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REACTION OF PHENYLHYDRAZINE WITH ERYTHROCYTES

CROSS-LINKING OF SPECTRIN BY DISULFIDE EXCHANGE WITH OXIDIZED HEMOGLOBIN

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Abstract—Phenylhydrazine causes deleterious oxidations of components of erythrocytes. These reactions and their effects on the mechanical properties of rabbit erythrocytes are investigated to provide insight into the mechanisms leading to destruction of oxidatively damaged erythrocytes. After 1 hr of incubation with phenylhydrazine, precipitated denatured protein (Heinz body protein) amounts to 25–30% of membrane protein, but deformability of erythrocytes as measured by filtrability is unchanged. After 4 hr of incubation filtrability drops sharply. Polymerization of spectrin and covalent binding of hemoglobin to spectrin, but no peroxidation of membrane lipids is observed. Precipitated protein amounts to 85–95% of membrane protein. It contains Fe, porphyrin and globin peptide in the proportion 1:1:1. Heinz body protein precipitated when hemoglobin is incubated under similar conditions has 90% of its sulfhydryl groups oxidized and no other amino acids than cysteine are destroyed. Addition of this Heinz body protein to erythrocyte ghosts causes polymerization of spectrin. Incubation of erythrocytes with tetrathionate, a specific cross-linking agent, causes filtrability to drop sharply after about 80 min. This effect is similar to that observed after 4 hr incubation with phenylhydrazine, and is accompanied by polymerization of spectrin and band 3. The results indicate that cross-linking of membrane proteins by disulfide exchange with precipitated hemoglobin may play a major role in decreasing deformability during incubation with phenylhydrazine.

Exposure of erythrocytes to oxidative stress may cause oxidations that lead to deleterious changes of properties [1]. The targets for these oxidations may be membrane lipids [2], sulfhydryl groups of membrane proteins [3], and Fe(II) ions and sulfhydryl groups of hemoglobin [4, 5]. A great variety of drugs and other chemical compounds cause intracellular precipitation of Heinz bodies (i.e. denatured hemoglobin) with concomitant hemolysis [6, 7], and the cause is generally believed to be an excess of oxidation over the reducing power of the cell [5]. Phenylhydrazine is the most extensively studied of these compounds. It produces O_2^- and H_2O_2 with oxyhemoglobin [8–10], and injury of the red cell may occur via these oxidizing compounds [8, 9]. It has also been suggested that phenylhydrazine itself is converted into reactive species capable of damaging erythrocytes [10]. Moreover, it is possible that precipitated bodies cause mechanical and chemical damage of the cell membrane, which may ultimately lead to removal of the erythrocytes from the circulation [11, 12]. The present study investigates the nature of the damage caused by phenylhydrazine. An important mechanism appears to be the formation of cross-linked spectrin by disulfide exchange with denatured hemoglobin. Our findings are probably representative of pharmaca known to cause precipitation of Heinz bodies and may also have a bearing on other conditions, such as the hemoglobinopathies, glucose 6-phosphate dehydrogenase deficiency and aging [13] in which denatured hemoglobin is produced.

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MATERIALS AND METHODS

Chemicals

A calibration kit of molecular weight standards for SDS-electrophoresis was obtained from Pharmacia, Sweden. Butylated hydroxytoluene (BHT), 2,6-di-tert-butyl-*p*-cresol, was purchased from Sigma. Globin (rabbit) was prepared according to Hill *et al.* [14]. Other chemicals were of analytical grade.

Erythrocytes

Blood was obtained from anaesthetized rabbits (25–30 mg pentobarbital/kg, intravenous) weighing 2–2.5 kg by bleeding. Acid Citrate Dextrose (A.C.D.) was used as anticoagulant, 7 ml per 40 ml blood. After centrifugation for 20 min at 1000 *g* plasma and buffy coat were removed by aspiration. Washing was then performed three times with cold 0.15 M saline containing 5 mM phosphate (pH 7.4) by centrifugation for 10 min at 2200 *g*. Washed erythrocytes were resuspended in isotonic phosphate buffer, pH 7.4 to a hematocrit of 50%, and incubated with phenylhydrazine at a concentration of 0.25 mg/ml cell suspension. Incubation with potassium tetrathionate was performed as described by Haest *et al.* [15]. All incubations were carried out in uncovered test tubes at 37° with continuous shaking.

Deformability

(a) *Filtrability*. Erythrocytes were suspended in isotonic sodium chloride, 0.5% albumin, 5 mM phosphate, pH 7.4 corresponding to a hematocrit of 1%. Four millilitres of 1% erythrocyte suspension was filtered through a Millipore filter (Catalogue

No. SCWP 02500, Lot No. H2A67351C) 2.5 cm in diameter and 8 μ pore size under a negative pressure of 20 cm H_2O. The percentage passage of erythrocytes is taken as a measure of deformability [12].

(b) *Resistance to packing.* Resistance to packing was determined by centrifugation at 750 *g* and 1500 *g* in Winthrobe hematocrit tubes. Hematocrit was read at 5–10 min intervals during centrifugation [16].

Preparation of erythrocyte membranes (ghosts)

Erythrocytes were hemolysed by 20 mOsM phosphate buffer, pH 7.4. Ghosts were isolated by centrifugation and washed three times as described by Dodge *et al.* [17].

Preparation of Heinz body protein

A rabbit hemoglobin suspension of 30% (w/v) (i.e. the same as the concentration of hemoglobin in erythrocytes) was incubated for 5 hr with phenylhydrazine as described for erythrocytes. Heinz body protein was subsequently isolated by centrifugation and washed three times with 20 mOsM phosphate buffer, pH 7.4.

Gel chromatography on sephacryl S-300 *superfine*

Ghosts were solubilized by adding sodium deoxycholate (approx. 15 mg/mg ghost protein) and the buffer used for gel chromatography to obtain a protein concentration of 2.5–3.5 mg/ml. Ten millilitres were subjected to gel chromatography in 10 mM sodium deoxycholate, 0.1 M Na_2SO_4 and 0.05 M sodium borate (pH 8.50) as previously described [18].

