Effect of 2,3-Diphosphoglycerate and Inositol Hexaphosphate on the Stability of Normal and Sickle Hemoglobins†

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ABSTRACT: The effects of 2,3-diphosphoglycerate and inositol hexaphosphate on the mechanical stability of normal and sickle hemoglobins were investigated under various pH and buffer conditions. The rates of precipitation of the oxy and deoxy forms of these hemoglobins were significantly reduced by the addition of 2,3-diphosphoglycerate or inositol hexaphosphate, particularly in neutral or acid conditions. This protective effect is attributed to the conformational change which occurs upon binding of these phosphates to the hemoglobin molecule. It was also found that 2,3-diphosphoglycerate and inositol hexaphosphate coprecipitates with hemoglobin at these pH's. The 2,3-diphosphoglycerate/hemoglobin ratio in the precipitate was 0.3 at pH 7.0 and 0.85 at pH 6.5. At pH 7.5, no 2,3-diphosphoglycerate was detected in the precipitate although 2,3-diphosphoglycerate still showed a considerable protective effect. From the degree of this protective effect, we estimated the association constant of 2,3-diphosphoglycerate for oxyhemoglobin as $4.2\text{–}4.6 \times 10^2$ M^{-1}. Further studies with hemoglobin subunits showed that 2,3-diphosphoglycerate and inositol hexaphosphate inhibit precipitation of oxy and deoxy forms of both β_A and β_S subunits. The precipitation of α_A subunits, on the other hand, was accelerated by these organic phosphates in acid, while it was inhibited in alkali. The deoxy form of α subunits was extremely stable. These results suggest that the rate of precipitation of hemoglobin and its subunits depends on the protein conformation in solution. The clinical significance of the 2,3-diphosphoglycerate effect on the stabilization of sickle oxyhemoglobin is also discussed.

Although the gross structure of oxyhemoglobin S is believed to be similar to that of oxyhemoglobin A (Perutz and Mitchison, 1950; Perutz *et al.*, 1951), the ease of precipitation of oxyhemoglobin S during mechanical shaking suggests that the conformation of oxyhemoglobin S in solution is different from that of oxyhemoglobin A (Asakura *et al.*, 1973, 1974a). This difference may be located in the abnormal β_S subunits since the oxy form of the β_S subunits are more labile than that of the normal β_A subunits (Asakura *et al.*, 1974b).

Recently, we have found that the rates of precipitation of oxyhemoglobins A and S were inhibited significantly by 2,3-diphosphoglycerate and inositol hexaphosphate. These phosphate compounds are known to bind to deoxyhemoglobin and to change the oxygen binding properties of hemoglobin (Benesch *et al.*, 1968a, 1969; Chanutin and Hermann, 1969). They also bind to oxyhemoglobin to a less extent (Chanutin and Hermann, 1969; Garby *et al.*, 1969; Luque *et al.*, 1969; Benesch *et al.*, 1971; Berger *et al.*, 1973). It was shown by X-ray analysis that a molecule of 2,3-diphosphoglycerate is held in the opening of the central cavity with valine (β-1), lysine (β-82), and histidine (β-143) of both deoxy β chains complementing the negative charge of 2,3-diphosphoglycerate (Bunn *et al.*, 1970; Perutz, 1970; Arnone, 1972). These results suggest that the binding of phosphates changes the conformation of hemoglobin and changes its oxygen binding property as well as its stability.

In the present manuscript, therefore, we describe the effect of 2,3-diphosphoglycerate and inositol hexaphosphate on the rates of mechanical precipitation of the oxy and deoxy forms of normal and sickle hemoglobins and their subunits under various conditions. The results clearly indicate that the stabilization occurs upon binding of these organic phosphates to hemoglobin. The mechanical shake test appears to be a sensitive indicator of conformational changes of the proteins in solution. The physiological significance of the stabilizing effect of 2,3-diphosphoglycerate is discussed with reference to the increased formation of Heinz bodies (Schneider *et al.*, 1972) and elevated 2,3-diphosphoglycerate levels in sickle erythrocytes (Charache *et al.*, 1970; Seakins *et al.*, 1973).

Materials and Methods

Chemicals and Enzymes. 2,3-Diphosphoglycerate and inositol hexaphosphate and other materials were purchased from Sigma. Enorase, pyruvate kinase, lactate dehydrogenase, and phosphoglycerate mutase were purchased from Boehringer. 2,3-Diphosphoglycerate (pentacyclohexylammonium salt) was converted into the free acid by treatment with Dowex-50. Buffers used are potassium phosphate buffer and bis-tris[1] buffer.

Preparation of Hemoglobin Solution. Red cells from fresh heparinized blood were washed three times with 0.9% NaCl and hemolyzed by adding 5 volumes of distilled water. After addition of one-tenth volume of 9% NaCl, the stroma were removed by centrifugation at 10,000 rpm for 20 min. Before each experiment the stock hemoglobin solution was desalted with Sephadex G-25, and diluted to an

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[1] Abbreviations used are: bis-tris, *N,N*-bis(2-hydroxyethyl)iminotris(hydroxymethyl)methane; DPG, 2,3-diphosphoglycerate; Hb, hemoglobin.

appropriate concentration with the buffers indicated. The concentrations of oxy- and deoxyhemoglobins were determined by the use of extinction coefficients of 15 mM^{-1} cm^{-1} at 578 nm and 13.5 mM^{-1} cm^{-1} at 556 nm, respectively.

