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SPECTRIN DEGRADATION IN INTACT RED BLOOD CELLS BY PHENYLHYDRAZINE

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Abstract—The effects of phenylhydrazine on intact red cells and on red cell ghost membrane proteins were studied by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). In intact red cells 1 mM phenylhydrazine induced a marked decrease in intensity of the α- and β-bands of spectrin without the formation of high molecular weight materials. Phenylhydrazine was also responsible for cross-linking of hemoglobin, which is apparent by the appearance of two new broad bands on the gel. Membrane glycoproteins were unaffected. Electrophoretic patterns of cytoskeletal proteins from phenylhydrazine-treated red cells obtained on two-dimensional SDS-polyacrylamide gels and stained with Coomassie blue or fluorescently labeled with monobromobimane indicated the presence of a new band between bands 4.2 and 5 at 60–65 kilodaltons (K). An immunoelectrophoretic blotting procedure utilizing polyclonal IgG antibodies for α- and β-spectrin of the red cell cytoskeletal proteins revealed that the band observed at 60–65 K in the two-dimensional SDS-PAGE studies reacted with the antibodies. The presence or absence of glucose in the incubation medium and modification of oxyhemoglobin to met- or carboxyhemoglobin in the red cells did not protect the phenylhydrazine-mediated degradation of the major cytoskeletal proteins. Metal chelators and antioxidants had no effect on membrane protein changes. Ghost red cell proteins did not undergo changes at 1 mM phenylhydrazine in the presence or absence of hemoglobin, although at 5 mM phenylhydrazine the appearance of a faint high molecular weight band was observed. These results indicate that spectrin degradation without significant polymerization can be induced by phenylhydrazine.

Phenylhydrazine is useful for studying oxidant damage in the red cell. Treatment of red cells with phenylhydrazine causes hemoglobin breakdown and membrane damage leading to hemolysis [1]. Phenylhydrazine undergoes autoxidation to reactive intermediates, and these reactive intermediates may react with oxygen to form superoxide [2] and hydrogen peroxide [3, 4]. These oxygen-derived products could be involved in the cellular damage due to phenylhydrazine [5, 6], but the most compelling evidence favors that a reactive intermediate of phenylhydrazine, such as the phenyl radical, plays an important role [7, 8].

Phenylhydrazine induces changes in reticulocyte membranes prepared from rats treated with phenylhydrazine. These changes are manifest as a decrease in the content of spectrin, an increase in high molecular weight proteins, and the possible formation of polymerized products of lipid peroxidation [9, 10]. The protein changes observed in red cell ghosts treated with phenylhydrazine are mentioned [11] as similar to those seen with a variety of oxidative agents and systems.

In this work, we present experiments in which intact human red cells were treated with phenylhydrazine and the nature of their protein changes was followed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). We observed that the diminution in intensity of spectrin staining by Coomassie blue by SDS-PAGE was not associated with the formation of significant amounts of high molecular weight material. To further characterize this observation, we conducted two-dimensional SDS-PAGE and either stained the gels with Coomassie blue or labeled the protein bands with the sulfhydryl reactive fluorescent indicator monobromobimane. In addition, we used a polyclonal antibody of spectrin to identify potential spectrin degradation products by an immunoblot technique.

EXPERIMENTAL PROCEDURES

Materials. Glycylglycine, phenylhydrazine, D-glucose and diethylenetriamine pentaacetic acid (DETAPAC) were purchased from the Sigma Chemical Co., St. Louis, MO. Deferoxamine mesylate and 2,6-di-*tert*-butyl-4-methylphenol were from the CIBA Pharmaceutical Co., Summit, NJ, and the Aldrich Chemical Co., Milwaukee, WI, respectively. Monobromobimane was obtained from Calbiochem Behring, San Diego, CA. Electrophoretic reagents were obtained from the Bio-Rad Co., Richmond, CA. All other reagents were of reagent grade quality.

