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Phenylhydrazine mediated degradation of bovine serum albumin and membrane proteins of human erythrocytes

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Phenylhydrazine in solution has been shown to produce hydroxyl radicals, as measured by 2-deoxyribose degradation assay. In vitro incubation of bovine serum albumin with phenylhydrazine leads to extensive degradation of the former which, however, is not inhibited by hydroxyl-radical scavengers like mannitol, Tris or *n*-butanol. Metal chelators like EDTA, however, inhibits the breakdown of BSA. Erythrocyte ghosts incubated in vitro with phenylhydrazine also show extensive loss of membrane cytoskeletal proteins, inhibited by 5 mM EDTA but not by mannitol. In both the occasions a hydroxyl-radical mediated damage to protein takes place by a 'site-specific mechanism'. Further, such a damage to erythrocyte membrane proteins by phenylhydrazine may be related to well known action of this compound in producing accelerated aging of erythrocytes.

Introduction

The phenomenon of aging of erythrocytes in vivo is being investigated extensively [1,2]. Phenylhydrazine is known to cause accelerated senescence of erythrocytes and often used to replace the circulating mature erythrocytes with a population of new red blood cells for experimental studies [3,4]. An understanding of the changes caused to erythrocytes on exposure to phenylhydrazine may offer clues to elucidate the mechanism of in vivo aging of red blood cells. It is reported that phenylhydrazine causes denaturation of hemoglobin, clusturing of band 3 proteins in erythrocytes and binding of autologous IgG to red blood cell membranes [5]. Moreover, phenylhydrazine when added to erythrocyte ghosts causes peroxidation of endogenous lipids and an alteration of membrane fluidity [6]. This peroxidative damage to erythrocytes is probably initiated by active oxygen species like $O_2^{\cdot -}$, $OH^{\cdot}$ and H_2O_2 which are generated in solution from autooxidation of phenylhydrazine [6]. Several studies carried out recently document that oxygen free-radicals cause fragmentation of proteins or inactivation of enzymes in vitro [7–9]. The actual damaging species in such cases of protein degradation is probably $OH^{\cdot}$ generated by a transition metal catalysed Haber-Weiss reaction

$$O_2^{\cdot -} + H_2O_2 \xrightarrow{\text{transition metal}} O_2 + OH^{\cdot} + OH^-$$

at the sites where the transition metal like copper remains loosely bound to the protein [9–11]. In this paper we present evidences that phenylhydrazine in vitro causes extensive degradation of commercially available purified proteins like bovine serum albumin (BSA) and also membrane proteins of erythrocyte ghosts. Such degradation of erythrocyte membrane cytoskeletal proteins by phenylhydrazine may be related to the action of this compound in causing accelerated aging of red blood cells.

Materials and Methods

Materials. 2-Deoxyribose, 2-thiobarbituric acid, BSA and D-mannitol were from Sigma. Phenylhydrazine hydrochloride was purchased from Loba-Chemie, India. Chemicals for SDS-gel electrophoresis were obtained from Sisco Research Laboratory, Bombay. All other chemicals were of the highest purity available.

2-Deoxyribose degradation assay. Hydroxyl radical generation by phenylhydrazine has been measured by the 2-deoxyribose degradation assay of Halliwell and Gutteridge [12] in 50 mM phosphate buffer (pH 7.4) containing 1 mM deoxyribose, 0.2 mM phenylhydrazine hydrochloride and other additions as necessary in a

Abbreviations: SDS, sodium dodecyl sulfate; BSA, bovine serum albumin.

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total volume of 1.6 ml. Incubation was terminated after 1 h or 4 h and 1 ml each of 2.8% TCA and 1% (w/v) thiobarbituric acid were added to the reaction mixture and heated for 10 min in a boiling water bath. The tubes were cooled briefly and absorbance taken at 532 nm.

