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Research Article

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Precipitation of oxyhemoglobin and carboxyhemoglobin in all cases gave nonspecific oxidation of approximately two of the six hemoglobin sulfhydryl groups to give intra- and intermolecular disulfide bonds. Single α- and β-chains, plus polymers of up to five or six chains linked by disulfide bridges, were demonstrated by polyacrylamide gel electrophoresis. This disulfide oxidation was not observed with deoxy- or methemoglobin and did not appear to influence the rate of precipitation. These findings fit the theoretical prediction that autoxidation of oxy- and carboxyhemoglobin is […]

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ABSTRACT The sequential changes that occur during the precipitation on mild heating of the unstable hemoglobins, Hb Christchurch, Hb Sydney, Hb Köln, and Hb A, were examined with particular attention to the possibility of an accompanying oxidative process. Hb Christchurch, Hb Sydney, and Hb A precipitated with equal amounts of α- and β-chains and full heme complement. Hb Köln, however, was one-half heme-depleted and showed a slight excess of precipitated β-chains. In all cases the spectrum of the precipitated material was typical of a hemichrome. There was no evidence that sulfhydryl oxidation contributed to the precipitation process. Reduced glutathione was unable to protect the hemoglobin against precipitation, and mixed disulfide formation between the precipitating hemoglobin and glutathione was insignificant, even in the presence of excess glutathione. No blockade of β93 cysteines could be demonstrated in the unstable hemoglobins.

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Together, these results are in keeping with predictions based on the known structural abnormalities of the unstable hemoglobins, all of which result in greater molecular flexibility. Our findings support the conclusion that the usual precipitating event is altered bonding at the heme to give the formation of hemichromes. There is no evidence of an accompanying oxidative process that could pose a threat to the integrity of the red cell.

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INTRODUCTION

The intracellular precipitation of hemoglobin to form Heinz bodies occurs in hemolytic anemias associated with the unstable hemoglobins. The defect in these unstable hemoglobins usually involves important internal bonding amino acids, particularly those forming bonds with the heme group. The reason for the instability of the hemoglobin is therefore quite well understood at the molecular level, but there is still a lack of knowledge of the changes occurring during precipitation and the ultimate cause of hemolysis (1–3).

The similarity to events observed in the oxidative hemolytic anemias has led to proposals that the precipitation of the unstable hemoglobins is accompanied by oxidative changes. The oxidative hemolytic anemias arise either from formation of excess oxidizing products (as with acetylphenylhydrazine administration) or from breakdown of protective mechanisms against oxidants (as in glucose-6-phosphate dehydrogenase deficiency). In either case, the end result is the same as with the unstable hemoglobins; the precipitation of hemoglobin, the formation of Heinz bodies, and hemolysis. In the case of the oxidative hemolytic anemias, the presence of free oxidants as well as precipitated hemoglobin could pose a direct threat to the cell membrane.

Two proposals have been made that could explain similar oxidative effects in the unstable hemoglobin hemolytic anemias. Jacob (4) has put forward a general mechanism of Heinz body formation based on sulfhydryl oxidation that has received support in other reviews (5, 6). He proposed that there is initial loss of

the heme groups from the affected chains, with oxidation of specific (β93) sulfhydryl groups to give mixed disulfides with glutathione, followed by precipitation of the abnormal chains to give Heinz bodies. He also proposed that a major contribution to the accompanying hemolysis is disturbance of the cell membrane by mixed disulfide bond formation between membrane proteins and the precipitated hemoglobin. Alternatively, Carrell (7) has proposed that the defects in the unstable hemoglobins could allow water to gain access to the oxygenated heme iron, resulting in the formation of methemoglobin and highly reactive superoxide ions. The end result could be the release of superoxide with effects analogous to those seen with acetylphenylhydrazine. In the present study, we have looked for evidence of oxidative processes accompanying hemoglobin precipitation, and also examined in detail the sequence of events proposed by Jacob.

The molecular defects that cause unstable hemoglobins to precipitate intracellularly to form Heinz bodies also cause their accelerated precipitation on mild heating, and in many ways the two processes appear to be similar. Detailed information on the changes that accompany heat precipitation should therefore contribute to the understanding of the mechanism of Heinz body formation. We have studied the heat precipitation of three unstable hemoglobins: Hb Christchurch[1] (β71 Phe → Ser), Hb Sydney (β67 Val → Ala), and Hb Köln (β98 Val → Met) and also Hb A, which behaves like the unstable hemoglobins when subjected to proportionately greater heat stress. We have determined the heme content and subunit composition of the precipitates, the extent of thiol oxidation, and nature of the disulfide bonds, and have examined the effects of some external factors on precipitation rate and constitution of precipitates.

METHODS

Blood was obtained from normal human donors, patients with Hb Christchurch and Hb Sydney, and four unrelated patients with Hb Köln. Red cells were separated, washed three times with isotonic saline, and hemolysed with 1.5 vol distilled water. Ghosts were removed by shaking with carbon tetrachloride and centrifuging. Where required, glutathione and other low molecular-weight materials were removed by passage through a column of Sephadex G25. Deoxyhemoglobin was prepared by repeated evacuation of hemolysates, carboxyhemoglobin by bubbling carbon monoxide through the solution, and methemoglobin by adding slightly more than 4 mol potassium ferricyanide and removing the excess on a column of Sephadex G25. Renatured globin was prepared by the method of Rossi Fannelli, Antonini, and Caputo (8). Hemoglobin with glutathione bound by disulfide bonds to the β93 cysteines was prepared by disulfide exchange with 10 mol oxidized glutathione (GSSG) at pH 8 and room temperature overnight. The hemoglobin solution was passed through Sephadex G25 before reaction to remove glutathione reductase substrates, and afterwards to remove excess GSSG. Hb Köln was purified by column chromatography on DEAE Sephadex by the method of Huisman and Dozy (9).

