A new turbidimetric method for the direct measurement of the solubility of oxy- and deoxyhemoglobins (Hb) in concentrated phosphate buffer has been established. The principle of the method is the formation of a homogeneous emulsion when hemoglobin is introduced in concentrated phosphate buffer. The solubility of the oxy and deoxy forms of Hb A, Hb S, Hb C, Hb F, and Hb C_Harlem (β^8_Glu→Val, β^3_Asp→Am) has been studied. The solubility of deoxy-Hb S was the lowest and the solubility curve was broader than those of the other hemoglobin, indicating that the aggregates of deoxy-Hb S require more water to be dissolved. The solubility of oxy- and deoxy-hemoglobin depends on temperature and pH. The solubility of hemoglobin is increased as the temperature is lowered and the pH is raised. The pH dependency of the solubility of deoxy-Hb S in high phosphate buffer was opposite to that of the minimum gelling concentration of deoxy-Hb S. The order of the solubility of Hb C_Harlem, Hb F, Hb AS, Hb CS, and Hb S in concentrated phosphate buffer corresponds to the order of minimum gelling concentration of these hemoglobins or hemoglobin mixtures. Solubility studies of a 1:1 mixture of deoxy-Hb A and deoxy-Hb S show that deoxy-Hb A aggregates in 2.42 M phosphate buffer in which pure deoxy-Hb A is totally soluble. This result indicates that deoxy-Hb S interacts with deoxy-Hb A and decreases its solubility.

Deoxy-Hb S is less soluble than oxy-Hb S and the oxy and deoxy forms of other hemoglobins (1, 2). This low solubility in red blood cells causes gel formation which leads to red cell sickling (3).

The two methods most often used for determining the solubility of hemoglobin are those which measure hemoglobin concentration in relatively diluted buffers (1, 4) and in concentrated phosphate buffer (2, 5, 6). The former method appears to be more physiological but requires considerable skill as well as a large amount of hemoglobin. The latter method requires less hemoglobin, but the continuous analysis of the formation and solubilization of hemoglobin aggregates is difficult because aggregated hemoglobin has to be removed prior to each measurement of the concentration of soluble hemoglobin.

This paper describes a new turbidimetric method for the direct measurement of the solubility of oxy- and deoxyhemoglobins in concentrated phosphate buffers. The solubility properties of the aggregates of the oxy and deoxy forms of Hb

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MATERIALS AND METHODS

Hemoglobin—Blood was collected in heparin, and red cells were washed three times with 0.9% saline solution. Packed cells were hemolyzed in 5 volumes of 50 mM potassium phosphate buffer, pH 7.4, containing 0.5 mM sodium EDTA, and stromata were removed by centrifugation at 27,000 x g for 10 min (7). Hb C and Hb C_Harlem were isolated from Hb SC and Hb AC_Harlem blood by the standard chromatographic technique on a CM-Sephadex column by gradient elution from 10 mM phosphate buffer, pH 6.0, to 20 mM, pH 8.0 (8). Hb S, Hb A, and Hb F were isolated from Hb AS and cored blood, respectively, on a DEAE-Sephadex column by gradient elution from 40 mM Tris-HCl, pH 8.3, to 40 mM Tris-HCl, pH 7.3. The Hb C was identified by fingerprints after tryptic and chymotryptic digests and amino acid analysis methods (6). Concentrations of oxy- and deoxyhemoglobins were determined spectrophotometrically using the millimolar coefficient of mE_{577} = 15 x 4 for oxyhemoglobin and mE_{577} = 12.5 x 4 for deoxyhemoglobin, respectively.

Solubility Test—Three different concentrations of phosphate buffers, 2.86, 2.59, and 2.48 M (2), were prepared for these tests. The solubility of deoxyhemoglobin in various phosphate buffers was determined turbidimetrically using an anaerobic photometric cuvette with a screw cap as described elsewhere (9). Under these conditions insoluble hemoglobin formed a homogeneous emulsion and the turbidity was measured with a Perkin Elmer 124 double beam spectrophotometer at the wavelength of 700 nm and recorded continuously with a Hamilton gas-tight syringe into the cuvette. The cuvette was inverted gently four to five times for mixing. The turbidity of hemoglobin suspensions gradually decreased as the phosphate buffer was diluted with water until the hemoglobin was completely dissolved. The absorption spectra between 500 and 700 nm were then recorded in order to confirm that the hemoglobin was totally in the deoxy form (cf. Fig. 1). The solubility of oxyhemoglobin can be examined by the same procedure under aerobic conditions. After each measurement, the temperature of the solution is determined with a thermometer. The pH of the solution is also determined with a pH electrode after 10-fold dilution of the solution.

