Abstract. Mutant, unstable hemoglobins precipitate as Heinz bodies in circulating red blood cells resulting in their premature hemolysis. We stress that generally these hemoglobins contain amino acid substitutions in the \( \beta \)-chain of globin near the heme pocket, and demonstrate that heme binding suffers thereby. Four genetically unstable hemoglobins lost roughly half their heme content while precipitating into Heinz bodies. Conversely, repletion of hemes \textit{in vitro} corrected the characteristically aberrant electrophoretic mobilities of these hemoglobins and concomitantly prevented their excessive denaturation into Heinz bodies. From the finding that heme-containing \( \alpha \)-chains accumulate in solution during Heinz body formation, we propose that heme loss occurs predominantly from mutant \( \beta \)-chains, which then precipitate. This mechanism of Heinz body formation is valid in most, but not all, the unstable hemoglobinopathies.

A number of mutant hemoglobins are unstable, denaturating in circulating red cells into Heinz bodies and precipitating \textit{in vitro} when heated to 50°C. Patients harboring these hemoglobins manifest a syndrome termed congenital Heinz body hemolytic anemia (CHBHA),\(^1\) 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 large amounts of pyrrolic pigments loosely termed “dipyrrroles.”\(^2\) Hemoglobin Köln (\( \beta \)-98 valine \( \rightarrow \) methionine) is the most prevalent of the mutant hemoglobin underlying CHBHA, and one of us has presented evidence that this hemoglobin binds its heme groups less avidly than normal hemoglobin A.\(^3\) From the observation that the amino acid substitution in Köln, as well as in two other unstable hemoglobins (Hammersmith and Zürich), are all in close apposition to the heme group of the beta chain of hemoglobin, we suggested that heme loss from genetically unstable hemoglobins may be the primary underlying mechanism in Heinz body formation in CHBHA.\(^4\) Since then the mutations in many CHBHA hemoglobins have been elucidated, and nearly all involve amino acid substitutions in the beta chain closely neighboring the heme pocket (Fig. 1). The present studies indicate that heme loss from beta chains underlies the instability of all the CHBHA hemoglobins available to us for study.

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Unstable Hemoglobins: The Role of Heme Loss in Heinz Body Formation

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Materials and Methods. Hemoglobins Köln, Hammersmith, San Francisco, and Zurich, from hemolysates of heterozygous donors were separated from hemoglobin A by column chromatography on CM-Sephadex at 4°C; Köln at pH 6.0 with a linear gradient of NaCl from 0 to 0.4 M; the others with a nonlinear gradient of 0.01 M phosphate from pH 6.8 to 8.0. Hemoglobin containing hemes only on its α-chains, α_{hem}β_{2}, was synthesized as previously described. Briefly, half-stoichiometric amounts of crystalline hemin are added to purified globin and the partially heme-deficient protein is then isolated by column chromatography on CM-Sephadex. Reflecting the fact that α-chains of globin have an eightfold greater affinity for hemes than do β-chains, the purified synthetic material has been shown to have the structure α_{hem}β_{2}.

Isolated CHBHA oxyhemoglobins were incubated as 0.01 M phosphate-buffered (pH 7.4) solutions at 50°C. This temperature leads to precipitation of unstable hemoglobins (but not of normal hemoglobin A) into typical coccoid Heinz bodies, which underlies its use as a convenient screening procedure for CHBHA. Heme loss from incubated hemoglobins was assessed by recording spectrophotometry of the clear supernates after removal of Heinz bodies by centrifugation at 20,000 g. The ratio of optical density at 540 mμ (mainly heme) to that at 280 mμ (mainly protein) was used as a measure of heme:globin ratio. Decreasing values of the 540:280 ratio accurately reflects heme loss from globin, as only globin-bound hemes absorb significantly at 540 mμ, and methemoglobin formation is negligible during the short incubation periods utilized. The ratio falls from 0.44 to 0.29 when half the hemes of hemoglobin are removed as in synthetic α_{hem}β_{2}.

Heme-deficient CHBHA hemoglobins were converted into fully heme-saturated components by the addition of excess hemin solution prepared by the method of Labbe and Nishida. The desired concentration of hemin was obtained by dilution in 0.01 Na_{2}HPO_{4} containing 100 mg KCN/l adjusted to pH 7.5 with H_{2}PO_{4}. Hemes in excess of the stoichiometric amount (four per hemoglobin tetramer) were removed by chromatography on DEAE columns with 0.1 M phosphate buffer (pH 7.0) as previously described.

