Our earlier studies suggested an association between \( \alpha \)-thalassemia and hypertension. We postulated that this association might involve trapping of the vasodilator nitric oxide (NO) by hemoglobin (Hb). Hb A has recently been shown to carry NO on its sulfhydryl groups in addition to its hemes. In this report we studied the interaction of purified Hb H as well as Hb A with NO. The number of reactive sulfhydryls were determined spectrophotometrically with bis-dithionitrobenzoate. Spectral studies and nitrosothiol measurements after treatment with NO or nitrosothiols indicated that all eight reactive sulfhydryls of Hb H were capable of binding NO. Hb A, however, was only able to bind and transfer two molecules of NO per tetramer. These findings support the biochemical basis for the association between \( \alpha \)-thalassemia and hypertension.

**INDEXING TERMS:** hemoglobin • thalassemia • sulfhydryl compounds • nitroso compounds • methemoglobin

\( \alpha \)-Thalassemia is the most prevalent genetic trait in the world [1], yet little is known about its impact on other clinical conditions. This is partially due to the difficulty of testing for this condition in clinical laboratories. Recently developed DNA methods make it possible to detect not only the most prevalent deletions that give rise to \( \alpha \)-thalassemia [2–4], but also to determine the number and types of deletions present [4]. On the basis of the chronic mild to significant hemolysis seen in various \( \alpha \)-thalassemia syndromes, we hypothesized that \( \alpha \)-thalassemia may be a cause of hypertension, since hemoglobin (Hb) is known to cause transient hypertension when introduced into the bloodstream [5]. The mechanism for this effect is believed to be due to the essentially irreversible binding of the vasodilator, nitric oxide (NO), by the hemes of Hb.

We used a multiplex PCR method [4] to test samples for the presence of the \( \alpha \)-3.7 deletion, the most prevalent \( \alpha \)-thalassemia deletion in the US. These data demonstrate a significant correlation between the presence of \( \alpha \)-thalassemia deletion(s) and the prevalence of hypertension [5, 6] in hospitalized African-American adults; we have proposed a model incorporating the mechanisms described above [6]. Recently, Jia et al. [7] demonstrated that NO has an important function in the normal regulation of blood pressure. Although these data support the mechanism we propose, they do not address the potential impact of the ability of Hb H (a tetramer of Hb \( \beta \)-chains present in \( \alpha \)-thalassemias) to trap NO. In this study we provide biochemical data that support the involvement of Hb H in the trapping of NO. We propose a model to explain the possible impact of the trapping of NO by Hb H in \( \alpha \)-thalassemia.

**Materials and Methods**

**SPECIMENS**

Aliquots of EDTA-anticoagulated whole blood were obtained from blood specimens that remained after diagnostic testing had been performed in the clinical laboratories at Evanston Hospital. This protocol was approved through the Institutional Review Board of Evanston Hospital.

**HB PREPARATION**

Hb A was prepared by centrifuging whole blood from healthy individuals (no \( \alpha \)-3.7 deletion) at 1500 \( g \), removing the plasma, and washing the erythrocyte pellet three times with isotonic saline. The erythrocytes were then hemolyzed by adding an equal volume of deionized water. The hemolsate was adjusted to a Hb concentra-
tion of 130 g/L with deionized water and passed through a Sephadex G25 gel filtration column (2.5 × 30 cm; Pharmacia Biotech, Uppsala, Sweden) that had been equilibrated with 0.1 mol/L NaCl. This procedure removes ~98% of 2,3-diphosphoglyceric acid, an allosteric effector for Hb [8].

Hb H was prepared by separating the α- and β-chains of Hb A and allowing the isolated β-chains to spontaneously form tetrameric Hb H [9]. In this method, sodium nitrite/ribenzoate (PMB) is allowed to react with the sulfhydryls of Hb A. The modified chains have sufficiently different charges and can be easily separated by ion-exchange chromatography on CM cellulose (Sigma Chemical Co., St. Louis, MO). After chain separation on a 2.5 × 30 cm column, the free sulfhydryls were regenerated by passing the free β-chains through a 2.5 × 30 cm Sephadex G10 gel filtration column equilibrated with 0.1 mol/L β-mercaptoethanol. The tetrameric Hb H was eluted and checked for purity by electrophoresis on agarose pH 8.6 (Beckman Paragon, Brea, CA) and by globin chain electrophoresis.