Preparation of α- and β-Hemoglobin Subunits. The α and β chains of hemoglobins A and S were prepared by the method of Bucci and Fronticelli (1965). After treatment with carbon monoxide gas, the *p*-chloromecuribenzoate was removed from the separated chains by stirring with dodecanethiol according to the method of DeRenzo *et al.* (1967). The purity of these preparations were checked by electrophoresis on a cellulose acetate strip.

Preparation of Deoxyhemoglobin. Experiments with deoxyhemoglobin were carried out using an anaerobic photometric cuvet with a screw cap (Capcell, Technical Consulting Services (TCS), Southampton, Pa.). A buffer solution in this anaerobic cuvet was first bubbled with argon gas in order to remove most of oxygen in the liquid and gas phases. A trace of oxygen was removed by adding a freshly prepared sodium dithionite solution through a syringe needle to make a final concentration of 1 mM. The vial was shaken for about 30 sec to remove remaining oxygen in the gas phase. A concentrated solution of deoxyhemoglobin, which was previously deoxygenated by passing argon gas over the surface, was then added to the anaerobic cuvet using a Hamilton gas-tight syringe. The absorption spectrum of the hemoglobin solution was measured with a Perkin-Elmer Coleman 124 spectrophotometer to check that the hemoglobin was completely deoxygenated.

Shaking Experiments. A hemoglobin solution (2 ml) (heme concentration, 40–50 μM) in a 5 × 45 mm vial or Capcell was shaken with a TCS shaker (Model 150) at a shaking frequency of 28 Hz for various time intervals at room temperature. After shaking, the vials were centrifuged at 4000 rpm for 5 min and the absorption spectra of the supernatant solutions were recorded between 500 and 700 nm with a Perkin-Elmer double beam spectrophotometer. The proportion of hemoglobin denatured during the shaking was calculated as described in previous reports (Asakura *et al.*, 1974a).

The Measurements of 2,3-Diphosphoglycerate and Inositol Hexaphosphate. 2,3-Diphosphoglycerate was determined on perchloric acid extracts by the colorimetric method of Bartlett (1959) and by the enzymatic method of Rose and Liebowitz (1970). Inositol hexaphosphate was determined by analysis of total inorganic phosphate by the ascorbic method after ashing (Chen *et al.*, 1956).

Results

Effect of 2,3-Diphosphoglycerate and Inositol Hexaphosphate on the Rate of Precipitation of Oxyhemoglobins A and S. As shown in the previous reports (Asakura *et al.*, 1973, 1974a), oxyhemoglobin S precipitates at about a tenfold faster rate than does oxyhemoglobin A during mechanical shaking (Figure 1). The rates are markedly slowed by the addition of 1 mM 2,3-diphosphoglycerate or inositol hexaphosphate. Addition of inositol hexaphosphate produces an initial lag phase which is always observed when the rate of precipitation is slowed down either by lowering the shaking rate or by lowering the temperature (Asakura *et al.*, 1974a). It should be pointed out that if we used 2,3-diphosphoglycerate in the commercially available pentacyclohexylammonium salt form, the rates of precipitation of oxyhemoglobins A and S were conversely accelerated several fold above pH 7.2. This acceleratory effect was found to be due to pentacyclohexylammonium ions and not due to 2,3-diphosphoglycerate. For this reason, pentacyclohexylammonium ions were removed from 2,3-diphosphoglycerate with cation exchange resin before use. Removal of this compound can easily be checked by the mechanical shake test with hemoglobin in 0.1 M bis-tris buffer (pH 8.0). If the compound is removed, 2,3-diphosphoglycerate inhibits the rate of precipitation of hemoglobin, while the rate accelerates if the compound is present.

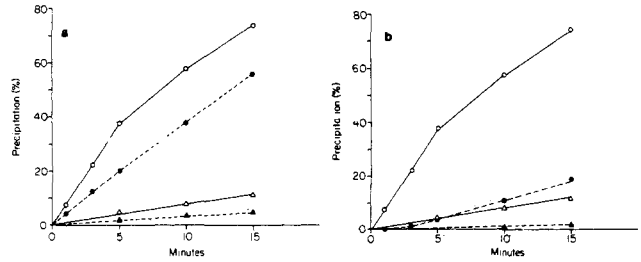

FIGURE 1: (a) Effect of 2,3-diphosphoglycerate on the rate of mechanical precipitation of oxyhemoglobins A and S. A two-ml solution of oxyhemoglobin A (11 μM) or oxyhemoglobin S (12 μM) in 0.1 M bis-tris buffer (pH 7.2) was shaken in a 1.5 × 4.5 cm vial in the presence and absence of 1 mM 2,3-diphosphoglycerate (DPG) with the TCS shaker at a frequency of 28 Hz at 21°. The total volume was adjusted to 2 ml. After shaking the vials were centrifuged at 6000 rpm for 5 min and the proportion of the remaining hemoglobin in the supernatant was measured spectrophotometrically. (O—O) Oxyhemoglobin S; (● - - - - ●) oxyhemoglobin S + DPG; (Δ — Δ) oxyhemoglobin A; (▲ - - - - ▲) oxyhemoglobin A + DPG. (b) Effect of inositol hexaphosphate on the rate of mechanical precipitation of oxyhemoglobins A and S. The concentrations of oxyhemoglobin A, oxyhemoglobin S, and inositol hexaphosphate (IHP) were 11 μM, 12 μM, and 1 mM, respectively. The other conditions were the same as those described in Figure 1a. (O—O) Oxyhemoglobin S, (● - - - - - ●) oxyhemoglobin S + IHP; (Δ — Δ) oxyhemoglobin A; (▲ - - - - - ▲) oxyhemoglobin A + IHP.