Protein analysis

Protein was determined according to Lowry *et al.* [19] and in some cases also by total N analysis involving wet combustion with perchloric acid [20] and colorimetric determination by Nessler's reaction.

Heinz body protein was calculated as the difference between protein of ghosts from equal volumes of erythrocyte suspension incubated in the presence and absence of phenylhydrazine.

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-electrophoresis)

SDS-electrophoresis was performed as previously described [18] using 7.5 and 4.5% running gels and a buffer containing 0.02 M sodium acetate, 0.18 mM Na_2 EDTA, 0.04 M Tris/acetic acid (Tris concentration), pH 7.4, and 2% (w/v) sodium dodecyl sulfate. Samples were prepared for electrophoresis without and with mercaptoethanol (0.9%). Gels were stained with Coomassie blue [21]. Gels were scanned on a LKB 2202 Ultra Scan Laser Densitometer. Integration was performed by cutting out recorded peaks and weighing.

Analysis for lipid peroxidation

Lipid peroxidation in ghosts as well as in the whole erythrocyte suspension was examined by the TBA analysis [22, 23]. Aliquots of 0.6 ml 50% erythrocyte suspension was added to 3.0 ml of reagent (0.375% (w/v) TBA; 15% (w/v) TCA; 0.25 N HCl), and precipitated protein was removed by centrifugation before development of colour by heating at 100° for 15 min. The absorption was read at 535 nm, and in some cases absorption spectrum from 400 to 550 nm was recorded (cf. Fig. 10). Controls of hemoglobin incubated similarly were analyzed in the same way to correct for background absorption arising from hemoglobin and phenylhydrazine (cf. Fig. 10).

Lipid peroxidation was also examined by recording u.v.-absorption spectrum [24] and fluorescence spectra [25, 26] of lipid extracted [27] from 1 ml erythrocyte suspension and dissolved in 2 ml ethanol/diethylether (2:1,v/v). Fluorescence spectra were recorded with excitation monochromator set at 365 nm and emission monochromator at 435 nm [25, 26].

Light absorption and fluorescence measurements

Absorption spectra were recorded with a Unicam SP 800B spectrophotometer. Fluorescence was measured on an Aminco–Bowman spectrophotofluorometer. Slit arrangement was 1 = 2 mm; 2 = 4 mm; 3 = 2 mm; 4 = 2 mm; 5 = 4 mm; 6 = 2 mm; 7 = 2 mm. Standardization was done on quinine sulfate in 0.1 N H_2SO_4 (1 μg/ml) with emission and excitation at 450 and 350 nm, respectively, by adjusting sensitivity to obtain a reading of 2.0.

Sulfhydryl (SH) group analysis

Sulfhydryl groups were determined by titration with dithionitrobenzoic acid [28]: 100–150 μl ghost suspension (2–3 mg protein/ml) in 20 mOsM phosphate (pH 7.4) was solubilized by addition of 400 μl 25 mM sodium deoxycholate, 0.25 M Na_2SO_4, 0.125 M sodium borate (pH 8.5), 100 μl 33% sodium dodecyl sulfate (w/v), 15 mg sodium deoxycholate, and 450–500 μl H_2O. Absorbance was read before and 10 min after addition of 20 μl dithionitrobenzoic acid (39.6 mg in 10 ml 0.1 M phosphate, pH 7.0). Sulfhydryl groups were calculated from the increase in absorbance at 412 nm using a molar absorbance of 13,600.

Ghosts incubated for 5 hr gave a background of 0.4–0.5, and the ΔE_{412} measured was never less than 0.125. The corresponding background in suspension of Heinz body protein formed by treatment of hemoglobin for 5 hr with phenylhydrazine was 0.6–0.7 and the ΔE_{412} measured was 0.016–0.02. This should be compared to values of 0.09–0.1 measured in samples incubated for 5 hr in absence of phenylhydrazine (corresponding to approx. 2 moles of titrable SH per mole hemoglobin).

Iron (Fe) analysis

Fe of precipitated protein was determined from analyses on ghost preparations: Ghosts corresponding to 25–40 mg protein were digested with 3 ml 65% nitric acid/70% perchloric acid (5:1, v/v), and Fe was then determined by atomic absorption spectrophotometry.

Amino acid analysis

Acid hydrolysis was carried out with 6 N hydrochloric acid at 110° for 22 hr under nitrogen. Basic hydrolysis was performed according to Ray and Koshland [29]. The hydrolysates were analyzed with Beckman 120 C amino acid analyzer. Tryptophan of basic hydrolysates was analyzed on the short column.

Methionine sulfoxide, the likely oxidation product of methionine, is destroyed in acid hydrolysis. It was determined from analysis of basic hydrolysates using the long column. Contents of the oxidatively labile amino acids, tryptophan and histidine in native hemoglobin and Heinz body protein were normalized on the basis of alanine content.

Microscopy

Ghosts were fixated in 0.1% glutaraldehyde and examined as a wet preparation in a 0.1% potassium-phosphate buffered glutaraldehyde solution, pH 7.4, 290 mOsM, with the oil-immersion objective (× 1000) of Leitz interference-phase-contrast microscope with Nomarshi optics [30].

RESULTS

Microscopy

Ghosts from erythrocytes incubated with phenylhydrazine for 1 hr were found to contain numerous small particles with a diameter about 1/100–1/50 of that of the erythrocytes. These particles have a similar appearance as those dense bodies consisting of denatured hemoglobin which are generally termed Heinz bodies [11, 31].

