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Research Article

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A B S T R A C T Several unstable mutant hemoglobins have alterations which affect areas of the molecule involved in the attachment of heme to globin. Loss of heme from globin has been demonstrated during the denaturation of some of these unstable mutants. The importance of heme ligands for the stability of hemoglobin was illustrated in the present experiments on the denaturation of several hemoglobins and hemoglobin derivatives by heat, oxidative dyes, and alkali. Heating of normal hemolysates diluted to 4 g of hemoglobin per 100 ml at 50°C for 20 hr in 0.05 M sodium phosphate, pH 7.4, caused precipitation of 23–54% of the hemoglobin. Dialysis against water or dilution of the sample decreased denaturation to 12–20%. Precipitation was decreased to less than 3.5% by the presence of 0.015 M potassium cyanide. Increasing the ionic strength of the medium increased precipitation. Cyanide prevented the formation of inclusion bodies when red cells containing unstable hemoglobin Philly, $\beta35$ tyr → phe, were incubated with the redox dye new methylene blue. Conversion to methemoglobin increased the rate of alkali denaturation of hemoglobin but the presence of potassium cyanide returned the denaturation rate to that of ferrohemoglobin. The ability of cyanide to decrease heat precipitation of hemoglobin may depend on a dimeric or tetrameric state of the hemoglobin molecule. Purified β-chains, which exist as tetramers, were stabilized but purified monomeric α-chains were not rendered more heat resistant by the ligand. Stabilization of hemoglobin by cyanide required binding of the ligand to only one heme of an $\alpha\beta$-dimer. Hemoglobin Gun Hill, an unstable molecule with heme groups present only on the α-chains was quite heat stable in the presence of cyanide. The binding of cyanide to the iron atom in methemoglobin is thought to be associated with increased planarity of the heme group and increased stability of the heme-globin complex. The stabilizing effect of cyanide in the above experiments suggests that Heinz body formation, heat precipitation of hemoglobin, and the increased alkali denaturation of methemoglobin depend on changes of heme-globin binding.

INTRODUCTION

Certain mutations of the human hemoglobin molecule decrease the stability of this protein in solution (1). Some of the unstable hemoglobins are associated with hemolytic disease of varying severity, and occasionally with the presence of spontaneous intra-erythrocytic inclusion bodies formed from precipitated hemoglobin (2, 3). Formation of inclusion bodies upon exposure of red blood cells to redox dyes and increased precipitation of hemoglobin solutions upon heating to 50°C are characteristic findings in patients with unstable hemoglobins. These denaturing procedures can be useful means of detection of unstable mutants in cases of apparent idiopathic hemolytic anemias where abnormal hemoglobins cannot be demonstrated by electrophoresis (4).

This paper reports observations on the different rates of precipitation of several hemoglobins and hemoglobin derivatives upon exposure to heat, oxidant dyes, and alkali. The results emphasize the importance of ligands which affect the binding of heme to globin on the stabilization of the hemoglobin molecule.

METHODS

Measurement of hemoglobin. The concentrations of hemoglobin solutions were determined by comparing the optical

density at 540 mμ of the cyanmethemoglobin derivatives to that of a reference standard (Acuglobin, Ortho Pharmaceutical Corp.). The pyridine hemochromogen determination was performed according to the method of Vernon and Kamen (5). Methemoglobin was measured according to the method of Evelyn and Malloy (6). The Folin protein determination was done by the method of Lowry, Rosebrough, Farr, and Randall (7). Deoxyhemoglobin was estimated by the method of Benesch, MacDuff, and Benesch (8).

Purified hemoglobins. Hemoglobin A and hemoglobin Gun Hill (9) were purified by starch-block electrophoresis in barbital buffer, pH 8.6 (10). Hemoglobin Gun Hill solutions prepared in this manner contained approximately 10% hemoglobin A_2.

