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Harry S. Jacob, ... , Michael C. Brain, John V. Dacie

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Research Article

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Reflecting the previously reported excessive flux of hemes from hemoglobin Köln, the expected heme/globin ratio in this hemoglobin was reduced by 30%. The further increment in heme loss that occurs with heat (50°C) underlies the unique heat precipitability of CHBHA hemoglobins; it was retarded if detachment of heme was inhibited by cyanide or carbon monoxide.

Heinz bodies were attached to red cell membrane thiol groups presumably through mixed disulfide bonds, being released by mercaptoethanol. Binding of hemoglobin Köln-⁵⁹Fe to red cell ghosts, which was markedly enhanced when Heinz bodies were generated at 50°C, was inhibited if membrane thiols were preblockaded by PMB. The depletion of membrane thiols by their reaction with Heinz bodies rendered CHBHA red cells hypersusceptible to membrane sulfhydryl inhibitors, as manifested [...]

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Altered Sulfhydryl Reactivity of Hemoglobins and Red Blood Cell Membranes in Congenital Heinz Body Hemolytic Anemia

HARRY S. JACOB, MICHAEL C. BRAIN, and JOHN V. DACIE

From the Medical Research Council Group for Research in Haemolytic Mechanisms, Royal Postgraduate Medical School, London, England; and St. Elizabeth's Hospital and Department of Medicine, Tufts University School of Medicine, Boston, Massachusetts 02111

ABSTRACT The mechanisms of hemoglobin precipitation into Heinz bodies and hemolytic anemia that characterize congenital Heinz body hemolytic anemia (CHBHA) were studied in patients with the unstable hemoglobins, Köln (β -98 valine \rightarrow methionine) and Hammersmith (β -42 phenylalanine \rightarrow serine). The cysteines in the 93rd position of the β -chains of CHBHA hemoglobins bound glutathione excessively in mixed disulfide linkage. The resulting diminished "free" GSH within the cell accelerated hexose monophosphate shunt metabolism. The unique precipitability of CHBHA hemoglobins when heated at 50°C could be induced in normal hemoglobin A by artificially blocking its sulfhydryl groups with paramercuribenzoate (PMB).

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Address requests for reprints to Dr. H. S. Jacob, Department of Medicine, University of Minnesota Medical School, Minneapolis, Minn. 55455.

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We conclude that both cellular and membrane thiols bind β -93 sulfhydryls of CHBHA hemoglobins as mixed disulfides. Concomitantly, heme avidity to β -92 lessens, suggesting that degradation of the resulting excessively freed heme may produce the pigmented dipyrroluria of this syndrome. Heinz bodies, reflecting the heightend precipitability of heme-deficient globin, attach to, thereby depleting, membrane sulfhydryl groups. This, as shown previously, could underlie the hemolytic anemia of this syndrome by causing membrane hyperpermeability, premature splenic entrapment, and ultimately osmotic destruction of red blood cells.

INTRODUCTION

The syndrome of congenital Heinz body hemolytic anemia (CHBHA) is defined by a clinical triad consisting of: (a) chronic hemolytic anemia; (b) the presence of circulating red cells with inclusion (Heinz) bodies more clearly evident after splenectomy; and (c) the excretion of urine

darkened by the presence of large quantities of dipyrrolic pigments (1-4). The disease is often familial, and is transmitted as an autosomal dominant trait. The presence of an abnormal hemoglobin, generally hemoglobin Köln β -98 valine \rightarrow methionine has been detected in affected individuals. Sporadic cases involving other abnormal hemoglobins have been reported and are usually of greater clinical severity (5), whereas patients with the more stable hemoglobin, Zürich, are healthy unless exposed to oxidant drugs such as the sulfonamides and aminoquinoline antimalarials (6). The presence of an unstable hemoglobin, associated with CHBHA, can be simply detected by a recently proposed screening test in which hemolysates are heated at 50°C for brief periods causing the copious precipitation of the unstable hemoglobins (1).

The mechanism by which unstable hemoglobins precipitate either as Heinz bodies *in vivo* or in the heat denaturation test has not been elucidated. The formation of Heinz bodies from normal hemoglobin A exposed to oxidant chemicals such as the phenylhydrazines has been investigated, and a sequence of oxidative denaturation of hemoglobin proposed (7, 8) as follows: (a) ferrohemes are initially oxidized to ferrihemes (methemoglobin); (b) if the oxidant stress continues, the two titratable sulfhydryl groups of hemoglobin, present as cysteines at the 93rd position of the β -chains, are then oxidized; (c) glutathione (GSH), present in large amounts in red blood cells, may bind to the β -93 thiols during their oxidation, in what has been presumed to be a protective process (8);

(d) finally, further oxidation ultimately causes an alteration in the conformation of both α - and β -polypeptide chains of globin, exposing the four other previously buried, sulfhydryl groups of hemoglobin; their oxidation, in turn, leads to precipitation of the proteins into coccoid aggregates, morphologically and tinctorially indistinguishable from Heinz bodies.

The relevance of these observations to the mechanism by which the mutant hemoglobins in CHBHA spontaneously precipitate in circulating red cells or upon being heated to 50°C was sought in the present studies, in which blood from several patients from two families harboring hemoglobin Köln was utilized.

The structure of the β -polypeptide chain of hemoglobin as described by Perutz (9) is shown in Fig. 1. The sharp folding and helical structure of the β -chain in the region of the mutation in hemoglobin Köln (the 98th position) places the mutant amino acid in close apposition to the groups that undergo oxidation in the aforementioned sequence; that is, to the hemes attached to histidines at β -92 and to the cysteines at β -93 which provide the two free sulfhydryl groups of hemoglobin (10). It will be shown that the mutation thereby crucially affects the reactivity of both of these groups in such a way as to enhance the propensity of this hemoglobin to precipitate into Heinz bodies. In addition, a mechanism by which the hemolytic anemia and dipyrroluria of CHBHA can be related to the denaturative precipitation of hemoglobin Köln *in vivo* has been proposed. The