Effect of pH on the Stabilization of Oxyhemoglobin by 2,3-Diphosphoglycerate and Inositol Hexaphosphate. The affinity of 2,3-diphosphoglycerate for hemoglobin has been shown to decrease with increasing pH and this indicates ionic binding between negatively charged phosphate group and positively charged protein side chains. The half-maximal binding of 2,3-diphosphoglycerate occurs at pH 8.3 for deoxyhemoglobin and at pH 7 for oxyhemoglobin at the 5 mM 2,3-diphosphoglycerate concentration (Garby *et al.*, 1969). The rate of precipitation of oxyhemoglobin A and S also depends on pH and on the type of buffers used (Asakura *et al.*, 1974a). Therefore, we examined the effect of 2,3-diphosphoglycerate and inositol hexaphosphate on the rate of precipitation of oxyhemoglobins A and S under various pH and buffer conditions. As shown in Table I, stabilizing effects of 2,3-diphosphoglycerate and inositol hexaphosphate were clearly seen in both bis-tris and phosphate buffers at neutral and acidic pH's, where organic phosphates bind to hemoglobin. At pH 8.0, on the other hand, these stabilizing effects are almost completely abolished, though inositol hexaphosphate still shows a considerable effect on oxyhemoglobin S in bis-tris buffer. These results are consistent with the idea that stabilization is due to the binding of organic phosphates to hemoglobin and that the rate of mechanical precipitation sensitively reflects protein conformational change due to the phosphate binding. No significant differences between the proportions of these phosphate effects for oxyhemoglobin A and oxyhemoglobin S were noticed except that oxyhemoglobin S always denatures at a faster rate than does oxyhemoglobin A.

TABLE I: The Effect of pH on the Stabilization of Oxy- and Deoxyhemoglobins S and A by 2,3-Diphosphoglycerate (DPG) and Inositol Hexaphosphate (IHP).[a]

		Rate of Precipitation (%/3 min)						Rate of Precipitation (%/30 min)		
		Oxy-Hb S			Oxy-Hb A			Deoxy-Hb S		
Buffer	pH	–	DPG	IHP	–	DPG	IHP	–	DPG	IHP
Bis-tris	6	13.7	9.7	2.7	5.4	4.6	4.5	6.0	4.2	1.0
Phosphate	6	12.4	10.0	8.7	6.5	5.7	5.5	6.4	6.3	3.9
Bis-tris	7	24.8	16.3	5.0	4.6	3.0	1.8	16.2	7.1	1.5
Phosphate	7	14.3	10.0	12.4	6.1	6.0	6.1	11.4	10.2	3.6
Bis-tris	8	17.5	17.5	9.4	1.0	1.0	1.0	19.0	7.6	0
Phosphate	8	36.7	36.5	36.5	10.0	10.0	9.5	30.7	26.1	26.1

[a] A 2-ml solution of 10 μM hemoglobin was shaken in the presence and absence of 1 mM organic phosphates with TCS shaker for 3 min (oxyhemoglobin) or 30 min (deoxyhemoglobin). Buffers used were 0.1 M Bis-tris and 0.1 M phosphate buffer with three different pH's.

Effect of pH on the Stabilization of Deoxyhemoglobin by 2,3-Diphosphoglycerate and Inositol Hexaphosphate. 2,3-Diphosphoglycerate, inositol hexaphosphate, and other phosphate compounds are known to bind tightly with deoxyhemoglobin. In the presence of 2,3-diphosphoglycerate or inositol hexaphosphate, the deoxyhemoglobin molecule is more stable in terms of its deoxy quaternary structure. The mechanical shake test showed that the deoxy forms of hemoglobins A and S are far more stable than their oxy forms (Asakura *et al.*, 1974a). Deoxyhemoglobin S is so stable that it is necessary to shake more than 30 min to measure the rate of denaturation at room temperature. As shown in Table I, the addition of 2,3-diphosphoglycerate or inositol hexaphosphate further stabilizes the molecule of deoxyhemoglobin S in all pH ranges examined. The effect was seen even in phosphate buffer at pH 8.0. This may be explained by the shift of pK of 2,3-diphosphoglycerate binding with deoxyhemoglobin to the higher pH's, so that much more 2,3-diphosphoglycerate can combine with deoxyhemoglobin at pH 8.0 than in the case of oxyhemoglobin.