Preparation of red cells. Venous blood was collected daily from healthy volunteers into a test tube containing a small mount of 3.8% sodium citrate solution. Red cells were isolated by centrifugation at 1000 *g* for 10 min. After centrifugation, plasma and white cells were removed, and red cells were washed three times with 4 vol. of 0.9% NaCl. Red cells containing methemoglobin were prepared by sus-

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pending packed red cells in an equal volume of 0.5% sodium nitrite, 0.45% NaCl and were incubated for 10 min at 25°. The red cells were washed five times with 4 vol. of 0.9% NaCl and centrifuged at 1000 *g* for 5 min. Red cells containing carboxyhemoglobin were prepared by blowing CO over a 50% (v/v) red cell suspension in Krebs–Ringer glycylglycine buffer (120 mM NaCl, 4.7 mM KCl, 2.5 mM $CaCl_2$, 1.2 mM KH_2PO_4, 1.2 mM $MgSO_4$, 50 mM glycylglycine, pH 7.4) until the visible spectra of red cell lysates reached a maximum at 569 nm.

Preparations of red cell ghosts with and without hemoglobin. After the washing step of the red cells, the hemoglobin-containing ghosts were prepared by hypotonic lysis of washed cells in 30 vol. of 5 mM NaH_2PO_4 (pH 7.4) buffer. The hemoglobin-free ghosts were prepared in the same way as reported above with the only difference being that the hemolysis buffer was 5 mM NaH_2PO_4 (pH 8.0) [12]. Both hemolysate preparations were washed five times in the respective buffer reported above and centrifuged at 6000 *g* for 15 min.

Incubation conditions. Incubations were carried out using 10-ml Erlenmeyer flasks containing 1 ml of 5% (v/v) red cell suspensions or red cell ghosts (about 2 mg/ml) in Krebs–Ringer glycylglycine buffer. Flasks were sealed with rubber stoppers and incubated in a shaking water bath at 37° for 1 hr. Phenylhydrazine was always added last after all other additions to the incubation mixture. The other variable additions were 5 mM glucose, 0.5 mM deferoxamine mesylate, 0.5 mM DETAPAC, 13 mM thiourea, 0.1 or 10 mM ascorbic acid, 0.25 mM butylated hydroxytoluene, and 1% ethanol. Butylated hydroxytoluene was added to the incubation mixture as stock solution dissolved in ethanol. The presence of 1% ethanol in a control experiment did not affect the electrophoretic patterns of red cell membrane proteins.

Preparation of red cells and red cell ghosts for SDS-PAGE. After incubations, red cell suspensions were diluted with 5 vol. of 0.9% NaCl and centrifuged for 5 min at 2000 *g*; red cell pellets were washed twice with 0.9% NaCl. One volume of packed red cells was lysed in 30 vol. of 5 mM NaH_2PO_4 (pH 8.0) buffer, and the red cell ghosts were washed three times with the same buffer. Red cell ghosts were dissolved to a concentration of about 1 mg protein/ml in SDS sample buffer [1% sodium dodecyl sulfate, 1% β-mercaptoethanol, 10% (v/v) glycerol, 63 mM Tris–HCl pH 6.5] and incubated at 95° for 5 min followed by removal of undissolved material by centrifugation at 6000 *g* for 10 min. After the incubation of the red cell ghosts with or without hemoglobin, the suspensions were diluted with 5 vol. of 5 mM NaH_2PO_4 (pH 7.4) buffer and 5 mM NaH_2PO_4 (pH 8.0) buffer, respectively, and washed three times with the same buffers. The red cell ghost pellets were dissolved to a concentration of about 1 mg protein/ml in the same SDS sample buffer and treated in the same way as reported above for red cell ghosts. SDS-PAGE was carried out as described by Laemmli [13]. Staining of protein bands with Coomassie blue or by the periodic acid-Schiff reagent (PAS) was carried out as described by Fairbanks *et al.* [14]. The numbering system for the periodic acid-Schiff bands (1 through 4) was as described [15]. Gel densitometry was performed by using the Hoefer scanning densitometer (GS 300) set in the transmittance operational mode.

Monobromobimane labeling of red cells. Monobromobimane was solubilized in acetonitrile (CH_3CN) at a final concentration of 60 mM. After the treatment of red cells with phenylhydrazine the samples were washed three times with 0.9% NaCl. Then, 20 μl of the original concentration of monobromobimane was added to 1 ml of red cells (5%) [16] resuspended in Krebs–Ringer glycylglycine buffer. The incubation for the labeling procedure was carried out in a 10-ml Erlenmeyer flask sealed with rubber stoppers in a shaking water bath at 37° for 30 min. After the incubation with the fluorescence compound, the samples were washed three times with 0.9% NaCl and then 30 vol. of 5 mM NaH_2PO_4 (pH 8.0) buffer was added to 1 vol. of packed red cell pellets. The red cell ghosts were then washed three times with the same buffer before the SDS-PAGE studies.