Preparation of erythrocyte membranes (ghosts). Human blood was freshly collected from healthy volunteers in 3.2% sodium citrate. The leucocytes and platelets were removed by a cotton-wool filtration technique [13]. Briefly, the cotton-wool (12 g dry wt. for 6–8 ml of whole blood) was boiled in distilled water with five changes and packed in a 50 ml plastic syringe clamped vertically. The cotton pack was washed extensively with isotonic phosphate buffered saline (pH 7.4). The blood was poured over the cotton-pack and washed down with the same ice-cold phosphate-buffered saline. The eluate from the column was collected in a total volume of 100 ml (for 8 ml of blood) and red cells centrifuged down at $1000 \times g$ for 5 min. The cells are free from white blood cells (> 99%) and platelets (> 90%) as seen microscopically. Erythrocyte ghosts were prepared by the method of Fairbanks et al. [14].

Degradation of protein by phenylhydrazine. Incubation of bovine serum albumin (1 mg/ml) or erythrocyte ghosts (0.4 to 0.8 mg/ml) in 50 mM phosphate buffer (pH 7.4) were carried out for varying periods of time (from 30 min to 4 h) in presence of 0.2 mM phenylhydrazine hydrochloride with other additions as necessary in a total volume of 500 μl. All additions were made up in 50 mM phosphate buffer (pH 7.4). For some experiments erythrocyte ghosts were solubilized in 1.5% SDS and heated for 5 min in a boiling water bath before being used for incubation. At the end of incubation, 250 μl of electrophoresis sample buffer containing 3.3% SDS and 5% mercaptoethanol was added in each tube and samples were immediately put for SDS-polyacrylamide gel electrophoresis. SDS-polyacrylamide gel electrophoresis was done following the method of Laemmli [15] using 4.5% stacking and 10% separating gel. The gels were stained by Coomassie brilliant blue R followed by destaining till the background is clear.

Several different incubation protocols were used with erythrocyte ghosts preparations in order to investigate the effects of metal chelating agent like EDTA on phenylhydrazine induced damage to erythrocyte membrane proteins. In one set of such experiments, erythrocyte ghosts were pre-incubated with 5 mM EDTA for 90 min at 37°C, followed by three washings in 50 mM phosphate buffer (pH 7.4) at 4°C to remove EDTA and finally the pelleted membranes were incubated with phenylhydrazine for a further period of 4 h as described earlier. In some other experiments, erythrocyte membranes after an initial incubation in presence of phenylhydrazine (0.2 mM) for a period of 4 h, were collected by centrifugation at $30000 \times g$, washed several times in 50 mM phosphate buffer (pH 7.4) at 4°C and subsequently incubated again in the same phosphate buffer at 37°C in presence of 5 mM EDTA for 1 h. In all these experiments proper controls were kept and erythrocyte membranes after the final incubation were analysed by SDS-polyacrylamide gel electrophoresis.

Protein estimation. Protein in the erythrocyte membrane was estimated by the method of Lowry et al. [16] after solubilizing in 1% SDS.

Results

2-Deoxyribose degradation

Results presented in Table I indicate that phenylhydrazine in solution generates $OH^{\cdot}$ radicals as measured by 2-deoxyribose degradation assay. The scavengers of $OH^{\cdot}$ radicals like mannitol, *n*-butanol and Tris each at a concentration of 20 mM can inhibit the production of thiobarbituric acid reactive material significantly over periods of 1 h or 4 h.

