INTRODUCTION
The Human Erythrocyte Membrane

The human erythrocyte membrane is the best understood among the biological membranes, in view of the ready availability of erythrocytes and ease of membrane preparation. It has long served as a convenient model for the testing of new concepts and methodologies in membrane biochemistry.

The erythrocyte membrane is composed of about equal amounts of lipids and proteins. The carbohydrate components of the red blood cell membrane are confined to the exterior surface as oligosaccharide moieties of glycolipids and glycoproteins (Sweeley and Dawson, 1969). They are for the most part responsible for the surface charge and surface determinant properties. It is well known that the rbcs have a large negative charge at their outer surface (Cook et al., 1961), contributed primarily by carboxyl groups of the sialic acid residues which are associated with the major rbc membrane glycoproteins (Eylar et al., 1962). Glycolipids constitute about 5-10% by weight of the total lipids.

The first systematic separation of rbc membrane proteins was described by Fairbanks et al. (1971). These workers designated the various polypeptide components by numbers, according to their mobility in polyacrylamide gel in the presence of SDS. This nomenclature is now widely accepted. Extensive literature is available on the nature and function of the principal polypeptides of the human erythrocyte membrane (Juliano, 1973; Haest, 1982; Bennett, 1985). Only a brief account of the available information on these polypeptides is, therefore, presented.
Membrane Polypeptides

Spectrin (band 1 and 2) - Spectrin is the major component of the human rbc membrane and occurs as a stable dimer of two similar but chemically distinct polypeptides. The two polypeptides, bands, 1 (Mr 240,000) and 2 (Mr 220,000) are oriented as parallel and intertwined chains (Marchesi, 1979) forming 10 nm long and 2.5 nm wide rods (Shotton et al., 1979). Spectrin is believed to be at least tetrameric in situ and the association of dimers occurs end to end (Morrow and Marchesi, 1981). Dissociation of spectrin to dimers leads to the fragmentation of cytoskeleton (Liu and Palek, 1979).

Ankyrin (band 2.1, 2.2 and 2.3) - Band 2.1 (Mr 210,000) is found in situ in the monomeric form (Bennett and Stenbuck, 1980). The band 2.1 polypeptide is believed to connect membrane skeleton with band 3 of intrinsic domain (Morrow et al., 1980). However, not much is known about the structure and associations of bands 2.2 (Mr 183,000) and 2.3 (Mr 105,000) (Siegel et al., 1980).

Band 3 - Band 3 is the major intrinsic protein consisting about 50-60% of the total intrinsic proteins. It is an inorganic anion transporting protein of Mr 95,000 (Cabantchik et al., 1978) and is believed to exist as dimers and to some extent as tetramers in the membrane (Nakashima et al., 1981). This protein transverses the rbc membrane and has an outer glycosylated domain and an intracellular domain, which is not glycosylated. Extensive literature is available on the structure, organization and function of this protein (Jay, 1976; Low, 1986).

Band 4.1 - The band 4.1 of Mr 78,000 is a globular molecule of
6 nm diameter (Tyler et al., 1979). Band 4.1 is believed to participate in the interaction of spectrin with actin (Wolfe et al., 1980). Each spectrin dimer has one binding site for band 4.1, located at the free end of dimer-dimer complex (Tyler et al., 1979).

Band 4.2 - This corresponds to the protomer (Mr 72,000) of a tetrameric protein of unknown function. Band 4.2 polypeptides remain associated with the cytoplasmic region of band 3 (Land and Nermut, 1980).

Band 4.9 - A further putative component of membrane skeleton is band 4.9 (Mr 48,000). Liu and Palek (1979) have reported its in situ association with band 2 of spectrin.

Actin (Band 5) - Band 5 polypeptide (Mr 43,000) is also a component of membrane skeleton and forms short oligomers containing about 10 molecules in filamentous form (Brenner and Korn, 1980). Each spectrin tetramer has two actin binding sites at the free end. This polypeptide has been shown to play a major role in shape change of rbcs.

Band 6 - This is the monomer of glyceraldehyde-3-phosphate dehydrogenase. This enzyme is a tetramer of Mr 35,000 subunits which binds to spectrin-actin complex and to band 3 (Yeltman and Harris, 1980; Murthy et al., 1981). It binds loosely to the membrane and can be readily eluted with high ionic strength solution (Fairbanks et al., 1971).