Two-dimensional SDS-PAGE. The two-dimensional SDS-PAGE experiments were carried out according to the method of Thompson and Maddy [17]. Red cell ghosts were dissolved to a concentration of 1 mg protein/ml in SDS sample buffer [5% SDS, 5% β-mercaptoethanol, 10% (v/v) glycerol, 63 mM Tris–HCl, pH 6.8, and 0.5 mM EDTA, pH 7.5]. The continuous Fairbanks gel system was employed in a slab gel for resolution in the first dimension, and a discontinuous Laemmli gel system was employed in a slab gel for resolution in the second dimension. After electrophoresis, the gels were fixed in methanol/acetic acid/water, the proteins labeled with monobromobimane were excited with u.v. light (360 nm), and the fluorescence emission of the protein bands was photographed using a Polaroid camera equipped with a Kodak 2 E filter. The same gels were then stained with Coomassie blue, washed, and photographed again.

Immunoelectrophoretic blotting procedures. Cytoskeleton membrane proteins were first subjected to electrophoresis as described above and were transferred from the gels to nitrocellulose paper according to the method of Towbin *et al.* [18]. Blotted proteins were incubated with polyclonal IgG antibodies (a gift of Dr. V. Marchesi) for the α- and β-spectrin. Spectrin and/or spectrin fragments were localized on the blotted sheets by the avidin–biotin–peroxidase complex (ABC) method of Hsu *et al.* [19].

RESULTS

Effect of increased concentration of phenylhydrazine on red cell membrane proteins. An SDS-PAGE of membrane proteins from red cells treated with increasing concentrations of phenylhydrazine at 37° for 1 hr appears in Fig. 1. Red cells treated with 1 mM phenylhydrazine showed diminution in intensity of spectrin bands without formation of high molecular weight material. At 5 mM phenylhydrazine, red cells showed a further diminution in intensity of the spectrin bands together with the initial appearance of high molecular weight material (>240K). In addition, two new broad bands with an

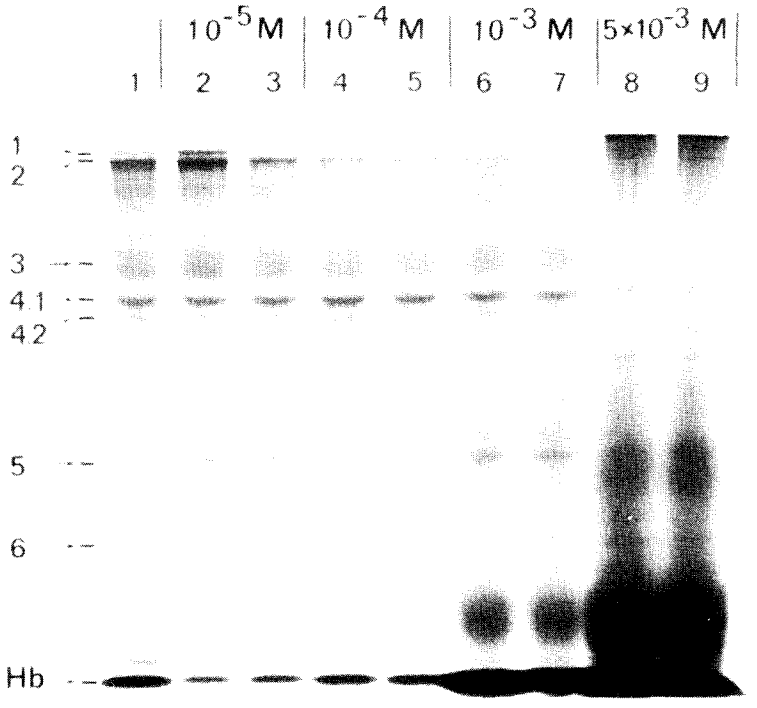

Fig. 1. SDS-PAGE of membrane proteins from red cells exposed to various concentrations of phenylhydrazine at 37° for 1 hr. Column 1, control in the presence of 5 mM glucose. Columns 2, 4, 6 and 8, red cells incubated with 0.01, 0.1, 1 and 5 mM phenylhydrazine respectively. Columns 3, 5, 7 and 9, red cells incubated with 0.01, 0.1 and 5 mM phenylhydrazine plus 5 mM glucose respectively.