Effect of 2,3-Diphosphoglycerate and Inositol Hexaphosphate Concentrations on the Rate of Precipitation of Oxyhemoglobins A and S. In order to study the mode of molecular interaction between hemoglobin and 2,3-diphosphoglycerate (or inositol hexaphosphate), different concentrations of organic phosphates were mixed with hemoglobin and the rates of precipitation of the mixture are compared. As shown in Figure 2a, the stabilizing effect of inositol hexaphosphate reaches maximum when the ratio of inositol hexaphosphate to hemoglobin becomes 1. This result strongly suggests a 1:1 molecular interaction between oxyhemoglobin and inositol hexaphosphate. The affinity of 2,3-diphosphoglycerate for oxyhemoglobin appears to be smaller than that of inositol hexaphosphate, since the maximum effect of 2,3-diphosphoglycerate was obtained when the ratio of 2,3-diphosphoglycerate to hemoglobin is 100 or more as shown in Figure 2b. This ratio naturally decreases as the concentration of hemoglobin increases.

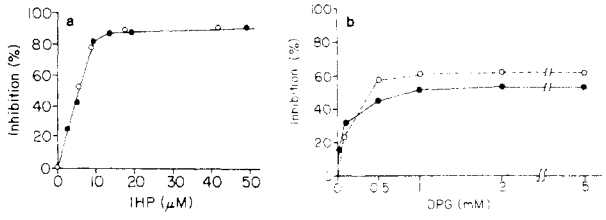

FIGURE 2: (a) Effect of inositol hexaphosphate concentration on the rate of precipitation of oxyhemoglobins A and S. A 2-ml solution of oxyhemoglobin A (12.3 μM) or oxyhemoglobin S (10.3 μM) in 0.1 M bis-tris buffer (pH 7.2) was shaken in the presence of inositol hexaphosphate (IHP) for 6 min at 23°. The per cent inhibition by inositol hexaphosphate was calculated by measuring the remaining hemoglobin in the supernatant. (○) Oxyhemoglobin A; (●) oxyhemoglobin S. (b) Effect of 2,3-diphosphoglycerate concentration on the rate of precipitation of oxyhemoglobin A and S. The concentration of oxyhemoglobin A and oxyhemoglobin S were 10.8 and 10.2 μM, respectively. The other conditions were the same as those described in Figure 2a, except that 2,3-diphosphoglycerate (DPG) was used. The shaking time was 3 min. (○) Oxyhemoglobin A; (●) oxyhemoglobin S.

Effect of 2,3-Diphosphoglycerate Concentration on the Rate of Precipitation of Deoxyhemoglobin S. The protective effect of 2,3-diphosphoglycerate on the precipitation of deoxyhemoglobin S is obtained at a lower concentration of 2,3-diphosphoglycerate than in the case of oxyhemoglobin S (Figure 3). The protective effect reaches maximum when approximately 1 mol of 2,3-diphosphoglycerate is added to 1 mol of deoxyhemoglobin S, indicating that deoxyhemoglobin has a higher affinity for 2,3-diphosphoglycerate than has oxyhemoglobin.

Effect of Salt on the Stabilization of Deoxy- and Oxyhemoglobin S by Organic Phosphates. Benesch *et al.* (1969) reported that the concentration of neutral salt has a decisive influence on the extent of 2,3-diphosphoglycerate binding with deoxyhemoglobin. At low salt concentrations

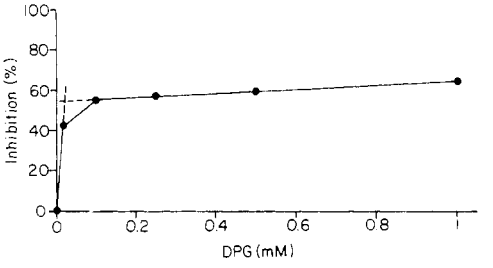

FIGURE 3: Effect of 2,3-diphosphoglycerate concentration on the rate of precipitation of deoxyhemoglobin S. A 2-ml solution of oxyhemoglobin S (12 μM) was deoxygenated in an anaerobic photometric cuvet as described in the text and was shaken in the presence of various concentrations of 2,3-diphosphoglycerate (DPG). Since deoxyhemoglobin is more stable than oxyhemoglobin, the solution was shaken vigorously with TCS shaker (Model 2500) at a frequency of 30 Hz for 15 min.

TABLE II: The Effects of Salt on the Stabilization of Deoxy- and Oxyhemoglobins S by Organic Phosphates.[a]

Salt (NaCl) (mM)	Rate of Precipitation: Deoxy Form (% precipitation/30 min)		Rate of Precipitation: Oxy Form (% precipitation/3 min)	
0	–	21.0	–	27.8
	DPG	12.5	DPG	20.5
			IHP	4.5
10	–	15.0	–	31.6
	DPG	6.5	DPG	21.8
			IHP	4.6
100	–	15.0	–	31.5
	DPG	6.7	DPG	21.0
			IHP	26.0
500	–	15.0	–	49.0
	DPG	15.0	DPG	42.0
			IHP	42.0

[a] A 2-ml solution of deoxyhemoglobin (11.0 μM) or oxyhemoglobin S (9.7 μM) in 0.05 M bis-tris buffer (pH 7.3) was shaken in the presence and absence of 1 mM organic phosphates and various concentrations of NaCl, at a frequency of 28 Hz for 30 min (deoxyhemoglobin) or 3 min (oxyhemoglobin) at 21°.

