We have previously shown that excess unpaired α- and β-globin chains in severe α- and β-thalassemia interacting with the membrane skeleton induce different changes in membrane properties of red blood cells (RBCs) in these two phenotypes. We suggest that these differences in membrane material behavior may reflect the specificity of the membrane damage induced by α- and β-globin chains. To further explore this hypothesis, we sought in vitro models that induce similar membrane alterations in normal RBCs. We found that treatment of normal RBCs with phenylhydrazine produced rigid and mechanically unstable membranes in conjunction with selective association of oxidized α-globin chains with the membrane skeleton, features characteristic of RBCs in severe β-thalassemia. Methylhydrazine, in contrast, induced selective association of oxidized β-globin chains with the membrane skeleton and produced rigid but hyperstable membranes, features that mimicked those of RBCs in severe α-thalassemia. These findings suggest that consequences of oxidation induced by globin chains are quite specific in that those agents that cause α-globin chain accumulation at the membrane produce rigid but mechanically unstable membranes, whereas membrane accumulation of β-globin chains results in rigid but mechanically stable membranes. These in vitro experiments lend further support to the hypothesis that membrane-associated α- and β-chains induce oxidative damage to highly specific different skeletal components and that the specificity of this skeletal damage accounts for the differences in material membrane properties of these oxidatively attacked RBCs and perhaps of α- and β-thalassemic RBCs as well.

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MATERIALS AND METHODS

Phenylhydrazine and phenazine methosulfate were purchased from Sigma Chemical Company (St Louis, MO). Methylhydrazine was purchased from Eastman Kodak (Rochester, NY). Thiocarbamoyl agarse was purchased from Pharmacia Chemical (Piscataway, NJ). A specially pure form of sodium dodecyl sulfate (SDS) required for solubilizing membrane proteins for analysis by thiol-disulfide exchange chromatography was purchased from BDB (Poole, England). Rabbit polyclonal anti-human hemoglobin antibody was obtained from Dako (Carpenteria, CA) and further purified using a human hemoglobin affinity column. Rabbit polyclonal antibodies against protein 4.1 and spectrin were generated as previously described. Rabbit polyclonal antibody against protein 3 was a generous gift from Dr Phillip Low of Purdue

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University, Lafayette, IN. All other reagents were the best analytical grade available. All experiments were performed on freshly drawn heparinized venous blood obtained from volunteers according to procedures and protocols established at the Stanford University and University of California Committees on Human Experimentation.

**Oxidation of normal RBCs.** To determine the nature of membrane abnormalities induced by oxidant damage to normal RBCs, human RBCs washed three times in Tris-buffered saline (pH 7.0, 290 mOsm) were treated with varying concentrations of phenylhydrazine (0.1 to 0.5 mg/mL), methylenehydrazine (0.1 to 1.0 mg/mL), and phenazine methosulfate (0.015 to 0.15 mg/mL) at a final hematocrit of 10% at 37°C for 1 hour. At the end of this period, the RBCs were washed five times with Tris-buffered saline and resuspended to a final hematocrit of 40% for analysis of their cellular and membrane properties.

**Membrane mechanical stability and deformability measurements.** Resealed membranes for stability and deformability measurements were prepared by a procedure adopted from Johnson. The washed RBCs were lysed in 40 vol of 7 mmol/L NaCl and 5 mmol/L Tris (pH 7.4). The membranes were then pelleted by centrifugation, resuspended in 10 vol of 5 mmol/L Tris and 140 mmol/L NaCl (pH 7.4) and incubated 30 minutes at 37°C for rescaling.

**For deformability measurements,** resealed membranes, prepared as described above, were suspended in 3 mL of Stractan II (290 mOsm, 22 centipoise, pH 7.4; St Regis Co, Lumber and Plywood Div, Libby, MT) and exposed to increasing shear stress in the ektacytometer. Analysis of the deformability index curve generated by the ektacytometer provides a measure of membrane deformability.

For mechanical stability measurements, 100 µL of a 40% membrane suspension was mixed with 3 mL of dextran (40,000 molecular weight, 35 g/100 mL in phosphate-buffered saline, 290 mOsm, pH 7.4, viscosity 95 centipoise) and subjected continuously to 750 dyne/cm² in the ektacytometer. Under this stress, the membranes progressively fragment, generating undeformable spheres. This process is detected as a time-dependent decrease in deformability index. The time required for the deformability index to decrease to 60% of its maximum value is taken as a measure of membrane mechanical stability.