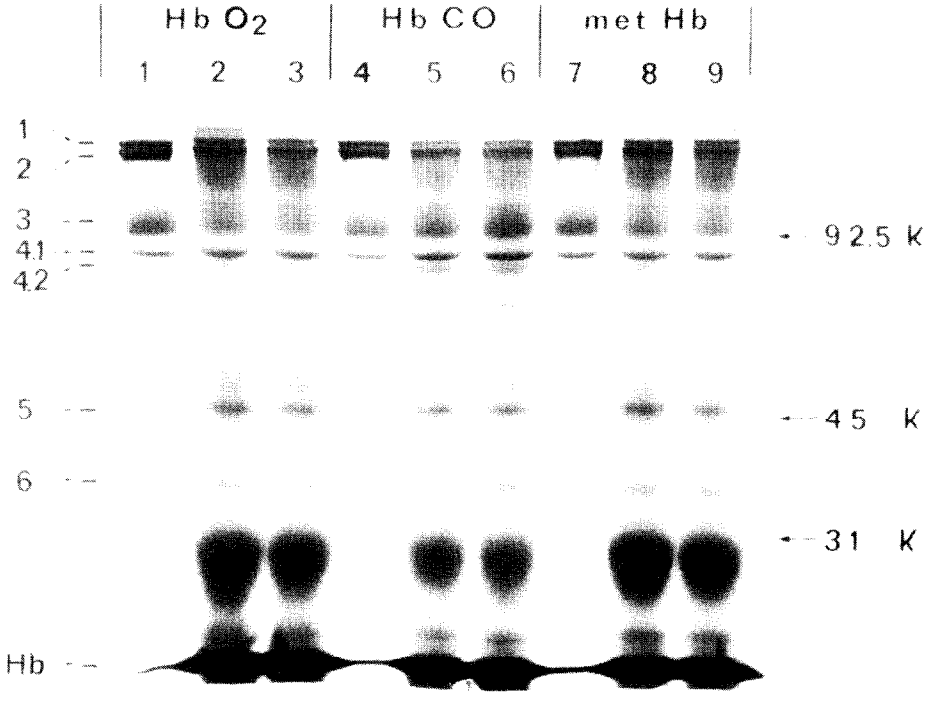

Fig. 2. SDS-PAGE of membrane proteins from red cells containing oxy-, met- and carboxyhemoglobin exposed to 1 mM phenylhydrazine at 37° for 1 hr. Protein (100 μg) was loaded on each column. Columns 1, 4 and 7, controls in the presence of 5 mM glucose. Columns 2, 5 and 8, red cells incubated with 1 mM phenylhydrazine. Columns 3, 6 and 9, red cells incubated with 5 mM glucose plus 1 mM phenylhydrazine. Approximate molecular weights, right ordinate.

apparent molecular weight below 45K and 31K were noted at the two higher concentrations of phenylhydrazine. These two broad bands are attributed to the crosslinking of hemoglobin subunits [20, 21]. The two highest concentrations of phenylhydrazine produced a generalized decrease in all the other major cytoskeletal protein bands. Exposure of red cells to the lowest concentrations of phenylhydrazine (0.1 mM) induced only a slight decrease in intensity of the spectrin bands; the remainder of the cytoskeleton protein pattern was unaffected. The presence of glucose in the incubation mixture did not protect the major cytoskeletal proteins from degradation induced by phenylhydrazine (Fig. 1), though a partial protection was observed in the cross-linking of the hemoglobin. Additional SDS-PAGE experiments in nonreducing conditions did not show any further modification in our system (data not shown). A duplicate of the gel shown in Fig. 1 stained with the periodic acid-Schiff reagent showed no change in the periodic acid-Schiff bands 1, 2, 3 and 4 with increasing concentrations of phenylhydrazine (data not shown).

