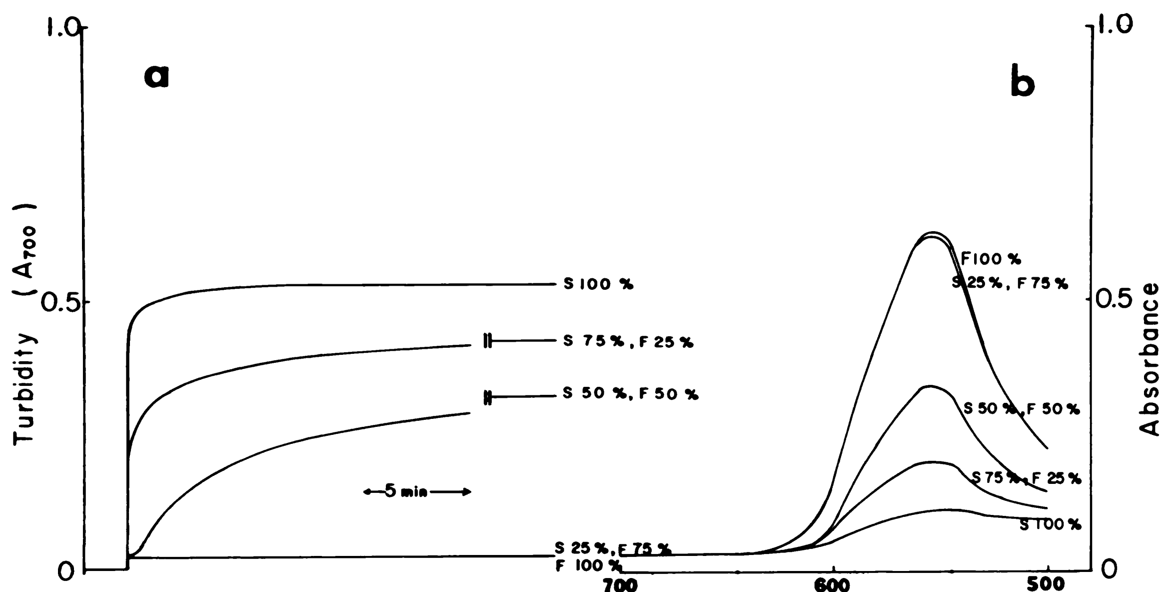

Fig. 2. Aggregation of mixtures of different ratios of HbS and HbF in 2.4 *M* potassium phosphate buffer, pH 6.95, at 22°C. Total amount of hemoglobin in each solution is 75 mg/dl. Absorption spectra of the solute after centrifugation are shown on the right side of the figure.

rate of aggregation of HbS and HbF is much slower than that of HbS with HbA (Fig. 2A). Second, HbF prevents, to some extent, the aggregation of hemoglobin. For instance, under the experimental condition described above with a 25:75 mixture of HbF and HbS, only 83% of the total hemoglobin aggregates; with a 50:50 mixture, there is 67% aggregation; and with a 75:25 mixture, there is no aggregation at all. These results were confirmed by measuring both the turbidity of the suspension and the absorption spectrum of the solution after centrifugation (Fig. 2B).

Electrophoresis of the aggregates and solute of a 50:50 mixture of HbS and HbF showed that the ratio of HbS/HbF in the aggregates was 68/32 and that in the solute was 23/77, indicating that part of HbF coaggregated with HbS and a portion of the HbS remained in the solution. Since pure HbS is totally insoluble in 2.4 *M* phosphate buffer, the HbS in solution may have been modified by interaction with HbF.

Solubilization Curve of a Mixture of HbS and Non-S Hemoglobin

The advantage of the turbidimetric method is that the rate of solubilization of aggregated hemoglobin can also be measured after successive additions of water into the cuvette. As shown in Figs. 3 and 4, the solubilization curve of aggregated HbS is shifted to the left as the fractions of HbA or HbF are increased. In these experiments, the turbidity at zero time was taken as 100% regardless of the presence of soluble hemoglobin, which has a slight absorption at 700 nm. The curve, therefore, indicates the ease of solubilization of the aggregated portion of hemoglobin in 2.4 *M* potassium phosphate buffer (Figs. 3 and 4).

Another type of solubilization curve was obtained after adding a 50:50 mixture of HbS and HbF in 2.8 *M* potassium phosphate buffer as the starting buffer.