**RBC membrane-protein analysis.** RBC membranes and membrane skeletons were prepared from washed RBCs as previously described. Ghosts were prepared by lysing 1 vol of packed RBCs with 40 vol of lysing buffer (5 mmol/L phosphate, 1.0 mmol/L diisopropylfluorophosphate [DFP], pH 8.0). The resulting membrane pellet was washed three times with the lysing buffer. Membrane skeletons were prepared by the addition of 25 mg of Triton X-100 to 1 mL of packed RBC suspension. The resulting skeletons were processed according to the protocol originally outlined by Hackett except for the use of 0.6 mol/L hypertonic KCl solution as the extraction buffer to maximize the release of protein 3 from the membrane skeletons.

**Ghost membranes and membrane skeletons were analyzed by one-dimensional SDS-polyacrylamide gel electrophoresis (PAGE) using a 5% to 18% polyacrylamide gradient SDS slab gel and the discontinuous buffer system of Laemmli, as previously described.** Globin chain separation and analysis was performed by Triton acid urea gel electrophoresis as previously outlined. In establishing Triton acid urea gel electrophoresis, the standard method of Triton acid urea gel electrophoresis was used initially, with appropriate standards of adult and cord blood. To further confirm the identification of the β-globin chain, we used the anti-β-globin monoclonal antibody generously provided to us by Dr L.M. Synder of the University of Massachusetts. All gels were stained with Coomassie blue and the various polypeptides quantitated by laser densitometry using the LKB Ultrascan Excel Enhance laser densitometer (Pharmacia, Bromma, Sweden). When further identification of the various peptide bands was required, Western blotting using monoclonal antibodies was performed as previously described.

To identify membrane protein oxidation, thiol-disulfide exchange chromatography was performed on solubilized proteins to document loss of free thiols in various protein components of the RBC membrane. When solubilized normal RBC membrane components are chromatographed, only two proteins, glycophorin A and protein 7, which have no free thiols, pass through the column and appear in the effluent. All other protein components are retained with the gel matrix. As these proteins lose their free thiols due to oxidative damage, they begin to appear in the unbound filtrate. SDS-PAGE analysis of the bound and unbound fraction shows which of the different membrane protein components are oxidized following treatment with the different oxidizing agents.

**Precipitation of oxidized hemoglobin.** To determine the globin chain composition of hemoglobin precipitates that form following oxidative damage, hemoglobin solutions were treated with phenylhydrazine and methylenehydrazine. Hemoglobin solutions obtained from hemolysates of normal RBCs was adjusted to a concentration of 3.3 g/dL and incubated with either phenylhydrazine (0.1 mg/mL) or methylenehydrazine (0.25 mg/mL) for 45 minutes at 37°C. Following this incubation, the solution was centrifuged at 50,000 rpm at 4°C for 30 minutes, and the precipitated pellet was recovered. The pellet was washed three times, solubilized, and analyzed by Triton acid urea gel electrophoresis to determine the globin-chain composition.

**RBC morphology.** Ten microliters of variously treated RBC suspensions was mixed with 200 µL of 1% glutaraldehyde in phosphate-buffered saline (10 mol/L phosphate, pH 7.4, 290 mOsm). The fixed RBCs were examined by phase-contrast microscopy. The extent of Heinz body formation following oxidant damage was quantitated by staining the cell suspension with methyl violet using standard clinical laboratory methods.

**RESULTS**

**Morphologic examination.** Phenylhydrazine at 0.1 mg/mL (0.7 mmol/L) and methylenehydrazine at 0.25 mg/mL (5.7 mmol/L) produced multiple Heinz bodies in all of the RBCs, but phenazine methosulfate produced no such changes at concentrations up to 0.15 mg/mL (0.5 mmol/L). Following incubation with all three agents, the RBCs exhibited predominantly discocytic morphology (data not shown).