Degradation of bovine serum albumin

Electrophoretic studies presented in Figs. 1 and 2 clearly indicate that phenylhydrazine causes significant degradation of BSA during in vitro incubation over a period of 4 h as seen by the loss in the intensity of starting BSA band (lane c, Fig. 1, lanes a, b and d, Fig. 2). Moreover, a number of faint but sharp bands of lower molecular mass could be detected as a result of such proteolysis (arrow-marks, Figs. 1 and 2). These bands are absent in the control lanes which contain BSA incubated with buffer alone. However, time-course of BSA degradation reveals (Fig. 2) that at no stage these newly generated minor polypeptides could completely account for the considerable loss of the native BSA band, as judged from the intensities of different

TABLE I

Hydroxyl-radical detection by 2-deoxyribose degradation assay

Reactions carried out with incubation mixtures containing 2-deoxyribose (1 mM) with or without phenylhydrazine (0.2 mM) in presence of other additions, as described in Materials and Methods. Value (absorbance at 532 nm after 1 h and 4 h) are from one typical experiment from a set of four similar experiments.

Incubation system	Absorbance at 532 of reaction mixture	
	1 h	4 h
2-Deoxyribose alone	0.007	0.005
2-Deoxyribose/phenylhydrazine	0.208	0.253
2-Deoxyribose/phenylhydrazine/ mannitol (20 mM)	0.043	0.055
2-Deoxyribose/phenylhydrazine/ Tris (20 mM)	0.043	0.045
2-Deoxyribose/phenylhydrazine/ *n*-butanol (20 mM)	0.052	0.078

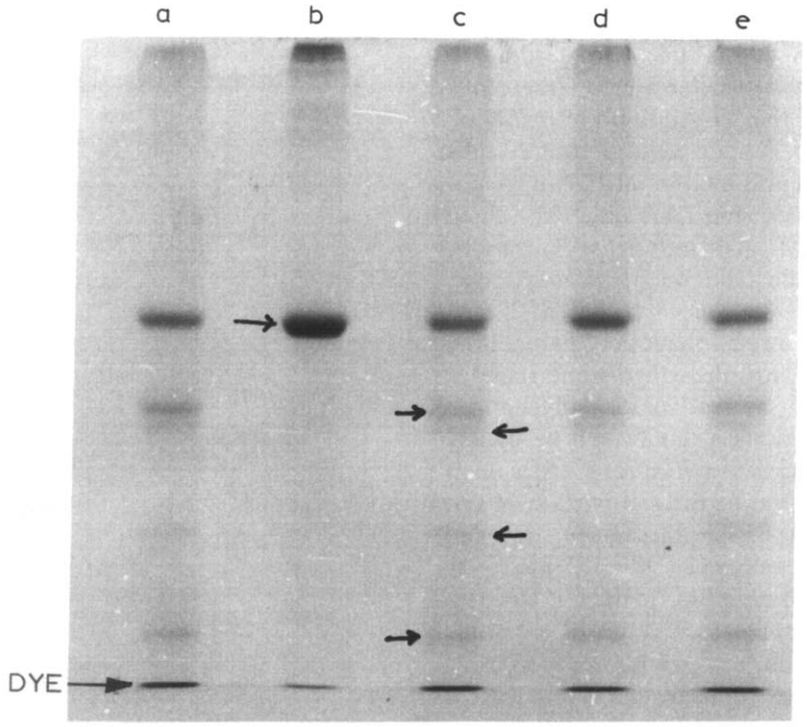

Fig. 1. Degradation of BSA during a 4 h incubation with phenylhydrazine. Experimental conditions are as described in Materials and Methods. Each lane contains 20 μg of protein. Lane b: BSA with buffer (control). Lane c: BSA with phenylhydrazine. Lanes a, d and e: BSA with phenylhydrazine in presence of Tris, 20 mM (a), mannitol, 20 mM (d) and *n*-butanol, 20 mM (e). The native BSA band is shown by an arrow in lane b, while the arrows in lane c indicate the degraded fragments of BSA. High molecular-weight proteins, possibly higher oligomers of BSA are visible in the top part of the gel, especially in lane b.