Heat precipitates of hemoglobin and various derivatives were prepared by incubating 2% hemolysates in pH 7.4 phosphate buffer at 50°C (precipitation of unstable hemoglobins) or 60°C (precipitation of Hb A). Precipitates were separated by centrifugation, washed twice with water, and dissolved in 3% sodium dodecyl sulfate (SDS) immediately before analysis. When removal of an early-precipitating nonhemoglobin protein was desired, hemolysates were preincubated at 60°C for 15–30 min, and the initial precipitate was discarded. At intervals during the precipitation of unstable hemoglobins, supernates were examined for the presence of α-chains by starch gel electrophoresis at pH 8.6 (Tris-citrate/borate system) followed by staining with *o*-tolidine (10).

Protein was estimated by the Lowry modification of the Folin-Ciocalteu method (11). Sulfhydryl groups were measured with 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB) as described by Ellman (12). Available sulfhydryl groups in hemoglobin were determined in pH 8.0 phosphate buffer, and total sulfhydryl groups in the same buffer containing 1% SDS. Reduced glutathione (GSH) was estimated by the DTNB method described by Prins and Loos (13). Heme groups were determined by adding the solution of native or precipitated hemoglobin to a known excess of cold acetone containing 1% HCl, centrifuging to separate the precipitated globin, and determining the absorbance of the acetone solution at 538 nm. The method was calibrated against standard hemoglobin solutions. Spectra of acid acetone solutions obtained from native and precipitated hemoglobin were identical. Hemoglobin was determined as cyanmethemoglobin.

To examine precipitated hemoglobin for the presence of covalently bound cysteine or glutathione, globin was prepared by adding the material dissolved in SDS to acid acetone, and was oxidized with performic acid. Protein was precipitated at pH 7.4 with 4 vol ethanol, and supernates were examined by high voltage paper electrophoresis (pH 6.5, 3.5 kV, 20 min). Papers were stained with ninhydrin, and cysteic acid and performic acid-oxidized glutathione were run as standards. Hemoglobin with glutathione bound to β93 cysteines was subjected to the same procedure.

The location of internal disulfide bonds in heat-precipitated hemoglobin was examined by diagonal electrophoresis. Free sulfhydryl groups in the hemoglobin precipitate were carboxymethylated by reacting with a 60-fold excess of iodoacetate at pH 8.0 in 8 M urea at room temperature for 30 min. This excess was necessary for carboxymethylation without any significant disulfide interchange. Excess iodoacetate was removed by dialysis against three changes of 0.04 M HCl, and globin was prepared and digested with pepsin. Electrophoresis and performic acid oxidation were carried out as described by Brown and Hartley (14). Native hemoglobin and hemoglobin with cysteine bound to β93 cysteines were subjected to the same procedure.

Molecular weights of polypeptides were determined by polyacrylamide gel electrophoresis in the presence of SDS, in 0.1 M Tris-citrate buffer, pH 6.8 (10.3 g citric acid, 6.2 g Tris, 1 g SDS, 97.5 ml N NaOH to 1 liter). Gel and sample preparation were as described by Weber and Osborn (15), and electrophoresis was carried out for about 4 h at 8 mA/gel. Protein bands were either stained with

[1] *Abbreviations used in this paper:* DTNB, 5,5′-dithiobis-(2-nitrobenzoic acid); Hb, hemoglobin; SDS, sodium dodecyl sulfate.

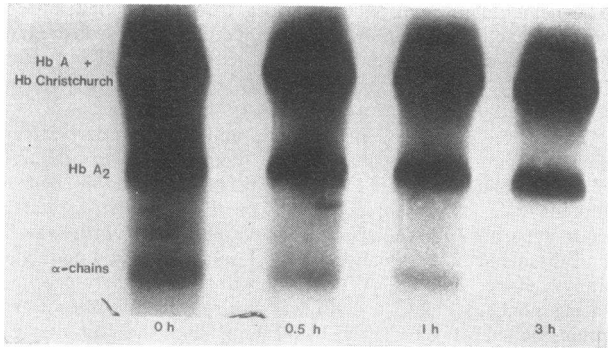

FIGURE 1 Starch gel electrophoresis of supernate at intervals during heat precipitation of Hb Christchurch at 50°C, stained with *o*-tolidine/peroxide.

Coomassie Blue and destained electrolytically, or stained with *o*-tolidine/hydrogen peroxide. The amount of protein in the bands was determined by cutting out the relevant discs of gel and eluting the dye into methyl Cellosolve (Union Carbide Corp., Chemicals & Plastics, New York). The ground-up gel was extracted for at least 24 h with occasional mixing, and the absorbance of the extracts measured at 596 nm.

Isolation of the different molecular weight fractions of heat-precipitated hemoglobin was carried out on a Sephadex G200 column in 0.1 M citrate buffer, pH 6.8, containing 0.2% SDS. Each peak was concentrated by ultrafiltration, and its purity was examined electrophoretically. Total amino acid analysis and peptide mapping of tryptic digests of the protein in each peak were carried out. In some cases, tryptic peptides $\alpha\beta 7$ and 7–8 were eluted from the paper and analyzed to obtain the ratio of α- to β-chains. Standard techniques for digestion and peptide mapping were used (16).

RESULTS

Subunit composition of heat-precipitated hemoglobin. Fingerprints of heat precipitates of Hb A, Hb Christchurch, and Hb Sydney showed peptides from both α- and β-chains present in apparently similar amounts. Quantitative amino acid analysis of the total protein showed that in each case there was no significant difference in the number of α- and β-chains present. With Hb Köln, fingerprints also showed comparable yields of α- and β-peptides, but amino acid analysis suggested that there could have been a slight excess of β-chains precipitated. In all cases no differences between precipitates collected after heating for a short or long period were detected.

Electrophoretic examination of hemolysates containing Hb Christchurch or Hb Köln showed small amounts of free α-chains. With Hb Köln, more α-chains were present in older hemolysates. Examination of the supernate at intervals during heat precipitation of Hb Christchurch (Fig. 1) and Hb Köln showed that the number of α-chains gradually declined, and after 3 h, none were detectable. Both chains of the abnormal hemoglobin, plus the small amount of free α-chains, must therefore have precipitated.