α and β-hemoglobins. The α- and β-chains of hemoglobin A were prepared by the method of Bucci and Fronticelli (11). The *p*-mercuribenzoate (PMB) was removed from the separated chains by stirring with dodecanethiol according to the method of DeRenzo and associates (12) or by exposure to 2-mercaptoethanol on carboxymethyl cellulose (CMC) and diethylaminoethyl cellulose (DEAE) columns according to the method of Geraci, Parkhurst, and Gibson (13). When titrated with PMB (14), β-chains treated with dodecanethiol showed 1.4–2.5 free regenerated sulfhydryl groups per molecule. Similarly treated α-chains often gave indeterminate values for free -SH groups. α-Chains treated with 2-mercaptoethanol on CMC columns showed approximately 0.8 regenerated free -SH groups per molecule. The purity of these preparations was checked by starch-gel electrophoresis (15).

Heat denaturation. The heat stability test was performed by a modification of the procedure of Grimes, Meisler, and Dacie (16). Hemolysates, purified hemoglobins, and hemoglobin derivatives, adjusted to 2–8 g/100 ml, were diluted 1:1 in 0.1 M potassium phosphate, pH 7.4 or pH 7.0, or 0.1 M potassium phosphate–0.4 M NaCl, pH 7.4, and incubated in stoppered test tubes in a water bath at 50°C or 65°C. The effect of cyanide was evaluated by incorporating 0.015 M potassium cyanide (final concentration with the hemoglobin) in the buffer. The pH of the buffer was adjusted to 7.4 after the addition of cyanide.

Erythrocyte inclusion body formation. 0.5 ml of whole blood was incubated at 37°C with 1 ml of a solution of either brilliant cresyl blue, methylene blue, or new methylene

TABLE I

Heat Denaturation of Normal Hemoglobin at 50°C

Sample No.	0.05 M potassium phosphate, pH 7.4*		0.05 M potassium phosphate, pH 7.4,* 0.2 M NaCl	
	−KCN	+0.015 M KCN	−KCN	+0.015 M KCN
	% precipitated			
1	50.4	3.3	58.7	5.2
2	50.3	1.5	67.7	3.5
3	23.7	1.5	49.5	0
4	48.8	3.0	63.5	3.5
5	38.1	3.5	62.3	3.8
6	54.1	0.6	60.6	0

* The buffers containing cyanide were adjusted to pH 7.4 after the addition of KCN.

TABLE II

Effect of Dialysis and Dilution with H_2O on Heat Denaturation of Hemoglobin at 50°C

Hb concn.	0.05 M potassium phosphate, pH 7.4		0.05 M potassium phosphate, pH 7.4, 0.2 M NaCl	
	Undialyzed	Dialyzed	Undialyzed	Dialyzed
g/100 ml	% precipitated			
4	42.3	16.4*	50.3	27.6*
2	24.8	14.2*	44.8	32.4*
1	19.9	12.0*	37.5	27.2*
4		5.3‡		

* Dialyzed 5 hr against H_2O.
‡ Dialyzed 24 hr against H_2O. 98% conversion to methemoglobin after 20 hr at 50°C.

blue N, 10 mg/ml, containing sodium citrate 4 mg/ml, and sodium chloride, 8.5 mg/ml. To this mixture was added either 0.5 ml of sodium chloride, 8.5 mg/ml, or 0.5 ml of 0.1 M KCN, brought to pH 7.6 with HCl. Blood films were prepared at 2, 4, and 24 hr.

In other experiments, one volume of washed red cells was mixed with one volume of a solution containing 5 or 10 mg/ml acetylphenylhydrazine and either 0.1 M sodium phosphate, pH 7.4, or 0.05 M sodium phosphate, pH 7.4. The mixtures were incubated at 37°C. Aliquots were removed at 2 and 15 hr, stained with new methylene blue N, and blood films were prepared.

Alkali denaturation. A modification of the method described by Singer, Chernoff, and Singer was used (17, 18). 0.2 ml of a hemoglobin solution, 3–6 g/100 ml, in 0.13 M sodium chloride–0.13 M sodium phosphate buffer, pH 7.4, or 0.13 M potassium cyanide–0.13 M sodium phosphate buffer, pH 7.4, was added to a series of test tubes containing 1.6 ml of a denaturing buffer, pH 12.8–13.4, prepared by mixing varying proportions of 0.4 M KOH and 0.4 M K_2HPO_4. All solutions were maintained at 0°C in an ice bath. The reaction was stopped by the addition of 3.4 ml of a 50% saturated ammonium sulfate solution containing 2.5 ml of concentrated HCl per liter. The neutralized solution was filtered through Whatman No. 50 filter paper. The percentage of undenatured hemoglobin was determined by comparing the optical density at 540 mμ of the filtrate to that of a "zero time" sample in which the denaturing buffer was replaced by an equal volume of the sodium chloride–sodium phosphate buffer and the ammonium sulfate solution contained no HCl.