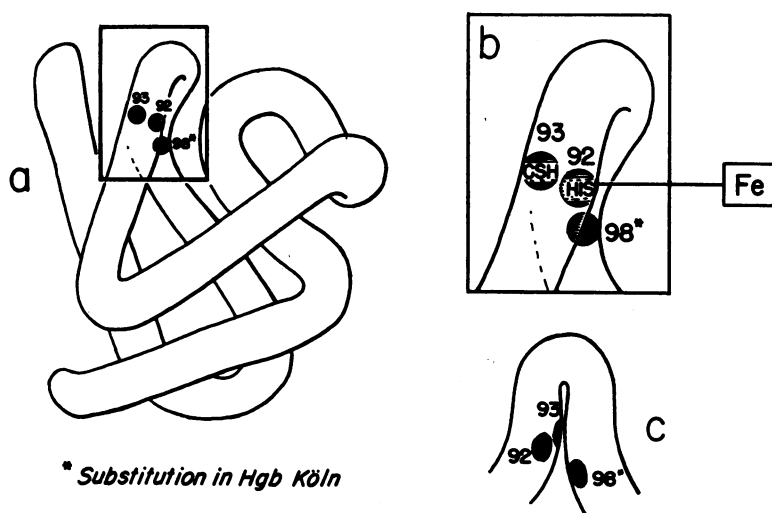


FIGURE 1 The approximate structure of the β -chain of hemoglobin after Perutz (9). The close proximity of the heme-binding histidine (HIS-position 92) to the titratable sulfhydryl group in cysteine (CSH-position 93), and to the mutant amino acid, methionine, in hemoglobin Köln (position 98), is emphasized in the three views shown.

results have been presented in preliminary form elsewhere (11).

METHODS

Subjects. Blood from four patients from two families with hemoglobin Köln was available for study. One of the patients who had been splenectomized provided red cells that were filled with Heinz bodies when stained vitally with methyl violet (12). The clinical and hematological courses of these patients have been described in detail elsewhere (1). The abnormal hemoglobin, which constituted approximately 20% of the total hemoglobin content of hemolysates, was identified by column and paper chromatographic purification, and peptide fingerprinting techniques performed and described by Carrell, Lehmann, and Hutchison (13). In addition, blood from one other splenectomized patient with a more severe anemia whose Heinz body red cells contained another unstable hemoglobin, Hammersmith²⁻⁴² phenylalanine → serine (14), was used in some studies. Observations on this patient's blood suggest that the mechanisms of Heinz body formation, hemolytic anemia, and dipyrroluria proposed for hemoglobin Köln have relevance for other unstable hemoglobins as well. This patient's clinical course has also been previously published (5). "Control" red cells were available from healthy volunteers or, when appropriate, from patients manifesting reticulocytosis with Coombs-positive hemolytic anemias or after acute hemorrhages.

Studies utilizing intact red cells. Heparinized whole blood was diluted with large volumes of phosphate-buffered saline¹ (pH 7.4) containing 0.022 M glucose, centrifuged at 3000 *g*, and the buffy coat removed. After a second dilution, recentrifugation, and removal of any remaining buffy coat the red cells were resuspended in buffered saline to hematocrits of 40%. The leukocytes in these suspensions numbered less than 1000, and the platelets less than 20,000/mm³. In studies involving prolonged incubation these preliminary steps were done aseptically, and penicillin, 500 U, and streptomycin, 250 µg, were added to each milliliter of washed red cells.

"Free" reduced glutathione was assayed on protein-free extracts of red cell hemolysates utilizing 5,5'-dithio-bis-(2-nitrobenzoic acid)² as described by Ellman (15). Cellular glutathione was titrated with *N*-ethylmaleimide (NEM)³ as described previously (16). This compound reacts in near-stoichiometric fashion with free GSH in normal red cells so that approximately 1.1 mole of NEM is required to fully blockade each mole of intracellular GSH (16). The red cell sodium and potassium levels were measured by flame photometry as described previously (17). Over-all glucose consumption (by the glucose oxidase method) and the proportion metabolized through the hexose monophosphate shunt (utilizing

¹ Three parts isotonic saline plus one part isotonic phosphate buffer, pH = 7.4; final K⁺ = 5 mmoles/liter.

² Aldrich Chemical Company, Inc., Milwaukee, Wisc.

³ Schwarz Bioresearch, Inc., Mt. Vernon, N. Y.

glucose-1-¹⁴C⁴) were assessed by our previously described methods (18).

Studies utilizing red cell ghosts. Red cell ghosts were prepared by osmotic lysis by a slight modification of the method described by Dodge, Mitchell, and Hanahan (19); the lysing and washing solution was 30 mOsmol and contained 1.0 mM EDTA and 10 mM NaHCO₃. The resulting ghosts appeared yellowish grey when prepared from the blood of splenectomized CHBHA patients, and yellowish-white from nonsplenectomized, CHBHA patients. As measured by a modification of the benzidine method described by Dacie and Lewis (12), less than 0.1% of original heme-protein was present in the ghosts. In this method 25 µl of ghost preparation or hemoglobin standard are added to the benzidine-H₂O₂ reagent and allowed to stand for 1 hr at room temperature in the dark before dilution with acetic acid and reading at 515 mµ. The shape of ghosts so prepared was well maintained, and under phase microscopy in platelet counting chambers, Heinz bodies were apparent as coccoid bodies attached to the membranes. In preparations from splenectomized subjects, approximately 70% of the ghosts contained inclusions (Heinz bodies) with diameters of 0.5–1.5 µ, whereas in ghosts from nonsplenectomized subjects a lesser, but definite, proportion of ghosts contained Heinz bodies of roughly ½ this size. A 20-fold dilution in 1% ammonium oxalate of a preparation containing roughly 4 × 10⁶ ghosts/mm³ allowed precise (±5% variation with three observers) quantitation of Heinz body-containing ghosts in platelet counting chambers under phase optics.

Normal red cell ghosts with their sulfhydryl groups blockaded were prepared by adding equal volumes of 4 mM paramercuribenzoate (PMB)⁵ in isotonic saline to a suspension of 4 × 10⁶ ghosts/mm³ prepared from normal erythrocytes. After incubation for 1 hr at 37°C, the ghosts were washed twice in the same 30 mOsmol media used in the preparation of the ghosts. Another aliquot of ghosts was treated identically except isotonic saline replaced PMB. To assess binding of hemoglobin Köln to red cell membranes, ⁵⁹Fe-labeled Köln, prepared as described below, was incubated for 2 hr with either control or PMB-treated ghosts at 37 or 50°C. The final 7 ml of suspension contained 75 mg of labeled hemoglobin and 6 × 10⁶ ghosts. After incubation the centrifuged (10,000 *g*) ghost pellets were washed three times in 30 mOsmol ghost buffer and counted in an automated well gamma spectrometer.

Studies utilizing hemoglobin solutions. Hemoglobin solutions were obtained by lysing thrice-washed red cells in 4 volumes of distilled water, followed by 1 volume of 5 g/100 ml NaCl; they were finally clarified by removing stroma and Heinz bodies through centrifugation at 10,000 *g*. These solutions were generally dialyzed for 2 hr against distilled water before use. Hemoglobin Köln and A were separated from hemolysates, containing approximately 20% of the former, by paper and column chromatography of the carbonmonoxy deriva-

⁴ New England Nuclear Corporation, Boston, Mass.