**Sulfhydryl Determinations**

The number of reactive sulfhydryl groups on Hb A and Hb H were determined with bis-dithionitrobenzoate (DTNB) [10]. The molar concentration of reactive sulfhydryls was determined by measuring the corrected absorbance at 412 nm after reaction with a minimum 10-fold molar excess of DTNB over total Hb sulfhydryls available. The corrected absorbance at 412 nm was determined by subtracting the absorbance contributed by Hb and unreacted DTNB (obtained by measuring the absorbance of the respective blanks) from the total absorbance at 412 nm.

**Nitrosylation of Hemoglobins**

Nitrosylation of Hb A and Hb H was achieved by in situ generation of NO with sodium nitrite (10 mg) and buffered ascorbate (30 g/L ascorbic acid in 0.1 mol/L phosphate, pH 7.4) [11]. Nitrosylation of Hb with NO gas was accomplished by generating NO gas from sodium nitrite/buffered ascorbate in a closed container (2 × 5 cm screw-capped vial). A small centrifuge tube (1.5-mL capacity) containing the Hb solution, along with a separate centrifuge tube containing buffered ascorbate, were then placed in the vial. The generation of NO gas was initiated by adding sodium nitrite crystals to the tube containing buffered ascorbate. The vial was quickly capped and the reaction was allowed to proceed for 10 min. Selective modification of only the reactive sulfhydryls of Hb A and Hb H was achieved with S-nitroso-N-acetylpenicillamine (SNAP) [12]. Nitrosylated Hbs formed by this method were analyzed spectrophotometrically for the presence of heme-bound NO [7] and chemically for the presence of sulfhydryl-bound NO with the Saville reaction [13].

**Results**

To compare the binding properties of NO with Hb A and Hb H, Hb A was "stripped" of the allosteric effector, 2,3-diphosphoglyceric acid, by gel filtration chromatography. This procedure was not necessary for Hb H because the preparation of Hb H involved dissociating the globin chains, which simultaneously removed diphosphoglycerate.

Figure 1 shows the migration patterns of PMB-treated Hb obtained after electrophoresis at pH 8.6 on agarose. Lane 4 shows the separation of PMB-derivatized α- and β-chains. Note that the more negatively charged β-chains move faster toward the anode (left) while the α-chains do not migrate significantly and remain near the application point (shown by arrow). Lanes 5–7 show the purity of products eluted from the ion-exchange column at different pH values. Fractions that eluted between pH 6.2 and 6.6 were essentially free of any α-chains and were used for subsequent analyses.

To determine the number of sulfhydryls in these purified fractions of Hb A and Hb H, we performed spectrophotometric titrations with DTNB. This reagent reacts with protein sulfhydryls accessible to the aqueous environment. When it reacts, it gives rise to a chromophore with an absorption maximum at 412 nm. Although Hb A (oxygenated conformation) has one sulfhydryl on each α-chain and three on each β-chain, the reaction is limited to the two α-sulfhydryls with the oxygenated conformation, resulting in a maximum absorbance of 0.9. The molar concentration of reactive sulfhydryls was determined by subtracting the absorbance contributed by Hb and unreacted DTNB (obtained by measuring the absorbance of the respective blanks) from the total absorbance at 412 nm.

**Fig. 1. Agarose electrophoresis of Hbs and modified Hbs.**

Electrophoresis was carried out on agarose gels at pH 8.6 with a Paragon electrophoresis cell and power supply. Lanes 1, 2, and 3 contain Hb controls for A and F; A, S, and C; and A, respectively. Lane 4 contains the products from the treatment of Hb A with PMB before ion-exchange chromatography on CM cellulose. Lane 5 contains the eluate obtained at pH 6.2 as the peak begins to elute from the column. Lane 6 contains the PH 6.2 eluate after concentration by membrane ultrafiltration. Lane 7 contains the eluate obtained at pH 6.6. The anode is to the left and the cathode is to the right. The application point is indicated by the arrow at the bottom of the figure. The PMB-modified α-chains (lanes 4, 5, 6, and 7) are to the left of the figure and the PMB modified α-chains (lane 4) are toward the right.
a-chain and two sulfhydryls on each β-chain, only one sulfhydryl on each β-chain is “reactive” with iodoacetamide [14]. However, both sulfhydryls are reactive on each of the four β-chains of Hb H [14]. Our data obtained with DTNB confirm these findings (Table 1).