TABLE III: 2,3-Diphosphoglycerate (DPG) and Inositol Hexaphosphate (IHP) in Precipitated Hemoglobin.[a]

Phosphate Compd	pH	Ratio of Phosphate Compd to Precipitated Hemoglobin (mol/mol)
DPG	6.0	1.51
	6.5	0.85
	7.0	0.3
	7.15	0.2
	7.5	0
	8.0	0
IHP	7.2	0.79

[a] DPG: A 2-ml solution of oxyhemoglobin S (42 μM) in 0.1 M bis-tris buffer was shaken in the presence of 2 mM DPG for 30 min with TCS shaker (30 Hz). The proportion of denatured hemoglobin calculated by measuring the remaining hemoglobin in the supernatant solution was 90%. IHP: A 2-ml solution of oxyhemoglobin S (21.7 μM) in 0.1 M bis-tris buffer (pH 7.2) was shaken in the presence of 20 μM IHP for 35 min with TCS shaker (30 Hz). The proportion of denatured hemoglobin calculated by measuring the remaining hemoglobin in the supernatant solution was 87%.

and at pH 7.3, the binding of 2,3-diphosphoglycerate with deoxyhemoglobin is tight and the binding ratio is 1 mol of 2,3-diphosphoglycerate/mol of hemoglobin. Binding was inhibited by 500 mM NaCl. Thus, if the protective effect of 2,3-diphosphoglycerate on the rate of mechanical precipitation is due to the phosphate binding to deoxyhemoglobin, this effect must be diminished in the presence of high salt concentrations. In order to confirm this, we investigated the stabilizing effect of 2,3-diphosphoglycerate on deoxyhemoglobin S in 0.05 M bis-tris buffer (pH 7.3), in the presence of different concentrations of salt. The result is shown in Table II. In the presence of 100 mM NaCl, 2,3-diphosphoglycerate still shows a stabilizing effect, while this effect was lost in the presence of 500 mM NaCl.

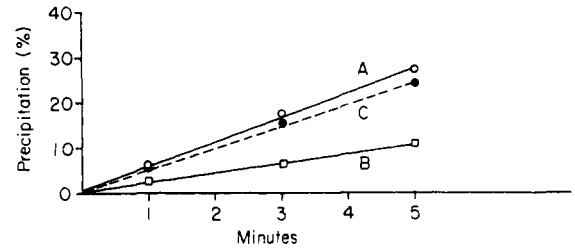

FIGURE 4: Removal of the protecting factor in the supernatant solution after precipitation of hemoglobin. A 2-ml solution of hemoglobin S (13 μM) in 0.1 M bis-tris buffer (pH 7.2) was shaken in the presence of 10 μM inositol hexaphosphate (IHP) for 25 min with TCS shaker (30 Hz). After shaking, the precipitate was removed by centrifugation. To the supernatant solution a concentrated hemoglobin was added to make a final concentration of 13 μM and a final volume of 2 ml. The time course of the hemoglobin precipitation was measured as described in the legend to Figure 1a. (A) Oxyhemoglobin S (○ — ○) (C) supernatant + oxyhemoglobin S (● - - ●); (B) oxyhemoglobin S + IHP (□ — □).

The effects of salt on the stabilization of oxyhemoglobin S by organic phosphates were also studied under the same buffer conditions and the results are shown in Table II. The stabilizing effect of inositol hexaphosphate was greatly weakened by increasing the NaCl concentration up to 100 mM or more, while the stabilizing effect of 2,3-diphosphoglycerate was not significantly affected by the salt concentration up to 100–200 mM.

The Measurement of 2,3-Diphosphoglycerate and Inositol Hexaphosphate in the Precipitated Hemoglobin. In order to find out if 2,3-diphosphoglycerate or inositol hexaphosphate coprecipitates with oxyhemoglobin during mechanical shaking, we measured the content of 2,3-diphosphoglycerate or inositol hexaphosphate in the precipitated hemoglobin. This experiment was carried out by shaking oxyhemoglobin S in the presence of 2,3-diphosphoglycerate or inositol hexaphosphate at various pH's. The precipitates formed were collected by centrifugation and washed several times with water. The contents of 2,3-diphosphoglycerate or inositol hexaphosphate in the acid extract of the precipitated hemoglobin was then measured enzymatically or colorimetrically. The results showed that 2,3-diphosphoglycerate coprecipitates with oxyhemoglobin S when it is shaken under acidic conditions (Table III). Above pH 7.15, however, no detectable amount of 2,3-diphosphoglycerate was found in the precipitate although 2,3-diphosphoglycerate still shows a protective effect. The molar ratio of 2,3-diphosphoglycerate to hemoglobin in the precipitates was 1.5 mol per oxyhemoglobin tetramer at pH 6.0. The value approached zero as the pH of the medium was raised above 7.0. Inositol hexaphosphate, on the other hand, coprecipitates with oxyhemoglobin even in alkaline pH's. This agrees well with the experimental results (shown in Figure 1a) that inositol hexaphosphate shows a protective effect even at pH 8.0. Coprecipitation of inositol hexaphosphate with hemoglobin was first demonstrated by measuring the stabilizing activity of the supernatant (Figure 4). This experiment was