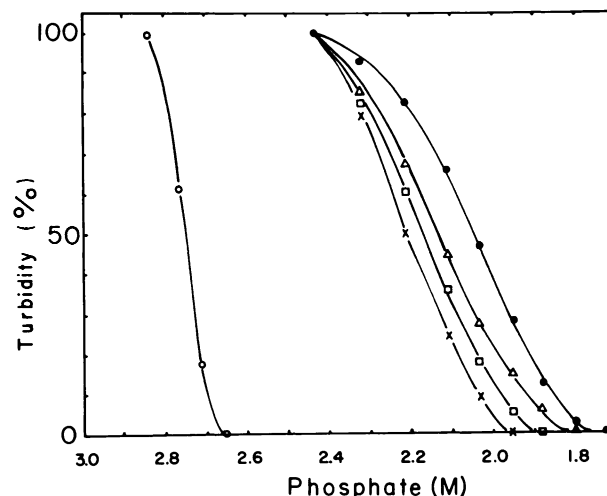

Fig. 3. Solubilization curves of different mixtures of deoxy HbA and deoxy HbS in concentrated phosphate buffer, pH 6.95, at 22°C. A small amount of oxy HbS was introduced into a deoxygenated cuvette containing 2.4 *M* potassium phosphate buffer and 10 mg of sodium dithionite.[19,20] The suspensions of deoxy HbS were titrated by adding aliquots (50–100 μl) or water.[19] The experiment with 100% HbA was started in 2.85 *M* potassium phosphate buffer. Total hemoglobin concentration in each solution was 70 mg/dl. (•—•) 100% HbS; (Δ—Δ) 75% HbS and 25% HbA; (□—□) 50% HbS and 50% HbA; (x—x) 25% HbS and 75% HbA; (O—O) 100% HbA.

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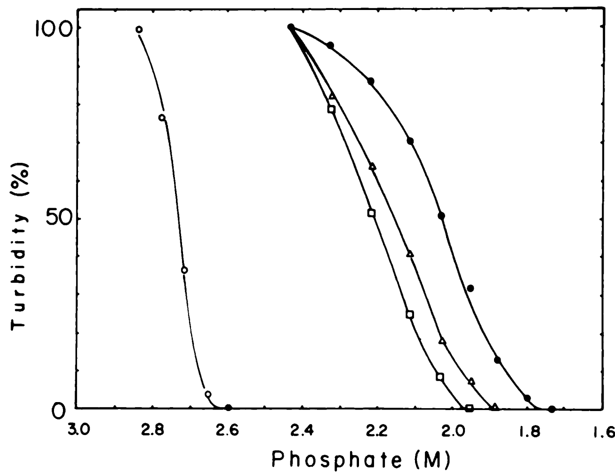

Fig. 4. Solubilization curves of different mixtures of deoxy HbS and deoxy HbF in concentrated phosphate buffer. Experiment with HbF was started in 2.8 *M* potassium phosphate buffer. Other conditions were the same (2.4 *M* potassium phosphate buffer) as those in Fig. 3. Total hemoglobin concentration in each solution was 75 mg/dl. (●—●) 100% HbS; (△—△) 75% HbS and 25% HbF; (□—□) 50% HbS and 50% HbF; (○—○) 100% HbF.

Under this condition, all hemoglobins, including HbA and HbF, totally aggregate (Fig. 5). It is interesting to note that the solubilization curve of a mixture of deoxy HbS and HbF, when it was started from 2.8 *M* phosphate buffer, showed a biphasic curve with an initial rapid drop followed by a slow phase. This result suggests that a part of the aggregated HbF in 2.8 *M* phosphate buffer can be dissolved quickly, but the HbF that remains may be coaggregated with HbS and dissolves more slowly.

The solubilization curves of aggregates of deoxy HbS, deoxy HbC_{Harlem}, and a mixture of sickle and nonsickle hemoglobins were determined after the formation of the aggregates in 2.2 *M* potassium phosphate buffer, pH 7.4.[17] In this experiment, the turbidity of the hemoglobin suspension in the starting buffer (2.2 *M*) was taken as 100% regardless of the presence of soluble hemoglobin. As reported in a previous article,[18] aggregates of deoxy HbC_{Harlem} were dissolved more readily than those of deoxy HbS. The ease of solubilization increases in the order of HbS, $HbAC_{Harlem}$, HbAS, HbC_{Harlem}, HbFS, and $HbFC_{Harlem}$. It is interesting to note that the solubilization curve for $HbAC_{Harlem}$ is more right-shifted than that for pure HbC_{Harlem}.