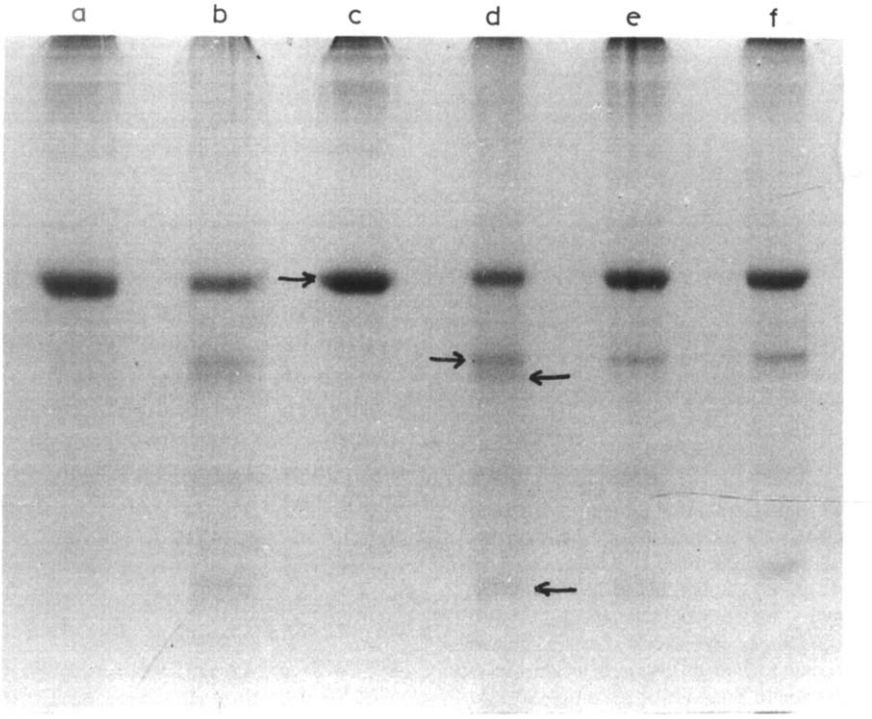

Fig. 2. Time-course of phenylhydrazine mediated degradation of BSA and inhibition by EDTA. Each lane contains 20 μg of protein. Experiments have been carried out as described in Materials and Methods. Lane c contains BSA with buffer alone (control) incubated for 4 h. Lanes a, b and d contain BSA with phenylhydrazine incubated for 1/2 h, 1 h and 4 h, respectively. Lanes e and f contain BSA with phenylhydrazine incubated for 4 h in presence of 5 mM and 1 mM EDTA, respectively. Arrow in lane c indicates the native BSA, while those in lane d mark the positions of degraded fragments of BSA.

protein bands in the gel. This points out that proteolysis of BSA probably occurs at multiple sites leading to formation of many low-molecular-weight fragments, which are not retained in the gel. Addition of mannitol, or Tris or *n*-butanol each at a concentration of 20 mM fails to show any significant inhibition of BSA degradation by phenylhydrazine (Fig. 1) although these scavengers of $OH^{\cdot}$ radicals at identical concentrations considerably inhibit the degradation of 2-deoxyribose. On the otherhand, EDTA at concentrations of 1 mM and 5 mM significantly prevents the loss of native BSA band during incubation with phenylhydrazine (lanes e and f, Fig. 2). However, several minor polypeptides (products of BSA fragmentation) could still be clearly seen in EDTA containing lanes (Fig. 2). A few high molecular weight bands seen at the top portion of the gel above the BSA band (possibly the higher oligomers of BSA present in the commercial sample) also gets degraded by phenylhydrazine (Figs. 1 and 2). No new high-molecular-weight proteins as a result of cross-linking of native BSA monomer could be detected after exposure of BSA to phenylhydrazine.