Effect of phenylhydrazine on oxy-, met-, and carboxyhemoglobin-containing red cells. The SDS-PAGE of membrane proteins from red cells containing oxy-, met-, and carboxyhemoglobin exposed to 1 mM phenylhydrazine at 37° for 1 hr is shown in Fig. 2. The common factor found on the gels linking these three cell types after treatment with phenylhydrazine was a decrease in the intensity of spectrin without high molecular weight material at the origin of the gel and the appearance of a broad band below 31K. A slight decrease in band 3, 4.2, 5, and 6 and a generalized increase in background staining in all of the hemoglobin ligand states were observed. In the carboxyhemoglobin-containing red cells the effect of phenylhydrazine on the cytoskeletal protein bands seemed not as pronounced as in the two other cell types. Glucose partially prevented the appearance of the broad band below 31K, but no protection was observed in the remainer of the gel.

Antioxidants and chelators. Red blood cells exposed to 1 mM phenylhydrazine showed no further change in the cytoskeletal protein bands in the presence of 0.25 mM butylated hydroxytoluene, 13 mM thiourea, 0.1 or 10 mM ascorbic acid, 0.5 mM deferoxamine mesylate and 0.5 mM DETAPAC incubated with or without glucose (data not shown).

Effects of phenylhydrazine on ghost red cells with or without hemoglobin. To verify whether or not the observed effects on the cytoskeletal protein bands in the intact red cells treated with phenylhydrazine were the same in the absence of the cytosol components, we utilized red cell ghosts which contained or did not contain hemoglobin. As shown in Fig. 3, the densitometric profiles of membrane proteins of the phenylhydrazine-treated red cell ghosts containing hemoglobin at two different concentrations of phenylhydrazine (1 and 5 mM) showed almost no difference with respect to the untreated red cells. In comparison to red cells, no changes were observed in the spectrin of red cell ghosts treated with 1 mM phenylhydrazine. The only relevant change was the appearance of the broad peak below 31K as in the intact red cells. Hemoglobin free red cell ghosts

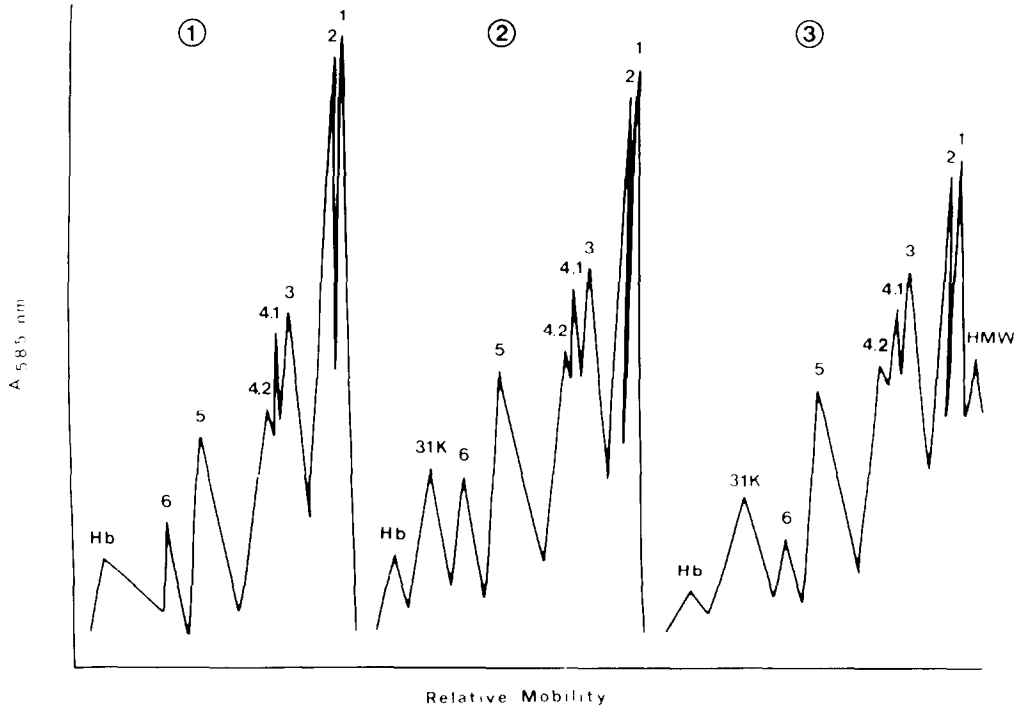

Fig. 3. Densitometric scan at 585 nm of proteins from red cell ghosts containing hemoglobin on an SDS-PAGE after staining with Coomassie blue exposed to two concentrations of phenylhydrazine at 37° for 1 hr. Key: (1) control, (2) red cell ghosts incubated with 1 mM phenylhydrazine, and (3) red cell ghosts incubated with 5 mM phenylhydrazine.