RESULTS

Heat precipitation. Several of the unstable hemoglobins are known to denature rapidly and precipitate from solution upon heating to 50°C (1, 16). Jacob, Brain, Dacie, Carrell, and Lehmann have demonstrated that the heat precipitation of hemoglobin Köln and hemoglobin Hammersmith is associated with loss of heme groups and that this loss can be prevented by cyanide (19). It was decided to investigate whether heat precipitation of normal hemoglobin was also asso-

ciated with heme loss and could be prevented by cyanide. Normal hemolysates heated to 50°C demonstrated less than 5% precipitation of hemoglobin during short periods (less than 3 hr) of incubation. However, when normal hemolysates, adjusted to a hemoglobin concentration of 4 g/100 ml, were heated at 50°C for 20 hr in 0.05 M sodium phosphate, pH 7.4, precipitation of 24–54% of the hemoglobin occurred (Table I). The addition of 0.015 M potassium cyanide reduced the precipitation of hemoglobin to less than 3.5%. Upon increasing the salt content of the mixture to 0.2 M NaCl–0.05 M sodium phosphate, pH 7.4, there was significantly greater precipitation ($P < 0.005$) (Table I). When 0.015 M KCN was added the amount of hemoglobin precipitated in the higher ionic strength medium decreased to 0–5.2%. Dilution or dialysis of the hemolysate with distilled water, before the addition of the phosphate buffer, resulted in a decrease in the amount of hemoglobin denatured (Table II). Removal of trace amounts of divalent cation or metal did not appear to be the basis of this effect. The addition of 0.001 M sodium EDTA to an undialyzed hemolysate having a hemoglobin concentration of 4 g/100 ml did not decrease the denaturation. In all these experiments, the precipitates formed were reddish-brown and heme and globin appeared to be removed from solution in approximately equal proportions. No significant deviations in apparent loss of hemoglobin were noted when the hemolysates were compared, before and after heating, by the cyanmethemoglobin method at 540 mμ, the pyridine hemochromogen method at 420 mμ, the optical density at 280 mμ, or by the Folin-Ciocalteu technique at 700 mμ. Evidence that cyanide prevented denaturation and not just precipitation was demonstrated by the failure of

TABLE III
Heat Denaturation of Hemoglobin Derivatives at 65°C

Hemoglobin derivative	Concn.	Time	0.05 M potassium phosphate, pH 7.4	
			−KCN	+0.015 M KCN
	g/100 ml	min	% precipitated	
Methemoglobin	3.6	5	55	0
		15	77	6
		60	91	9
Carbonmonoxy-hemoglobin	4.5	150	0	0
Oxyhemoglobin	4.5	150	91	18
Deoxyhemoglobin*	4.0	150	8.4	

* Complete deoxygenation determined by spectral analysis. Heating was done *in vacuo* and conversion to methemoglobin was 1.8% after heating.

TABLE IV
Heat Denaturation of Various Hemoglobins at 50°C

Hemoglobin type	Concn.	Time	0.05 M potassium phosphate, pH 7.0	
			−KCN	+0.015 M KCN
	g/100 ml	hr	% precipitated	
α^A	2.8	3	93	86
		20	98	92
β^A	2.5	3	49	6
		20	81	15
A	3.3	20	11	6
Gun Hill	2.3	20	84	15
	1.2	20	94	0

50% ammonium sulfate to cause precipitation of the cyanide-treated samples after heating (20).