**Measurement of deformability and mechanical stability.** The effects of various oxidant agents on RBC membrane properties were assessed by measuring membrane deformability and mechanical stability of treated cells. Treatment of RBCs with all three agents—phenylhydrazine, methylenehydrazine, and phenazine methosulfate—resulted in a marked increase in membrane rigidity (Fig 1). The increase in membrane rigidity was dose-dependent for all three compounds. In contrast to the similar effects of these three oxidants on membrane rigidity, the membrane mechanical stability was differently affected (Fig 2). Phenylhydrazine treatment resulted in a dose-dependent decrease in membrane mechanical stability, as evidenced by a faster rate of decline of deformability index for membranes of treated cells compared with normal membranes. In contrast, the rate of decline of deformability index for membranes of RBCs treated with either methylenehydrazine or phenazine...
Membrane-associated globin. Protein constituents of ghosts and membrane skeletons prepared from normal and oxidant-treated RBCs were analyzed using 6% to 18% concave gradient SDS-PAGE. The concentrations of the three oxidants used for this analysis were chosen because they produced approximately equivalent degrees of membrane rigidity. Moreover, the extent of oxidant-induced increase in membrane rigidity of these samples was the same magnitude as that we previously reported for RBCs in severe α- and β-thalassemia. Membranes and membrane skeletons prepared from RBCs treated with phenylhydrazine and methylhydrazine, but not those from RBCs treated with phenazine methosulfate, showed protein bands that had mobility corresponding to monomeric and dimeric globin (Fig 3). Globin association with the membrane skeleton thus appears to be a feature of hydrazine-treated RBCs, but not that of phenazine methosulfate–treated RBCs. Western blot analysis using an affinity-purified rabbit anti-human hemoglobin antibody confirmed the identity of the assigned bands to be globin (Fig 4). A monomeric band, as well as a broad band, with approximate mobility of dimeric globin could be visualized in membranes and membrane skeletons prepared from hydrazine-treated RBCs. No such bands could be seen in membrane preparations from phenazine methosulfate–treated RBCs, over a broad range of drug concentrations (0.01 to 0.1 mmol/L).

Membrane protein analysis showed an additional difference between phenazine methosulfate–treated RBCs and hydrazine-treated RBCs. Phenazine methosulfate treatment resulted in the generation of a high–molecular weight protein complex that appeared as an indistinct hazy band above α-spectrin and also as a band at the top of the separation gel (Fig 3). The amount of complex formed increased as RBCs were treated with increasing concentrations of the drug. Western blot analysis using polyclonal anti-spectrin and anti-protein 3 antibodies confirmed that spectrin is the principal component of this complex (data not shown). Interestingly, no such complexes were seen in membranes prepared from RBCs treated with either phenylhydrazine or methylhydrazine.

Oxidation status of membrane-associated globin. Thiol-disulfide exchange chromatography was used to determine if the globin chains bound to the membranes of hydrazine-treated cells were unmodified native globin or oxidized globin. As shown in Fig 5, much of the membrane-associated globin in phenylhydrazine and methylhydrazine
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Fig 3. SDS-PAGE analysis of membranes and membrane skeletons prepared from RBCs treated either with 0.1 mg/mL phenylhydrazine (PHZ), 0.25 mg/mL methylhydrazine (MHZ), or 0.03 mg/mL phenazine methosulfate (PMS). Note protein bands corresponding to monomeric and dimeric globin chains in membrane preparations from PHZ- and MHZ-treated cells, but not in those derived from PMS-treated RBCs.

RBCs appeared in the unbound filtrate fraction, suggesting loss of free thiols in the globin as a result of oxidant damage. No protein bands corresponding to globin were found in the unbound filtrate fraction of membranes from phenazine methosulfate–treated RBCs. Western blot analysis using anti-human hemoglobin antibody confirmed the identity of globin monomers and dimers in the unbound filtrate fraction of membranes from hydrazine-treated cells and absence of globin in that of phenazine methosulfate–treated red cells (Fig 6). These findings imply that while phenylhydrazine and methylhydrazine induce oxidative damage to globin, phenazine methosulfate fails to do so at the concentrations used.

Nature of membrane-associated globin. The globin-chain composition of membrane-associated material in phenylhydrazine- and methylhydrazine-treated RBCs was determined using Triton acid urea gel electrophoresis. This analysis enabled us to resolve membrane bound globin into its individual globin-chain components. As shown in Fig 7, globin associated with membranes and membrane skeletons of phenylhydrazine-treated RBCs was composed predominantly of α-globin chains. In contrast, globin associated with membranes and membrane skeletons of methylhydrazine-treated RBCs was composed predominantly of β-globin chains. Thus, there appears to be selectivity in the type of globin chain that becomes associated with membrane skeletons following oxidant damage induced by these two types of hydrazines.

To determine the basis for the selective association of specific globin chains with the membrane following oxidative damage, we determined the globin-chain composition of hemoglobin precipitates generated by the treatment of pure hemoglobin solutions with different oxidants. Hemoglobin precipitates could be generated following incubation of hemoglobin solution with either phenylhydrazine or methylhydrazine, but not following incubation with phenazine methosulfate. The precipitates generated by the hydrazines were isolated and analyzed for globin-chain composition by Triton acid urea gel electrophoresis. As shown in Fig 8, precipitates generated by phenylhydrazine consisted predominantly of α-globin chains, whereas those generated by methylhydrazine were composed of β-globin chains. Thus, the globin-chain specificity appears to be at the level of the particular hydrazine used to induce oxidative damage to hemoglobin.