treated with phenylhydrazine showed no differences when compared to untreated red cell ghosts (data not shown). At 5 mM phenylhydrazine, a slight decrease in the spectrin peaks and in the 4.2 peak, together with the appearance of a relatively small high molecular weight peak (>240K) and of the usual broad peak (<31K) were observed (Fig. 3). In the phenylhydrazine-treated hemoglobin free red cell ghosts, there was essentially no difference when compared to the phenylhydrazine-treated hemoglobin containing red cell ghosts except for the lack of the broad peak at 31K, which corresponds to a polymerized product of hemoglobin (data not shown).

Two-dimensional SDS-PAGE studies. To obtain more information about the cytoskeleton protein changes induced by phenylhydrazine in intact red cells, we carried out two-dimensional SDS-PAGE experiments in red cells labeled with monobromobimane. Monobromobimane labels red cell membrane protein thiol groups [22], which appear as fluorescent bands by SDS-PAGE. The pattern of the fluorescent bands corresponds to almost all of those stained with Coomassie blue. The two-dimensional SDS-PAGE gels of membrane proteins of intact red cells treated and untreated with 1 mM phenylhydrazine and stained with Coomassie blue appear in Fig. 4. No substantial differences were observed when the two-dimensional gels were compared with the respective monodimensional experiment reported in Fig. 1. Band 3 seemed to be affected by the phenylhydrazine treatment. This was apparent as a decrease in intensity of the Coomassie blue stain in the area coinciding with band 3. In the corresponding monobromobimane-labeled gel in Fig. 4, the membrane proteins appearing as fluorescent spots were easily recognizable as those stained with Coomassie blue. We also found that there was not only a decrease in the fluorescence associated with spectrin and band 3 and the appearance of a broad spot related to the cross-linking of hemoglobin subunits [20, 21] in the phenylhydrazine-treated red cells, but there were also well defined and intense fluorescent spots in the area between bands 4.2 and 5 with apparent molecular weight of 60–65 K.

Immunoblotting of red cell membrane proteins. The technique of immunoblotting was employed to detect changes in spectrin composition. Cytoskeletal protein extracts of phenylhydrazine-treated and untreated red cells were separated by SDS-PAGE, transferred to nitrocellulose sheets, and reacted with polyclonal antibody directed against α- and β-spectrin. As shown in Fig. 5, the spectrin antibody reacted with protein fragments between bands 4.2