Precipitation of Heinz body protein and deformability of erythrocytes

Precipitation of Heinz body protein in erythrocytes incubated with phenylhydrazine is shown in Fig. 1 together with filtrability. There is a gradual precipitation of protein corresponding to 0.25–0.3 mg Heinz body protein per mg membrane protein after 1 hr, and about 1 mg Heinz body protein per mg membrane protein after 5 hr. Filtrability decreases moderately from about 1 hr to about 4 hr and then drops sharply to very low values. Similar results were obtained when the antioxidant, butylated hydroxy-

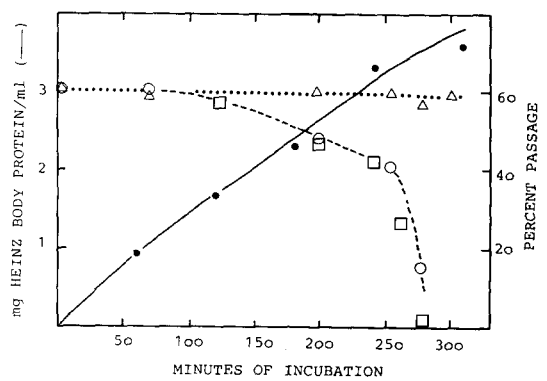

Fig. 1. Deformability and precipitation of Heinz body protein in erythrocytes incubated with phenylhydrazine. Incubation was performed at 37° in isotonic saline containing 5 mM phosphate, pH 7.4, hematocrit 50%. Incubations used for measurements of filtrability in addition contained 0.5% albumin. Deformability is expressed as percentage passage of erythrocytes filtered through an 8 μ filter, phenylhydrazine-exposed (--○--), phenylhydrazine and BHT-exposed (--□--), control (··Δ··). Precipitated Heinz body protein (——·——), determined as difference between ghost protein from equal volumes of erythrocyte suspension, is given as mg/ml incubation.

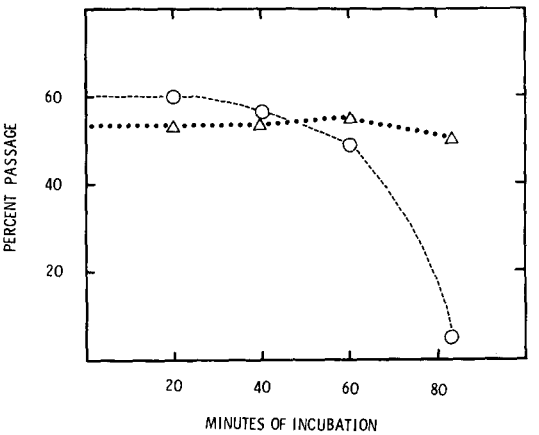

Fig. 2. Deformability of erythrocytes incubated with tetrathionate. Incubation was performed at 37° in isotonic saline containing 5 mM phosphate, pH 8.0, at a hematocrit of 8% and with 0.5% albumin. Deformability is expressed as percentage passage of erythrocytes filtered through an 8 μ filter, tetrathionate-exposed (--○--), control (··Δ··).

toluene (BHT), was included in the reaction mixture together with phenylhydrazine (also shown on Fig. 1). BHT was used at a concentration of 1%, which is known to protect membrane lipids from peroxidation [32, 33]. Figure 2 shows filtrability of erythrocytes incubated with tetrathionate, which is an efficient cross-linker of membrane proteins [15]. After about 80 min of incubation filtrability drops to zero.

Figure 3 shows relative resistance to packing of erythrocytes incubated with phenylhydrazine for various periods of time. Resistance increases and reaches a maximum after about 5 hr of incubation

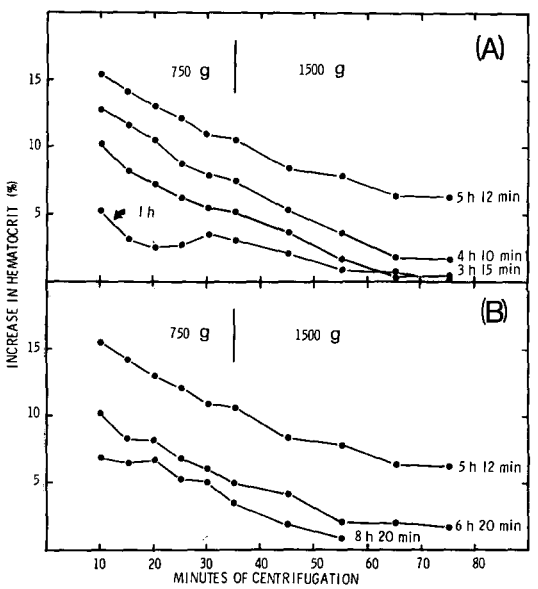

Fig. 3. Resistance of erythrocytes to packing after incubation with phenylhydrazine for various periods of time. Hematocrit of phenylhydrazine-exposed and control erythrocytes were read at intervals during centrifugation and the increase relative to control is given in % hematocrit of control centrifuged at 2550 *g* for 25 min.

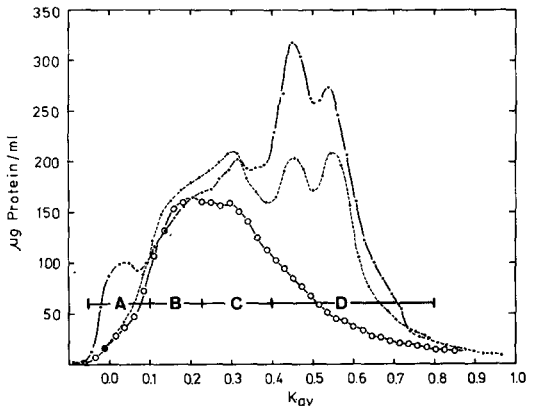

Fig. 4. Gel chromatography on Sephacryl S-300 Superfine of ghost protein from erythrocytes incubated with phenylhydrazine for 1 hr (--○--), 5 hr (——·——) and from control erythrocytes (——○——). Solubilized ghosts corresponding to 11.0 ml erythrocyte suspension were applied. Column dimensions were 2.6 × 93 cm; flow rate was 18–20 ml/hr and 5–6 ml fractions were collected.

(A). With prolonged incubation it decreases to approximately initial values (B).