Precipitation of the hemolysates upon heating was preceded by conversion of most of the hemoglobin to methemoglobin. The instability of methemoglobin and the ability of certain heme ligands to stabilize methemoglobin and hemoglobin even during severe heat stress, were illustrated by experiments performed at 65°C. When a solution of methemoglobin formed by treating an hemolysate with 1.2 equivalents of potassium ferricyanide per heme equivalent was heated to 65°C, there was immediate precipitation, with loss of over 50% of the protein within 5 min (Table III). The results were the same whether the potassium ferricyanide was allowed to remain in the hemoglobin solution or was removed by dialysis or Sephadex G-25 filtration. The addition of cyanide stabilized the methemoglobin so that loss was reduced to 9% even after heating for 60 min at 65°C. Oxyhemoglobin was more stable but over 90% precipitated after 150 min at 65°C. The precipitation was reduced by cyanide to less than 20% (Table III). Carbon monoxide, which binds to the heme of ferrohemoglobin, conferred great stability. No precipitation of a solution of carbon monoxyhemoglobin occurred after 150 min of heating at 65°C (Table III). Carbon monoxide inhibited conversion of hemoglobin to methemoglobin in this experiment. Deoxyhemoglobin was resistant to heat denaturation, and in the absence of oxygen, there was little conversion to methemoglobin (Table III).

The great stability at elevated temperatures conferred by cyanide appeared to depend partly on a dimeric or tetrameric state of the hemoglobin polypeptide chains. Purified β^A-chains were stable when incubated in the presence of cyanide for 20 hr at 50°C (Table IV). These chains can form the tetramer, hemoglobin H (21). Purified α^A-chains, which exist primarily as monomers (22), were unstable when heated in the
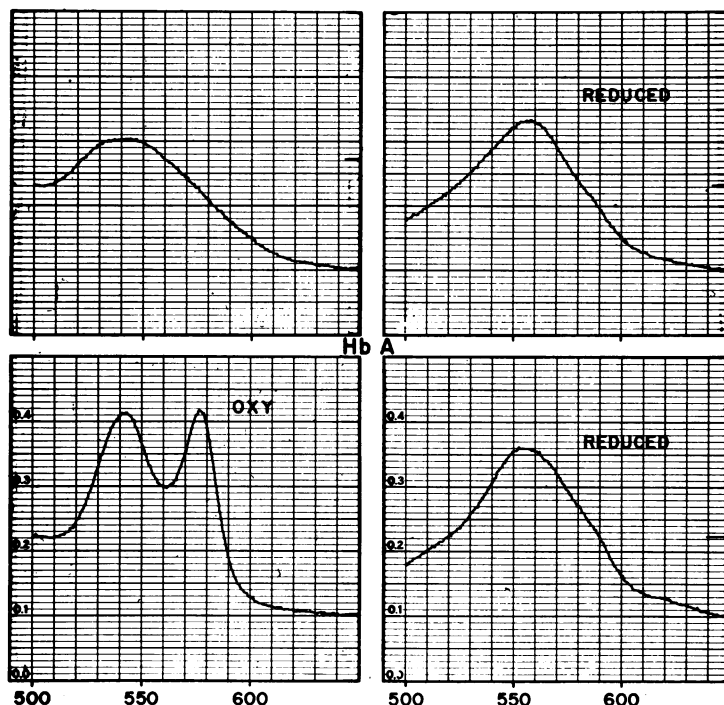
Hb GUN HILL
50°C, 20 HOURS, 0.015 M KCN

FIGURE 1 The upper portion of the figure shows the absorption spectrum from 500 to 650 mμ of a solution of hemoglobin Gun Hill, heated in the presence of KCN, before and after reduction with dithionite. No hemochromogen peaks are present in the specimen after reduction. The bottom of the figure shows the same portion of the absorption spectrum of a solution of unheated hemoglobin A before and after dithionite reduction. The deoxyhemoglobin A spectrum is identical with the heated deoxyhemoglobin Gun Hill pattern.

presence of cyanide (Table IV). α-chains whose free -SH groups were regenerated with either dodecanethiol or mercaptoethanol gave similar results. Upon exposure to 50°C the solutions of α^A-chains rapidly became turbid and precipitation of approximately 90% of the protein occurred after 3 hr of heating. The results could also be explained on the basis of an inherent difference in stability of α- and β-chains unrelated to the differences in quaternary structure.