Band 7 - Band 7 protein of Mr 29,000 has not yet been fully characterized and it is also not clear as to where and how it is arranged in erythrocyte membrane. The phosphatidyl serine (PS)
transporter protein of human erythrocyte membrane has been shown to co-migrate with band 7 protein (Connor and Schroit, 1988).

In addition to these polypeptides, there are a number of glycopeptides embedded into lipid bilayer of rbc membrane. They are designated as PAS-1, PAS-2, PAS-3 and PAS-4 (Fairbanks et al., 1971). Among PAS-positive polypeptides, glycophorin A, is the major one and constitutes about 75% of the total sialoglycopeptides of the membrane (Furthmayr et al., 1975). The work has also been extensively reviewed (Marchesi et al., 1976; Bennett, 1985).

Cytoskeleton

The human rbc membrane is decorated at the cytoplasmic surface with a densely packed spectrin-actin skeleton. From microscopic pictures and calculations it can be derived that the membrane skeleton covers 50-70% of the membrane surface. The membrane skeleton was originally defined as the protein filamentous matrix of the same dimensions as the originating ghost that remains after extraction of the lipids and intrinsic proteins from intact erythrocytes or ghosts using Triton X-100 (Yu et al., 1973). Subsequent purification of the skeleton gave a lipid-poor (1-2%) protein band containing beside spectrin (band 1 and 2), a number of peptides and band 5 (Sheetz, 1979). This composition represents the minimal number of bands necessary to maintain stability of the skeleton. Stabilization of the skeleton components within the network probably occurs via non-covalent bonds of varying affinity as well as by electrostatic repulsions of negatively charged spectrin molecules.

Although the membrane skeleton restricts lateral mobility
of intrinsic proteins, cytoskeleton is a highly shear deformable lattice. This is evidenced by the extreme deformations of the cell in circulation as well as by deformations induced by drugs and other substances. The membrane skeleton can be viewed as being constructed around spectrin. Spectrin tetramers are involved in two independent classes of protein associations that are both essential for the final structure for (a) formation of the two dimensional meshwork underlying the lipid bilayer, by associations with actin oligomers, band 4.1 and possibly other spectrin molecules and (b) linkage of the spectrin actin-meshwork to integral membrane proteins via association with ankyrin and possibly band 4.1 (Fig. 1).

Assembly of a meshwork requires some types of polymerization reactions leading to branched structures. A frequently discussed arrangement of proteins (Cohen and Langley, 1984) involves a basic structural unit composed of actin filaments, containing 12-17 actin monomers and accessory proteins such as band 4.9 and tropomyosin, which are attached at the ends of multiple spectrin tetramer/band 4.1 complexes (Fig. 1). These actin-spectrin complexes can then assemble to form a branching polymeric structure by interconnections between the free ends of spectrin tetramers and other actin oligomers. It has been proposed that self assembly of spectrin into hexamers and higher order oligomers from erythrocyte membrane initiates polymerization of G-actin by a cytochalasin inhibitable reaction and thus these actin filaments have free fast growing ends (Lin and Lin, 1979; Brenner and Korn, 1980; Pinder and Gratzer, 1983; Shen et al., 1989). In cells such as developing erythrocytes and other tissues with related forms of spectrin, the spectrin-actin meshwork could have extended actin filaments cross-linked along their length by spectrin or other actin-binding proteins to form three dimen-
Fig. 1

Schematic model of the human erythrocyte membrane skeleton. The barbed end of the actin filament is the fast-growing end while the pointed end is the slow-growing end.

(Reproduced from Bennett, 1985)
sional structures. Presumably during erythrocytes maturation, actin in excess of the final $5 \times 10^5$ copies per cell is lost, perhaps as the consequence of a band 4.1 severing activity and the red cell is left with a shell of short actin filaments adjacent to the membrane (Pinder et al., 1984).