⁵ Sigma Chemical Company, St. Louis, Mo.

tives as described by Carrell, Lehmann, and Hutchison (13), whose generous gifts of these materials are gratefully acknowledged. Labeled purified hemoglobins were obtained by identical separation of the hemoglobins from hemolysates prepared from reticulocyte-rich red cell suspensions which had been exposed to transferrin-bound $^{59}\text{FeCl}_3$ as described by Borsook (20).

Met- and sulfhemoglobin were assayed by the method of Evelyn and Malloy (21). Precipitated hemoglobin was quantified by measurements of turbidity in NH_4OH -treated hemolysates measured at $620\text{ m}\mu$ as described previously (35), or by gravimetric analysis. For the latter the precipitate from a known quantity of hemoglobin was collected by centrifugation at $10,000\text{ g}$, washed twice with distilled water, and dried to a constant weight in vacuo over P_2O_5 ; a method similar to that used by Jandl, Engle, and Allen (7).

Two methods of assaying the relative degree of binding of glutathione to hemoglobins A and Köln were utilized. In the first, 3.6×10^{-8} moles of chromatographically purified hemoglobin A or Köln were reacted with 8.1×10^{-8} moles of G^{35}SH^* (about $0.6\text{ }\mu\text{c}$) in a final volume of 0.5 ml of 0.1% EDTA buffered to $\text{pH } 7.4$ with phosphate buffer. After 90 min at 37°C , hemoglobin was precipitated with ice cold 10% TCA, the precipitate washed twice with cold 5% TCA, and once each with absolute ethanol and ether. Weights of the dried precipitates indicated nearly complete ($> 90\%$) recoveries for both hemoglobins. The precipitates were dissolved in 1 ml of 0.1 N NaOH, and aliquots were pipetted in triplicate into aluminum planchets and counted with $20\text{--}25\%$ efficiency in an automated gas-flow counter. Triplicate determinations agreed to within $\pm 3\%$. In the second method fresh clarified hemoglobin solutions, containing roughly 20% Köln and 80% A were prepared from water hemolysates obtained from nonsplenectomized patients. To 6×10^{-7} moles of this hemoglobin mixture in 0.1% EDTA-phosphate buffer ($\text{pH } 7.4$) were added 8×10^{-7} moles of G^{35}SH and the mixture was incubated at 37°C for 60 min . It was then applied to a starch block and electrophoresed overnight in 0.05 M barbital buffer, at $\text{pH } 8.6$. The well-separated zones of A and Köln hemoglobins were eluted with the developing barbital buffer, concentrated by ultrafiltration, assayed for hemoglobin content, and finally precipitated with TCA, redissolved with NaOH, and counted in planchets as described above.

RESULTS

Blockade of hemoglobin Köln sulfhydryls by GSH. We have recently presented evidence utilizing two separate titration techniques for thiol groups that globin sulfhydryl activity is diminished or even absent in hemoglobin Köln (22). This, despite the fact that the usually titratable

* Schwarz Bioresarch Company, Inc., Mt. Vernon, N. Y. Available as 85% radiochemically pure G^{35}SH , $25\text{ }\mu\text{c}/\text{mg}$.

β -93 cysteines are present by fingerprint analyses of degraded hemoglobin Köln (13). It follows that this group is blockaded in the native molecule. Further support for this is shown in Fig. 2. Hemoglobin precipitates at 37°C into Heinz bodies when its sulfhydryl groups are inhibited as with paramercuribenzoate (PMB), an organomercurial used throughout these studies as a sulfhydryl blocker. Hemoglobin A precipitates only when PMB is present in molar ratios in excess of 2; that is, after the 2 easily titratable β -93 sulfhydryl groups are blockaded (black circles, Fig. 2). Thereafter, as shown previously by Allen and Jandl (8), conformational changes occur in the globin molecule which expose previously buried sulfhydryl groups; their blockade by PMB or their oxidation by oxidant chemicals results in irreversible denaturation and precipitation of the protein into Heinz bodies. However, in the case of hemoglobin Köln (white circles, Fig. 2) in which β -93 sulfhydryls are evidently naturally blockaded, precipitation occurs with abnormally low levels of PMB. In fresh hemolysates containing hemoglobin Köln, precipitation occurs when as little as an equimolar concentration of PMB is added, suggesting that blockade of Köln sulf-

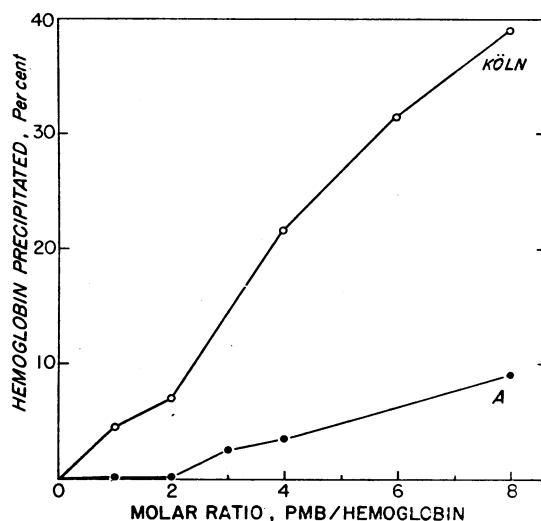


FIGURE 2 Precipitation of hemoglobins by SH blockade. Hemoglobin A (black circles) precipitates during 2 hr of incubation at 37°C only when more than two SH groups/molecule are blockaded by paramercuribenzoate (PMB). In contrast, hemoglobin Köln, whose sulfhydryl activity is normally diminished or absent, precipitates with any added PMB (white circles).

hydrils exists in vivo and is not simply a manifestation of isolation and purification procedures.