RESULTS

Solubility of the Aggregates of Deoxy- and Oxy-Hb S—As reported by Itano (2), deoxy-Hb S is insoluble in 2.24 M phosphate buffer, pH 7.0. We found that, under this condition, deoxy-Hb S forms a stable and homogeneous emulsion. As shown in Fig. 1A, the addition of stock hemoglobin solution to the deoxygenated 2.24 M phosphate buffer increases the optical readings at 700 nm due to the light scattering of aggregates of deoxy-Hb S. The optical readings are unchanged for more than 30 min. Upon successive additions of 50 or 100 μl of distilled water into the cuvette, the optical reading gradually decreases until the hemoglobin is totally dissolved. Careful observation of the initial point of the solubilization curve

1 The abbreviations used are: Hb AC_Harlem, Hb AC_Harlemb, MGC, minimum gelling concentration; Sso, the phosphate concentration in which 50% of the aggregates of Hb are dissolved.
Solubility of Hemoglobin

A, titration of the aggregates of deoxy-Hb S with H2O. A stock hemoglobin solution (0.65 mM, 35 μl) was introduced into the CAPCEL containing 10 mg of Na2S2O4, and 2 ml of 2.48 M potassium phosphate buffer, pH 7.0, at 27°C. Aliquots (50 or 100 μl) of H2O were successively added as indicated in the figure. The cuvette was gently inverted four to five times after each addition of H2O. B, titration of the aggregates of oxy-Hb S with H2O. A stock hemoglobin solution (0.65 mM, 35 μl) was introduced into the CAPCEL containing 2 ml of 2.86 M potassium phosphate buffer, pH 7.0, at 27°C. Other conditions were the same as those in A.

showed that the turbidity drops rapidly after the addition of the first few aliquots of water and that the curve then increases slowly. This initial drop is due to the solubilization of aggregated deoxy-Hb S by the addition of water and the gradual increase that follows is due to the reaggregation of dissolved hemoglobin molecules under the new phosphate condition. The aggregation of hemoglobin in high phosphate buffer is not due to denaturation because hemoglobin is totally redissolved with a total restoration of the normal absorption spectrum of deoxyhemoglobin (Fig. 1B). The same technique can be used under aerobic conditions to measure the solubility of the aggregates of oxyhemoglobin. Since oxy-Hb S is more soluble than deoxy-Hb S, the titration of oxy-Hb S should be started in 2.86 M phosphate buffer at pH 7.0 (Fig. 1B). It should be noted that the total fluid volume increases after successive additions of distilled water. This factor can be easily corrected according to the volume of water added. It is clear from Fig. 2 that the initial small decrease in the turbidity of deoxy-Hb S is due to the dilution effect, while the decrease in turbidity below 2.3 M phosphate buffer is mainly due to the solubilization of deoxy-Hb S (Fig. 2). To study the relationship between the turbidity of the suspensions of hemoglobin aggregates and the concentration of hemoglobin, the optical densities at 700 nm in 2.8 M phosphate buffer, pH 7.0, of different concentrations of deoxy-Hb S were compared. There

successively until the aggregates were totally dissolved. The phosphate concentrations are shown on the top of the figure. C, similar experiment for oxy-Hb S.
Solubility of Hemoglobin

Effect of Temperature—The effect of temperature on the solubility of the deoxy forms of Hb A and Hb S is shown in Fig. 3A and that for oxy-Hb A and Hb S in Fig. 3B. As already shown by several workers (6, 10), the solubility of hemoglobin increases as the temperature is lowered. Similar results were obtained by the turbidimetric method.

Effect of pH—The solubility of the oxy and deoxy forms of hemoglobin depends on the pH of the medium (6, 11). In contrast to the effect of pH on minimum gelling concentration (MGC) of deoxy-Hb S and sickling (12), the solubility of all hemoglobins decreases as the pH of the medium is increased. There is a clear difference between the titration curves of the aggregates of deoxy-Hb S and those of other hemoglobins at all pH values (Fig. 4, A and B). The slopes of deoxy-Hb S curves are broad, indicating that the aggregates of deoxy-Hb S are solubilized at a wide range (0.6 M) of phosphate concentrations. Oxy-Hb S, in contrast, was found to be solubilized in a relatively narrow range of phosphate concentrations (0.3 M). The \( S_{50} \), the phosphate concentration in which 50% of the aggregates of Hb are dissolved, versus the pH of deoxy-Hb S is also different from that of other hemoglobins (Fig. 5). The \( S_{50} \) of oxy-Hb A and deoxy-Hb A are the same at pH 7.0, while the \( S_{50} \) of deoxy-Hb S and oxy-Hb S varies widely at pH 7.0. However, the opposite is true at pH 8.0.