Gel chromatography and SDS-electrophoresis

Proteins of ghosts prepared from erythrocytes incubated for 1 and 5 hr with phenylhydrazine, and from controls incubated in the absence of phenylhydrazine, were investigated by gel chromatography and SDS-electrophoresis. Figure 4 shows gel chromatography of ghosts prepared after incubation of erythrocytes in the presence and absence (controls) of phenylhydrazine. After 1 hr of incubation with phenylhydrazine there is an increase in protein eluted in K_{av} interval 0.2–0.8 (Heinz body protein), but not in K_{av} interval −0.05–0.2. In contrast, incubation for 5 hr significantly increases the amount of protein eluted in K_{av} interval −0.05–0.1. Protein eluted in K_{av} interval 0.2–0.8 is further increased after 5 hr of incubation reflecting the gradual precipitation of protein. The protein corresponding to 5 hr of incubation was pooled over K_{av} intervals A, B, C and D (Fig. 4) and examined by SDS-electrophoresis (Fig. 5A–D). Comparison of sample 1 and 2 of Fig. 5A shows that exposure to phenylhydrazine for 5 hr produces very high molecular weight protein in the erythrocyte membrane. Reduction with mercaptoethanol converts this protein to two compact and closely migrating bands and a third band at the lower border of the gel (compare 1 with 1 M). Molecular weights of the closely migrating compounds, as estimated by the use of molecular weight standards, were similar to spectrin polypeptide chains. The third band at the border of the gel has the same mobility as hemoglobin peptide. High molecular weight proteins are almost absent from the control incubated in the absence of phenylhydrazine. The composition of protein of K_{av} interval B is identical in ghost from phenylhydrazine exposed erythrocytes and control (Fig. 5B, compare 1 with 2 and 1 M with 2 M). Protein eluting in K_{av} interval C from phenylhydrazine exposed erythrocytes differs from control (Fig. 5C, compare 1 with 2) by a band with the same mobility as dimer hemoglobin peptide. Mercaptoethanol converts this band to a band with the same mobility as monomer hemoglobin peptide (Fig. 5C, compare 1, 1 M and Hb). The protein eluting in K_{av} interval D from phenylhydrazine exposed erythrocytes is a mixture of monomer and dimer hemoglobin peptide (Fig. 5D, compare 1, 1 M and Hb). The control contains one component only, migrating close to monomer hemoglobin peptide. Based on gel scanning of proteins from K_{av} interval C and D, Heinz body protein consists of 60% monomer and 40% dimer hemoglobin peptide.

No polymerization of membrane proteins was detected when ghost protein corresponding to 1 hr of incubation was likewise pooled and examined by SDS-electrophoresis (not shown). The protein from K_{av} intervals C and D consists of a mixture of monomer and dimer hemoglobin similar to that found after 5 hr of incubation.

To evaluate quantitatively the relative amount of high molecular weight material on the top of the gel, the solubilized membranes were examined directly by electrophoresis without prior gel chromatography. Figure 6 shows scans of gels corresponding to membranes from erythrocytes incubated for 5 hr with and without phenylhydrazine. The decrease in the amount of spectrin induced by incubation with phenylhydrazine amounts to 30–35% as evaluated by integration of the spectrin peaks. A similar decrease in spectrin peaks was observed on examination of erythrocytes incubated with phenylhydrazine in the presence of BHT (cf. Fig. 1).

Proteins of ghosts prepared from erythrocytes incubated 1/2, 1 and 2 hr with and without tetrathionate were also investigated by SDS-electrophoresis. Figure 7 shows that exposure to tetrathionate produces increasing amounts of high molecular weight proteins not able to penetrate the gel. Reduction with mercaptoethanol converts it to two closely migrating compact bands, and a third diffuse band. The two compact bands migrate corresponding to spectrin polypeptide chains, and the third band migrates corresponding to band 3 of erythrocyte membrane proteins, as estimated by the use of molecular weight standards.

In order to investigate directly the effect of Heinz body protein on polymerization of erythrocyte membrane proteins, ghosts were incubated 5 hr with an equal amount of preformed Heinz body protein. After incubation the sample and corresponding controls were gel chromatographed as described above and protein of K_{av} interval −0.05–0.1 was analyzed by SDS-electrophoresis. Figure 8 shows that incubation with Heinz body protein produces high molecular weight proteins not able to penetrate the gel (sample 1). Such proteins are almost absent in the control sample (sample 2) incubated in a similar way, but without Heinz body protein. Reduction with mercaptoethanol converts the high molecular weight proteins to two compact bands migrating corresponding to spectrin polypeptide chains (sample 1 M). Furthermore, Heinz body protein incubated in the absence of ghosts does not contain high molecular weight proteins, as indicated by the gel electrophoretic pattern (not shown).