Presence of heme groups in unstable hemoglobins. Hb Köln purified by chromatography on DEAE Sephadex contained only 2.1–2.6 heme groups/tetramer. Heat precipitated Hb Köln was also heme-depleted (Table I), and the material that precipitated first contained fewer hemes than later-precipitating material. However, heat precipitates of Hb Christchurch and Hb Sydney contained all or almost all of their theoretical four heme groups, and no accumulation of excess hemes in the supernate was evident (Table I). Values for precipitates are probably slightly low, as there appeared to be a small amount of heme that did not dissociate from globin on addition to acetone. Heme groups in precipitated Hb A approached the theoretical 4.0/molecule if most of the hemoglobin was precipitated or if hemolysates were preincubated and the early precipitate discarded (Table I). Otherwise the presence of a nonhemoglobin protein that precipitated during the first few minutes' incubation gave rise to apparently low ratios.

Like Jacob and Winterhalter (17), we found that the abnormal electrophoretic mobility of Hb Köln disappeared on the addition of excess hemin.

Presence of hemichromes in heat precipitates. Hemichromes are derivatives of ferric heme in which the sixth coordination position contains a nitrogen base derived from protein, and can be recognized spectrally (18). We examined precipitated Hb Christchurch, Hb Köln, and Hb A dissolved in 6-M urea within 5 min of dissolving the precipitates, and observed typical hemichrome spectra. No features typical of other hemoglobin derivatives were evident.

No soluble hemichromes could be detected during the heat precipitation of Hb A. The spectrum showed predominantly oxyhemoglobin with increasing amounts of

TABLE I

Ratios of Heme:Globin in Precipitates and Supernates after Incubation of Hemoglobin Solutions

Hemoglobin	Incubation time	No. of estimations	Hemes per mole globin	
			Precipitate	Supernate
	min			
Hb Köln	60	3	1.2±0.4	
	120	3	2.1±0.1	
Hb Christchurch	30–120	7	4.1±0.2	4.2±0.2
Hb Sydney	80	2	3.3±0.1	3.9±0.1
Hb A	10–60	22		3.98±0.28
	30	2	3.4	
	60	2	3.7	

Hemolysates containing Hb Köln, Hb Christchurch, and Hb Sydney in pH 7.4 phosphate buffer were incubated at 50°C. Hb A was incubated at 60°C. In experiments in which precipitated Hb A was analyzed, hemolysates were preincubated at 60°C for 10 min.

methemoglobin as incubation continued. Precipitation of methemoglobin was much more rapid, and the precipitate gave a typical hemichrome spectrum. However, no soluble hemichromes could be detected, even after long periods of incubation.

Rates of oxidation of the thiol groups of oxyhemoglobin and methemoglobin dissolved in 8 M urea were compared. Gradual oxidation occurred in each solution, but only methemoglobin was converted to hemichrome, so the processes of hemichrome formation and thiol oxidation appear to be unrelated.

Sulfhydryl group oxidation. Hb Köln from two sources was purified, and in each case none of the six sulfhydryl groups was found to be blocked (Table II). Analyses of whole hemolysates containing Hb Christchurch or Hb Sydney indicated that these hemoglobins also contained the normal number of sulfhydryl groups. Although the abnormal hemoglobins made up only 20–30% of the total, the results would be significantly lower if two of their sulfhydryl groups had been blocked.

Heat precipitation of each hemoglobin was accompanied by oxidation of 1.6–2.7 of its six sulfhydryl groups (Table III). With Hb Köln it is possible that there were more free sulfhydryl groups in the material that precipitated first, but with the other hemoglobins, the amount of oxidation was independent of the length of incubation, and in the case of Hb A, of the incubation temperature. No decrease in sulfhydryl groups in the soluble hemoglobin could be detected, even after extended periods of incubation. The oxidized sulfhydryl groups could be reduced again by reacting the precipitate dissolved in SDS with dithiothreitol, indicating that oxidation to disulfides had occurred.

The nature of the disulfide bonds in precipitated hemoglobin. Heat-precipitated hemoglobin from fresh hemolysates was oxidized with performic acid and examined electrophoretically for the presence of low molecular weight thiols that could be present as mixed disulfides. No glutathione sulfonic acid or other small ninhydrin-positive molecules could be detected with either Hb Christchurch, Hb Sydney, or Hb A. A very low-intensity spot corresponding to glutathione sulfonic acid was observed with Hb Köln. However, comparison with electrophoretograms of known amounts of GSH, and hemoglobin with β93 cysteines bound to GSH, which had been oxidised with performic acid, indicated that no more than 2% of the β93 cysteines in Hb Köln, and fewer in the other hemoglobins, could have formed mixed disulfides with glutathione.

The nature of the disulfide bonds in precipitated Hb A was investigated by diagonal electrophoresis. In this technique (14), electrophoresis of a peptic digest of the protein is followed by performic acid oxidation and electrophoresis in a perpendicular direction. On oxidation, peptides containing disulfides are split, and each gains an additional negative charge and lies off the diagonal in the second dimension. The patterns obtained with carboxymethylated heat precipitates of Hb A and Hb Christchurch were compared with those of carboxymethyl Hb A, and Hb A in which the β93 cysteines

TABLE II

Hemoglobin Sulfhydryl Groups in Purified Hb Köln and Hemolysates Containing Hb Christchurch and Hb A

Hemoglobin	Abnormal Hb	Hb Sulfhydryl groups	
		Total	Available
	%		
Hb Köln	100	6.0	1.7
Hb Christchurch	20	6.6	2.1
Hb Sydney	30	6.0	2.0
Hb A		6.3±0.3	2.0±0.1

Measurements of available sulfhydryl groups were made on the native hemoglobins, and total sulfhydryl groups were measured on the proteins denatured by SDS. Hb Köln was purified by DEAE-Sephadex column chromatography. Nonprotein thiols were removed on Sephadex G25 columns.