TABLE IV: Effect of 2,3-Diphosphoglycerate (DPG) and Inositol Hexaphosphate (IHP) on the Rate of Precipitation of Hemoglobin Subunits.[a]

pH	Organic Phosphate	Rate of Precipitation (%/30 sec)				
		Oxy Form			Deoxy Form	
		β_S	β_A	$\alpha_{A(S)}$	β_S	β_A
6	–	29.8	28.2	6.7 (6)	29.6	29.3
	DPG	11.3	21.8	12.5 (12)	20.2	21.4
	IHP	8.4	21.5	19.5	12.2	29.0
7	–	25.3	23.6	15.0	27.0	27.8
	DPG	14.4	14.6	26.4	17.3	18.7
	IHP	17.7	17.1	21.8	18.0	18.0
8	–	20.7	11.1 (12)	25.2	18.3	13.9
	DPG	12.6	10.7	13.0	15.0	13.0
	IHP	12.5	4.5 (6.5)	8.0	12.7	0

[a] Two milliliters of a solution of subunits (26–30 μM, as heme) in Capcell were shaken in the presence and absence of 1 mM organic phosphates with the TCS shaker (28 Hz) for 30 sec. The other conditions were the same as those described in Table I. Values in parentheses are the per cent of hemichrome formed in the supernatant during shaking.

carried out first by shaking 13 μM inositol hexaphosphate at 30 Hz for 25 min at pH 7.2, which precipitated 78% of total hemoglobin. After removal of the precipitate by centrifugation, a concentrated hemoglobin solution was added to the supernatant solution to make the final concentration 13 μM. The final volume was also adjusted to 2 ml. Observation of the time course of the precipitation of oxyhemoglobin S in this solution showed that the supernatant had lost the protecting factor. Further evidence was obtained by measuring the inositol hexaphosphate content as total phosphate. As shown in Table III, inositol hexaphosphate coprecipitates with oxyhemoglobin S and the molar ratio of inositol hexaphosphate to oxyhemoglobin S in the precipitate was 0.79 mol/mol of tetrametric oxyhemoglobin S at pH 7.2.

Effect of 2,3-Diphosphoglycerate and Inositol Hexaphosphate on the Rate of Precipitation of Oxy and Deoxy Forms of Hemoglobin Subunits. As shown in the previous paper (Asakura *et al.*, 1974b), the isolated α_A, β_A, and β_S subunits showed different pH profiles for the ease of precipitation during mechanical shaking. The α_A and β_A subunits had opposite pH profiles; the α_A subunits precipitated more rapidly in acidic pH's than alkaline pH's. While β_A subunits precipitated more rapidly in alkaline pH's. The oxidizability of the hemoglobin subunits in solution also showed opposite properties. The pH profiles of the β_S subunits were similar to those of β_A subunits, though β_S subunits were more unstable than β_A subunits in alkaline pH's. These results are shown in Table IV. The effect of 2,3-diphosphoglycerate and inositol hexaphosphate on the stability of hemoglobin subunits also differed among the various subunits. The β_A and β_S subunits are generally stabilized by the addition of inositol hexaphosphate or 2,3-diphosphoglycerate. On the other hand, these organic phosphates accelerate the precipitation of α subunits markedly at pH 6 and 7, while they show an inhibitory effect at pH 8.0. At pH 7.4, the effects of these organic phosphates were slightly inhibitory (2,3-diphosphoglycerate 7.3%, inositol hexaphosphate 15.8%). It is very interesting to compare the stability of deoxy subunits. Although the β_A and β_S subunits were not stabilized significantly upon deoxygenation, the deoxy form of the α subunits was so stable that it did not precipitate at all during shaking for 30 min.

Discussion

Although 2,3-diphosphoglycerate and inositol hexaphosphate were originally assumed to bind only to deoxyhemoglobin, it is now generally accepted that these phosphates bind also to oxyhemoglobin (Chanutin *et al.*, 1969; Garby *et al.*, 1969; Luque *et al.*, 1969; Benesch *et al.*, 1971; Berger *et al.*, 1973).

The association constant of 2,3-diphosphoglycerate for hemoglobin depends on pH, ionic strength, and temperature of the medium and also on the method of determination. The reported values of association constants for 2,3-diphosphoglycerate to hemoglobin have varied widely (Benesch *et al.*, 1968a; Lo and Schimmel, 1969; Chanutin and Herman, 1969; Garby *et al.*, 1969; Riggs, 1971; Garby and DeVerdier (1971); DeBruin and Janssen, 1973; Gerber *et al.*, 1973; Imai and Tyuma (1973). Generally, it is believed that the affinity for deoxyhemoglobin is one or two orders of magnitude larger than that for oxyhemoglobin. Inositol affinity for both oxy- and deoxyhemoglobins (Jänig *et al.*, 1971). The present results obtained by the new mechanical shake method clearly show that the binding of these organic phosphates stabilizes both oxy and deoxy forms of hemoglobin molecule. In addition, from the rates of precipitation of hemoglobin, it is possible to estimate the binding constant of 2,3-diphosphoglycerate to hemoglobin as $4.2\text{–}4.6 \times 10^2$ M^{-1}.[2]