The Solubility of a Mixture of Hb A and Hb S—It is known
that gel formations occur in a 1:1 mixture of deoxy-Hb A and deoxy-Hb S, requiring a higher concentration than does pure deoxy-Hb S (13, 14). To study the effect of deoxy-Hb A on the solubility of deoxy-Hb S, titration experiments were carried out in 2.4 M potassium phosphate buffer, pH 6.95, at room temperature (26°C). Under this condition, deoxy-Hb A alone is completely soluble while deoxy-Hb S forms aggregates. If deoxy-Hb A has no effect on the solubility of deoxy-Hb S, the turbidity of the mixture should only be attributed to Hb S. However, the results showed that the turbidity of the 1:1 mixture of Hb A and Hb S was twice that of pure deoxy-Hb S. This result indicates that, in the presence of deoxy-Hb S, deoxy-Hb A forms aggregates which are similar to deoxy-Hb S in 2.42 M phosphate buffer. Complete aggregation of deoxy-Hb A occurs only when Hb A and Hb S are mixed before hemoglobin solutions are introduced into the cuvette. The same results were obtained whether Hb A and Hb S were mixed in the oxy or the deoxy form. If deoxy-Hb A is added after the aggregation of Hb S, the increase in turbidity at 700 nm is negligible. When deoxy-Hb S was added to the deoxy-Hb A that was previously dissolved in 2.42 M phosphate buffer, the turbidity increased only 50% (Fig. 6). These results indicate that deoxy-Hb A does not interact with already aggregated deoxy-Hb S.

Solubility of Other Hemoglobins and the Mixture of Sickle and Non-sickle Hemoglobins—Bookchin et al. (15) reported that erythrocytes containing Hb C(marm) sickled less readily than SS cells. They also found that the minimum gelling concentration (MGC) of Hb S was increased by the presence of Hb C(marm) (14). These results suggest that Hb C(marm) had an inhibitory effect on the gelation of Hb S. The solubilities of the deoxy forms of Hb C, Hb F, and Hb A and the 1:1 mixtures of these hemoglobins with deoxy-Hb S are shown in Fig. 7. In the absence of deoxy-Hb S, the solubilities increase in the order of Hb C(marm), F, A, and C. In contrast, in the presence of deoxy-Hb S, solubilities increase in the order of Hb C and S, Hb A and S, and Hb F and S. This order corresponds to the order of the minimum gelation concentrations of these hemoglobin mixtures (16) but is completely opposite to that of the pure form of these hemoglobins.

**DISCUSSION**

The sickling phenomenon and the gelation of deoxy-Hb S must be explained by the substitution of the hydrophobic valine residue for the hydrophilic glutamic acid residue at the sixth position of the β chain. Perutz and Mitchison (1) showed that oxygenated Hb S and Hb A at low phosphate concentrations have similar solubilities. However, upon deoxygenation, the solubility of Hb A falls by one-half, whereas the solubility of deoxy-Hb S is 100 times less than its oxygenated form. Itano (2) reported that deoxy-Hb S is less soluble than deoxy-Hb A in high phosphate concentrations. Recently Cottam and Waterman (6), by using the filtration technique, showed that the solubility of deoxy-Hb S in 1.96 M phosphate buffer responds to changes in temperature and oxygenation in a manner similar to in vitro gelation or sickling of erythrocytes containing Hb S. Although these methods provided us with important information about the solubility of sickle and other hemoglobins, they are inconvenient to use. Cottam and Waterman (6) pointed out that Itano's method requires a large amount of hemoglobin and a considerable centrifugation time. With the filtration method of Cottam and Waterman (6), there are problems involving filtration under anaerobic conditions and foaming. Also, with these methods, the amount of aggregated hemoglobin is determined indirectly by measuring the remaining dissolved hemoglobin after the removal of aggregated hemoglobin. For this reason, experiments have to be done by a point-to-point manner. The turbidimetric method described in this paper not only allows us to measure directly the turbidity of aggregated hemoglobin but allows us to measure the absorption spectrum of dissolved hemoglobin after centrifugation of the cuvette. Thus, we can check the possible denaturation during reoxygenation or deoxygenation. In addition, the kinetics of formation and deformation of aggregates can be monitored continuously on a recorder (9). The test requires only a small amount of hemoglobin, and all procedures can be carried out in a spectrophotometric cuvette with a cap, making the measurements quite simple under controlled temperature conditions.