TABLE III

Sulfhydryl Groups in Heat-Precipitated Hemoglobin

Hemoglobin	Incubation time	No. of determinations	Hb sulfhydryl groups		
			Before incubation	After incubation	
				Precipitate	Supernate
	min				
Hb Christchurch	30–120	17	6.0±0.3	4.4±0.5	
Hb Sydney	60–90	4	6.0±0.1	3.3±0.3	
Hb Köln	60–120	5	6.1±0.2	4.4±0.6	
Hb A	10–60	20	6.0±0.3	3.8±0.6	6.3±0.4

Sulfhydryl groups were measured by the DTNB method calibrated against a standard hemoglobin solution having 6.0 sulfhydryl groups/mol.

were present as mixed disulfides with cysteine. With Hb A, the only significant nondiagonal peptide (in low yield) was β15–22, which contains no cysteine but an NH_2-terminal tryptophan, which placed it off the diagonal. Hemoglobin bound to cysteine gave two prominent nondiagonal spots in addition to this peptide. Amino acid analysis showed that these spots were derived from the mixed disulfide of cysteine and β92–96. There was also a small amount of nondiagonal cysteic acid, in a position consistent with its arising from cystine used in the preparation of the material and incompletely removed. In contrast to this picture, the heat precipitates produced patterns in which β92–96 was not a major component, but instead there were a number of nondiagonal peptides, all present in relatively low yields.

Degree of polymerization of the precipitated hemoglobin chains. Heat precipitates of Hb A and Hb Christchurch were examined by polyacrylamide gel electrophoresis in the presence of SDS. Patterns obtained after Coomassie Blue staining are shown in Fig. 2. Staining with *o*-tolidine/peroxide, which is specific for heme-containing proteins, gave essentially similar patterns except that band X did not stain. In contrast to normal hemoglobin (Fig. 2*a*), which was dissociated into subunits and ran as a single band with molecular weight about 17,000, both precipitates gave patterns in which this band was the major component, but a series of bands with increasing molecular weights were also present (Figs. 2*b*, *c*, and *d*). Heinz bodies separated from cells containing Hb Christchurch gave a similar electrophoretic pattern (Fig. 2*f*). This pattern was obtained for ghosts prepared from cells containing Hb Christchurch incubated at 37°C for 2 days and containing 48 large Heinz bodies/100 cells. Heinz body proteins were present in sufficiently large amounts for ghost proteins to be scarcely visible. Some high molecular weight ghost proteins that were too large to penetrate the gel can be seen.

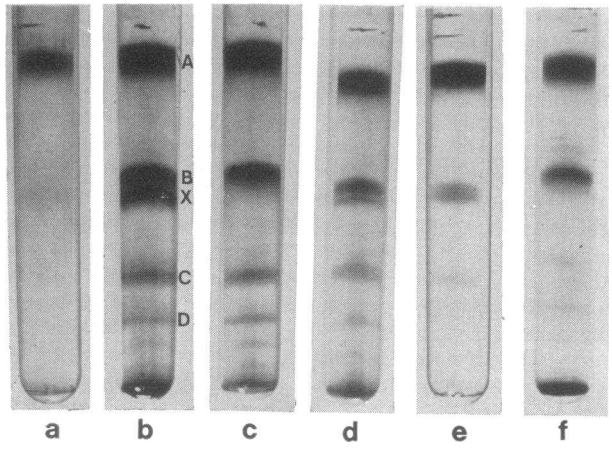

FIGURE 2 Polyacrylamide gel electrophoresis in the presence of SDS. *a*, Hemoglobin A; *b*, heat-precipitated Hb A, 20 min at 60°C; *c*, heat-precipitated Hb A, hemolysate preincubated 10 min at 60°C and initial precipitate discarded, then incubated 20 min; *d*, heat-precipitated Hb Christchurch, 2 h at 50°C; *e*, heat precipitate of preincubated Hb A, treated with mercaptoethanol; *f*, Heinz bodies from incubated red cells containing Hb Christchurch. All stained with Coomassie blue. Bands A–D and X are referred to in the text and in Table IV.

TABLE IV

Molecular Weights and Percentage Distribution of Protein Bands Obtained by Polyacrylamide Gel Electrophoresis of Heat-Precipitated Hemoglobin

Band	Molecular wt	Percentage distribution	
		Hb A (6)	Hb Christchurch (3)
A	17,000	52	56
B	35,000	16	18
X	40,000	—	—
C	60,000	9	8
D	75,000	23	5
	>75,000		13

Bands are identified in Fig. 2. Molecular weights were estimated by comparing mobilities with those of standard proteins. Distributions were determined by eluting the stained gels. Figures in brackets refer to the number of analyses performed.

The relative amounts of the different polypeptides (apart from band X) were similar for Hb Christchurch and Hb A, and were independent of incubation time (Table IV). The molecular weights of the bands were consistent with their being monomers, dimers, trimers, tetramers, and higher polymers of the polypeptide chains of hemoglobin. After treatment of the redissolved precipitated hemoglobin with mercaptoethanol to reduce disulfide bonds, almost all the bands corresponding to polymers were eliminated, to leave only the monomer band (Fig. 2*e*).

The protein with molecular weight about 40,000 (band X) was almost entirely precipitated from hemolysates by heating at 60°C for 10 min, and was not reduced by mercaptoethanol. It was separated on a Sephadex G200 column in the presence of SDS, after treatment at the redissolved precipitate with mercaptoethanol. Inspection of a peptide map of a tryptic digest confirmed that the material was not a hemoglobin derivative.