Below pH 7.2, a considerable amount of 2,3-diphosphoglycerate coprecipitates with hemoglobin. This may be due to a rise in the value of k_2 at low pH. Thus, k_2 appears to be pH dependent. At pH 6.0, the ratio of 2,3-diphosphoglycerate to hemoglobin in the precipitate exceeds 1. The nature of 2,3-diphosphoglycerate binding at low pH may be different from that at neutral pH's. The 2,3-diphosphoglycerate content in the precipitate of deoxyhemoglobin was not measured because of the marked stabilization. The binding constant of 2,3-diphosphoglycerate for deoxyhemoglobin is assumed to increase by a factor of 2 since a smaller amount of 2,3-diphosphoglycerate is required for the stabilization of deoxyhemoglobin than oxyhemoglobin. Inositol hexaphosphate appears to have a higher affinity for both oxy- and deoxyhemoglobin than 2,3-diphosphoglycerate. The hemoglobin is stabilized when 1 mol of inositol hexaphosphate is added/mol of hemoglobin (*cf.* Figure 2a). This result is consistent with that reported by Jänig *et al.* (1971). Since hemoglobin bound to inositol hexaphosphate is stabilized, the mechanical shake method may be used for the rapid estimation of inositol hexaphosphate in solution if no other phosphate compounds are present.

As we have shown elsewhere (Asakura *et al.*, 1974b), the α_A and β_A subunits of hemoglobin show completely different pH profiles when they are subjected to a mechanical shake test. The α_A subunits are stable in acidic pH's, while the β subunits are more stable in alkaline pH's. The effect of 2,3-diphosphoglycerate on the mechanical stability of hemoglobin subunits is also different. In 0.1 M bis-tris buffer

[2] A discussion of the precipitation reaction can be found in the Appendix.

(pH 7.4), 2,3-diphosphoglycerate showed a stabilizing effect on the oxy and deoxy forms of β_A chains, while it had little effect on α_A chains. These results agree well with those reported by Benesch *et al.* (1968b). The β_A subunits of hemoglobin bind with 2,3-diphosphoglycerate to the same extent in oxy and deoxy forms. The β chains are known to form tetramers and β_4 is reported to have a structure which closely resembles that of deoxyhemoglobin (Perutz and Mazzarella, 1963). At acidic or alkaline pH, however, 2,3-diphosphoglycerate appears to interact more strongly with the oxy form of α chains as 2,3-diphosphoglycerate accelerates the rate of precipitation at acidic pH's, while it inhibits at alkaline pH's (*cf.* Table IV). The deoxy form of α chains is extremely stable and no precipitation occurs during shaking for 30 min at room temperature. This property is completely different from that of β_A subunits which precipitate at a similar rate ragardless of the oxygenation state. The β_S subunit showed essentially similar properties to those of β_A subunits except that β_S subunits precipitated more rapidly at alkaline pH's.

It was reported that sickle erythrocytes contain a higher level of 2,3-diphosphoglycerate than normal erythrocytes (Charache *et al.*, 1970; Seakins *et al.*, 1973). The increased formation of 2,3-diphosphoglycerate may be bad for sickling of erythrocytes as 2,3-diphosphoglycerate shifts the oxygen equilibrium curves to the right. On the other hand, the increase of 2,3-diphosphoglycerate may be good for the stabilization of sickle oxyhemoglobin. As reported by Schneider *et al.* (1972), sickle erythrocytes contain a higher amount of Heinz bodies (intraerythrocytic aggregation of denatured hemoglobin). Further studies are necessary to evaluate the physiological significance of the increased 2,3-diphosphoglycerate for the stabilization of sickle hemoglobin.

Acknowledgment

The authors thank Dr. J. A. McCray for the mathematical treatment of the kinetic method for determining the DPG association constant to oxyhemoglobins, and Drs. A. F. W. Coulson and P. D. Smith for their helpful discussions. They also thank Drs. E. Schwartz and S. Freedman for generous supply of blood samples.

Appendix

The precipation of Hb and Hb-DPG may be formulated

$$\begin{array}{ccc} \mathrm{Hb} & \xrightarrow{k_1} & \mathrm{Hb_{ppt}} \\ {\scriptstyle +\mathrm{DPG}}\uparrow\downarrow{\scriptstyle -\mathrm{DPG}} & \left(\begin{array}{c}k_3\\ \rightleftharpoons\\ k_4\end{array}\right) & \\ \mathrm{Hb-DPG} & \xrightarrow{k_2} & \mathrm{Hb-DPG_{ppt}} \end{array}$$

where k_1 and k_2 are the rate constants of precipitation of hemoglobin and its complex with DPG and $K = (k_3/k_4)$, association constant of DPG binding to hemoglobin.

The rate constant k_1 follows first-order kinetics (Asakura *et al.*, 1974a) and can be determined by measuring the rate of precipitation of hemoglobin in the absence of DPG. The amount of DPG in the precipitate can also be determined from the acid extract enzymatically. Since no DPG precipitates in 0.1 M bis-tris buffer at pH 7.4, the k_2 at this pH is considered to be very small relative to k_1. Thus, if we assume k_3, $k_4 \gg k_1$, k_2 the apparent rate of precipitation V is described as

$$V = k_1(\mathrm{Hb}) + k_2(\mathrm{Hb-DPG}) \simeq k_1(\mathrm{Hb})$$

and association constant K can be calculated from

$$K = \frac{(\mathrm{Hb-DPG})}{(\mathrm{Hb})(\mathrm{DPG})} = \frac{[(\mathrm{Hb_t}) - (V/k_1) - (\mathrm{Hb_{ppt}})]}{(V/k_1)(\mathrm{DPG})}$$

where $\mathrm{Hb_t}$ is total hemoglobin used, $\mathrm{Hb_{ppt}}$, amount of hemoglobin precipitated, and (Hb-DPG) = ($\mathrm{Hb_t}$ − Hb − $\mathrm{Hb_{ppt}}$), thus, we can estimate $K = 4.6 \times 10^2\ \mathrm{M}^{-1}$.