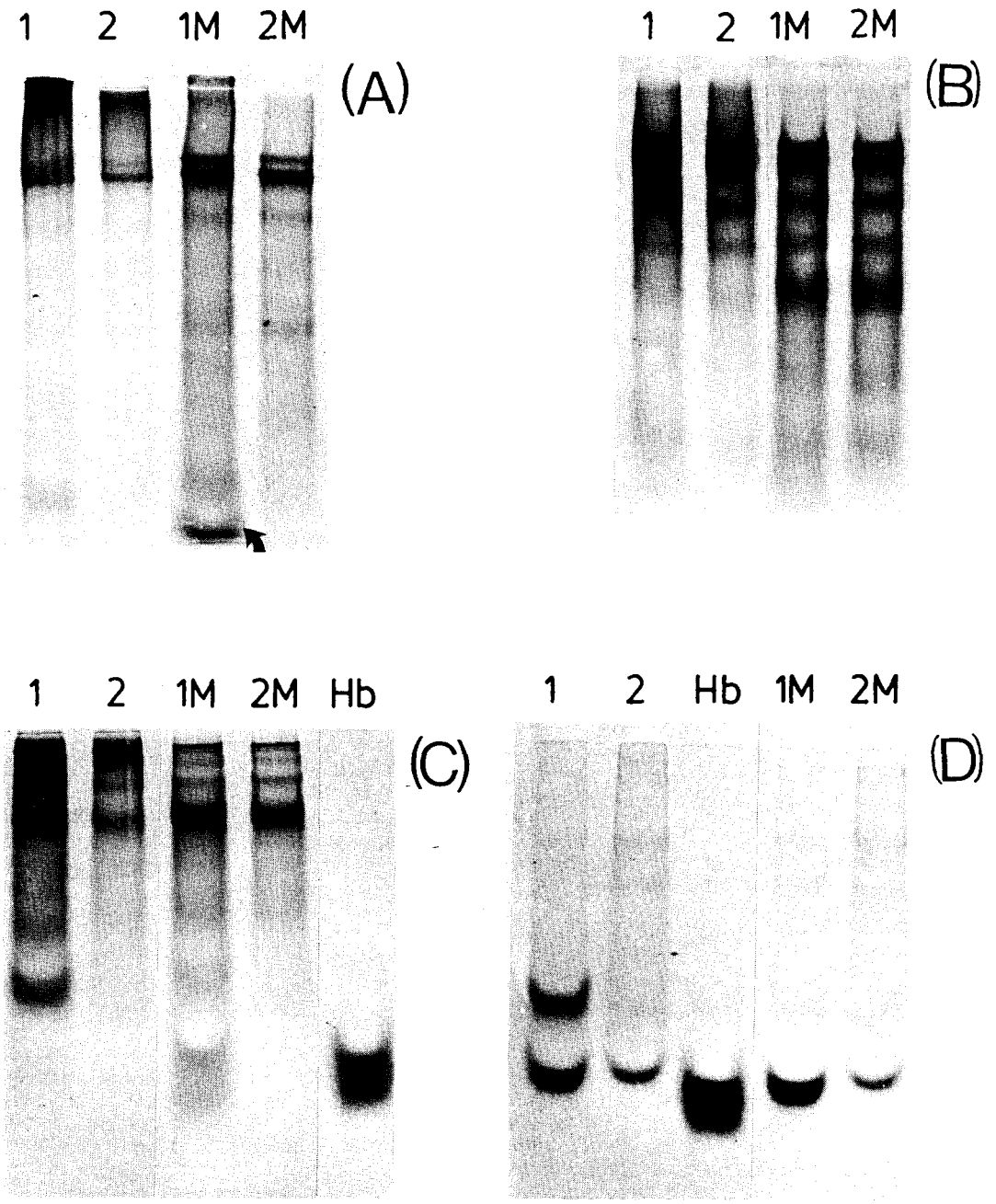

Fig. 5. SDS-electrophoresis of ghost protein from erythrocytes exposed to phenylhydrazine for 5 hr (sample 1) and control ghosts (sample 2). Protein investigated in A, B, C and D was obtained by pooling over correspondingly lettered K_{av} intervals of Fig. 4. "M" indicates that samples were reduced with mercaptoethanol prior to electrophoresis. The arrow indicates hemoglobin released after reduction with mercaptoethanol. In A and B running gels are 4.5%, and in C and D 7.5%. Samples 1 and 2 are applied corresponding to identical volumes of erythrocytes to allow direct comparison of quantities.

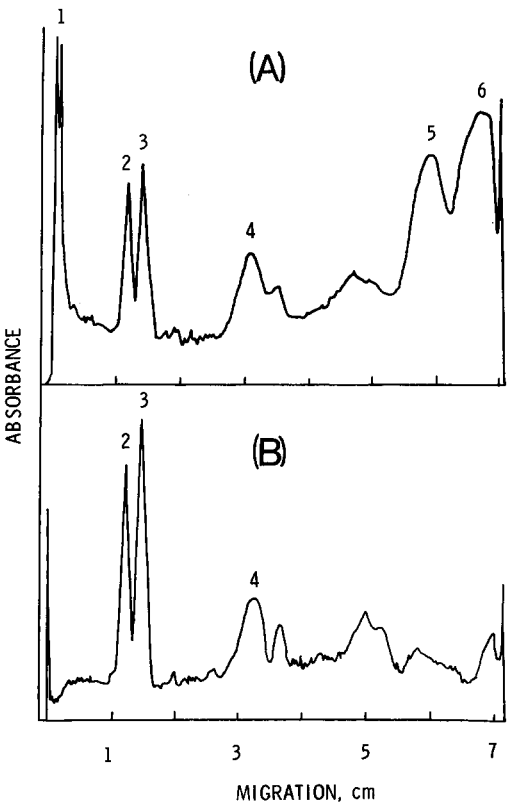

Fig. 6. Scans of 4.5% gels corresponding to unfractionated ghost protein from erythrocytes incubated for 5 hr in presence (A) and absence (B) of phenylhydrazine. Identical amounts of membrane protein (calculated according to the results of Fig. 1) were applied on the gels to allow direct comparison of quantities. "1", high molecular weight material on top of the gel; "2" and "3", spectrin; "4", band 3, "5", dimer hemoglobin and "6", monomer hemoglobin. The initial peak at the origin of the control membrane proteins (B) is an interphase artefact.

Sulfhydryl (SH) group analyses

Sulfhydryl content of ghosts from erythrocytes incubated with phenylhydrazine for 1 and 5 hr and corresponding control ghosts (A, Table 1) was determined, as was SH-content of precipitates (Heinz body protein) from solutions of rabbit hemoglobin treated in the same way with phenylhydrazine (B, Table 1). The precipitated hemoglobin contains 0.3–0.4 SH-groups per mole, which means that more than 90% of the four sulfhydryl groups is oxidized. Assuming the same extent of oxidation of SH-groups in hemoglobin precipitated in erythrocytes and in hemoglobin solutions [13], the apparent membrane sulfhydryl content in the presence of precipitated hemoglobin can be calculated (D, Table 1). There is no decrease in the apparent membrane sulfhydryl content after 1 and 5 hr of incubation with phenylhydrazine. Therefore all disulfide formation in the membrane must be compensated for by a corresponding increase in reactive sulfhydryls in Heinz body protein. This suggests that cross-linking of membrane sulfhydryls occurs by exchange with disulfides in Heinz body protein and not by primary oxidation (see "Discussion").