DISCUSSION

Aggregation of Hemoglobin in Concentrated Phosphate Buffer

The solubility of deoxy HbS in concentrated phosphate buffer is much lower than that of other hemoglobins.[12,22] Cottam and Waterman[23] showed that the solubility of deoxy HbS in 1.96 *M* phosphate responds to changes in temperature and oxygenation in a manner similar to gelation in vitro or to sickling of erythrocytes containing HbS. Briehl[24] also reported

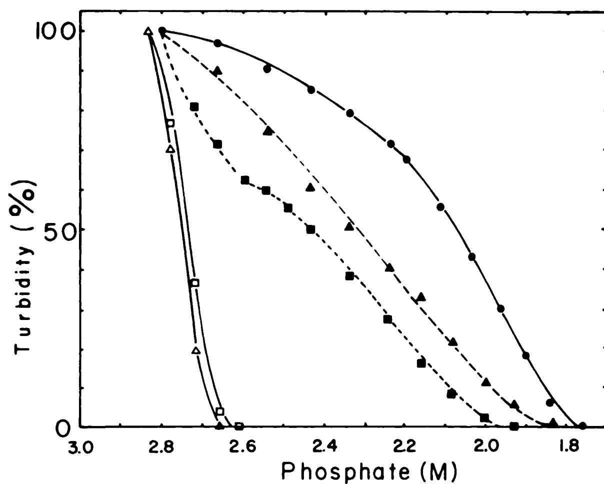

Fig. 5. Solubilization curves of all mixtures of deoxy HbS and deoxy HbA or deoxy HbF in 2.8 *M* potassium phosphate buffer, pH 6.95, at 26°C as the starting buffer. The hemoglobin concentration in each solution was 75 mg/dl. (△—△) HbA; (▲—▲) HbAS (50% HbA and 50% HbS); (●—w) HbS; (□—□) HbF; (■—■) HbFS (50% HbF and 50% HbS).

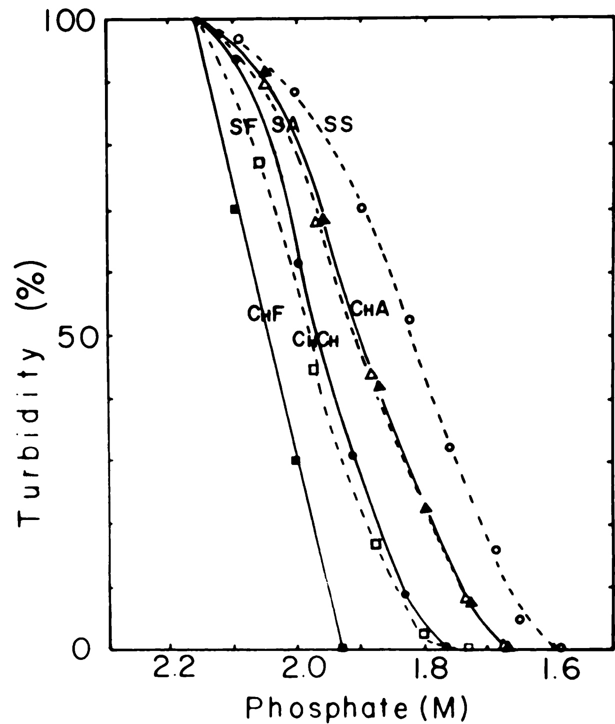

Fig. 6. Solubility of a mixture of sickle and nonsickle hemoglobins in 2.2 *M* potassium phosphate buffer, pH 7.4, at 22°C. The hemoglobin concentration in each solution is 70 mg/dl. The ratio of mixture of sickle and nonsickle hemoglobin was 50:50. (○—○) HbS; (△—△) HbSA; (□—□), HbSF; (●—●), HbC_H; (▲—▲) HbC_HA; (■—■) HbC_HF.