**Effect of Temperature and pH** The studies of Murayama (10) indicated by the evidence of negative temperature coefficient that the aggregation of sickle Hb molecules involves hydrophobic bonding. Cottam and Waterman (6) reported...
that the solubility of deoxy-Hb S at high phosphate concentrations also has a negative temperature coefficient. They also reported that the solubility of deoxy-Hb S changes sharply between 10 to 15°C. Our results showed a negative temperature coefficient but not the sharp increases in temperature which were observed by Cottam and Waterman (6) (Fig. 3).

As to the nature of the interactions between hemoglobin molecules, the substitution of the hydrophilic glutamic acid residues by the hydrophobic valine appears to play an important role in gel formation. Bookchin and Nagel (14), however, claimed that ionic bonds are also involved in the polymerization of sickle hemoglobins because the sickling phenomenon and the MGC of deoxy-Hb S depend upon pH; namely, that lowering the pH causes an increased sickling tendency and a decrease in the MGC. The pH dependency of the aggregate formation of deoxy-Hb in high phosphate concentrations is completely opposite to the pH dependency in sickling. The solubility of deoxy-Hb S in low phosphate concentrations is lowest at pH 6.5 and increases with the increase in pH (4). The solubility of deoxy-Hb S in high phosphate concentrations increases as the pH of the medium is lowered (Fig. 4, A and B). Cottam and Waterman (6) reported similar results by the filtration method. Briehl (17) pointed out that in studies of solubility in high phosphate concentrations the increasing concentration of the HPO₄²⁻ is responsible for the enhanced condensation rather than pH itself, since it is the HPO₄²⁻ rather than H₂PO₄⁻ which is primarily responsible for the salting out effect. These data suggest that the difference in the mechanism of the solubility of deoxy-Hb S in high and low phosphate concentrations are related more to the amount of HPO₄²⁻ than pH of the phosphate buffer.

Interaction of Sickle Hemoglobin with Other Hemoglobins—The shapes of the solubility curves for oxy-Hb S, deoxy-Hb A, and oxy-Hb S resembled each other, but the curves for deoxy-Hb S were clearly different (Fig. 3). The titration curves for deoxy-Hb S are broad, indicating that the aggregates of deoxy-Hb S require more water to be dissolved. The titration curves may be expressed more conveniently by the Hill plot (Fig. 8). The Sₙ₀ indicates the overall solubility of the aggregates of hemoglobin in phosphate buffer, and the n value may be assumed to be the parameter of strength in the intermolecular interaction between hemoglobin molecules. In other words, the higher the n values, the smaller the amount of water required to disrupt the interaction between hemoglobin molecules. The Sₙ₀ and n values for various hemoglobins are summarized in Table I. The mean value of Sₙ₀ of deoxy-Hb S was 2.048 ± 0.0083 (n = 10). The comparison of Sₙ₀ and n values between the aggregates of the deoxy forms of Hb S and Hb Cₙ indicates that the aggregates of deoxy-Hb Cₙ are not only more soluble than these of deoxy-Hb S but that the interactions of the hemoglobin molecules are weaker. The high solubility of deoxy-Hb Cₙ has been explained by contamination by Hb A₂ (15) or by the effect of the surface structure in the vicinity of β₇₃ (18). Our results suggest that the higher solubility of deoxy-Hb Cₙ is due to the weakened interaction between the molecules of deoxy-Hb Cₙ. The substitution of Asp at β₇₃ for Asn in Hb Cₙ inhibits gel formation by eliminating the interaction of hemoglobin molecules at β₇₃ in gelation (18). The increased solubility of deoxy-Hb Cₙ compared to deoxy-Hb S in high phosphate buffer may also be attributed to the elimination of the interaction of Hb molecules at β₇₃ in deoxy-Hb Cₙ.

The interaction of Hb S with other hemoglobins has been studied by measuring the minimum gelling concentration (14, 19), viscosity (20), and solubility (2, 11, 21). All of these results suggest that the polymerization of deoxy-Hb S is affected by other types of hemoglobin molecules. Recently, Goldberg et al. (11) reported that of concentrated mixture of Hb A and Hb S in low phosphate buffer was not affected by the presence or absence of hybrid forms but mixtures of Hb S and Hb F were affected by the hybrid formation. In concentrated phosphate buffer, we also did not recognize any difference regardless of whether Hb A and Hb S were mixed in the oyx or deoxy form. Further studies on the effect of fetal hemoglobin are currently in progress in our laboratory.

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REFERENCES

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TABLE I

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FIG. 8. Hill plots of solubility curves shown in Fig. 7. Y is the fraction of turbidity relative to the turbidity of starting suspensions.
Solubility of Hemoglobin

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The solubility of sickle and non-sickle hemoglobins in concentrated phosphate buffer.

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