Figure 2 shows the absorption spectra of purified oxygenated (solid line) and deoxygenated (dashed line) Hb A. In comparison, Fig. 3 shows the spectra of oxygenated Hb H (solid line), highly deoxygenated Hb H ($P_{O_2} = 32.9$ mmHg, dashed line), and Hb H fully deoxygenated by the use of dithionite (dashed/dotted line). Although the spectra for oxyhemoglobin A and Hb H are very similar, there are small shifts in the absorption maxima near 415 and 540 nm for Hb H. The spectra for fully deoxygenated Hb A and Hb H are nearly identical. A major difference between the two is that Hb H has a very high oxygen affinity and remains almost fully oxygenated at the $P_{O_2}$ values present in the venous circulation (Fig. 3, dashed line). Hb H has been shown to have a $P_{50}$ (oxygen partial pressure resulting in 50% saturation) of $<1$ mmHg (pH 7, 30 °C), whereas Hb A had a $P_{50}$ of 16.6 mmHg under the same conditions [15]. Therefore, to achieve full deoxygenation of Hb H, dithionite must be used.

When NO gas or sodium nitrite is allowed to react with Hb H in the presence of oxygen, methemoglobin H is formed in a manner similar to that described for Hb A [7]. The spectrum obtained after exposure of oxyhemoglobin A to NO gas is shown in Fig. 4 (dashed line). The reaction occurred in a closed vial as described in Materials and Methods. Note the presence of the characteristic peak for

---

**Table 1. Determination of reactive sulfhydryl groups.**

<table>
<thead>
<tr>
<th></th>
<th>Hb</th>
<th>DTNB detected (× 10⁻⁶ mol/L)</th>
<th>DTNB per Hb tetramer</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(× 10⁻⁶ mol/L)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hb A</td>
<td>3.09</td>
<td>4.89</td>
<td>1.56</td>
</tr>
<tr>
<td></td>
<td>3.40</td>
<td>6.66</td>
<td>1.96</td>
</tr>
<tr>
<td></td>
<td>2.89</td>
<td>4.50</td>
<td>1.56</td>
</tr>
<tr>
<td>Mean (±SD)</td>
<td>1.70 (±0.23)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hb H</td>
<td>10.1</td>
<td>77.8</td>
<td>7.7</td>
</tr>
<tr>
<td></td>
<td>10.3</td>
<td>89.0</td>
<td>8.6</td>
</tr>
<tr>
<td></td>
<td>9.01</td>
<td>77.0</td>
<td>8.4</td>
</tr>
<tr>
<td>Mean (±SD)</td>
<td>8.23 (±0.47)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

---

**Fig. 2. Absorption spectra of Hb A.**

Absorption spectrum of oxyhemoglobin A (110 μmol/L) in 0.1 mol/L phosphate buffer, pH 7.4 (solid line). The sample was placed in a closed 1-mm pathlength cuvette (Precision Cells, Hicksville, NY) for spectral measurements. Absorption spectrum of deoxyhemoglobin A (92.3 μmol/L) in 0.1 mol/L phosphate buffer, pH 7.4 (dashed line); Hb A was deoxygenated (1.0 mL, 2.2 mmol/L) with nitrogen for 15 min with an IL Model 237 tonometer (Instrumentation Laboratory, Lexington, MA). The sample was then transferred anaerobically with a gas-tight syringe and diluted ~20-fold into a 1-mm pathlength cuvette containing phosphate buffer that had also been deoxygenated in the tonometer.