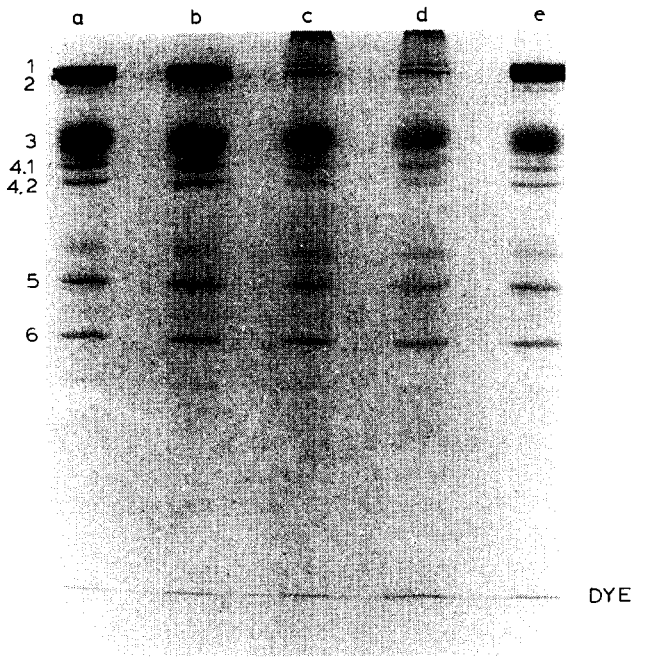

Fig. 3. Phenylhydrazine effects on membrane proteins of human erythrocytes during a 4 h incubation. Experiments have been carried out as described in Materials and Methods. Each lane contains 60 μg of protein. Lane a: erythrocyte ghosts without any incubation (unincubated control). Lane b: erythrocyte ghosts incubated (4 h) with buffer alone (incubated control). Lane c: erythrocyte ghosts incubated (4 h) with phenylhydrazine. Lanes d and e: erythrocyte ghosts incubated (4 h) with phenylhydrazine in presence of mannitol, 20 mM (d) and EDTA, 5 mM (e). The bands in Lane a have been numbered according to Fairbanks et al. [14]. A distinct high molecular weight band is visible at the top margin of separating gel in lanes c and d. Two controls (lanes a and b) have been used to check the protein degradation in incubated ghosts as a result of endogenous protease activity.