RBC Lipid Composition and Organization

Extensive literature is available on the composition of erythrocyte membrane lipids (Stephen et al., 1972; Opden Kamp, 1979). The amount of total lipid present in $10^{10}$ cells is about 5 mg, approximately, 30% of which comprises of neutral lipids, 60% of phospholipids and 5-10% of glycolipids (Cooper, 1969). Cholesterol, the major constituent of the neutral lipids, appears to be relatively randomly distributed in the bilayer (Higgins, et al., 1973). Freeze fracture studies, however, indicate that outer layer may contain more cholesterol than the inner layer of the bilayer (Fischer, 1975). Phosphatidyl choline (PC), PS, phosphatidyl ethanolamine (PE) and sphingomyelins are the principal subclasses of the phospholipids (Cooper, 1969) and they constitute 32.06%, 13.54%, 30.28% and 22.15% respectively of the total phospholipids. Other minor phospholipids sub-classes are phosphatidic acid and phosphatidyl inositol (PI) which respectively constitute about 1.91% and 1.06% of the total phospholipids (Jain and Shohet, 1981). The fatty acid composition of the membrane lipids has also been investigated and those ranging from $C_{14}$ to $C_{24}$, with varying number of double bonds have been reported to occur in erythrocytes (Peuchant et al., 1989). Phospholipids positioned in the inner leaflet of the membrane contain fatty acid that are more unsaturated than those present in the outer layer (Van Deenen, 1981).
Detailed investigations, carried out using chemical or enzymatic modification, phospholipid exchange techniques and a few immunochemical procedures using intact cells, leaky and or resealed ghosts and inside out vesicles, have established conclusively that the phospholipids in the human erythrocyte membrane are asymmetrically distributed (Op den Kamp, 1979; Van Deenen, 1981). The most convincing data obtained using phospholipases suggest that 80% of the total sphingomyelin, 75% of PC and 20% of PE are located in the outer leaflet of the bilayer (Verkleij et al., 1973; Zwaal et al., 1975). Apparently aminophospholipids, particularly PS are concentrated on the cytoplasmic side of the membrane. A possible fraction of such asymmetric distribution may be related to the ability of PE and PS to influence the blood coagulation process (Op den Kamp, 1979).

Among the various phospholipid classes of the erythrocyte membrane PS is exclusively located on the cytoplasmic leaflet (Gordesky et al., 1972; Zwaal et al., 1977). Insertion of exogenously supplied fluorescent lipid analogues of PS into mouse or human rbcs stimulated their binding and concomitant phagocytosis by cultured syngenic macrophage and allogenic monocytes respectively (Tanaka and Schroit, 1983; Schwartz et al., 1985). Schroit et al. (1985) demonstrated the rapid uptake of rbcs containing fluorescent PS in their membranes and their rapid accumulation in the spleen. Earlier studies gave evidence of the vasooculussion and splenomegaly associated with exposure of endogenous PS in sickle cell anemia (Chui et al., 1981) and in chronic myeloid leukemia patients (Kumar and Gupta, 1983). A number of other investigations have also demonstrated that condition leading to the transfer of PS to the outer leaflet of the bilayer cause a dramatic decrease in circulating half-life.
of the cells (Arduni et al., 1986; 1989). The decrease is attributed to the recognition and binding of the erythrocytes with exposed PS. Working with reversibly sickled cells, Middle Koop et al. (1988) inferred that asymmetric localization of PS requires both interaction with cytoskeleton and an ATP dependent translocation. The authors believed that increased availability of PS to phospholipase A2 digestion is the result of the increase in transverse dynamics rather than static redistribution of phospholipids. The work of Bitbol and Devaux (1988) indicated that a single protein may be involved as inward and outward aminophospholipid translocase. Calvez et al. (1988) questioned the role of spectrin in maintaining PS asymmetry in view of their studies on severely spectrin-depleted heat-induced vesicles from human erythrocytes, the vesicles accumulated PS in the inner layer. The role of band 4.1 in binding and retention of PS in the inner layer was indicated by the studies of Chandra et al. (1987), using calcium loaded human erythrocytes and more recently by Bitbol and Devaux (1988) and Cohen et al. (1988) using PS vesicles. An interesting correlation between 4.1a, 4.1b ratio and erythrocyte life span was observed during a comparative study of several mammalian erythrocytes by Inaba and Maeda (1988). Suzuki and Dale (1989) also observed an increased 4.1a/4.1b ratio in senescent cells. An interesting study by Allen et al. (1988) using liposomes has shown that uptake of the vesicles with exposed PS is not mediated via alteration in phospholipid packing and suggest PS as a recognition marker of RES. These authors also gave the evidence of the requirement of a threshold concentration of PS in outer layer for the uptake of the rbc by the RES system.

