Hemoglobin H is of particular interest since it consists only of \( \beta \) chains (1). This abnormal hemoglobin, therefore, presents a unique opportunity for investigating the influence of \( \alpha-\beta \) chain interactions on some of the structural and functional properties of hemoglobin.

The hemoglobin H used for these studies was obtained from two patients. The first patient, L. G., was a 25-year-old woman of Negro and American Indian ancestry. The second patient, N. H., was a 51-year-old woman of Chinese ancestry. The evidence that the hemoglobin H of both of these patients is identical with hemoglobin II studied by others is summarized below.

1. Patient L. G. had a moderate microcytic hypochromic anemia; after incubation with brilliant cresyl blue, the erythrocytes showed characteristic inclusion bodies (2). Patient N. H. had been aware of anemia for many years and underwent a splenectomy at age 35; after incubation with brilliant cresyl blue, her erythrocytes showed the giant inclusion bodies noted in other patients with hemoglobin H who have undergone splenectomy (3).

2. A family study of patient L. G. showed an absence of hemoglobin H in both parents. This is usually the case in this disease. Examination of the cord blood of the baby of L. G., in April 1961, revealed Bart's hemoglobin (four \( \gamma \) chains).

3. Starch granule electrophoresis at pH 8.6 in barbital buffer revealed an abnormal rapid hemoglobin component in both L. G. and N. H. This component comprised 6 to 10\% of the total pigment in L. G. and 35 to 40\% in N. H. At pH 6.5 the abnormal hemoglobin from both patients migrated toward the anode, only hemoglobin H exhibits this property (2).

4. The amino acid composition of globin prepared from hemoglobin H of each patient was compared with that reported by Hill and Craig (4) and by Braunitzer et al. (5) for isolated \( \beta^A \) chains as shown in Table I.

As can be seen from these analyses, the correspondence between the composition of the hemoglobin H from the two patients and that of \( \beta^A \) chains is indeed very close. An exception is the occurrence of small amounts of isoleucine. It is unlikely that this is due to the presence of a small peptide which could be mistaken for isoleucine, since the amount of this material was not diminished after 72-hour hydrolysis. Since the amount of isoleucine found in the hemoglobin H from the two patients differs markedly and amounts to less than one-half a residue per chain in the case of N. H., it is probable that isoleucine represents an impurity rather than a substitution. Impurities of isoleucine have been encountered previously in hemoglobins separated by starch electrophoresis (7).

**EXPERIMENTAL PROCEDURE**

**Methods**

**Isolation of Hemoglobin H**—Erythrocytes were washed five times with 0.9\% sodium chloride solution and lysed by the addition of 2 volumes of water and 0.5 volume of toluene. After high speed centrifugation, the hemolysates were subjected to starch granule electrophoresis in phosphate buffer, pH 7.5, ionic strength 0.1, for 16 hours.

Under certain conditions, the hemoglobin H split into two components on electrophoresis. This has also been described recently with several cases of hemoglobin H by Silvestroni, Bianco, and Muzzolini (8). The results of an investigation of this phenomenon will be reported in a separate communication.

The instability of hemoglobin H made it necessary to complete all measurements within 24 to 48 hours after withdrawal of the blood from the patient and to perform the isolation and concentration at 4\°. The hemoglobin components were eluted from the starch either with water or with 0.1 \( n \) phosphate buffer, pH 7.0. The hemoglobin concentration of the eluates was approximately 0.1\%. When required, more concentrated solutions (up to 1\% ) were prepared by ultrafiltration through collodion bags, 5 \( \mu \) porosity, Schleicher & Schuell Company. Protein concentration was determined spectrophotometrically by conversion to cyanomethemoglobin, with a molar extinction coefficient of 4.6 \( \times 10^4 \) at 540 nm.

**Determination of Reactive —SH Groups**—The \(-\text{SH} \) groups of hemoglobin fall into two sharply defined categories in their reactivity with a variety of reagents. The methods given below were chosen because, under the conditions described, only the reactive —SH groups are determined.

1. Titration with \( p \)-mercuribenzoate at pH 7.0 (9): The high absorption of hemoglobin at 250 \( \mu \) makes it desirable to use hemoglobin itself as a blank for this titration. This can be done most effectively by using hemoglobin in which the —SH groups have been irreversibly blocked by alklylation. In this case, \( p \)-mercuribenzoate can be added to both cells and titration...
curves such as those shown in Fig. 1 are obtained. The alkyated hemoglobin was prepared from hemoglobin A by the reaction of a 6.6 × 10⁻⁴ M solution with a 10-fold molar excess of iodoacetamide for 5 hours at pH 8.0 followed by exhaustive dialysis.