---

**Fig. 3. Absorption spectra of Hb H.**

Absorption spectra of oxyhemoglobin H (110 μmol/L; solid line), highly deoxygenated Hb H (110 μmol/L, $P_{O_2} = 32.9$ mmHg; dashed), and fully deoxygenated Hb H (86.4 μmol/L, with 0.01 mol/L sodium dithionite; dashed/dotted line). Because of significant absorption of dithionite, the spectrum of dithionite-treated Hb H is not shown below 400 nm. All samples were dissolved in 0.1 mol/L phosphate buffer, pH 7.4 and all spectral measurements were made in a 1-mm pathlength cuvette.
methemoglobin at 630 nm. Similar spectra are obtained after exposure of Hb H to NO gas (data not shown). If NO is generated in situ by adding solid sodium nitrite to buffered solutions of oxyhemoglobin A or Hb H containing ascorbate, methemoglobin formation is prevented. Under these conditions, NO is bound to heme iron and the absorption spectrum shown in Fig. 4 (solid line) is obtained. The reaction of NO with reactive sulfhydryls has no significant effect on the absorption spectra above 500 nm [7]. Therefore, the spectral changes in Fig. 4 (solid line) are related to the binding of NO to heme groups. Interestingly, if Hb A is allowed to react with sodium nitrite in the absence of ascorbate, methemoglobin is also formed (spectra not shown) and the spectra show no significant change for at least 1 h. If, however, Hb H is treated with sodium nitrite in the absence of ascorbate, methemoglobin is formed initially (Fig. 5, dashed line) but is rapidly converted to (ferrous) nitrosylated Hb (Fig. 5, solid line). Note that the spectrum of Hb H obtained ~45 min after treatment of Hb H with sodium nitrite is almost identical to that of nitrosylated Hb H (Fig. 5, dashed/dotted line) and nitrosylated Hb A (Fig. 4, solid line) produced by sodium nitrite-buffered ascorbate.

Jia et al. [7] have shown that NO also binds to the reactive sulfhydryls of Hb A. Nitrosylation of these Hb sulfhydryls is most readily achieved by transfer of the nitroso group from a nitrosothiol to the protein sulfhydryls. To test whether the sulfhydryls of Hb H could be nitrosylated in an analogous fashion, we treated Hb H with SNAP, a nitrosothiol shown to be effective in trans-nitrosylation reactions [12]. The reaction mixture was then passed through a G25 gel filtration column to remove excess SNAP. The absorption spectrum of this modified Hb H containing nitrosothiol groups on Cys residues was identical to Fig. 3 (solid line) because heme groups were not modified by this procedure. The nitrosothiolated Hbs were then analyzed for the number of nitrosothiols with the Saville reaction [13]. This method involved mercuric ion-assisted hydrolysis of nitrosothiols to yield nitrous acid. The nitrous acid produced was immediately reacted with sulfanilamide and N-(1-naphthyl) ethylenediamine to form an azo chromophore, which was monitored by its absorbance at 540 nm. The number of nitrosothiols formed by reaction of Hb A and Hb H with SNAP and detected by the Saville reaction is summarized in Table 2. In contrast to Hb A, Hb H forms nitrosothiols with both of...
the Cys residues on each of the four β-chains. Hb H is therefore capable of binding four times as many molecules of NO as Hb A.

**Discussion**

The ability of Hb solutions to affect blood pressure has been known for some time [16, 17]. For example, Malcolm et al. [17] demonstrated that cross-linked Hb solutions could increase mean arterial pressure as much as 25–30% in a dose-dependent manner within 15 min of administration and that the effect could last as long as 5 h. Although the effects are not fully characterized, they are believed to be due largely to the trapping of NO by Hb [18, 19]. In vivo experiments suggest that the endothelin-related pressor effect of cross-linked Hb involves the stimulation of the conversion of proendothelin to endothelin [19]. NO is a smooth-muscle relaxant that functions via activation of guanylate cyclase and the production of cGMP [20] or by direct activation of calcium-dependent potassium channels [21]. Therefore, the increased binding of NO, which could result from increased free Hb in thalassemic patients or from the presence of an intraerythrocytic Hb species that binds significantly more NO than Hb A, could result in transient or sustained increases in blood pressure.