Results. When heated to 50°C CHBHA hemoglobins precipitate into a mass of typical coccoid Heinz bodies. Heme loss occurs concomitantly with this instability as demonstrated in Table 1. That is, the ratio of optical density at 540 mμ (heme) to that at 280 mμ (predominantly globin) diminishes in all of the heated CHBHA hemoglobins but remains stable in hemoglobin A. The tendency of the unstable hemoglobins to lose hemes correlates well with the severity of the clinical CHBHA syndrome associated with them. Thus, hemoglobin
Table 1. *Heme loss from unstable hemoglobins.*

<table>
<thead>
<tr>
<th>Hemoglobin</th>
<th>Unincubated</th>
<th>3 Hr Incubation</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Heme de-</td>
<td>Heme de-</td>
</tr>
<tr>
<td></td>
<td>ficiency†</td>
<td>ficiency†</td>
</tr>
<tr>
<td></td>
<td>540/280 mµ</td>
<td>540/280 mµ</td>
</tr>
<tr>
<td></td>
<td>(%)</td>
<td>(%)</td>
</tr>
<tr>
<td>A</td>
<td>0.44 ± 0.02</td>
<td>0.44 ± 0.01</td>
</tr>
<tr>
<td>Zürich</td>
<td>0.44 ± 0.01</td>
<td>0.37 ± 0.01</td>
</tr>
<tr>
<td>Hammersmith</td>
<td>0.40 ± 0.02</td>
<td>0.33 ± 0.01</td>
</tr>
<tr>
<td>Köln</td>
<td>0.35 ± 0.02</td>
<td>0.30 ± 0.01</td>
</tr>
<tr>
<td>San Francisco</td>
<td>0.32 ± 0.01</td>
<td>0.30 ± 0.01</td>
</tr>
<tr>
<td>Synthetic α²β²</td>
<td>0.29 ± 0.02</td>
<td>0.25 ± 0.01</td>
</tr>
</tbody>
</table>

* Purified oxy-hemoglobin incubated at 50°C; 540/280 mµ values = means ± ranges of two or more observations.
† Heme deficiency approximated by comparison with 540/280 mµ ratio of synthetic α²β² (abbreviated α²β²) defined as 50% heme depleted.

Zürich causes no hemolytic anemia unless affected patients are stressed with certain oxidant drugs, such as the sulfonamides. Reflecting this, hemoglobin Zürich loses hemes only after an hour or more of heating. In contrast, hemoglobins Hammersmith, Köln, and San Francisco produce a chronic, moderately severe Heinz body hemolytic anemia, and these lose hemes immediately in vitro. As reported with another, unstable hemoglobin, Gun Hill, all three are partially heme depleted, even prior to incubation, simply following chromatographic isolation (Table 1). With incubation they progressively further diminish in heme: globin (540:280 mµ) ratio, and this ratio ultimately approaches that value noted for synthetic hemoglobin containing hemes only on its alpha chains, α²β².

Since the loss of heme groups from beta chains of CHBHA hemoglobins would generate compounds analogous to synthetic α²β² (abbrev. α²β²), it is of interest that we have presented evidence elsewhere that synthetic α²β² closely mimics the genetically unstable hemoglobins in several ways: that is, (a) α²β² precipitates into typical coccoid Heinz bodies when heated at 50°C, (b) it forms mixed disulfides with glutathione at the markedly accelerated rate characteristic of CHBHA hemoglobins, and (c) like the CHBHA hemoglobins, it has an inordinate propensity to attach to red cell ghosts through mixed disulfide bonding with membrane thiol groups. When electrophoresed at pH 8.6, α²β² migrates anodally more slowly than hemoglobin A (α²β²) (*first channel* Fig. 2). This observation suggested to us that heme deficiency might underlie the previously noted paradox that at least two unstable hemoglobins, Köln and Sabine (β²1 leucine → proline), migrate more slowly than A, frequently in multiple bands; this despite mutations that involve a single, neutral amino acid replacement which should lead to no net charge difference. This paradox is shown in the second channel of Figure 2 in which a split, electrophoretically slow hemo-