PHZ - Reticulocytosis and Heinz Body Anaemia

Observations in the last century indicated that PHZ
could induce dramatic changes in erythrocytes both \textit{in vitro} as well as \textit{in vivo}. Hoppe Seyler (1885) reported that blood from rabbits treated with PHZ was brown in colour and that addition of PHZ to the suspended erythrocytes also gave them a brown colouration. Subsequently Heinz (1890) found that mixing either nucleated (from cold-blooded animals) or non-nucleated erythrocytes with PHZ turned them brown. Heinz (1890) also discovered that inclusion bodies were formed in erythrocytes exposed to PHZ.

Heinz bodies are aggregates of denatured hemoglobin that bind to the inner surface of the erythrocyte membrane (Waugh and Low, 1985). Such patterns of denatured hemoglobin are frequently observed in aged cells (Sears et al., 1975), in erythrocytes from individuals with glucose-6-phosphate dehydrogenase deficiency disease and other maladies involving loss of intracellular reducing power (Jandl et al., 1960) as well as in cells containing unstable hemoglobins (Jacob and Winterhalter, 1972; Winterbourn and Carrell, 1974). Heinz bodies are believed to be responsible for the hemolysis which commonly accompanies the anemias associated with the latter two diseases (Waugh and Low, 1985).

Heinz bodies formed due to the exposure of rabbit erythrocytes to PHZ, were observed by phase contrast microscopy as small dense bodies 6.7 \mu in diameter within 5 to 8 min., of the exposure (Rifkind and Danon, 1965). Incubation of cells with either hydrazine or PHZ resulted in the accumulation of Heinz bodies. About 82\% of the cells contained such material after only 30 min., incubation with PHZ while only about 7\% of the hydrazine-treated cells contained Heinz bodies (Jain and Hochstein, 1979).

Good evidence indicates that whether natural or drug-
induced, Heinz body formation may occur by a similar mechanism
(Waugh and Low, 1985). Initially the native hemoglobin is oxidized
to methemoglobin (MHb) followed by, minor structural rearrange-
ments leading to formation of a reversible hemichrome. Under
appropriate conditions the hemichromes may revert back to MHb.
Otherwise, the reversible hemichromes denature further to form
irreversible hemichromes, which eventually aggregate to form the
Heinz bodies (Peisach et al., 1975).

It is well known that red cell loss either by repetitive
bleeding or by treatment with hemolytic agents results in reticu-
locyte accumulation in the circulation. Experiments with PHZ also
show that this agent induces reticulocytosis and is routinely
used for induction of reticulocytosis for a variety of experi-
ments. In fact by repetitive PHZ injection it is possible to
achieve reticulocytosis at a level of 98 % (Jain and Hochstein,
1980 b). By this technique it was possible to study the lipid
composition and osmotic fragility of reticulocytes unconta-
minated with mature erythrocytes (Jain and Subramanyam, 1976).

Administration of PHZ to animals results in hemolytic
anemia with reticulocyte counts of about 30 % and an accumulation
of fluorescent pigments in the membranes of the surviving cells
in the circulation (Jain and Hochstein, 1979). APHZ and hydrazine
which cause less severe anemia than PHZ, also elicit a lesser
reticulocyte response (< 10 %) and decreased amounts of
fluorescent pigments in the membranes of the surviving
cells(Hochstein and Jain, 1981). The reticulocytes formed after PHZ
exposure have a dramatically lowered half lives when compared to
those produced by bleeding of animals (Jain and Hochstein, 1980 b).