Heat precipitates of Hb A and Hb Christchurch were separated on a Sephadex G200 column in the presence of SDS into three fractions consisting of polymers of hemoglobin chains larger than dimers, dimers plus component X, and single chains. The dimers were separated from component X by rechromatographing after treatment with mercaptoethanol. Peptide maps of tryp-

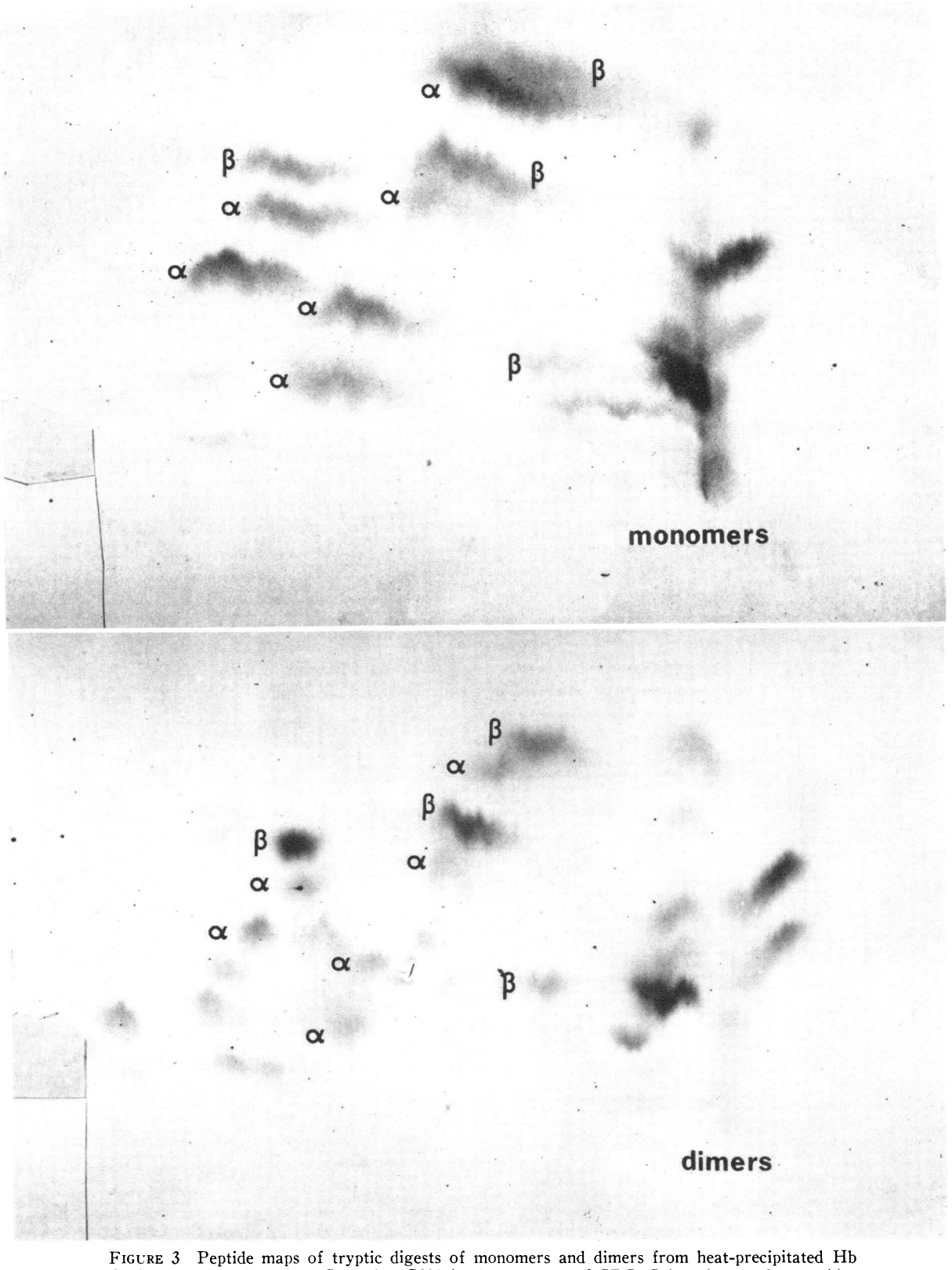

FIGURE 3 Peptide maps of tryptic digests of monomers and dimers from heat-precipitated Hb Christchurch, separated on Sephadex G200 in the presence of SDS. Selected α- and β-peptides have been marked to allow comparison of relative amounts of α- and β-chains.

TABLE V

Estimation of the Relative Amounts of α- and β-Chains in the Monomeric, Dimeric, and Polymeric Components of Heat-Precipitated Hb A and Hb Christchurch

	Percentage of β-chains	
	Hb A	Hb Christchurch
Total heat precipitate	52	52
Monomers	40	47
Dimers	73	86
Polymers	74	87

Results were obtained by determining the ratio of alanine to glycine in tryptic peptides αβ7 and 7–8. The α- and β-peptides differ only in one alanine or glycine and are not separated by peptide mapping.

tic digests of each hemoglobin fraction were examined visually for relative proportions of α- and β-peptides. As shown in Fig. 3, monomers of Hb Christchurch appeared to give slightly more α-chain than β-chain peptides, and dimers gave considerably more β-peptides. With the polymers, β-peptides appeared in even greater excess. Similar patterns were obtained for Hb A. These observations were substantiated by quantitative analysis (Table V).

Effect of reduced glutathione on heat precipitation of hemoglobin. Rates of precipitation of Hb Christchurch, Hb Sydney, and Hb A were little affected by the presence of GSH, and there was no difference in numbers of sulfhydryl groups oxidized (Table VI). Similar results were obtained whether or not NADPH and other substrates necessary for glutathione reductase activity were removed from hemolysates.

Precipitates prepared in the presence of GSH but absence of reductase activity were shown by performic acid oxidation and electrophoresis to contain small but significant amounts of bound glutathione. However, this amount corresponded to only 5% of the two sulfhydryl groups per molecule that were oxidized on precipitation of the hemoglobin.