Stabilization by cyanide of only one heme group on a heme dimer appeared to be adequate to prevent heat precipitation. When a solution of purified hemoglobin Gun Hill (8, 23), which lacks heme groups on the β-chains, was heated to 50°C for 20 hr in 0.05 M potassium phosphate, pH 7.0, over 90% denatured (Table IV). The presence of 0.015 M cyanide reduced precipitation to 0 and 15% in two experiments. Thus although cyanide could bind only to heme groups on the α-chains of the hemoglobin Gun Hill αβ-dimer, stabilization was essentially complete.

The stabilization of hemoglobin Gun Hill by cyanide appeared to be a true inhibition of denaturation and not just the prevention of precipitation by the formation of a soluble cyanide–irreversibly denatured hemoglobin complex. The heated hemoglobin Gun Hill solution did not precipitate in the presence of 50% saturated ammonium sulfate. The absorption spectrum of the heated solution, in 0.05 M potassium phosphate, pH 7.0, 0.015 M KCN, was that of cyanmethemoglobin with peaks at 540 and 420 mμ (Fig. 1). Upon reduction with dithionite, the cyanmethemoglobin peaks were replaced by deoxyhemoglobin peaks at 558 and 430 mμ. There was no evidence of the peaks at 530 and 565 mμ characteristic of a ferro-denatured globin hemochromogen or hemochrome (24).

Inclusion body formation. Erythrocytes containing hemoglobin Gun Hill or hemoglobin Philly (4) developed inclusion bodies when heated at 37°C with the oxidant dyes brilliant cresyl blue, methylene blue, or new methylene blue N. The prior addition of potassium cyanide to the incubation mixture inhibited the

formation of inclusion bodies. Fig. 2 shows this effect on erythrocytes containing hemoglobin Philly after 4 hr of incubation. Similar protection by cyanide was observed even after 24 hr of exposure to the dye. Cyanide also inhibited the production of inclusion bodies when normal erythrocytes were incubated for 15 hr with acetylphenyl-hydrazine (Fig. 2).

Alkali denaturation. The stability of several hemoglobin derivatives was examined by the alkali denaturation method. The pH values of the hemoglobin–alkali mixtures were controlled by using alkaline buffers as denaturing agents. In any single experiment, the final pH of the mixtures of a given denaturing buffer and the hemoglobin preparations under study varied by less than 0.1 pH unit.

The influence of pH on the rate of denaturation of normal hemoglobin is shown in Fig. 3. The denaturation had the appearance of a first-order reaction until the hemoglobin concentration was reduced below 10% of the initial value of 3–6 g/100 ml. The presence of potassium cyanide had no effect on the rate of alkali denaturation of oxyhemoglobin (Fig. 3).

Methemoglobin was much less stable than ferrohemoglobin in the presence of alkali (Fig. 4). Increasing the proportion of methemoglobin increased the rate of denaturation of the hemolysate. The addition of KCN to a sample containing 98% methemoglobin restored the rate of denaturation to that of oxyhemoglobin (Fig. 4).