Light absorption

Figure 9 compares light absorption of protein precipitated in erythrocytes incubated with phenylhydrazine (Heinz body protein) with absorption of methemoglobin and globin. Methemoglobin and Heinz body protein both have maximum at 405–410 nm corresponding to the Soret band of conjugated bonds of porphyrins. Ultraviolet absorption by globin is due to its aromatic amino acids. The corresponding absorption by methemoglobin and Heinz body protein is higher due to overlapping absorption by their prosthetic groups. Absorption by Heinz body protein is apparently higher than that of methemoglobin over the entire spectral range. This may be due to light scattering.

Table 1. SH-content in ghost membrane protein from erythrocytes before incubation, after incubation for 1 and 5 hr with phenylhydrazine and from corresponding control ghosts

SH content*		Before incubation	1 hr of incubation		5 hr of incubation	
		Control ghost	Sample ghost	Control ghost	Sample ghost	Control ghost
(A)	nmole SH/mg total protein	55.28 ±2.49	43.80 ±2.49†	55.89 ±2.37	31.85 ±1.96	56.50 ±2.77
(B)	nmole SH/mg Heinz body protein	–	6.19 ±00.402	–	6.06 ±0.390	–
(C)	mg Heinz body protein/ mg membrane protein	0	0.296	0	0.913	0
(D)	apparent membrane SH-content nmole SH/mg membrane protein	55.28 ±2.49	54.93 ±2.63	55.89 ±2.37	55.40 ±3.55	56.50 ±2.77

* A: SH content per mg of total protein (Heinz body protein and membrane protein).
B: SH content per mg Heinz body protein.
C: Ratio of Heinz body protein and membrane protein in ghosts.
D: Calculated as A(1 + C) − BC, where A, B and C are defined above, and 1 + C is the ratio between total protein and membrane protein.
† Mean ± S.D. $n = 10$.

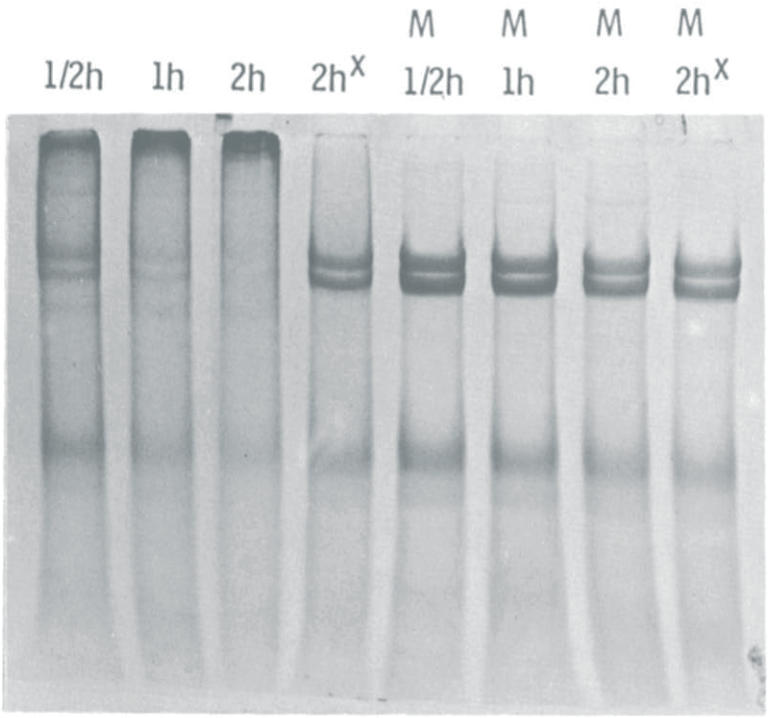

Fig. 7. SDS-electrophoresis of ghost protein from tetrathionate treated erythrocytes. Samples 1/2, 1 and 2 hr are ghost protein from erythrocytes incubated for 1/2, 1 and 2 hr with tetrathionate and $2h^x$ is ghost protein from erythrocytes incubated without tetrathionate for 2 hr "M" indicates that samples were reduced with mercaptoethanol prior to electrophoresis. Running gel was 4.5%. Samples and control are applied corresponding to identical volumes of erythrocytes to allow direct comparison of quantities.

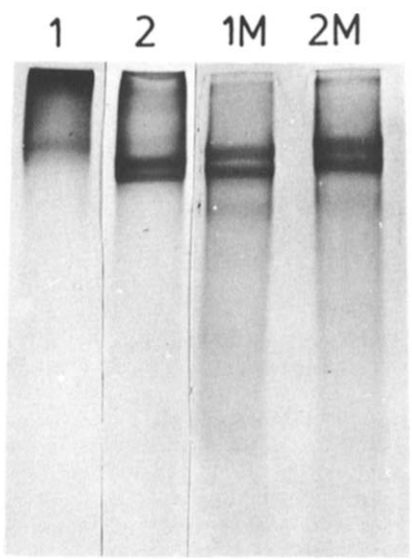

Fig. 8. SDS-electrophoresis of high molecular weight protein from ghosts incubated for 5 hr with (sample 1) and without (sample 2) preformed Heinz body protein. Protein isolated by gel chromatography (corresponding to K_{av} interval −0.05–0.1) was applied to 4.5% gel before and after ("M") reduction with mercaptoethanol.

Iron analyses

The iron content of protein precipitated in erythrocytes incubated with phenylhydrazine corresponds to Fe : peptide ratio of 0.8–1.1, assuming a molecular weight of 16,000 for peptide chains of the protein.