Heat precipitation of derivatives of hemoglobin. Carboxyhemoglobin and deoxyhemoglobin precipitated more slowly than oxyhemoglobin, and methemoglobin precipitated more rapidly (Table VII). Sulfhydryl oxidation occurred with carboxyhemoglobin, but with deoxyhemoglobin and methemoglobin, little, if any, was evident. No oxidation occurred on precipitation of isolated globin. The polyacrylamide gel electrophoretic pattern of precipitated carboxyhemoglobin was similar to that of oxyhemoglobin. Deoxyhemoglobin and methemoglobin showed markedly fewer dimers and hardly any higher-polymer bands. Deoxyhemoglobin Christchurch and deoxyhemoglobin Sydney (not shown) also precipitated more slowly, with very little oxidation. The deoxy derivatives of both hemoglobins were still less stable than deoxyhemoglobin A. Blocking the β93 cysteines in Hb A with glutathione caused a decrease in stability but did not affect the amount of sulfhydryl oxidation. However, since the initial material had only four rather than six free sulfhydryl groups, the precipitate was more oxidized than normal precipitated oxyhemoglobin.

The rate of methemoglobin precipitation was found to be not directly influenced by sulfhydryl oxidation. Precipitation of methemoglobin in the presence of ferricyanide was accompanied by sulfhydryl oxidation, but did not occur significantly faster (Table VIII).

DISCUSSION

This study of three unstable hemoglobins has shown that, although in most respects the events that accompany the precipitation of the different variants are similar, there are some notable differences. A major difference appears to be in affinity for heme groups. Hb Christchurch, Hb Sydney, and Hb A, which behaves similarly if subjected to proportionately greater heat stress, be-

TABLE VI

Precipitation of Hemoglobin in Presence of GSH

Hemoglobin	Incubation time	Total Hb precipitated		Sulfhydryl groups/mole Hb in precipitate		No. of estimations
		No GSH	10 mol GSH	No GSH	10 mol GSH	
	min	%	%			
Hb Christchurch	30	5.5	5.5	4.3	4.4	2
	60	10.5	12.0			
Hb Sydney	75	11.4	12.6	3.6	3.5	2
Hb A	10	6.8–7.5	8.1–9.5	3.3	3.4	3

2% hemolysates containing Hb Christchurch and Hb Sydney were incubated at 50°C, Hb A was incubated at 60°C.
No GSH: all GSH and other low molecular weight compounds removed by passing hemolysate through Sephadex G25. 10 mol GSH: 10 mol GSH/mole Hb was added to this hemolysate.

TABLE VII

Rates of Heat Precipitation and Thiol Oxidation of Different Hemoglobin Derivatives

	Amount of precipitation relative to oxyHb under same conditions	Decrease in SH groups per mole Hb on precipitation
OxyHb A	100	2.2±0.6
CarboxyHb A	38–54	2.3±0.2
DeoxyHb A	22–65	0.4±0.2
MetHb A	300–600	0.4±0.4
Hb SSG (β-93)	150–180	2.7
Globin (30 min at 37°C, 47% precipitated)	—	0
OxyHb Christchurch	100	1.4
DeoxyHb Christchurch	65	0.4

Apart from globin, solutions were incubated at 50–60°C until suitable amounts of material had precipitated. DeoxyHb solutions were incubated after evacuation in tonometers. CarboxyHb was prepared by bubbling carbon monoxide through a solution of oxyHb, and was incubated in air.

long to a group which shows little tendency to lose hemes, whereas with others, such as Hb Köln and probably Hb Hammersmith and Hb Sabine, dissociation of heme groups from the abnormal chains occurs much more readily (4, 19, 20). Members of each group can generally be distinguished electrophoretically. Hemoglobins of the former type run as a single band that cannot be separated from Hb A, whereas the others give a pattern of multiple bands that can be normalized by adding hemin. We found that heat precipitates of Hb Sydney and Hb Christchurch were visibly quite dark, and contained close to their theoretical four hemes per tetramer, but precipitated Hb Köln was much paler and contained only two or fewer hemes. We also found that chromatographically purified Hb Köln lacked two heme groups, presumably from the β-chains. Although altered hemoglobin binding may be a general phenomenon, only in some cases does it appear to result in actual dissociation of the heme groups.

In a previous report on the constitution of Heinz bodies from Hb Christchurch, we suggested a mechanism of Heinz body formation in which the intact, unstable hemoglobin precipitates rather than only the abnormal chain (21). The present findings of similar quantities of the α- and β-polypeptide chains in each heat precipitate and the lack of accumulation of soluble free α-chains during heating of Hb Köln and Hb Christchurch suggest that these hemoglobins precipitate on heating by a similar mechanism. Further support comes from the finding of equivalent amounts of each chain in precipitates of another unstable hemoglobin, Hb Abraham Lincoln (22). Jacob (4) has alternatively proposed that only the abnormal chains precipitate without heme groups. We have found no evidence for this with Hb Christchurch, Hb Sydney, or Hb A. However, with Hb Köln, although peptide maps of heat precipitates showed similar proportions of both chains, earlier precipitates contained fewer heme groups and possibly more sulfhydryl groups than later ones, which could indicate initial precipitation of mainly β-chains. This mechanism may be more significant for Hb Köln stored cold. We noted an increase in free α-chains in solutions stored under these conditions. It remains to be demonstrated which mechanism is more representative of the denaturation of Hb Köln in the red cell.

In addition, our results generally favor concurrent precipitation of both chains rather than dissociation and precipitation of the unstable β-chains, followed by precipitation of the α-chains. Firstly, the constitution of precipitates appeared to be independent of incubation time. Secondly, the relative amounts of α- and β-chains in the monomers and disulfide-bonded polymers of precipitated Hb A and Hb Christchurch were essentially the same. Also α-chains that contain only one cysteine must have been bound to β-chains to give trimers and larger aggregates, and therefore the chains must have precipitated together.