Fig. 2—Effect of heme repletion on electrophoretic mobility of hemoglobin Köln. Starch gel patterns at pH 8.6 are depicted.
globin Köln is noted. A similar pattern has been described for hemoglobin Sabine and an even slower, similarly split appearance has been observed in our laboratory with hemoglobin San Francisco (not shown). Addition of excess crystalline hemin to synthetic $\alpha^b\beta_2$ generates a hemoglobin both electrophoretically and functionally identical to hemoglobin A. Similarly, addition of crystalline hemin to hemoglobin Köln transforms it into a homogenous heme protein with mobility identical to hemoglobin A (third channel, Figure 2). Hemoglobin San Francisco behaves identically as well (not shown). Repletion of hemes in vitro also enhances the stability of CHBHA hemoglobins and of synthetic $\alpha^b\beta_2$. Thus, the addition of excess hemin to hemoglobins Köln, San Francisco, and to $\alpha^b\beta_2$ completely prevents their usual precipitation (about 50% in 2 hr) during heating at 50°C.

**Discussion.** The present studies indicate that heme is crucial to globin stability and that its loss from mutant beta chains is an important mechanism of hemoglobin denaturation in the congenital Heinz body hemolytic anemias. Others have demonstrated that heme attachment alters the conformation of globin; when present, the helical content of globin increases and conversely decreases when heme is removed. The heme-depleted polypeptide chains in CHBHA hemoglobins are therefore partially uncoiled, perhaps further “unwinding” during their precipitation into Heinz bodies. That heme-depleted, rather than heme-containing polypeptide chains, form the bulk of Heinz body precipitates in CHBHA can be inferred from observations with synthetic $\alpha^b\beta_2$. During its precipitation at 50°C, naked beta chains accumulate in the white Heinz body precipitate, while concomitantly, soluble heme-containing alpha chains progressively accumulate in the supernate. The CHBHA hemoglobins used in the present studies, all of which involve beta-chain mutations, almost certainly behave similarly, since by starch gel electrophoresis or column chromatography we have noted free $\alpha_\text{heme}$ chains in hemolysates from all affected patients. These steadily increase in quantity at 50°C at a time when whitish, heme-depleted precipitates of Heinz bodies are forming. No soluble free beta chains are demonstrable under these conditions.

It seems likely that precipitation of mutant CHBHA hemoglobins into Heinz bodies involves two steps: (1) a tendency for mutant polypeptide chains (usually beta chains) to lose their heme groups; this in turn reflects an alteration in their affinity for hemes due to amino acid substitutions neighboring the heme pocket. Presumably, the resulting excessively freed hemes are excreted as the dark urinary “dipyrrolic” pigments so characteristic of CHBHA; and (2) the partial uncoiling of the resulting heme-deficient polypeptide chain evidently disturbs its binding affinity for the nonmutant (usually alpha) chains, and the resulting heme-depleted monomeric mutant chains precipitate. Indeed purified $\alpha$- or $\beta$-chains of hemoglobin, when stripped of their heme groups, become extremely unstable at body temperature, precipitating into typical coccoid Heinz bodies. The importance of this second step is supported by independent observations on a new unstable hemoglobin, Philly, ($\beta^{\text{tyrosine}} \rightarrow \text{phenylalanine}$). Mutation in this hemoglobin is not near the heme pocket, but instead is at a contact point between $\alpha$- and $\beta$-chains. The bonding between $\alpha$- and $\beta$-chains
diminishes thereby, and \(\alpha\)- and \(\beta\)-chain monomers are found preceding the formation of Heinz bodies in this CHBHA hemoglobinopathy.\(^{19}\) Unlike most patients with CHBHA, those with hemoglobin Philly do not excrete dark "dipyrrolic" urine, and they produce reddish, rather than whitish, Heinz body precipitates in vitro. This suggests that excessive monomer formation, even in the absence of heme loss, may be another, albeit less common, cause of hemoglobin denaturation into Heinz bodies.

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\(^{6}\) Blood containing hemoglobin Köln an: Hammersmith was generously provided by Drs. M. C. Brain and J. V. Dacie from patients described in ref. 1; blood containing hemoglobin Zürich provided by Dr. P. G. Frick from patient described by Frick, P. G., W. H. Hitizig, and K. Betke, *Blood*, 20, 261 (1962); blood containing hemoglobin San Francisco was generously supplied by Dr. D. Heywood from a patient with chronic CHBHA associated with an unstable, previously unelucidated, mutant hemoglobin not identical with the other hemoglobins used in this study, but involving a \(\beta\)-chain mutation.


\(^{10}\) Bunn, H. F., and J. H. Jandl, these *Proceedings*, 56, 974 (1968).


\(^{18}\) Jacob, H. S., unpublished results.