DISCUSSION

We had previously shown that RBCs in severe α- and β-thalassemia are characterized by different cellular and
membrane rheological properties. A biochemical feature that distinguished these two phenotypes was the type of globin chain that became associated with the membrane skeletons. In α-thalassemia, the membrane skeleton-associated globin was predominantly β-chains, whereas in β-thalassemia it was predominantly α-chains. These various features of α- and β-thalassemic RBCs are summarized in Table 1. Based on these findings, we hypothesized that in the thalassemias, membrane-associated α-globin and β-globin chains might induce oxidation of different membrane components leading to different alterations in membrane properties.

In this study, we explored this hypothesis further by attempting to induce material behavior in normal RBCs that mimics the material behavior of α- and β-thalassemic RBCs, through use of different oxidant agents. We have shown that oxidative damage in normal RBCs induced by phenylhydrazine reproduces several features of β-thalassemic RBCs (Table 1). The induced changes include generation of rigid but mechanically unstable membranes with concomitant selective association of oxidized α-globin chains with the membrane skeletons. In contrast, methylhydrazine-induced oxidative damage reproduced several features of α-thalassemic RBCs (Table 1), including rigid but hyper-stable membranes in association with selective accumulation of membrane-bound oxidized β-globin chains. The data enable us to conclude that there is indeed a correlation between the type of globin chain that becomes associated with the membrane skeleton and the nature of perturbations induced in membrane material properties.

Our ability to document that phenylhydrazine and methylhydrazine reacting with pure hemoglobin solution can
generate precipitates that resemble Heinz bodies that are composed only of either \( \alpha \)- and \( \beta \)-globin chains, provides a rationale for the observations we have outlined. We suggest that each of these oxidants induce specific damage to globin, phenylhydrazine oxidizing \( \alpha \)-globin and methylhydrazine oxidizing \( \beta \)-globin. These oxidized globin chains then bind to specific membrane components and provide the nidus for oxidative attack on the bound component, as well as to adjacent membrane components. The specificity of the membrane alteration may be dictated by the specificity of the binding site for oxidized \( \alpha \)- and \( \beta \)-globin chains of the RBC membrane.

The different biochemical and biophysical sequelai we have documented for the three oxidant agents used evokes a note of concern and caution in interpretation of experiments using oxidizing agents. While all three agents produce rigid membranes, the biochemical basis appears to be strikingly different. Phenylhydrazine- and methylhydrazine-induced rigidity appears to be mediated through oxidative damage to membrane skeletal proteins caused, at least in part, by specific globin chains binding to the membrane skeleton. In contrast, phenazine methosulfate induces rigidity not through globin-mediated oxidation, but via the generation of superoxide that directly attacks the membrane skeleton cross-linking spectrin molecules.

A number of previous studies have outlined the various cellular and membrane alterations that accompany oxidative damage to the RBC, as well as the oxidant species responsible for inducing the damage.\(^{12,21}\) Directly relevant to the present study are the studies outlining rheological alterations induced by phenylhydrazine and phenazine methosulfate.\(^{12,13}\) Oxidant damage has been shown to markedly reduce cellular and membrane deformability of RBCs. The unique aspect of the present study is the unexpected finding that phenylhydrazine and methylhydrazine induce different membrane alterations, and that there exists distinctly different biochemical basis for these alterations.

In this regard, a study by Shinar et al,\(^{26}\) in which the functional competence of various skeletal proteins in thalassemic membranes was examined, has relevance to our studies. These investigators found different skeletal protein functional abnormalities in \( \alpha \)- and \( \beta \)-thalassemic RBCs: defective binding site for spectrin association with membrane was noted in \( \alpha \)-thalassemia and decreased ability of protein 4.1 to associate with spectrin and actin was found in \( \beta \)-thalassemia. We would like to suggest that these different structural defects identified may be the results of differential binding of oxidized \( \alpha \)- and \( \beta \)-chains to specific proteins in the membrane skeleton, which in turn induce localized structural defects to specific skeletal components. The in vitro model we have developed should enable the dissection of these specific molecular defects.

While we have been able to mimic in normal RBCs the biochemical and biophysical features of thalassemic RBCs, many questions remain unanswered. For example, it is unclear why the two hydrazines cause oxidation of different globin chains, and the nature of interaction between oxidized \( \alpha \)- and \( \beta \)-globin chains and specific RBC membrane components remains unknown. We hope that the availability of the model system we have outlined will enable the exploration of these issues, as well as provide further molecular insights into pathobiology of thalassemic RBCs.

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Globin-chain specificity of oxidation-induced changes in red blood cell membrane properties

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