The titrations were performed by placing 1 ml of hemoglobin H (10⁻⁴ M in 0.1 M phosphate buffer pH 7.0) in one cuvette and 1 ml of alkyated hemoglobin of approximately the same concentration in the other and adding 8 × 10⁻⁴ M p-mercuribenzoate in 0.02 ml increments to both. The measured difference in optical density was plotted as a function of the amount of p-mercuribenzoate added, as shown in Fig. 1.

(2) Reaction with iodoacetamide: This reaction was followed by measuring the acid liberated at pH 7.30 as described previously (10).

3. Reaction with N-ethylmaleimide: The method used for determining the binding of N-ethylmaleimide by hemoglobin H was identical with that described earlier (10).

Determination of Oxygen Dissociation Curves—The method was essentially that of Wyman, Guthe, and Allen (11) except that, because of the dilute solutions used, per cent oxygenation was measured at 500 mm.

Measurement of Bohr Effect—For this purpose both the different titration method described previously (10) and the determination of the oxygen dissociation curve as a function of pH value were used.

Amino Acid Analyses—Globin was prepared by the method of Anson and Mirsky (12). Three to 5-mg samples of the dried protein were hydrolyzed as described by Hirs, Stein, and Moore (13). Aliquots of the hydrolysates were analyzed for their nitrogen content by Kjeldahl and their amino acid composition determined in a Beckman/Spinco model 120 amino acid analyzer.

RESULTS AND DISCUSSION

The hybridization experiments of Jones et al. (1) and the analyses reported in this paper seem to establish beyond reasonable doubt that hemoglobin H consists of β chains only. Rigas has recently determined the molecular weight of hemoglobin H by sedimentation and diffusion and found it not to differ significantly from that of hemoglobin A.² We have found a molecular weight of 69,000 by sedimentation equilibrium for the hemoglobin H isolated from patient N. H. This, therefore, confirms Jones' (1) formulation of hemoglobin H as four β chains.

Normal adult human hemoglobin (Hb A), which consists of two α chains and two β chains, contains six cysteine residues (14), two on the α chains and four on the β chains (4). In the native protein only two of the —SH groups are reactive (cf. Table II) and these are located on the β chains (15). Each α chain, therefore, carries one unreactive —SH group and each β chain one unreactive and one reactive —SH group.

The number of reactive —SH groups in hemoglobin H (four β chains) was, therefore, determined by three different methods with the results shown in Table II. It is apparent that all the —SH groups present are fully reactive, i.e. that each β chain contains two reactive —SH groups. The unreactive nature of one —SH group on each β chain in hemoglobin A thus seems to be related to the presence of the α chains. It is therefore possible that these —SH groups are unreactive in hemoglobin A because they are concerned in interchain bonding. Support for this hypothesis derives from the fact that p-mercuribenzoate

² D. A. Rigas, personal communication.

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**Table I**

<table>
<thead>
<tr>
<th>Number of residues per 18,000 g</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td>Lysine</td>
</tr>
<tr>
<td>Histidine</td>
</tr>
<tr>
<td>Arginine</td>
</tr>
<tr>
<td>Aspartic acid</td>
</tr>
<tr>
<td>Thrreonine</td>
</tr>
<tr>
<td>Serine</td>
</tr>
<tr>
<td>Glutamic acid</td>
</tr>
<tr>
<td>Proline</td>
</tr>
<tr>
<td>Glycine</td>
</tr>
<tr>
<td>Alanine</td>
</tr>
<tr>
<td>Cysteine</td>
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<td>Valve</td>
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</tr>
<tr>
<td>Leucine</td>
</tr>
<tr>
<td>Tyrosine</td>
</tr>
<tr>
<td>Phenylalanine</td>
</tr>
<tr>
<td>Tryptophan</td>
</tr>
</tbody>
</table>

* Tryptophan was determined spectrophotometrically (6) on the isolated globin.

---

**Fig. 1.** Sulphydryl groups of hemoglobin H and hemoglobin A from patient L. G. ○—○, 1.0 ml of 1.5 × 10⁻⁴ M hemoglobin H in 0.1 M phosphate buffer, pH 7.0; ●—●, 1.0 ml of 1.5 × 10⁻⁴ M hemoglobin A in 0.1 M phosphate buffer, pH 7.0. Titrated with 8.0 × 10⁻⁴ M p-mercuribenzoate.

reacts with the unreactive —SH groups of hemoglobin A only under conditions where α-β dissociation is known to occur, i.e. below pH 5. This is reminiscent of the findings of Madsen and Cori (16) with the enzyme phosphorylase.

The oxygen dissociation curves of hemoglobin A and H are shown in Fig. 2. It should be stressed that both components were always isolated from the same blood sample by electrophoresis on a single starch block. Three major differences are apparent: (a) There is an enormous difference in the oxygen affinity of the two hemoglobins (approximately 12-fold). (b) The oxygen dissociation curve of hemoglobin H shows greatly reduced heme-heme interaction. The interaction constant n,
TABLE II

Reactive —SH groups in hemoglobin components of patient L. G.