TABLE VIII

The Effect of Ferricyanide on the Precipitation of Methemoglobin A

Excess ferricyanide per mole Hb	Hb precipitated	Decrease in −SH groups per mole Hb on precipitation
	%	
0	36	0.5
0.5	36	1.2
5	36	2.4

Potassium ferricyanide sufficient to oxidize oxyHb to metHb was added and the excess removed on a Sephadex G25 column. Additional ferricyanide was added as indicated.

Spectral examination of heat-precipitated Hb Christchurch, Hb Köln, and Hb A dissolved in 6 M urea suggested that the heme groups were present as hemichromes. Although methemoglobin dissolved in 6 M urea gradually converts to hemichrome, this occurs too slowly for the hemichromes to have arisen from this source. Hemichromes have also been demonstrated in the intracellular β-chain inclusions in α-thalassemia (23), and Rachmilewitz and White (24) have reported the formation of hemichromes in cold solutions of methemoglobin Köln. We were not able to detect soluble hemichromes during hemoglobin precipitation. This is likely to be due to their instability in solution (18), and the relative insensitivity of spectral analysis which would not detect low levels.

Oxidation of the β93 sulfhydryl groups has been proposed as an important factor in the precipitation of unstable hemoglobins, and it has been reported that in some unstable hemoglobins, particularly Hb Köln, the β93 cysteines are blocked, possibly by forming mixed disulfides with glutathione (25–27). However, we could detect no blockage of β93 cysteines either in two preparations of Hb Köln purified from different sources, or in hemolysates containing Hb Christchurch or Hb Sydney. The apparent finding by others of sulfhydryl oxidation may be explained by an unpublished result we obtained using a broad chromatographic peak containing Hb Köln, which had subsequently been allowed to stand for 10 days. This gave a substantial decrease in available sulfhydryls, but chain separation showed that this was due to a marked excess of α-chains. Correction for the changed $\alpha:\beta$ ratio showed that no sulfhydryl oxidation had occurred.

We also found that precipitation of the unstable hemoglobins and Hb A was accompanied by oxidation of approximately two of the six sulfhydryl groups, but the disulfide bonds formed were almost entirely within or between hemoglobin molecules. Bonding to small molecules, such as GSH, was insignificant. Even when precipitation was carried out in the presence of high concentrations of GSH, there was a little mixed disulfide formation, but by far the majority of the binding was internal. It is therefore our view that coupled oxidation of GSH and unstable hemoglobins does not occur to any great extent. It is probable that any mixed disulfides arose through exchange with GSSG rather than coupled oxidation with GSH. Other workers (28) have found the exchange mechanism to be more significant. Therefore, in the red cell where GSSG levels are low, mixed disulfide formation should be minimal.

Polyacrylamide gel electrophoretic patterns of precipitated Hb Christchurch and Hb A, consisting of single α and β-polypeptide chains plus decreasing amounts of disulfide-bonded dimers and higher polymers of predominantly β-chains, demonstrate nonspecific disulfide bonding between all three sulfhydryl groups of the hemoglobin. Had there been specific oxidation of the β93 cysteines, only α-chain monomers and β-chain dimers would have been formed. With polymerized α-chains being clearly demonstrable, and the existence of higher aggregates than dimers, some oxidation of β93, β112 and α104 cysteines must have occurred. It is unlikely that there could have been oxidation of a specific sulfhydryl group on precipitation of the hemoglobin with subsequent randomization before analysis. Precipitates were analyzed immediately after preparation and were exposed only to media with pH less than 7, at which disulfide exchange is very slow. It was also found with redissolved precipitates that full exchange of the disulfides with mercaptoethanol took several hours at pH 8. The nonspecific nature of the sulfhydryl oxidation is supported by the finding of several minor peptides, rather than one major peptide containing cysteic acid, on diagonal electrophoresis of carboxymethylated heat-precipitated hemoglobin.

The absence of sulfhydryl oxidation on precipitation of deoxyhemoglobin, or methemoglobin and isolated globin, even in the presence of molecular oxygen, compared with the oxidation of approximately two sulfhydryls in oxyhemoglobin and carboxyhemoglobin, suggests that oxidation is a by-product of the breakdown of certain ligands. Methemoglobin is a probable intermediate in the precipitation of oxyhemoglobin, and it has been postulated that the superoxide free radical (O_2^-) is formed in this reaction (29). Carboxyhemoglobin in air could either precipitate via an oxyhemoglobin intermediate or else could give rise to the CO^- radical on oxidation (29). The observed sulfhydryl oxidation could be explained by the role of these groups as scavengers of free radicals. This suggests that sulfhydryl oxidation is an incidental accompaniment rather than fundamental cause of precipitation. This conclusion is also supported by the precipitation of methemoglobin, which is considerably less stable than oxyhemoglobin, without forming disulfides, and in particular by the finding that the presence of small amounts of ferricyanide in methemoglobin solutions results in sulfhydryl oxidation but does not increase the rate of precipitation. The oxidation of an additional two sulfhydryl groups on precipitation of oxyhemoglobin in which the β93 cysteines were bound to glutathione shows that precipitation does not necessarily occur when a certain number of sulfhydryls are oxidized and also favors the idea that disulfide formation may represent the mopping up of an oxidizing agent, such as superoxide, formed during the precipitation process.

Our results support the conclusions of Rachmilewitz, Peisach, Blumberg, and others (18, 23, 24, 30) that the precipitation of unstable hemoglobins is generally a

result of the formation of irreversible hemichromes. These workers have shown that in the process of denaturation, hemoglobin first forms a reversible hemichrome, in which the heme iron forms a covalent bond with the nitrogen of the distal histidine, followed by formation of an irreversible hemichrome, in which the bond is to another amino acid side chain. The increased molecular flexibility of the unstable hemoglobins will accentuate the folding and unfolding processes that the normal molecule undergoes, increasing the likelihood of the molecule being trapped as a hemichrome in an unfolded or denatured state, and thus leading to precipitation. Rachmilewitz and White (24) have interpreted their finding of hemichrome formation by Hb Köln as evidence for dissociation of α- and β-chains. However, we feel that any alteration to the molecular conformation that allows greater flexibility about the heme group could accelerate hemichrome formation, and that the structural changes in the unstable hemoglobins could be sufficient without the additional requirement of subunit dissociation.