Two observations suggest that the blockade of the β -93 sulfhydryls of Köln results from the formation of mixed disulfide linkages between these groups and glutathione: (1) such interaction has been demonstrated to occur during the oxidative denaturation of hemoglobin A by phenylhydrazines (8); and (2) intracellular GSH levels are abnormally low in red cells containing hemoglobin Köln despite the appropriate functioning of metabolic pathways which should maintain normal levels of this compound (18). That this interaction of GSH with hemoglobin Köln is occurring at excessive rates is shown in Table I. When hemoglobins A and Köln are incubated with $G^{35}SH$ either as mixtures in clarified fresh hemolysates from patients (experiments 1 and 2, Table I) or separately as chromatographically purified fractions (experiment 3, Table I), the labeling of Köln exceeds that of A by 3–9 times. No labeling of precipitated protein occurred if $G^{35}SH$ was added to hemoglobin Köln or A immediately before TCA treatment. Previous evidence by others (8) has demonstrated that the protein-bound label in such experiments is specifically released by disulfide-splitting reagents, indicating that glutathi-

TABLE I
Binding of $G^{35}SH$ to Hemoglobins A and Köln*

Experiment	Specific activity		Ratio Köln/A
	A	Köln	
	<i>cpm/mg Hgb</i>		
1	340	2317	6.8
2	445	3850	8.7
3	2473	7480	3.0

* In experiments 1 and 2 hemolysates containing roughly 20% Köln and 80% A were incubated for 60 min at 37°C with $G^{35}SH$, the hemoglobins then separated electrophoretically, precipitated with TCA, and their specific activities measured as described in Methods. In experiment 3, chromatographically purified hemoglobin A and Köln were incubated in separate flasks with $G^{35}SH$ for 90 min at 37°C and specific activities of TCA precipitates then measured. Roughly 0.1 mole of GSH bound to each mole of Köln during the incubation periods.

one and globin sulfhydryls are in mixed disulfide linkage. When ^{35}S -labeled hemoglobin was separated into α and β chains (36) and these isolated by column chromatography, virtually all the label was on β -chains.⁷ Protein-bound ^{35}S -label could

⁷ These studies were performed with Dr. Kaspar Winterhalter whose collaboration is gratefully acknowledged.

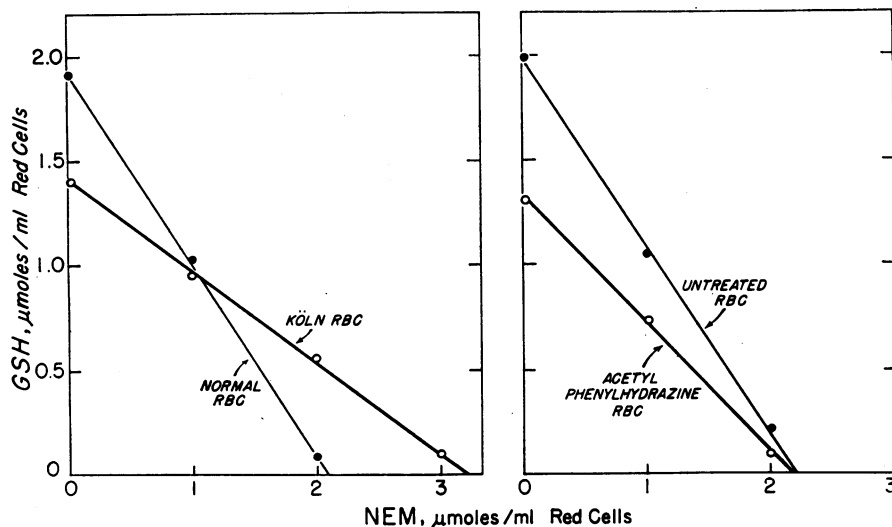


FIGURE 3 Titration of red cell GSH with N-ethylmaleimide (NEM). A roughly stoichiometric amount of NEM is required to fully titrate GSH in normal red cells (black circles, left and right). In contrast, cells containing Heinz bodies (white circles), either from splenectomized patients with Köln hemoglobinopathy (left) or after their exposure to acetylphenylhydrazine (right), contain lower levels of free GSH, but also contain a hidden pool of GSH as manifest by the greater than stoichiometric amount of NEM required to completely blockade total cellular GSH.

be exchanged with unlabeled GSH if the protein was dialyzed against a solution containing the unlabeled tripeptide. As further evidence of the increased interaction of GSH with hemoglobin Köln, this exchange occurred at rates several times that noted with hemoglobin A; that is the specific activity of $G^{35}S$ H-labeled hemoglobin Köln diminished 7–10 times faster than did labeled A during the dialysis (not shown).

Further evidence that a portion of cellular GSH is bound to hemoglobin Köln was derived from titrations of GSH with *N*-ethylmaleimide (NEM) as shown in Fig. 3. As previously described (16), NEM reacts in near stoichiometric fashion with GSH in normal red cells (black circles; left, Fig. 3); that is, roughly 2 μ moles of NEM is required to completely blockade the 2 μ moles of GSH normally present per milliliter of normal red cells. In contrast, red cells containing Heinz bodies from a splenectomized patient with hemoglobin Köln (white circles; left, Fig. 3) contain diminished levels of "free" GSH, about 1.4 μ moles/ml of red cells. When NEM is added to these cells, however, over twice the expected stoichiometric amount is required to titrate fully GSH. This suggests that a hidden pool of GSH is present in Heinz body-containing red cells that becomes available for measurement during titration of GSH with NEM. That this pool is probably bound to denaturing hemoglobin Köln is suspected. As shown above in binding studies of $G^{35}S$ H with hemoglobin, GSH attaches and detaches readily and continuously from hemoglobin, especially Köln. When NEM complexes unbound cellular GSH, bound GSH evidently shifts off of hemoglobin and becomes available for titration with NEM. Further support for the proposal that a portion of cellular GSH is bound to unstable hemoglobin is shown by the findings in the right portion of Fig. 3. A similar hidden pool of NEM-titratable GSH can be appreciated in normal red cells in which Heinz bodies have been generated by their exposure to acetylphenylhydrazine. Such exposure diminishes "free" GSH from 2.0 to 1.3 μ moles/ml of RBC, but total NEM-reactive GSH continues to be roughly 2.0.⁸

⁸ Another unstable hemoglobin, H, forms inclusion bodies found mainly in lower layers of centrifuged blood. Red cell GSH is diminished in these layers (37). A hidden pool of NEM-reactive GSH is observed in lower, but not upper-layer red cells in this condition.

A final experiment supporting the binding of intracellular GSH to denaturing hemoglobin is shown in Fig. 4. As described previously (1), precipitation of hemoglobin Köln occurs in hemolysates heated to 50°C. This also occurs in red cells containing Köln (white circles; left, Fig. 4), but not in normal red cells (black circles; left, Fig. 4). As shown in the right portion of Fig. 4, loss of free GSH accompanies this precipitation of Köln into Heinz bodies (white circles), a phenomenon not observed in normal red cells, in which Heinz bodies are not generated (black circles).

The diminution in free GSH within Köln red cells affects the metabolism of glucose by these cells. We have previously demonstrated that the metabolism of glucose through the hexose monophosphate (HMP) shunt is indirectly proportional to the amount of reduced glutathione within red cells (18). That is, diminished glutathione levels, which can be provoked in a variety of ways, accelerate HMP shunt activity. As shown in Table II the diminution in free GSH by its binding to Heinz bodies in red cells containing hemoglobin Köln (CHBHA patients 1–3, Table II) or another unstable hemoglobin, Hammersmith (CHBHA patient 4, Table II), also leads to potentiation of HMP shunt activity.