DISCUSSION

The heme moieties of normal hemoglobin lie in nonpolar pockets formed by the folding of the globin chains and each porphyrin group makes approximately 60 contacts, mainly nonpolar, with the protein portion of the molecule (25). One covalent bond links the iron atom
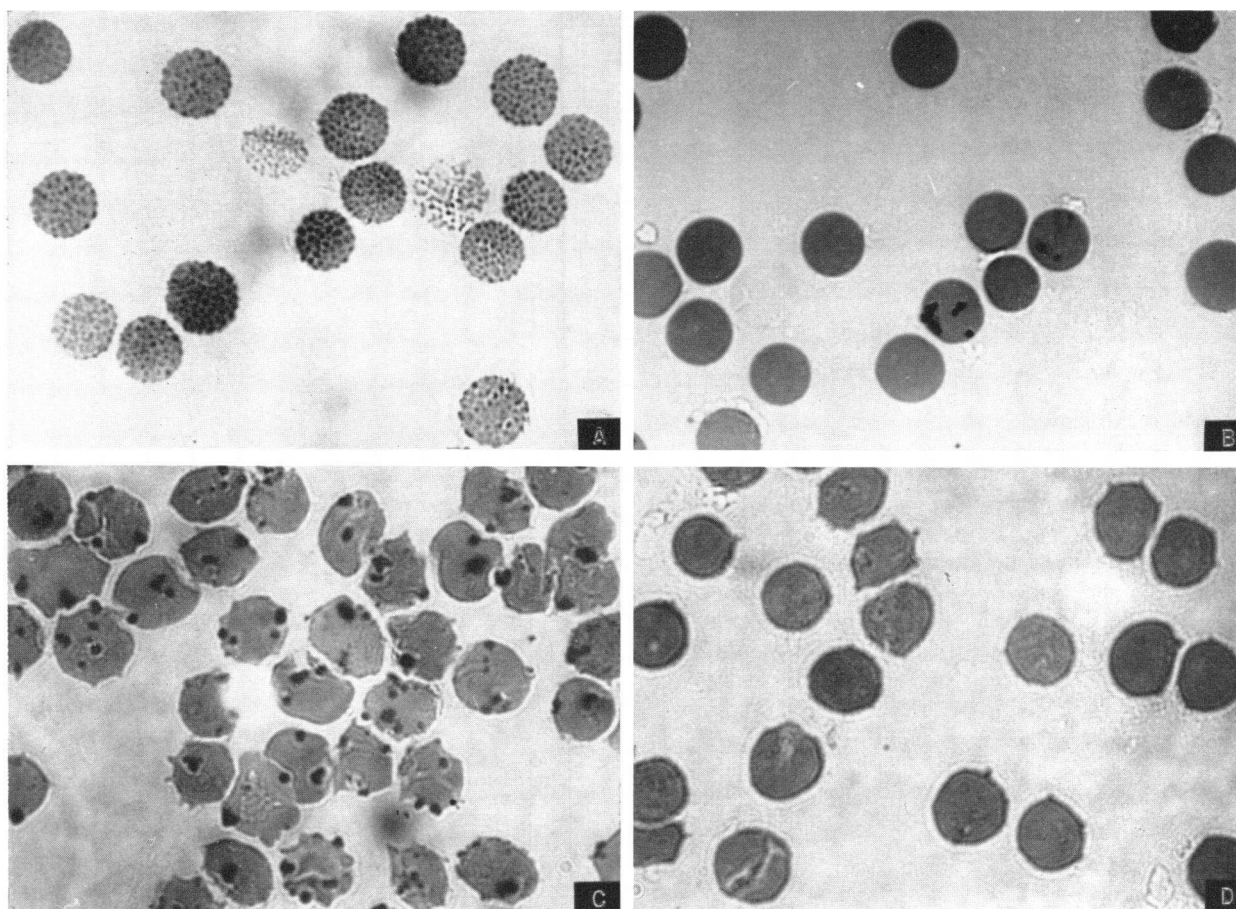