Measurement of lipid peroxidation by TBA-analysis, UV-absorption and fluorescence

Measurements of TBA reacting substances were performed on ghosts from erythrocytes exposed to phenylhydrazine for 1 and 5 hr and on non-exposed controls. There was no significant difference (0.344 ± 0.050 nmole malondialdehyde per mg membrane protein after 5 hr of exposure vs 0.298 ± 0.036 nmole/mg in control). This suggests that no lipid peroxidation had occurred (cf. that fully peroxidized ghosts contain about 30 nmole malondialdehyde per mg protein [34]).

In order to examine whether water soluble TBA-reacting substances are washed away during preparation of ghosts, TBA-analysis was also performed on whole erythrocyte suspensions. In this case it was necessary to include appropriate controls to correct for background arising from hemoglobin and phenylhydrazine in the TBA assay. Figure 10 shows absorption spectra obtained after reaction with TBA. The sample corresponding to whole erythrocyte suspension incubated in presence of phenylhydrazine (curve 1) gives rise to a somewhat higher absorbance than that incubated in its absence (curve 3).

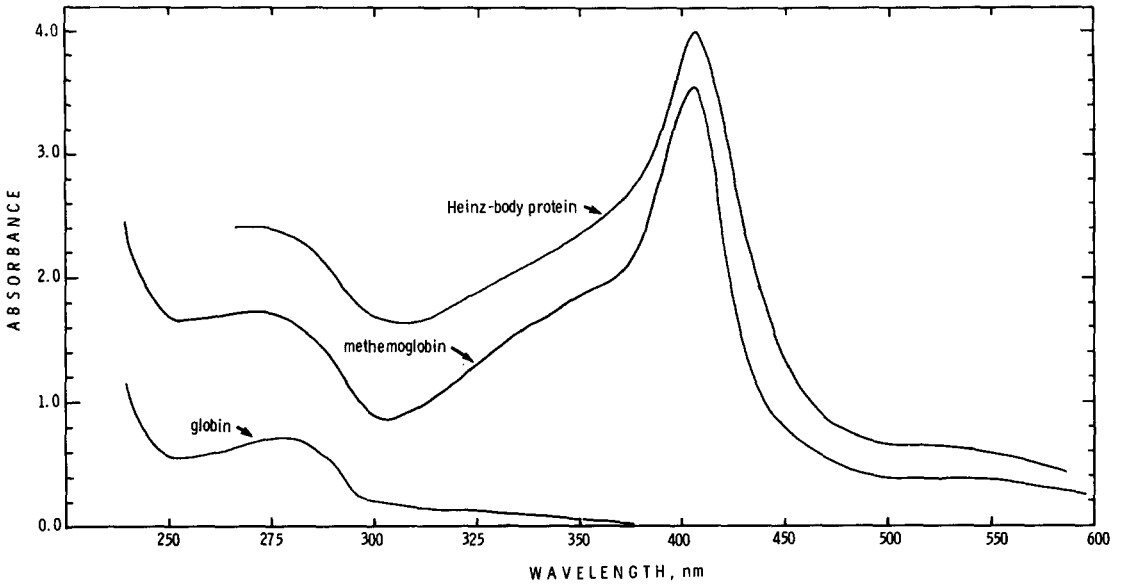

Fig. 9. Light absorption of protein precipitated in phenylhydrazine-exposed erythrocytes (Heinz body protein), methemoglobin and globin. The spectra correspond to 1.00 mg/l ml. Spectrum of Heinz body protein was obtained as difference spectrum: ghosts from phenylhydrazine-exposed erythrocytes were solubilized and read against solubilized ghosts from non-exposed erythrocytes.

However, the spectrum corresponding to incubation of hemoglobin with phenylhydrazine (curve 2) is identical to that corresponding to whole erythrocyte suspension (curve 1) in the region where the coloured malondialdehyde-TBA complex absorbs [22, 23]. For comparison spectrum of malondialdehyde reacted with TBA has been shown (Fig. 10, curve 4). The ordinate values have been calculated to make the curve representative of fully oxidized membrane lipid of curve 1. In conclusion the TBA-analysis does not give evidence for phenylhydrazine induced peroxidation of membrane lipids.

This was confirmed by measurements of u.v. light absorption and fluorescence of lipids extracted from ghosts of phenylhydrazine treated and control erythrocytes. Thus, neither absorption typical of diene conjugation [24] nor fluorophores characteristic of lipid peroxidation [25, 26] could be detected.

Amino acid composition of Heinz body protein

Apart from cysteine and cystine, amino acid composition of Heinz body protein and native hemoglobin is identical. Only trace amounts of methionine sulfoxides were detected in Heinz body protein as well as in native hemoglobin. The content of cystine in Heinz body protein was significantly higher than in native hemoglobin. The amount of NH_4^+ in acid hydrolysates from Heinz body protein was some 25% higher than in native hemoglobin.

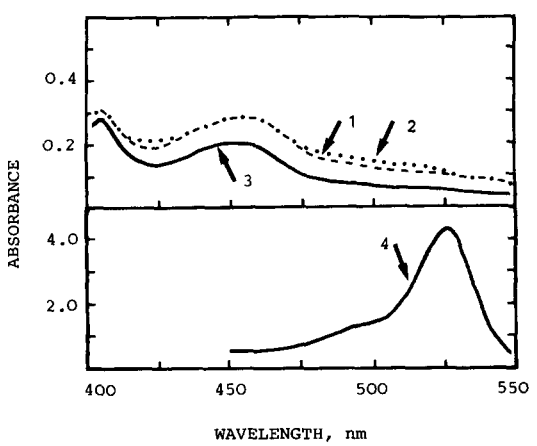

Fig. 10. Absorption spectra of TBA-developed supernatant from 0.6 ml whole erythrocyte suspension incubated for 5 hr with phenylhydrazine ("1") and without ("3") and from 0.6 ml control hemoglobin suspension similarly incubated with phenylhydrazine ("2"). "4" shows spectrum of the colored complex formed between MDA and TBA. The ordinate values have been calculated to make the curve representative of fully oxidized membrane of curve 1.