Heinz body formation as a manifestation of diminished avidity of hemoglobin Köln for hemes. Heinz bodies, whether collected by centrifugation from lysed red cells or by precipitation of CHBHA hemoglobins at 50°C, appear whitish-yellow rather than red. This suggested to us that hemes may

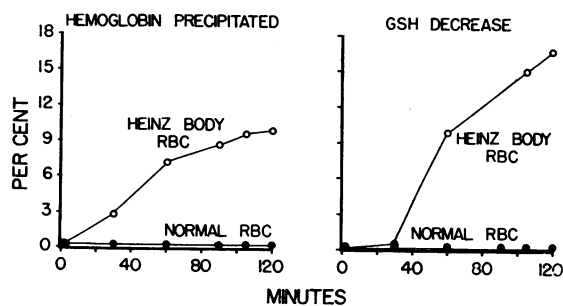


FIGURE 4 Destruction of hemoglobin and GSH in heated red cells. During heating at 50°C, red cells containing hemoglobin Köln progressively accumulate Heinz bodies as reflected by increasing precipitation of their hemoglobin (left). Concomitantly, free GSH diminishes within these cells (right). In contrast, normal red cells (black circles) neither form Heinz bodies nor lose cellular GSH.

TABLE II
Excessive HMP Shunt Metabolism in CHBHA Red Blood Cells*

Patient	Reticulocytes	GSH	Glucose consumption		% Via HMP Shunt	
			Total	HMP Shunt		
			$\mu\text{moles/ml RBC per hr}$			
CHBHA	1	4.8	37	3.37	0.33	9.8
	2	6.2	35	3.69	0.54	14.6
	3	5.2	43	3.37	0.34	10.2
	4	36.8	39	8.09	0.91	11.2
				Mean \pm SE		11.5 \pm 1.1
Control	1	6.8‡	82	3.09	0.16	5.1
	2	0.8	55	2.26	0.10	4.6
	3	0.2	74	1.98	0.10	5.2
	4	1.0	61	2.09	0.10	4.9
				Mean \pm SE		4.9 \pm 0.13

* Washed red cells from four patients with CHBHA (patients 1-3 = hemoglobin Köln; patient 4 = hemoglobin Hammersmith) and from four normal donors were incubated for 4 hr at 37°C with glucose-¹⁴C-1 to assay HMP shunt metabolism as described in Methods.

‡ Red cells from a patient with reticulocytosis following massive epistaxis were utilized. Per cent HMP shunt activity in red cells from two additional patients with hereditary spherocytosis was 5.8 and 6.3%, respectively, and from one patient with 12% reticulocytes accompanying a Coombs' positive hemolytic anemia, 4.8%.

be altered or lost during the denaturative process. We have presented evidence elsewhere (22) that hemoglobin Köln has diminished avidity for its hemes groups. As demonstrated by Bunn and Jandl (23) ferrihemes are in constant flux between globin molecules; in our hands this flux was found excessive from Köln. Thus when unlabeled fetal methemoglobin is incubated with ferrihemoglobin-⁵⁹Fe Köln or A, the fetal hemoglobin rapidly becomes labeled, but at much faster rates when Köln is the donor of labeled heme (22). It seemed reasonable to postulate that this diminished avidity of Köln for its hemes might underlie its propensity to precipitate. In fact we have found that globin from which hemes are removed by treatment with zinc acetate⁹ precipitates at 37°C, and that this precipitated material appears microscopically as aggregated coccoid bodies that are indistinguishable, tinctorially and morphologically from Heinz bodies.

Bunn and Jandl (23) have also demonstrated that the flux of hemes from hemoglobin is directly proportional to temperature. Thus the already excessive release of hemes from hemoglobin Köln is further accelerated by its incubation at 50°C in the aforementioned heat-denaturation test. Presumably a critical state is reached in which globin

depleted of its hemes, precipitates. This state is not reached with hemoglobin A in which heme avidity is inherently greater. To support this conjecture, advantage was taken of the observation that loss of hemes from globin is largely prevented by their

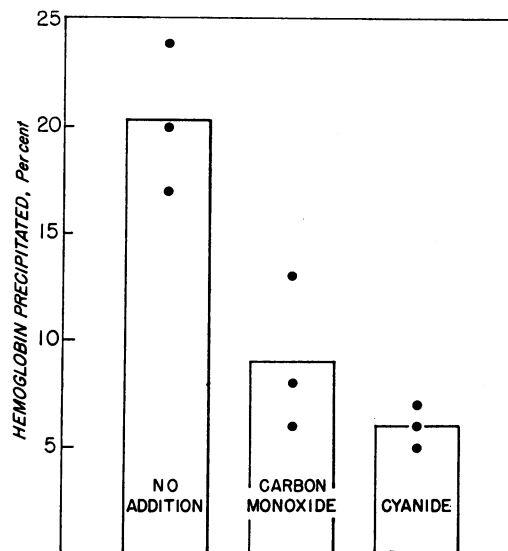


FIGURE 5 Inhibition of heat precipitation of hemoglobin Köln by cyanide or carbon monoxide. Copious precipitation of hemoglobin Köln occurs in hemolysates heated at 50°C for 2 hr (left bar). Addition of the heme ligands, cyanide (right bar), or carbon monoxide (middle bar), diminishes this heat denaturation.

⁹ Gerald, Park S. Personal communication.

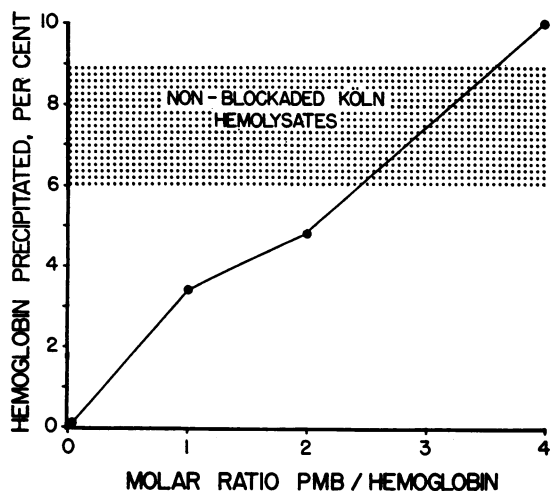


FIGURE 6 Induction of heat precipitability of hemoglobin A by globin sulfhydryl blockade. Although normally not precipitable when heated for 1 hr at 50°C, hemoglobin A becomes so when its SH groups are blocked by paramercuribenzoate (PMB). When more than two SH groups/mole of hemoglobin A are inhibited, precipitation by heat is equivalent to that observed when untreated hemoglobin Köln is heated (shaded areas).

reaction with cyanide or carbon monoxide (23). If hemoglobin Köln is reacted with either of these ligands, its precipitation when heated to 50°C is diminished (Fig. 5).