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that gelation of deoxy HbS occurred in 1.7 *M* phosphate buffer. Levine and others[25,26] have evaluated the effect of anions on the solubility of deoxy HbS measured both by MGC and by salting out in high phosphate buffer. More recently, Pillon and Bertles[27] systematically studied the effect of lyotropic salts on the polymerization of deoxy HbS. Roth et al.[28] reported that when HbS is treated with either nitrogen mustard or cyanate, there is no correlation between high ionic strength salting out and results of low ionic strength gelation studies. We also suggested previously[19] that the size of nuclei prior to aggregation in concentrated phosphate buffer is different from that in low phosphate buffer and that the mechanism of the aggregation in above 2.0 *M* phosphate may be different from that in below 1.8 *M* phosphate buffer. We have found that diluted solutions of hemoglobin in concentrated phosphate buffer above 2.4 *M* form homogeneous emulsions.[18] The degree of aggregation can be traced semiquantitatively by light scattering at 700 nm with an ordinary spectrophotometer. Since the addition of water to suspensions of aggregates decreases turbidity as aggregated hemoglobin melts, a solubilization curve can be drawn by plotting the turbidity (A_{700}) against the molarity of phosphate.[18] We found that the position and the shape of the solubilization curve are specific to the type of hemoglobin under study, and that the solubilization curves of deoxy HbS and deoxy HbC_{Harlem} are shifted to the right compared to those of non-S hemoglobins.[18] The advantages of this method are that (1) the degree of aggregation can be traced continuously without centrifugation or filtration; (2) the solubility of hemoglobin in any phosphate buffer can be accurately determined by measuring the hemoglobin remaining in the solute; and (3) since the aggregates can be separated easily from the solute, the composition of the aggregates can be determined by electrophoretic analysis.

Aggregation of Deoxy HbA With Deoxy HbS

It has been recognized that an increase in the minimum gelling concentration (MGC) of deoxy HbS due to the presence of non-S hemoglobin is related to the mildness of clinical symptoms in heterozyous patients.[10] Bertles et al.[14] reported that deoxy HbA was incorporated into a gel containing HbS. They suggested that HbA and HbAS hybrid molecules take part in the linear array of the gel. Gelation of deoxy HbA with deoxy HbS was also reported by Bookchin et al.,[29] Goldberg et al.,[16] and Cheetham et al.[30] Our results in concentrated phosphate buffer also demonstrate that HbA molecules clearly coaggregated with deoxy HbS. Of interest is the finding that most of HbA becomes insoluble when it is mixed with an equimolar amount of deoxy HbS, although pure deoxy HbA is totally soluble in 2.4 *M* potassium phosphate buffer. If deoxy HbA is mixed with lesser amounts of deoxy HbS, the excess HbA remains soluble in the supernatant, suggesting that coaggregation of deoxy HbA does not exceed the amount of coexisting deoxy HbS.

Aggregation of Deoxy HbF with Deoxy HbS

In contrast to deoxy HbA, data concerning coaggregation of deoxy HbF with deoxy HbS are conflicting. Some workers reported that deoxy HbF molecules coaggregate with deoxy HbS,[16,30] while others reported the opposite.[14,15] Our results using concentrated phosphate buffer show that a considerable amount of HbF coaggregates with HbS when equal amounts are mixed in 2.4 *M* phosphate buffer; surprisingly, some HbS remains in the supernate when HbF is present. The experiments were highly reproducible because the small volume of aggregates can be separated from the solute easily and completely. After the addition of equal amounts of HbS and HbF, electrophoretic analysis of the aggregates and the solute showed that the aggregates contained 32% HbF and 68% HbS, while the solute contained 23% of HbS. The result clearly indicates that HbF coaggregates with HbS and that the amount of HbF coaggregated is less than that of HbA. In contrast, a part of HbS that is totally insoluble in 2.4 *M* phosphate buffer becomes soluble in the presence of HbF. It appears that deoxy HbF interacted with deoxy HbS so that a mixture of HbS and HbF becomes more soluble than deoxy HbS. The solubilization curves of the FS mixture show a different pattern from those of SS and AS samples. The biphasic nature of the FS solubilization curve (Fig. 5) suggests that there are at least two types of aggregates containing different amounts of HbF: the ones that contain high amounts of HbF and low HbS are easily solubilized by a slight dilution of phosphate buffer, while the others that contain low amounts of HbF and high amounts of HbS are only solubilized with difficulty by dilution of phosphate buffer. If the experiments were started by using 2.4 *M* phosphate buffer rather than 2.8 *M*, a monophasic curve was obtained because the more soluble fraction does not aggregate in this buffer.