DISCUSSION

Incubation of erythrocytes with phenylhydrazine leads to precipitates of denatured hemoglobin designated Heinz body protein [31], and to chemical changes in the membrane [35]. The result of these events is a change in the mechanical and functional properties followed eventually by hemolysis. The present study demonstrates a close relationship between cross-linking of spectrin and decrease in deformability, both occurring abruptly after 5 hr of incubation with phenylhydrazine. Precipitation of hemoglobin which has been suggested to influence the mechanical properties of the cell directly [11, 12, 36] occurs gradually amounting to 25–30% of membrane protein after 1 hr and 85–95% of membrane protein after 5 hr. It has been demonstrated

by the present data and previously [37, 38] that formation of membrane polypeptide aggregates by artificial cross-linking agents in the absence of Heinz body protein leads to decreased deformability. The observed cross-linking of spectrin in erythrocytes exposed to phenylhydrazine is therefore likely to be the cause of the deformability change detected after 5 hr of incubation. Either in a direct way by affecting mechanical stability of the membrane, or more indirectly by disturbing ion fluxes across the membrane [39].

Cross-linked spectrin can be produced in a number of different ways [1]. However, in the present study the sensitivity of the cross-links to mercaptoethanol (Figs. 5 and 8) excludes mechanisms such as formation of amide linkages by transglutaminase [40], and Schiff base formation through products of lipid peroxidation [2]. The latter mechanism also is unlikely because lipid peroxidation could not be detected by any of three independent analyses applied (TBA-analyses and ultraviolet absorption and fluorescence of lipid). Furthermore, the effects of phenylhydrazine were not abolished or even influenced by the presence of the antioxidant, BHT, indicating that these effects are not dependent on lipid peroxidation. Thus, it can be concluded that phenylhydrazine does not induce lipid peroxidation in erythrocytes incubated under the present conditions. While some reports suggest lipid peroxidation in connection with the action of phenylhydrazine [35, 41–45], our results are supported by those of Winterbourn and Carrell [46], showing no lipid peroxidation in human erythrocytes exposed to acetylphenylhydrazine.

One mechanism which may be invoked to account for the polymerization of spectrin by disulfide formation observed in the present study is direct oxidation of membrane thiols resulting from H_2O_2 and O_2^- generation by phenylhydrazine. This would be in line with the suggestions by Johnson *et al.* [47] and Allen *et al.* [3] in studies of glucose-6-phosphate dehydrogenase deficient erythrocytes. However, if direct oxidation of membrane thiols is responsible for spectrin cross-linking in the present study, one would expect a decrease in the apparent membrane sulfhydryl content, which is not indicated by the data of Table 1. Even though a considerable fraction of spectrin is polymerized under the present conditions (30–35% as estimated by electrophoresis performed before gel filtration, see Fig. 6) sulfhydryl group determination with a S.D. of some 4% (Table 1) did not detect any net oxidation. Our data are therefore best explained by disulfide exchange between denatured hemoglobin and spectrin, resulting in formation of cross-linked spectrin and a corresponding increase in titrable globin sulfhydryl groups. This mechanism is supported by the presence of some globin peptide being bound covalently to spectrin through a mixed disulfide (Fig. 5). Also the polymerization of spectrin obtained by incubation of ghosts with preformed Heinz body protein (Fig. 8) suggests that disulfide exchange is a likely mechanism by which cross-linking of spectrin may take place in the erythrocyte.

Electrophoretic mobilities (Figs. 5C and 5D) show that the Heinz body protein formed in the presence of phenylhydrazine consists of 60% monomer and 40% disulfide linked dimer. All of the four sulfhydryl groups present per mole of hemoglobin are oxidized.

One of the characteristics of unstable hemoglobins appears to be decreased avidity of globin for the prosthetic group [48]. However, light absorption at 405–410 of Heinz body protein formed by incubation of erythrocytes with phenylhydrazine indicates that Heinz body protein and methemoglobin have approximately the same porphyrin content (Fig. 9). The slightly higher light absorption of Heinz body protein which occurs over the entire spectrum may be due to increased light scattering of Heinz body protein. Since, furthermore, Fe:globin ratio for Heinz body protein is close to 1, the prosthetic group remains bound to denatured hemoglobin precipitated as Heinz bodies by phenylhydrazine.

Our results may be compared with those of Winterbourn and Carrell [49] on composition of heat precipitated Hb A and heat precipitated, genetically unstable hemoglobins. They find polymerization by S–S cross-linking similar to the present observed dimerization. In addition, they find a similar heme content in precipitated Hb Christchurch and Hb A. However, in all the cases studied 3.3–4.4 sulfhydryl groups reside per mole after precipitation. Thus, oxidation of cysteine residues may be an important step in phenylhydrazine induced precipitation of hemoglobin as also suggested by Allen and Jandl [50], but not necessarily in precipitation of genetically unstable globins.

Amino acid analysis of Heinz body protein formed in the presence of phenylhydrazine show no oxidation of tryptophan, methionine and histidine. The substantial increase of NH_4^+ in hydrolysates of Heinz body protein may reflect binding to Heinz body protein of strongly reactive products of phenylhydrazine, such as a phenyldiazene, phenyldiazonium and phenylhydrazyl radical as suggested by Misra and Fridovich [10].

In conclusion we propose that the primary event in the reaction of phenylhydrazine with erythrocytes is formation of denatured oxidized hemoglobin. The changes in mechanical properties of the erythrocyte occur secondarily as a result of disulfide exchange between hemoglobin disulfides and spectrin. This encompasses the suggestions by previous investigators that either Heinz bodies *per se* [11, 12, 36] or chemical changes in the membrane [51] might be responsible for hemolysis. It appears likely that disulfide exchange with oxidatively damaged hemoglobin contributes to decreased stability of erythrocytes also in other drug induced hemolytic anemias, and possibly also in some of the chronic hemolytic anemias [48] as well as in aging [13].

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