The blockade of the β -93 SH groups of Köln

may be involved in the diminished avidity of the neighboring β -92 histidines for their heme groups. Supporting this, is the previous demonstration (24) that artificial blockade of the sulfhydryl groups of hemoglobin A leads to a diminished avidity of the molecule for its hemes. As shown in Fig. 6, such treatment also renders hemoglobin A precipitable at 50°C. In fact precipitation of hemoglobin Köln (shaded area, Fig. 6), in which sulfhydryl groups are naturally blocked, is exactly mimicked when more than two SH groups of hemoglobin A are blocked by PMB. As in the case of untreated hemoglobin Köln, the precipitation of PMB-treated hemoglobin A is diminished by cyanide or carbon monoxide (not shown).

Binding of Heinz bodies to red cell membrane thiols. Electron micrographic studies (25) of red cells after their exposure to phenylhydrazines have demonstrated that Heinz bodies are initially generated in the interior of red cells; thereafter they rapidly migrate and become fixed to the cell membrane. In our hands this attachment was obvious by examination under phase optics of red cell ghosts prepared from the splenectomized patient with hemoglobin Köln. Roughly 70% of such ghosts contained 0.5–1.0 μ inclusions that seemed firmly attached to the ghost membrane. We suspected that attachment might involve binding of

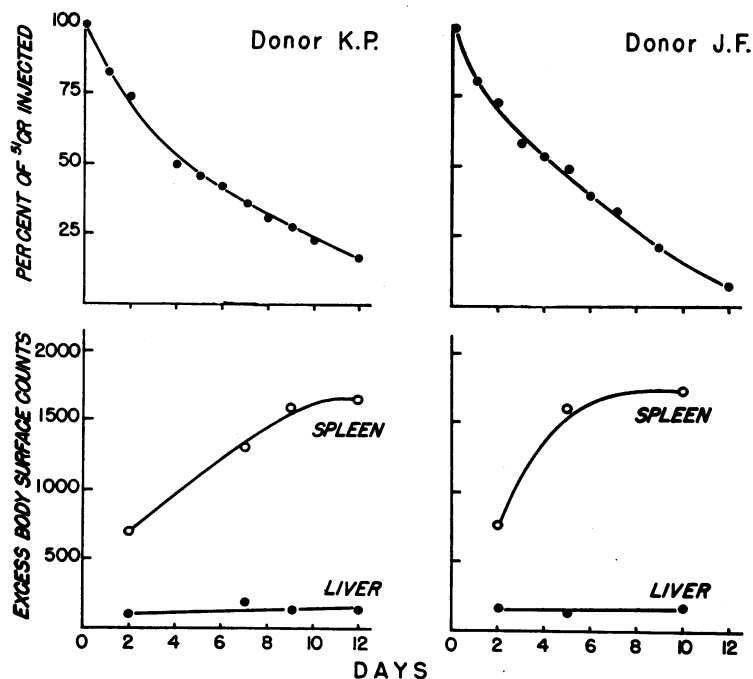


FIGURE 7 Survival and sites of sequestration of hemoglobin Köln red cells. ⁵¹Cr-labeled red cells from two patients with Köln hemoglobinopathy disappear from the circulation (upper) at rates roughly five times that of normal (normal half disappearance time = 25 days). Sequestration and destruction is almost entirely by the spleen (lower).

TABLE III
Release of Heinz Bodies from Red Cell Membranes
by Mercaptoethanol*

Experi- ment	Ghosts containing Heinz bodies		
	No addition	Ethanol	Mercaptoethanol
	%	%	%
1	73	71	41
2	66	62	35
3	70	69	30
4	60	65	20
Mean \pm SE	67.3 \pm 2.8	66.7 \pm 2.0	31.5 \pm 4.4

* Approximately 5×10^6 red cell ghosts/mm³ from a splenectomized patient harboring hemoglobin Köln were suspended in phosphate-buffered EDTA (pH 7.4). After incubation for 1 hr at 37°C alone or with 0.26 mM ethanol or mercaptoethanol, the ghosts were examined under phase microscopy for the presence of Heinz bodies in platelet-counting chambers.

the excessively reactive sulfhydryl groups of hemoglobin Köln to the sulfhydryl groups of the red cell membrane in mixed disulfide linkages. Red cells with membrane thiols blockaded, as would occur in the proposed scheme, have been studied previously using PMB to inhibit membrane sulfhydryl activity (16, 26). The unique characteristic of such SH-inhibited cells in vivo is their rapid removal from the circulation, and their strikingly specific sequestration and destruction by the spleen (26). As seen in Fig. 7 ⁵¹Cr-labeled red cells from two patients with hemoglobin Köln behave similarly; that is, their survival in the circulation is diminished to about $\frac{1}{3}$ that of normal, and by body surface monitoring of radioactivity, the spleen can be shown to be the major site of their destruction.¹⁰

Evidence that mixed disulfide linkages are, in fact, involved in the attachment of Heinz bodies to red cell membranes is shown in Table III. The disulfide-splitting reagent, mercaptoethanol, releases many of the Heinz bodies from their attachment to red cell ghosts. Ethanol, a closely similar molecule but unreactive toward disulfide bonds, is without effect.

Further evidence that denatured hemoglobin Köln attaches to membrane thiols is presented in Table IV. When normal red cell ghosts are incubated at 37°C with ⁵⁹Fe-labeled hemoglobin

¹⁰ The authors gratefully acknowledge the assistance of Dr. Leon Szur in these studies.

Köln, they bind significant radioactivity and appear pink after removal of unreacted hemoglobin. If however, their sulfhydryl groups are blockaded by treatment with PMB before their reaction with the labeled hemoglobin, labeling is substantially reduced and the ghosts appear only slightly colored. When Heinz body production is accelerated by performing the incubation at 50°C, binding of labeled Köln to ghosts is more than doubled, unless ghosts without free sulfhydryl groups are utilized. About 70% of the bound radioactivity in these experiments could be removed from the ghosts by mercaptoethanol; ethanol was without effect (not shown).

That Heinz body-containing red cells are functionally depleted of membrane sulfhydryls through their reaction with denatured hemoglobin is shown by their hypersusceptibility to PMB in Fig. 8. PMB inhibits only the thiol groups of the red cell membrane in the small doses used in these experiment (16). As compared with identically treated normal red cell (black circles), Heinz body-containing red cells from a splenectomized patient with hemoglobin Köln excessively leak cations (left, Fig. 8) and increase their osmotic fragility (right, Fig. 8) when incubated with PMB. In ancillary studies red cells from nonsplenectomized patients with CHBHA, whose cells contained few Heinz bodies, were intermediate between Heinz body-rich and normal cells in their susceptibility to PMB (not shown).