It is probable that with Hb Christchurch and Hb Sydney, it is hemichrome formation by the affected β-chains that leads to precipitation. However, with Hb Köln, which is substantially present as the heme depleted $\alpha_2^{heme}\beta_2^{o}$, it is likely that precipitation follows formation of hemichromes by the α-chains. The α-chain is particularly susceptible to hemichrome formation (30). With the loss of heme from the β-chain imposing a strain on the $\alpha\beta$-dimer interface and consequently distorting the α-chain, this tendency will be increased. Evidence for this is the stabilization of Hb Köln and heme depleted Hb A ($\alpha_2^{heme}\beta_2^{o}$) by carbon monoxide and cyanide (19), both of which must react with the α-chain hemes.

The inability of GSH, either in combination with GSH peroxidase or as a direct competitor with protein sulfhydryl groups, to reduce precipitation of the unstable hemoglobins or Hb A contrasts with the protective role of GSH when hemoglobin is exposed to acetylphenylhydrazine (31), and presumably the two processes do not proceed via a similar oxidative mechanism. Exposure to acetylphenylhydrazine results in the generation of peroxides capable of being detoxified by reaction with GSH. It appears, however, that any oxidizing agents formed during precipitation of unstable hemoglobins either react within the hemoglobin molecule, or if they are released, do not react with GSH in preference to hemoglobin sulfhydryl groups.

On the basis of these results, it is possible to propose a mechanism for the events that accompany the precipitation of unstable hemoglobins on mild heating. In a number of ways this process has been shown to be similar to the intracellular precipitation of hemoglobin to form Heinz bodies: the microscopic appearance of heat precipitates and Heinz bodies is very similar (27); precipitation of both chains of Hb Christchurch without apparent loss of heme groups occurs both on heating and in process of Heinz body formation (21); and the amount of interchain disulfide bonding, as shown by polyacrylamide gel electrophoresis, appears to be the same in both. The mechanism of Heinz body formation by these unstable hemoglobins, therefore, should not be too different from this model.

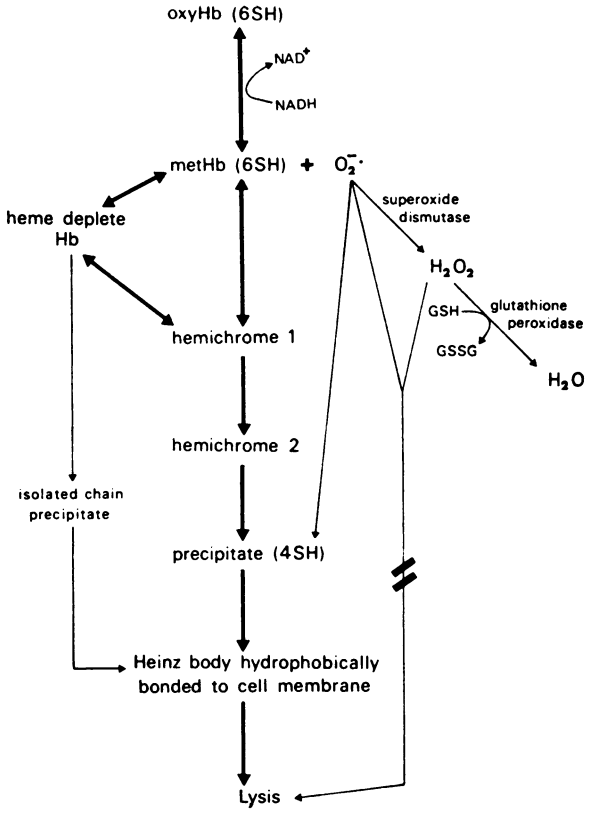

FIGURE 4 Proposed scheme for the intracellular denaturation of unstable hemoglobins to give Heinz bodies and hemolysis. The main sequence of events observed with the hemoglobins we have studied is demonstrated with dark arrows. Other pathways which may be applicable to other unstable hemoglobins are shown with lighter arrows. The diagram is explained further in the text.

Several of our results do not uphold the currently favored general mechanism for the denaturation of unstable hemoglobins proposed by Jacob (4). In particular, we would question the formation of mixed disulfides with glutathione and the necessary roles of heme loss and precipitation of only the abnormal chain. We have also shown (32) that precipitates and Heinz bodies of Hb Christchurch are not attached to the cell membrane by disulfide bonds.

We propose that Fig. 4 describes the events accompanying the precipitation of the hemoglobins we have examined, and may represent a more general mechanism.

The structural defects of the unstable hemoglobins should result in increased flexibility about the heme group (1). This will facilitate conversion to methemoglobin and the sequential formation of a reversible hemichrome (hemichrome 1), and then an irreversible hemichrome (hemichrome 2), with resulting precipitation. This may proceed directly, as with Hb Christchurch and Hb Sydney, or may be preceded by partial heme loss, as with Hb Köln. In addition, dissociation and precipitation of heme-depleted abnormal chains may be significant for some hemoglobins. It is postulated that superoxide is normally produced on autoxidation of oxyhemoglobin and is detoxified in the cell by the superoxide dismutase (33) and glutathione peroxidase systems. When there is distortion of the molecule to give a hemichrome, the superoxide may not be released as usual but may react with the precipitating protein and cause the consistently observed oxidation of two sulfhydryl groups. If these events occur in the circulating red cells, aggregation of this precipitated hemoglobin would give rise to Heinz bodies. These would migrate to the cell surface (34), where they become attached by hydrophobic interactions (32). Probably their physical presence, which reduces the deformability of the cell and leads to splenic entrapment and pitting (35, 36), is the ultimate cause of the accompanying hemolytic anemia.

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