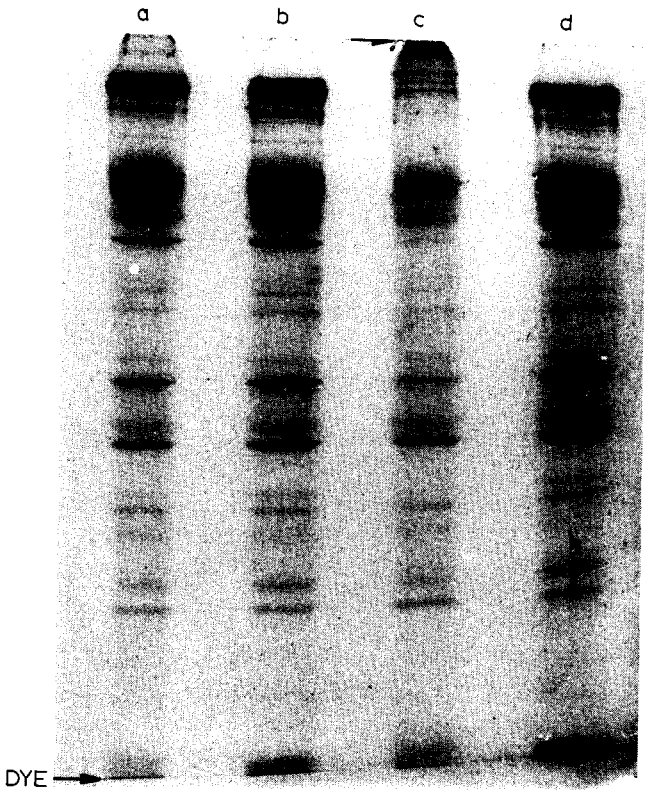

Fig. 4. Phenylhydrazine effects on EDTA-treated erythrocyte ghosts. Erythrocyte ghosts pretreated with EDTA (5 mM) at 37 °C for 90 min or control ghosts (without EDTA treatment) were incubated for 4 h in presence (lanes a and c) or absence (lanes b and d) of phenylhydrazine (0.2 mM) as described in Materials and Methods. Membrane proteins were subsequently analysed by SDS-polyacrylamide gel electrophoresis. All lanes contain equal amounts of protein. Lanes a and b: EDTA pre-treated ghosts. Lanes c and d: control ghosts. Phenylhydrazine induces extensive loss of major membrane proteins in control ghosts (lane c) along with the formation of a high molecular weight cross-linked product (arrow-mark), while similar changes are largely absent in EDTA-treated ghosts (lane a).

Degradation of erythrocyte membrane proteins

Results in Fig. 3 present the effects of phenylhydrazine on membrane cytoskeletal proteins of erythrocyte ghosts incubated for 4 h. The major proteins of erythrocyte membrane cytoskeleton could be easily identified in the lanes containing erythrocyte ghosts unincubated or incubated only with buffer (lanes a and b, Fig. 3). The bands are numbered according to electrophoretic mobility following Fairbanks et al. [14]. An incubation for a period of 4 h with phenylhydrazine leads to significant loss of bands 1 and 2 (spectrin), 3, 4.1, 4.2 and 5 (lane c, Fig. 3). Other minor bands are also degraded to variable extent. As in the degradation of BSA by phenylhydrazine, proteolysis in erythrocyte ghosts also is inhibited to a significant extent by 5 mM EDTA, while mannitol produces no inhibition (Fig. 3). Further, when erythrocyte ghosts treated with 5 mM EDTA at 37 °C for 90 min are subsequently incubated in presence of phenylhydrazine after thorough wash-

ings, the degradation of major membrane proteins is mostly prevented (Fig. 4). It may be emphasised here that EDTA pre-treatment of erythrocyte ghosts needs to be carried out at 37°C for a period of 45–90 min, since several other procedures like repeated washings with EDTA of membranes at 4°C or incubation of membranes at 4°C with EDTA for several hours seems to be ineffective. A high molecular weight protein band is always seen at the top margin of separating gel in the lanes containing erythrocyte ghosts with phenylhydrazine. We presume that this results from cross-linking of proteins and interestingly this phenomenon is also inhibited by EDTA. However, when the ghosts are first exposed to phenylhydrazine and subsequently treated with EDTA (5 mM) for 1 h, the latter fails to reverse either the degradation or cross-linking of membrane proteins (result not shown).

Erythrocytes are reported to have several types of membrane associated proteases, inhibitible to varying degrees by phenylmethanesulfonyl fluoride, EDTA, diisopropylphosphofluoridate etc. [17,18]. In order to demonstrate that the degradation of erythrocyte membrane proteins by phenylhydrazine and its inhibition by EDTA is not dependent on the presence of proteinase activity in red cell membranes, we solubilized the ghosts membranes in 1.5% SDS followed by heating in a boiling water bath for 5 min to inactivate the proteinases. This point is important since there are reports that protein exposed to oxygen free-radicals show increased susceptibility to digestion by proteolytic enzymes [7]. However, we noticed that phenylhydrazine causes significant and apparently identical degradation of proteins in detergent solubilized and heat-treated ghosts as in intact ghosts and this is also inhibitible by 5 mM EDTA (results not shown). But in detergent-solubilized membranes no high-molecular-weight cross-linked protein could be detected after exposure to phenylhydrazine unlike that in the case of intact ghosts.