TABLE IV
Diminished Binding of Hgb Köln to SH-Depleted
(PMB-Treated) Red Cell Ghosts*

Incubation temp	Experiment	Bound Hgb Köln- ⁵⁹ Fe		PMB Effect: % inhibition
		Untreated ghosts	PMB-Treated ghosts	
°C		cpm		
37	1	1792	982	45
	2	336	170	49
	3	1320	486	63
50	1	3496	1555	56
	2	556	265	52
	3	2893	1050	64

* Two aliquots of normal RBC ghosts, one of which with sulfhydryl groups blockaded by prior incubation with PMB, were incubated in identical amounts with hemoglobin Köln-⁵⁹Fe. After removal of unreacted hemoglobin by washing, ghost radioactivity was assessed. For details see text.

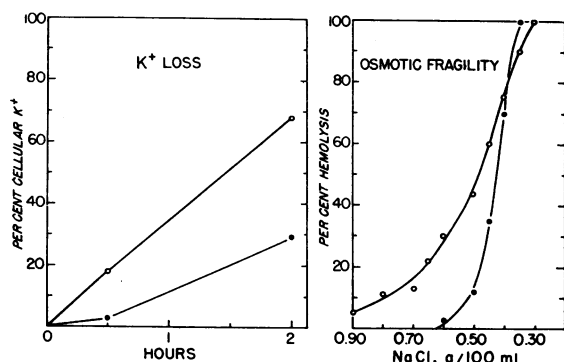


FIGURE 8 Hypersensitivity of hemoglobin Köln red cells to membrane SH blockade. As compared with normal red cells (black circles), Heinz body-containing cells from splenectomized patients with hemoglobin Köln (white circles) lose greater amounts of cellular K^+ (left), accumulate more Na^+ (not shown), and become more osmotically fragile (right) when exposed to the membrane SH inhibitor, paramercuribenzoate (2.5 μ moles of PMB/ml of red cells).

DISCUSSION

The present studies indicate that the mutation at β -98 of hemoglobin Köln critically affects neighboring sulfhydryl reactivity at β -93 and the avidity of β -92 histidines for their heme groups. These alterations in turn can be shown to underlie several of the clinical and biochemical manifestations of CHBHA.

The diminished-to-absent titratability of the β -93 sulfhydryls of hemoglobin Köln, despite their presence by fingerprint analyses of the degraded molecule, was suggested in our previous communication (22) to reflect their blockade by mixed disulfide linkage with glutathione. Since glutathione contains a free carboxyl group in its glutamate moiety, mixed disulfide linkage of hemoglobin with this compound should produce a molecule with increased anodal migration during electrophoresis at pH 8.6. This has been documented with aged hemoglobin A, reflecting its mixed disulfide interaction with glutathione (7, 8, 27). Since the mutation in hemoglobin Köln (methionine for valine) invokes no net charge alteration, the slower anodal migration of the compound relative to A at pH 8.6, even despite its carriage of glutathione, suggests that secondary conformational changes exist that expose new charged groups. The relevance of such alterations for the instability of the molecule is unknown. That, in fact, β -93 sulf-

hydryl blockade by glutathione does exist in Köln is supported by: (1) the hypersusceptibility of this hemoglobin to further blockade by sulfhydryl inhibitors (Fig. 2); (2) the diminished free, but not NEM-reactive levels of GSH in red cells containing Köln (Fig. 3) which decreases further as Heinz bodies are generated by heat (Fig. 4); (3) the increased flux of $G^{35}SH$ to and from the hemoglobin Köln molecule (Table I), specifically its β -chains;¹¹ and (4) our previous evidence that although the β -93 sulfhydryl groups which are normally titratable are not so in Köln, the usually buried other four globin thiols are freely titratable when the molecule is unfolded by 8 M urea or dodecyl sulfate (22). The decreased level of free GSH which results from its binding to Heinz bodies stimulates HMP shunt activity within the affected red cells (Table II). This is analogous to the heightened HMP shunt activity observed in red cells in which GSH has been partially blocked by NEM (18). The same combination of diminished free GSH and excessive HMP shunt metabolism has been observed in red cells from the CHBHA patient with hemoglobin Hamersmith also examined in these studies and in those from patients ingesting toxic doses of phenacetin or Azulfidine.¹²

The blockade of the β -93 sulfhydryls of hemoglobin Köln is relevant to its instability. Thus by artificially blockading these two thiols with PMB, one produces the identical heat-precipitability in hemoglobin A as found in untreated hemoglobin Köln (Fig. 6). Previous work by others has also demonstrated that such blockade diminishes the avidity of hemoglobin A for its heme groups (24, 40), a characteristic of hemoglobin Köln (22). Others have suggested (13) that the substitution of the larger amino acid, methionine, for valine in a very packed area of the hemoglobin molecule may in some unspecified manner bring about the instability of hemoglobin Köln. Regardless of cause the observation that heme-depleted globin is ex-

¹¹ Since the β -93 sulfhydryl group is normally reactive, whereas the other β -chain thiol is buried (9), it is assumed that flux of $G^{35}SH$ involves the β -93 position.

¹² Metabolic studies of red cells from patients with acute Heinz body hemolytic anemias after ingestion of phenacetin or Azulfidine have been performed by one of us (Dr. Jacob) and Dr. William Tyler and will be presented separately.

tremely unstable, precipitating rapidly at body temperature, makes it reasonable to suggest that the diminished avidity of hemoglobin Köln for its hemes underlies its instability. The ability of the heme ligands, cyanide, and carbon monoxide, which prevent heme loss from hemoglobin (23), to inhibit the generation of Heinz bodies from Köln supports this conjecture (Fig. 5). It is of interest in this regard that many other hemoglobins, that are associated with CHBHA contain mutations which are in close apposition to heme groups. Thus hemoglobin Gun Hill has recently been reported (28) to lack the five amino acids of the beta chain just distal to the heme-binding β -92 histidine (β -93-97). Of great interest in terms of our proposition that diminished avidity of Köln for its hemes underlies its instability is the observation that Gun Hill contains no hemes on its beta chains (28). Using similar techniques we have found that purified hemoglobin Köln is also deficient in hemes; thus heme activity measured as cyanometheme at 540 $m\mu$ is 30% less than expected from measurement of globin nitrogen by micro-Kjeldahl analysis.¹³ Since heme loss from beta chains of hemoglobin A has been demonstrated to be many times faster than from the alpha chains (23), it seems reasonable to suggest that the diminished avidity of Köln and Gun Hill for their hemes reflects a further weakening of the binding of the mutant beta chain for its heme group.