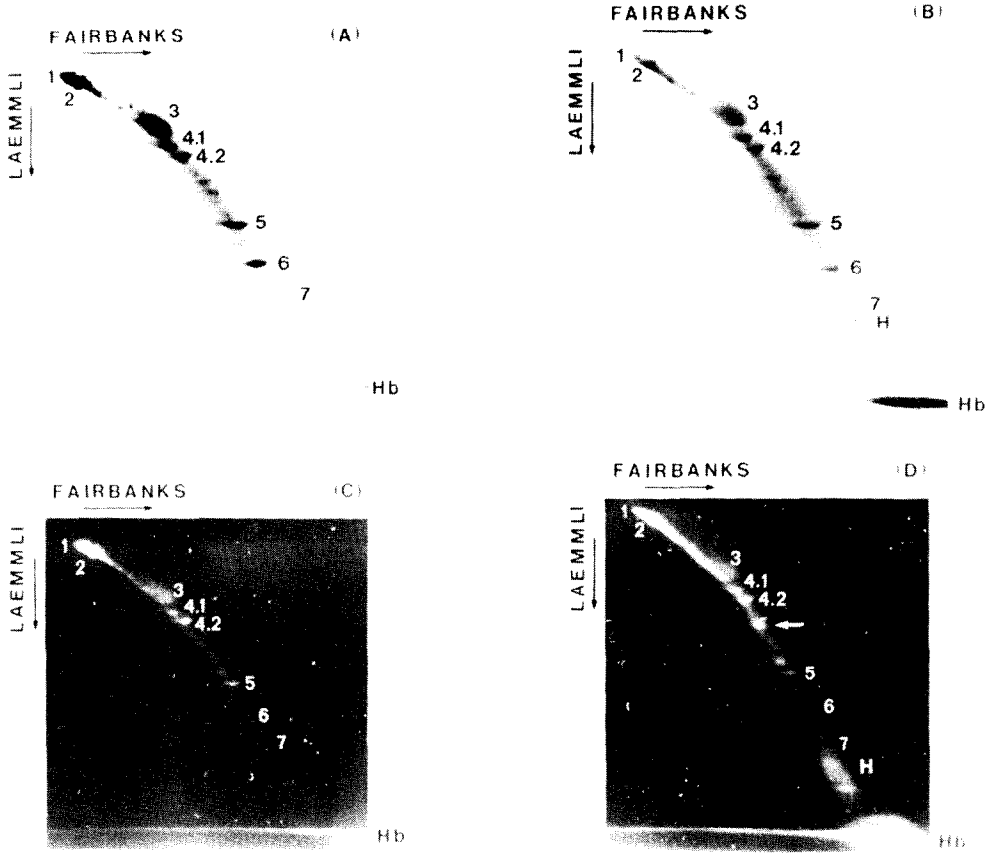

Fig. 4. Two-dimensional SDS-PAGE patterns of red blood cell membranes isolated from red cells after treatment with 1 mM phenylhydrazine at 37° for 1 hr. (A and B) patterns of Coomassie blue–protein complexes. (C and D) fluorescence patterns with monobromobimane. (A, C) untreated red cells and (B and D) phenylhydrazine-treated cells. The arrow indicates the presence of new fluorescent complexes with an apparent molecular weight of 60–65 K indicates cross-linked hemoglobin.

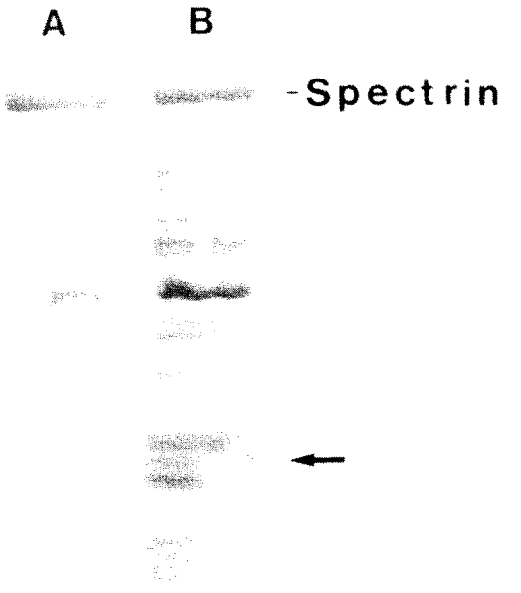

Fig. 5. Immunoelectrophoretic blotting detection of spectrin from membrane proteins of red cells: (A) untreated, and (B) treated with 1 mM phenylhydrazine at 37° for 1 hr. The arrow shows two new polyclonal spectrin antibody reactive bands with an apparent molecular weight of 60–65 K. Blotted proteins were also stained with Coomassie blue (data not shown), and the comparison with the blotted proteins reacted with polyclonal spectrin antibody showed that the two new bands were localized between bands 4.2 and 5.

and 5 in the lane corresponding to the phenylhydrazine-treated extracts. The absence of immunoreactivity with α-spectrin in our blot may be accounted for by the reduced affinity of this antibody for the α-subunit when compared to that of its β-counterpart.

DISCUSSION

The effects of oxidative compounds on cytoskeletal proteins of red cells in SDS-PAGE studies are manifest by the appearance of high molecular weight material and the disappearance of the α- and β-spectrin bands [23]. In addition, the mechanisms responsible for inducing protein changes, during the oxidative challenge, are different from those that give rise to changes in membrane lipids [21]. In this latter report, the red cell membrane protein changes induced by *t*-butyl hydroperoxide included changes in spectrin leading to the formation of high molecular weight material and increased membrane bound hemoglobin appearing on the gels as broad bands below 66K, 45K and 31K. In the present study, we observed that, despite the striking decrease in intensity of both spectrin monomers, a minor amount of high molecular weight material was present on the gels. Furthermore, an immunoelectrophoretic blotting study with a polyclonal IgG preparation against spectrin and a two-dimensional SDS-PAGE analysis of both Coomassie blue staining and fluorescent labeling of cytoskeletal protein sulfhydryls with monobromobimane indicated that spectrin undergoes a cleavage process in phenylhydrazine-treated red cells. A generalized decrease of the remainder of major cytoskeletal proteins was also observed, though the spectrin bands were those most affected. Two new bands related to hemoglobin degradation and polymerization appeared at the lower molecular weight region of the gel.