FIGURE 2 Inhibition of inclusion body formation by cyanide. (A) Erythrocytes containing hemoglobin Philly heated at 37°C for 4 hr in the presence of new methylene blue N. (B) The same incubation mixture as in A but containing 0.025 M KCN. (C) Normal erythrocytes incubated 15 hr with acetylphenylhydrazine. (D) Normal erythrocytes incubated with acetylphenyl-hydrazine in the presence of 0.05 M KCN.
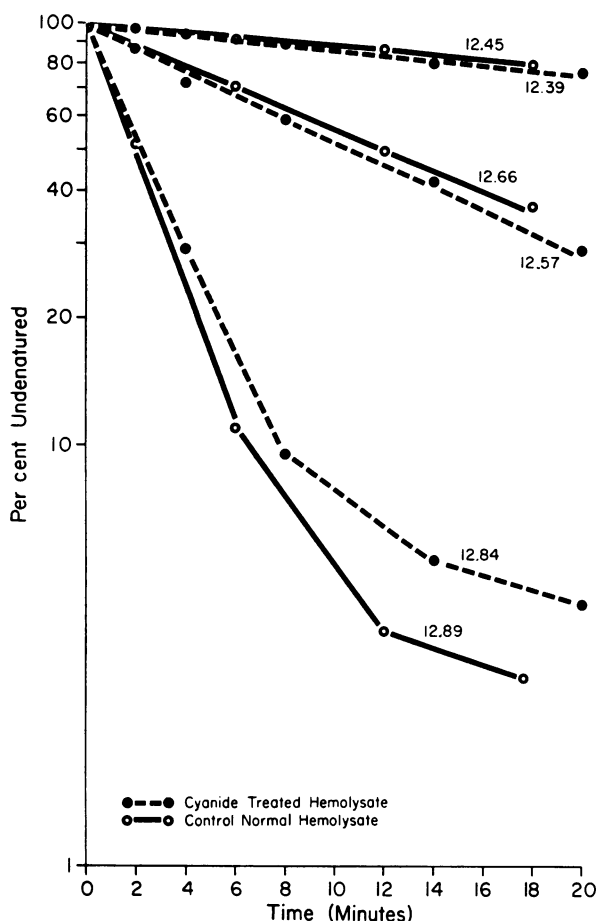
FIGURE 3 Alkali denaturation at varying pH values of hemolysates in the presence and absence of KCN. The pH value of each mixture is indicated next to the appropriate curve.

at the center of a heme group to the histidine at either position 92 of the β-chain or position 87 of the α-chain.

The importance of the normal α-β interaction for the stability of the hemoglobin molecule is suggested by the ease of denaturation of the separated α- and β-components. In vivo, the lability of free β-chains is illustrated by the occurrence of inclusion bodies in the erythrocytes of patients with hemoglobin H disease (26). The instability of α-chains results in inclusion body formation in erythrocytes having excess α-chains as in β-thalassemia (27). Mutations in amino acids involved in the contacts between the α- and β-chains can result in hemoglobin instability as illustrated by hemoglobin Philly (4), hemoglobin Tacoma (28), and hemoglobin Kansas (29).

The structural importance of the normal heme-globin contacts is indicated by the instability of many hemoglobin mutants having amino acid substitutions involving heme-binding residues (1). Some of these mutations

so weaken the binding of heme to globin that there is actual loss of heme from the molecule (19, 23, 30–33). Heme-free globin is unstable and precipitates rapidly at room temperature (34).

The oxidation state of the heme iron and the presence of ligands exert a pronounced effect on the resistance of the hemoglobin molecule to denaturation by a variety of agents. Hartridge measured the first-order rate constants for the heat denaturation of dog hemoglobin derivatives at temperatures between 50°C and 80°C (35). Methemoglobin was more rapidly precipitated than oxyhemoglobin. The attachment of CO to the heme ion also decreases the rate of alkali denaturation of hemoglobin, and heavy smokers have more alkali-resistant hemoglobin than nonsmokers (36, 37).

Steinhardt, Ona-Pascual, Beychok, and Ho (38) and Steinhardt, Polet, and Moezie (39) have shown that horse ferrohemoglobin is more resistant to acid denaturation than ferrihemoglobin. The addition of cyanide to
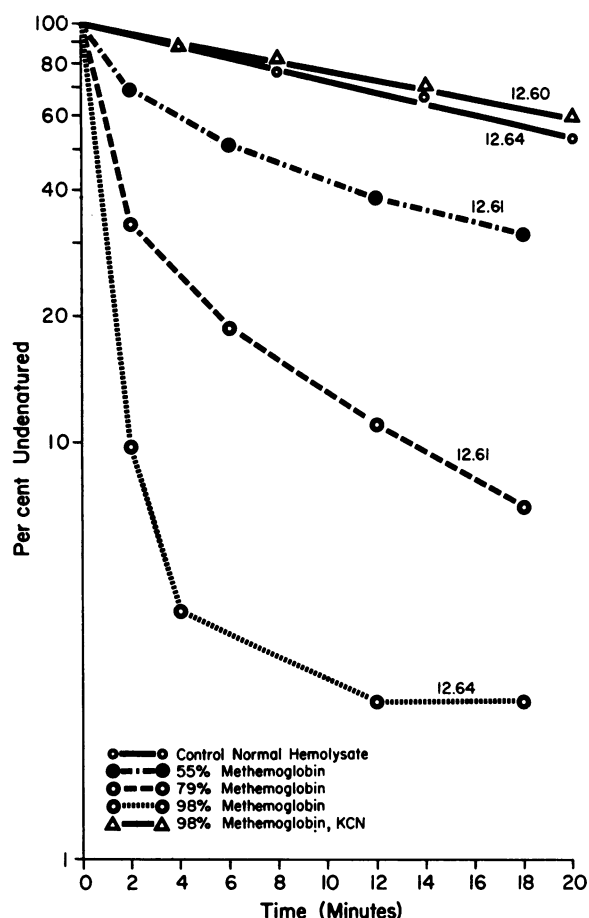