Jia et al. have demonstrated that NO is also bound to the reactive sulfhydryls of Hb A and is transported to and from the tissues in a manner analogous to the transport of oxygen by heme groups [7]. Our results indicate that Hb H also binds NO to heme iron (Figs. 4 and 5) as well as to reactive sulfhydryls (Table 2). Because the reaction of NO with reactive sulfhydryls of Hb to form nitrosothiols does not give rise to a significant change in the absorption spectrum in the region between 500 and 700 nm, direct spectral measurements cannot be used to monitor these reactions. However, hydrolysis of these nitrosothiols with mercuric ion results in the release of eight molecules of NO per Hb H tetramer (Table 2). Under similar conditions, only two molecules of NO are released from Hb A (Table 2). Therefore, Hb H traps four times as many NO molecules (in the form of nitrosothiols) as Hb A. Our data also suggest that Hb H is less susceptible to oxidation in the presence of nitrite (and presumably NO) since it has the ability to rapidly convert methemoglobin H to ferrous nitrosylated Hb H. This may be related to a more rapid rate of reduction of nitrosylated methemoglobin H because reactions of ferric Hbs are more subject to subtle changes in protein structure than ferrous Hbs [22, 23]. For example, the on and off rate constants for the binding of NO to ferric β-chains are greater than for binding to ferric α-chains [23].

On the basis of our observations and the data from Jia et al., we propose the following model to explain the potential relation between α-thalassemia and hypertension (Fig. 6). Fig. 6A summarizes the transport of oxygen and NO by Hb A [7]. Note that NO is trapped by deoxyhemoglobin A (e.g., in tissues) but that the reaction of NO with oxyhemoglobin A in the presence of oxygen (e.g., in the lungs) results in the formation of methemoglobin A. This oxidized Hb cannot transport NO or oxygen at its hemes and must be reduced in vivo by

### Table 2. Determination of Hb nitrosothiol content.

<table>
<thead>
<tr>
<th></th>
<th>Hb (× 10⁻⁵ mol/L)</th>
<th>Nitrosothiol (× 10⁻⁵ mol/L)</th>
<th>Nitrosothiol per Hb tetramer</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hb A</td>
<td>4.53</td>
<td>8.38</td>
<td>1.85</td>
</tr>
<tr>
<td></td>
<td>2.75</td>
<td>6.41</td>
<td>2.33</td>
</tr>
<tr>
<td></td>
<td>2.99</td>
<td>8.50</td>
<td>2.84</td>
</tr>
<tr>
<td>Mean (±SD)</td>
<td>2.34 (±0.49)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hb H</td>
<td>1.07</td>
<td>8.96</td>
<td>8.37</td>
</tr>
<tr>
<td></td>
<td>1.04</td>
<td>9.80</td>
<td>9.42</td>
</tr>
<tr>
<td></td>
<td>0.44</td>
<td>3.12</td>
<td>7.09</td>
</tr>
<tr>
<td>Mean (±SD)</td>
<td>8.29 (±1.17)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
methemoglobin reductase. However, oxyhemoglobin A can be nitrosylated at its sulfhydryls by transnitrosylation with nitrosothiols in blood (e.g., nitrosocysteine, nitrosoalbumin, etc.). The net result is that oxygen and NO (as nitrosothiols) can be picked up by Hb A in the lungs and released at tissue sites as the conformation of Hb changes from oxy to deoxy.

There is no significant change in protein conformation as Hb H (Fig. 6B) passes through the lungs or through the tissues, since it has such a high oxygen affinity. As a result, Hb H is unlikely to give up any NO (whether bound to heme groups or sulphydryls) as it passes through the tissues. NO can be trapped by the heme groups of Hb H (Fig. 5 dotted line) or it can be captured from nitrosothiols in plasma (Table 2, transnitrosylation of Hb H by SNAP). Therefore, in contrast to the reversible transport of NO to and from tissues by Hb A, NO is irreversibly trapped by Hb H. Although the amount of NO carried by Hb is only ~1/10 000 that of oxygen [7, 24], changes in this small fraction in vivo results in significant changes in vascular contractility and blood pressure [7]. Because Hb H has four times the capacity to bind NO and once bound should not give it up readily, small amounts of Hb H present in individuals with α-thalassemia could have a significant effect on mean blood pressure unless compensatory mechanisms are activated.

The data presented here do not establish proof of the proposed mechanism or describe all of the potential changes in α-thalassemia that could result in changes in hydrodynamic properties of blood or vasoactive substances and lead to hypertension. However, further studies of such changes could lead to a better understanding of the pathophysiology of α-thalassemia as well as its implications for other clinical conditions.

We are very grateful for the excellent technical assistance provided by Millicent Boykin and Darlene Morgan in assisting with some of the data collection.

References