Discussion

Fragmentation of proteins and inactivation of enzymes by hydroxyl radical generating systems have been widely documented although the exact mechanism of this damage is still unclear [8,9,19]. Our results show that phenylhydrazine gives rise to $OH^{\cdot}$ radicals in solution and can also cause breakdown of BSA or erythrocyte membrane proteins. However, this protein degradation is not inhibited by mannitol, *n*-butanol, Tris, etc. which are effective scavengers of $OH^{\cdot}$ radicals in uniform solutions. This indicates that a 'site-specific' mechanism for protein damage is probably involved here [8,20]. Superoxide radicals and H_2O_2, products of autooxidation of phenylhydrazine [6], reach the target protein molecules and lead to localised formation of $OH^{\cdot}$ radicals at sites where transition metal like Cu^{2+} or Fe^{3+} is loosely bound to protein and these highly reactive $OH^{\cdot}$ radicals subsequently attack the susceptible amino-acid residues at or near the site of their formation [8,11,20]. Transition metal like copper is known to bind to serum albumin or other proteins especially at the histidine residues [10] and catalytic amounts of iron also possibly remain attached to erythrocyte membranes and such protein-bound endogeneous metal ions result in the localized formation of $OH^{\cdot}$ radicals leading to extensive protein breakdown in our experimental system even in the absence of added Cu^{2+} or Fe^{3+}. This 'site-specific' mechanism further explains the inhibition of protein degradation in our systems by metal chelaters like EDTA which removes the metal from the target molecule. It may be suggested that a few minor fragments from BSA degradation that are visible in the lanes containing EDTA probably result from protein fragmentation at sites where copper is relatively strongly bound to albumin and, therefore, not removed by EDTA. This, however, accounts for only a minor portion of actual degradation of BSA in presence of phenylhydrazine. We suggest that endogenous metal ions remain loosely bound to BSA or erythrocyte membrane proteins at multiple sites and may, therefore, lead to fragmentation of protein molecule into multiple small peptides by 'site-specific' formation of $OH^{\cdot}$ radicals. Such small peptides are not retained in the gel during electrophoresis and this result in only a few minor (in case of BSA) or no detectable (in case of erythrocyte membrane protein) degraded products, even when extensive proteolysis is occurring. This conclusion of 'site-specific' damage to proteins by bound metal ions is further strengthened by the fact that in erythrocyte membranes pretreated with EDTA, protein degradation in presence of phenylhydrazine is largely prevented. It is interesting to point out that with some grades of BSA (extensively dialyzed preparation, as stated by the manufacturer) the degradation in presence of phenylhydrazine is much smaller than that presented in Figs. 1 and 2, presumably because extensive dialysis largely removes the loosely bound metal ions from albumin. A high-molecular-weight cross-linked protein is also formed when intact erythrocyte ghosts are incubated with phenylhydrazine. Phenylhydrazine also causes peroxidation of membrane lipids and cross-linking of proteins in presence of peroxidizing lipids has earlier been reported [7]. It is possible that disappearance of major protein bands of erythrocyte membranes exposed to phenylhydrazine is partly due to the formation of high-molecular-weight cross-linked proteins. However, as stated earlier, in detergent solubilized and heat-treated membranes incubated in presence of phenylhydrazine this cross-linked protein does not appear, even though a significant loss of several protein bands is seen in SDS-polyacrylamide gel indicating

thereby that the latter effect largely results from protein degradation.

The visco-elastic properties of erythrocyte membranes are determined to a great extent by the membrane cytoskeletal proteins like spectrin, ankyrin, etc. [21]. Phenylhydrazine induced fragmentation or cross-linking of such proteins may, therefore, lead to altered shape and deformability of cells enhancing their removal from the circulation. This could be one of the possible mechanisms of accelerated aging of circulating erythrocytes seen after administration of phenylhydrazine. Moreover, since the $OH^{\cdot}$ radical, the ultimate damaging species, is formed from $O_2^{\cdot -}$ and H_2O_2, the antioxidant enzymes superoxide dismutase and catalase are likely to play very important roles in preventing such damage to erythrocytes, since both superoxide radicals and H_2O_2 are generated in erythrocytes in vivo. It remains to be investigated whether such free-radical induced damage to cellular or membrane protein is involved in the aging of erythrocytes in vivo.

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