<table>
<thead>
<tr>
<th>Protein</th>
<th>Reactive —SH groups/68,000 g</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>( \text{p-Mercuro-} )</td>
</tr>
<tr>
<td></td>
<td>benzamidet ( ^\dagger )</td>
</tr>
<tr>
<td>Hemoglobin A</td>
<td>2.3</td>
</tr>
<tr>
<td>Hemoglobin H</td>
<td>7.9</td>
</tr>
<tr>
<td>Hemoglobin H</td>
<td>7.5</td>
</tr>
<tr>
<td>Hemoglobin H</td>
<td>7.0</td>
</tr>
<tr>
<td>Hemoglobin H</td>
<td>8.0</td>
</tr>
</tbody>
</table>

* At pH 7.
† Amount added: 5 moles per mole of Hb A and 29 moles per mole of Hb H.
‡ Amount added: 10 moles per mole of Hb A and 20 moles per mole of Hb H.

Fig. 2. Oxygen dissociation curves of hemoglobin components of subject N. H. The hemoglobins used for these experiments were derived from a single starch block. ——, pH 7.30, 0.1 M phosphate buffer; ——, pH 6.88, 0.1 M phosphate buffer.

TABLE III

Bohr effect of hemoglobin components of patient L. G.
The Bohr effect was determined by the differential titration method (10).

<table>
<thead>
<tr>
<th>Protein</th>
<th>pH Value</th>
<th>Bohr effect</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>protons/mole</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Hb</td>
</tr>
<tr>
<td>Hemoglobin H</td>
<td>7.30</td>
<td>0</td>
</tr>
<tr>
<td>Hemoglobin H</td>
<td>7.00</td>
<td>0</td>
</tr>
<tr>
<td>Hemoglobin H</td>
<td>7.30</td>
<td>2.4</td>
</tr>
</tbody>
</table>

calculated from Hill’s equation \( Y = \frac{K_p x}{1 + K_p x} \), was found to be 1.3 for the hemoglobin H and 2.7 for the hemoglobin A. (c) Fig. 2 as well as the data in Table III show that hemoglobin H has no Bohr effect, whereas the Bohr effect of the hemoglobin A from the same subject is 2.4 protons per mole.

These findings lead to several interesting conclusions:

1. The absence of a Bohr effect in hemoglobin H provides further evidence for Wyman and Allen’s conclusion (17) that the change in the electronic structure of the iron, which accompanies oxygenation, is quite insufficient to account for the Bohr effect.

Thus, changes in the conformation of the protein must be the reason for the change of pK value with oxygenation.

2. Since the \( \beta \) chains in hemoglobin A have been shown to make approximately the same contribution to the Bohr effect as the \( \alpha \) chains (10), the absence of a Bohr effect in a hemoglobin which consists only of \( \beta \) chains suggests that interactions between \( \alpha \) and \( \beta \) chains are essential for the change in conformation responsible for the Bohr effect.

3. The absence of “heme-heme interactions” in hemoglobin H likewise indicates that \( \alpha-\beta \) chain interactions, rather than direct interaction between the hemes, play an important role in determining the sigmoid shape of the oxygen dissociation curve of hemoglobin A. The very high over-all oxygen affinity of hemoglobin H also points to decreased interaction between the chains in this hemoglobin. These conclusions are in excellent agreement with the results of Rossi-Fanelli, Antonini, and Caputo (18) who found that hemoglobin A in increasing concentrations of urea shows a progressive loss of heme-heme interaction and an increase in the over-all oxygen affinity. In 6 M urea both the position and the shape of the oxygen dissociation curve of hemoglobin A are almost identical with those of hemoglobin H without urea (Fig. 2).

4. It should be recalled that the blood of patients with hemoglobin H disease is composed mostly of hemoglobin A. It is obvious that a hemoglobin with such gross functional abnormalities as hemoglobin H could not occur as the sole hemoglobin component, since under physiological oxygen tensions, its oxygen would be unavailable to the tissues.

SUMMARY

An investigation of hemoglobin H which consists of four \( \beta \)A polypeptide chains showed that:

1. The number of reactive —SH groups is two per \( \beta \) chain (i.e. eight per mole), although the same \( \beta \) chains in hemoglobin A contain only one reactive —SH group per chain.

2. Hemoglobin H has no Bohr effect.

3. Its oxygen affinity is approximately 10 times that of hemoglobin A and its oxygen dissociation curve shows no evidence of heme-heme interaction. This behavior is duplicated by hemoglobin A in 6 M urea as shown by Rossi-Fanelli et al.

4. It is concluded that interactions between \( \alpha \) and \( \beta \) chains are essential for the normal oxygen affinity of hemoglobin A as well as for the variation of this parameter with oxygenation and pH value.

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