Formation of Hybrid Hemoglobin

It is generally believed that a mixture containing both HbS and non-S hemoglobin (X) has an equilibrium of HbS, HbSX hybrid, and HbX.[31,32] The relative concentrations of these components are expressed by the equation $a^2 + 2\,ab + b^2 = 1$, where a, ab, and b are the fractions of HbS, HbSX hybrid, and HbS,

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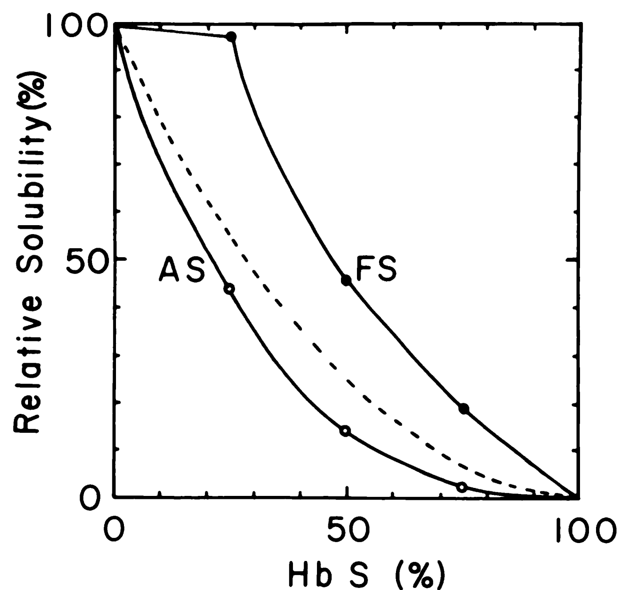

Fig. 7. Relation of the solubility of a mixture of sickle and nonsickle hemoglobin and the hybrid formation. Dotted line is the theoretical values of the solubility of SS and XS hybrid calculated from the equation in the text. Experimental results were denoted from the data of the Fig. 2B and 3B. (○—○) 1:1 mixture of HbA and HbS; (●—●) 1:1 mixture of HbF and HbS.

respectively. Bookchin et al.[29] reported that hybrid tetramers are capable of gelation and polymerization, which is similar or identical to that of HbS. Although it is not known if such hybrid is formed in concentrated phosphate buffer, the condition in concentrated phosphate buffer appears more favorable for the formation of hybrid, since the tetramer–dimer dissociation constant in high salt condition is slightly higher than in low salt condition.[33] Noren et al.[33] reported that deoxy hemoglobin still has a tetramer structure even at high salt and neutral pH conditions. To study if FS hybrid hemoglobin is involved in the aggregation of the mixtures of HbS and HbF, the experimental results obtained in concentrated phosphate buffer are compared with theoretical values obtained from the equation, provided FS hybrid in a 1:1 mixture of HbS and HbF has solubility similar to HbS. Of the total hemoglobin, 75% should aggregate and 25% HbF should remain in the supernatant because deoxy HbF is totally soluble in 2.4 *M* phosphate buffer. The same results should be obtained for a 1:1 mixture of HbS and HbA. As shown in Fig. 7, however, the theoretical values and experimental values differ quite strikingly. In addition, the theory cannot explain the existence of a considerable amount (23%) of deoxy HbS in the solute, because the pure form of deoxy HbS is totally insoluble in 2.4 *M* phosphate buffer. On the other hand, if the FS hybrid has the same solubility as that of deoxy HbF, the presence of 23% HbS in the solute can be explained, but the coaggregation of HbF cannot be accounted for.

Recently, Cheetham et al.[30] reported that no difference was recognized in the solubility of the mixtures of HbA and HbS, and HbF and HbS whether HbA or HbF and HbS were mixed in the oxy or deoxy form. On the other hand, Goldberg et al.[16] and Bookchin et al.[29] reported a difference in the solubility or MGC of S–F mixtures, depending on whether the hemoglobin was deoxygenated before or after mixing. Our results in concentrated phosphate buffer showed no difference in both the aggregation reaction and the solubilization curve for mixtures of HbA and HbS and HbF and HbS if these hemoglobins were mixed in the oxy or deoxy state. It appears that the simple hybrid theory cannot apply to the aggregation reaction of deoxy HbS and deoxy HbF in concentrated phosphate buffer.

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