If the concentration of DPG is high compared to that of hemoglobin and $k_2 = 0$, the kinetic equations may be

$$\begin{array}{ccccc} \mathrm{Hb-DPG} & \underset{k_3}{\overset{k_4}{\rightleftharpoons}} & \mathrm{Hb} & \xrightarrow{k_1} & \mathrm{Hb_{ppt}} \\ \mathrm{A} & & \mathrm{B} & & \mathrm{C} \end{array}$$

solved with the use of Laplace transform. The differential equations are

$$dA/dt = -k_4A + k_3(\mathrm{DPG})B = k_4A + \lambda_3B$$

$$dB/dt = k_4A - \lambda_3B - k_1B$$

$$dC/dt = k_1B$$

If the rate constants k_3 and k_4 are large relative to k_1, the solution is

$$A(t) = \left[\frac{k_4A_0 - \lambda_3B_0}{\lambda_3 + k_4}\right] e^{-(\lambda_3+k_4)t} + \frac{\lambda_3}{\lambda_3 + k_4}(A_0 + B_0)\ e^{-2k_4k_1t/(\lambda_3+k_4)}$$

where A_0 and B_0 are the initial concentrations, before shaking, of Hb-DPG and Hb, respectively.

If we start from the initial equilibrium for the first reaction, we have

$$dA/dt = k_4A_0 = \lambda_3B_0 = 0$$

thus

$$A(t) = A_0e^{-2k_4k_1t/(\lambda_3+k_4)}$$

Assuming the concentration of hemoglobin to be small compared with Hb-DPG, the initial velocity of precipitation may be found by differentiating $A(t)$ with respects to the time and evaluating the result at $t = 0$. The result is

$$V = \frac{-2k_4k_1A_0}{\lambda_3 + k_4} = \frac{-2k_4k_1A_0}{k_4 + k_3(\mathrm{DPG})}$$

If the reciprocal of the absolute value of V is plotted against the concentration of DPG, a linear curve results

$$\frac{1}{|V|} = \frac{1}{2A_0k_1} + \frac{k_3}{2A_0k_1k_4}(\mathrm{DPG})$$

A value for k_1 may be obtained from the intercept and a value for the equilibrium association constant $K = k_3/k_4$ may be obtained from the slope. From the results shown in Figure 2b, we obtain a value $K = 4.17 \times 10^2\ \mathrm{M}^{-1}$.

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Alkali and Urea Induced Conformation Changes in Concanavalin A†

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ABSTRACT: The urea- and alkali-induced transitions of concanavalin A are apparently irreversible, as judged by circular dichroic (CD) spectra and hemagglutinating activity, although the nature of the product depends on the pH at which reversal is attempted. The native protein exhibits a characteristic far-uv CD spectral band centered at 223 nm, which is blue shifted by alkali and abolished by urea. Lowering pH after denaturation with alkali leads to loss of protein by precipitation in amounts dependent upon pH. The remaining soluble protein has CD characteristics which are dependent on the pH at which reversal attempts are made. A similar pH dependence of the CD spectrum occurs on dilution of urea after denaturation, but this procedure leads to little or no precipitation, perhaps as a result of the lower final protein concentrations. Reversal at pH 6 leads to a CD band centered near 223 nm; products obtained at pH 7, however, show a band at 217 nm. In this same pH interval, native Con A undergoes changes in sedimentation coefficient reflecting a transition from dimer to tetramer. Native Con A, however, exhibits a constant CD spectrum over this pH interval. Succinylated Con A has a CD spectrum closely resembling that of the native protein, but is more resistant to alkali denaturation, suggesting that specific group titration, rather than general charge effects, are responsible for the alkaline lability.

Concanavalin A, a lectin isolated from jack beans (Sumner, 1919), undergoes an apparently irreversible conformational transition between pH 8 and 9 at room temperature (Pflumm *et al.,* 1971; Zand *et al.,* 1971). A similar transition occurs at pH 8 if the protein is allowed to stand at 37° (Cunningham *et al.,* 1972). The conformational changes are followed by precipitation if the pH of the solution is below 9. Turbidity arises even in pH 7 solutions of the lectin (McKenzie *et al.,* 1972) suggesting that a slow transition occurs also at neutral pH. We have now examined this transition in greater detail in an effort to determine whether it is indeed irreversible and, if it is, what structured features are responsible. Since the conformational changes involve elements of the protein's secondary and tertiary structure, the course of the transition may be monitored by changes in the circular dichroic (CD) spectrum of Con A.

Native Con A exhibits an unusual far-uv CD band cen-

† From the Department of Biological Sciences, Columbia University, New York, New York 10027. *Received May 23, 1974.* Supported in part by grants from the National Science Foundation (NSF GB 29481) and from the National Institutes of Health (5 RO1 CA 13014).