Completely analogous results to that presented for Köln hemoglobinopathy have been gathered in one patient (CHBHA patient 4, Table II), with hemoglobin Hammersmith. The mutation in this unstable hemoglobin involves the substitution of serine for phenylalanine at beta 42. This position, in the C-D helices of the beta chain also lies in extremely close proximity to the beta-linked heme. Referring to Fig. 1, this heme group projects to the right of the F and G helices that contain the beta

¹³ These measurements were kindly performed by Dr. J. M. White, whose assistance is gratefully acknowledged. The proposition that hemoglobin Köln loses hemes, and that these may exist free for a time within the cell, is further supported by the observation that specific activity of heme is abnormally low, perhaps by dilution with cold free heme, in Köln-containing red cells incubated with ⁵⁹Fe. Recent studies after glycine-¹⁴C administration in vivo show similar depressions of Köln-heme specific activity (38).

92, 93, and 98 groups. The beta-42 position is directly opposite, to the right, in the C and D helices and thus borders the heme group closely. As such, it seems reasonable to suggest that a mutation in this position may diminish avidity of the beta chain for its heme. Hemoglobin Zürich, which may lead to CHBHA in patients given oxidant drugs (6), contains arginine at beta 63, normally the secondary heme-binding histidine of the beta chain. Another mutation at this site in hemoglobin M_{Saskatoon} leads mainly to methemoglobinemia, but also to shortened red cell survival (39); the mechanism for the latter has not been elucidated. Finally hemoglobin Ube-1, which also causes CHBHA and which in fact may be identical to Köln, has been reported by Shibata and colleagues (41) to involve a β -chain mutation leading to blockade of β -93 sulfhydryl groups.

The excessive loss of hemes from CHBHA hemoglobin may also be relevant to the excessive urinary excretion of the pigmented dipyrroles observed in this syndrome. Similar dipyrroluria has been reported recently in patients with thalassemia (29) where, because of diminished availability of globin chains, excessive unattached hemes probably also occur. In these patients available evidence suggests that the dipyrroles are derived from catabolism of hemes in early erythroid precursors (29). Injection of labeled heme directly into the circulation of rats does not lead to dipyrroluria, but is instead recovered nearly quantitatively as biliary pigments, especially bilirubin (30); the dipyrroluria of CHBHA and thalassemia presumably results from other metabolic pathways. The possibility that degradation of hemes within erythroid-precursor cells differs from that within reticuloendothelial cells seems worthy of further investigation.

At least two mechanisms for the hemolytic anemia in CHBHA can now be offered. Rifkind (31) has presented convincing electron micrographs of Heinz body-containing red cells trapped while traversing the small apertures in walls of the splenic microvasculature. These apertures, between splenic cords and sinusoids, are sufficiently small to require extreme deformation of red cells desiring passage through them. The rigid Heinz body acts as a "sticking-point" and unless the remaining red cell can break away from its anchoring inclusion, its destruction is ordained. The pres-

ence of circulating fragmented red cells of diminished MCHC in Heinz body hemolytic anemias supports this sequence (1).

The present studies also demonstrate that membrane sulfhydryl groups may be depleted in Heinz body red cells by their binding in mixed disulfide linkage to sulfhydryl groups of denaturing hemoglobin. Previously buried sulfhydryl groups of hemoglobin become reactive when denaturation commences, i.e., after the easily titratable thiols have been lost (8). Since the usually free sulfhydryls of CHBHA hemoglobins are mostly reacted with glutathione, the binding of unmasked globin sulfhydryls to membrane thiols probably underlies Heinz body attachment to red cell membranes. That disulfide linkages are involved is supported by: (1) disulfide-splitting reagents remove Heinz bodies from CHBHA ghosts (Table III); (2) binding of denaturing hemoglobin Köln to red cell membranes is inhibited by prior blockade of the sulfhydryl groups of the ghosts (Table IV); and (3) Heinz body containing red cells are hypersusceptible to further blockade of their membrane sulfhydryls (Fig. 8). As previously shown, artificial blockade of membrane sulfhydryl groups has deleterious effects on red cells both in vitro (16) and in vivo (26). As such their blockade by interaction with Heinz bodies might be expected to have pathologic implications in CHBHA. One of the characteristics of red cells with blocked membrane thiols is their hyperpermeability to cations, leading ultimately to colloid osmotic hemolysis (16). Nathan and Gunn (32) have demonstrated such excessive permeability (to potassium) in inclusion-bearing red cells from patients with thalassemia. Excessive K leaks were documented in our patient as well, and this was potentiated manifold by increasing Heinz body generation with heat. Thus, red cells containing hemoglobin Köln lost 45–50 meq of K/liter of cells when heated for 2 hr at 50°C to generate Heinz bodies, whereas normal cells, in which Heinz bodies were not formed, lost 20–25 meq of K/liter. At 4 hr 60–70% of Köln red cells, but only 15% of normal cells had hemolyzed. The behavior of CHBHA red cells in vivo may also reflect the blockade of their membrane sulfhydryl groups. Red cells in which these groups are artificially blocked by PMB or NEM are rapidly and rather specifically entrapped and destroyed

in the spleens after reinjection (26). Similar specificity in destruction of CHBHA red cells is seen in Fig. 7, and presumably underlies the improvement although, not cure, of these patients following splenectomy.

Our findings can be synthesized into a scheme in which the formation of Heinz bodies, the dipyrroluria, and the hemolytic anemia in patients with hemoglobin Köln might be explained. Thus, the mutation at the 98 position of the beta chain of hemoglobin Köln (or in other analogous positions in other CHBHA hemoglobins) alters hemoglobin, and secondarily, red cell membrane stability through its effect on sulfhydryl metabolism. That is, the neighboring beta-93 sulfhydryl group is blocked by its reaction with glutathione in mixed disulfide linkage. Concomitantly, avidity of the juxtaposed beta-92 histidine for its heme group is diminished. We suggest that the resulting excessively freed hemes are metabolized by as yet obscure pathways to the pigmented dipyrroles, so prominent in this syndrome. The diminished avidity of hemoglobin Köln and other CHBHA hemoglobins for hemes also underlies their precipitation into Heinz bodies, reflecting the instability of heme-depleted globin at body temperature. The binding of this denatured hemoglobin to red cell membrane thiols through mixed disulfide linkages produces membrane hyperpermeability in a fashion analogous to that observed in other cells when proteins such as ADH (33) or insulin (34) bind membrane thiols in mixed disulfide linkage. In the latter instances a useful function is served, whereas with the red cell, osmotic damage and premature reticuloendothelial entrapment and hemolytic anemia result.

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