Phenylhydazine mediated cytoskeletal protein changes were unaffected by either the ligand state of hemoglobin or glucose metabolism. This is in contrast to *t*-butyl hydroperoxide induced cytoskeletal protein changes. This could be explained by the fact that the autoxidation products of phenylhydrazine are not directly metabolized by the glucose-metabolizing pathways, as is observed with *t*-butyl hydroperoxide [24–26], though a by-product of the autoxidation reaction of phenylhydrazine, hydrogen peroxide, is scavenged by the glutathione peroxidase step of the hexose monophosphate shunt [27]. Oxy- and methemoglobin serve a catalytic function for phenylhydrazine autoxidation [2, 28], and therefore the typical protective effect of methemoglobin in oxidative challenge is not observed [29]. Though hemoglobin catalysis could not account for the similar findings in carboxyhemoglobin containing red cells compared to either met- or oxyhemoglobin containing red cells, the lack of degradation of hemoglobin by the products of autoxidation of phenylhydrazine probably allows for the reactive intermediates of phenylhydrazine to principally attack the red cell membrane [7, 30]. The lack of effect of antioxidants and chelators on the observed protein changes can be accounted for by the fact that the autoxidation products of phenylhydrazine are not effectively scavenged by the antioxidants and that a metal-cation mediated reaction is of minor consequence.

Recently, Vilsen and Nielsen [31] reported cross-linking of spectrin by disulfide exchange with precipitated hemoglobin in rabbit red cells treated with phenylhydrazine. Similar cross-linking interactions between spectrin and hemoglobin have been observed in pathologically manifest or artificially dehydrated red cells [32]. In the report by Vilsen and Nielsen, the red cells were incubated with phenylhydrazine for 5 hr to obtain high molecular weight material. Incubation for 1 hr did not show any spec-

trin cross-linking with hemoglobin. Jain and Hochstein [9] have reported that, in rats treated by intraperitoneal injection of phenylhydrazine, membrane proteins of reticulocytes undergo diminution in intensity of spectrin bands concomitant with the appearance of high molecular weight material on SDS-PAGE. These two reports differ with the findings reported here because of the experimental conditions (incubation time, the presence of high phosphate, which can have significant effects on the metabolic events in the red cell [33, 34], the absence of calcium and magnesium in the media or whole animal study) and/or the species or cell difference. The observed differences could be resolved by envisioning a two-step process for the different fates of spectrin induced by phenylhydrazine. The first step in the degradation of spectrin could be a post-synthetic covalent modification that is manifest as high molecular weight material [9, 31]. The second step, which is the degradative step, involves the activation of a proteolytic pathway (see below) which removes the oxidatively damaged proteins.

The mechanism of phenylhydrazine-induced red cell membrane spectrin degradation is unknown. The red cell ghost experiments with or without the presence of hemoglobin show no change of the membrane proteins at the same concentration of phenylhydrazine that produced the degradation of spectrin in the intact red cell. This observation indicates that the interaction of phenylhydrazine or its intermediate products does not take place directly with the membrane protein, but some other mediator(s) in the soluble fraction is necessary for spectrin degradation. One plausible explanation could be related to the recent observation that the rate of protein degradation to amino acids in rabbit red cells exposed to 1 mM phenylhydrazine is increased 7- to 33-fold [35]. These authors found that the ATP-dependent proteolytic system is responsible for the observed phenomena. A family of calcium-activated thiol-proteases, the calpains, are also able to degrade native cytoskeletal proteins. Calpain I, the low calcium requiring form of the protease, is present in the cytosol of human red cells [36]. The association of a small but distinct portion of calpain I with the membrane fraction was demonstrated recently [37]. Although it would be premature to state unequivocally that proteolytic activity might have produced the observed changes in spectrin, our data strongly support this interpretation. In this context, a cellular system is available for removing proteins altered by oxidative damage.

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