FIGURE 4 Alkali denaturation of a normal hemolysate before and after conversion to varying proportions of methemoglobin. The addition of KCN reduced the rate of denaturation of a sample containing 98% methemoglobin to normal.

horse ferrihemoglobin decreased the velocity of denaturation in acid up to 300-fold.

Similar results were obtained with hemolysates of human blood in the present studies. Cyanide markedly reduced precipitation of hemoglobin even after 20 hr of exposure to 50°C. The ability of ligands to stabilize hemoglobin during severe, prolonged heat stress was demonstrated by the effect of carbon monoxide and cyanide on hemoglobin solutions heated to 65°C for 2.5 hr. Analogous results were also observed in the study of the alkali denaturation of methemoglobin in the presence and in the absence of cyanide.

Erythrocyte inclusion body formation appears to have some common basis with heat and alkali denaturation of hemoglobin. The present experiments demonstrated that ligand binding reduced the formation of Heinz bodies in normal red blood cells exposed to acetylphenylhydrazine and in erythrocytes containing hemoglobin Philly, incubated with redox dyes.

The stabilizing effect of cyanide on normal hemoglobin heated to 50°C appeared to extend also to hemoglobin H. However, monomeric α-chains were denatured by 5 min of heating even with cyanide in the buffer. These results suggest that some sort of interchain interaction may be strengthened by the addition of the cyanide ligand to methemoglobin. Evidence for such an interaction is also suggested by the study of the heat denaturation of hemoglobin Gun Hill. The binding of cyanide to only the α-chains strengthened the entire complex and prevented precipitation after prolonged heating. Steinhardt and associates (38), on the basis of stoichiometric considerations, have also postulated that stabilization can occur by combination of ligand with a single heme of each hemoglobin dimer.

The structural basis for the stabilization of methemoglobin by cyanide is not certain. Binding of cyanide is associated with a decrease in the ionic character and an increase in the covalent nature of the bond between histidine and the heme iron and between the iron atom and the four pyrrole rings of the porphyrin group (40, 41). The porphyrin group is drawn closer to the protein portion of the molecule and the iron atom moves to a position closer to the plane of the porphyrin (41). Such changes could result in tighter binding of heme to globin. Bunn and Jandl (42) have shown that cyanide inhibits migration of heme between methemoglobin molecules and between hemoglobin and albumin.

Steinhardt and associates (38) have postulated that the stabilizing effect of cyanide may be due to loss of a positive charge on the iron of ferrihemoglobin upon binding the ligand. The charged iron atom without ligand, together with the attached porphyrin, is thought to be situated further towards the outer portion of the hemoglobin molecule and thus more exposed to the solvent medium of high dielectric constant. In the present experiments a reduction in the ionic strength of the medium increased hemoglobin stability.

Although some unstable hemoglobins may lose heme during denaturation such loss of heme could not be demonstrated in the present studies on normal hemoglobin. Several authors have presented evidence that hemoglobin denaturation is accompanied by formation of a hemichrome in which the sixth coordination position of the heme iron becomes bound to a nitrogenous group, possibly the "distal" histidines at β63 and α57 (43–45). Prior binding of cyanide to the sixth coordination position could prevent hemichrome transformation and the configurational changes which accompany it.

ACKNOWLEDGMENTS

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