The processes, resulting from the in vivo interaction
of oxyhemoglobin A and APHZ to the destruction of the red cells, are summarized below. (Peisach et al., 1975).

\[
\begin{align*}
\text{Deoxyhemoglobin A} + \text{O}_2 & \rightarrow \text{Oxyhemoglobin A} + \text{APHZ} \\
\text{Ferrylhemoglobin A} + \text{e}^- & \rightarrow \text{Sulfohemoglobin}
\end{align*}
\]

\[
\begin{align*}
\text{Ferric hemoglobin A} + \text{Phenyldiimide} & \rightarrow \text{Ferric hemoglobin A-phenyldiimide} \\
& \rightarrow \text{Benzene + N}_2
\end{align*}
\]

formation of reversible ferrihemochrome, separation of unlike \(\alpha\) and \(\beta\) chains

formation of irreversible ferrihemochromes

formation of Heinz bodies (Precipitates of irreversible ferrihemochromes, globin and hemin)

Attachment of Heinz bodies to red cell membrane by mixed disulphide bonds

"Pitting" of red cells with Heinz bodies in the spleen

Accelerated red cell destruction

Waugh and Low (1985) reported that since hemoglobin binds to band 3, this band may also provide a binding site for hemichromes, and the cross-linking of band 3 by hemichromes can also account for the enhanced fragility of the membrane commonly observed in cells containing elevated levels of denatured hemoglobin. The cells containing denatured hemoglobin frequently
undergo isotonic hemolysis in vivo (Waugh and Low, 1985). In other cases, where isotonic hemolysis is not observed, the cells are found to experience hypotonic hemolysis at weaker osmotic stresses than normal cells (Goldberg and Stern, 1977). While oxidative damage remains a possible cause of this hemolysis (Hebbel et al., 1982), the lateral tethering and aggregation of band 3 molecules by hemichromes could also generate the mechanical strain in the membrane which leads to membrane weakening and ultimately to the hemolytic anemia (Waugh and Low, 1985).

Moderately damaged cells appear to be removed by the splenic and those with more severe damage by the liver RES. This appears to be true in case of rbcs damaged by a variety of mechanisms including those treated with PHZ (Rifkind, 1965).

Reactions of PHZ with Hemoglobin

Ever since the first observation of Hoppe-Seyler (1885) that oxyhemoglobin disappeared from blood of rabbits treated with PHZ and those of Heinz (1890) that the blood from nucleated/non-nucleated erythrocytes turned green-brown on exposure to the drug, a large body of information has accumulated on the effects of PHZ on hemoglobin.

Heinz (1890) was the first to observe in his classical study that treatment of oxyhemoglobin with PHZ produces MHb. The formation of MHb however depends strongly on relative concentration of PHZ and oxyhemoglobin (P/H ratio) and the time of observation after initial treatment, in a complex manner. While increasing the P/H ratio increased MHb formation at early time points, such correlation was not observed after longer incubation
especially at high P/H ratios (Harley and Mauer, 1960). Jandl et al. (1960) also observed a decrease in MHB formation at high P/H ratios after relatively long incubation periods. At P/H ratio of 20, about 33 % of initial hemoglobin was transformed to MHB in one min., but the quantity decreased first rapidly and subsequently more slowly to almost undetectable levels in about one hr. A second rise in MHB was observed during the next 11 hr., suggesting the participation of MHB in a complex series of reaction. In the absence of atmospheric oxygen, oxyhemoglobin was transformed to deoxyhemoglobin (Hb) with no evidence of MHB formation on treatment with PHZ. MHB was however observed as the only product when cyanide was used to trap the moiety (Castro et al., 1978). It was suggested that oxidation product of PHZ rather than PHZ itself was reacting rapidly with MHB in the absence of cyanide to reduce it back to Fe(II) state.

Phenylhydrazyl radical (PHZR) is the most probable candidate to carry out reduction of MHB to Fe(II) state. The electron transfers to oxygen from heme-Fe(II) and from PHZ would form PHZR and Fe(III) within the heme cavity. Since PHZR formation is likely to occur in the vicinity of heme iron and it may be a better reducing agent than PHZ itself, rapid reduction of MHB to Hb should ensue (Shetlar and Hill, 1985).

Accompanying to the changes that occur in the hemoglobin, a variety of small molecules both stable and unstable have been shown to be formed in the reaction of oxyhemoglobin and MHB with PHZ. Nitrogen was identified as a final product in the reaction of oxyhemoglobin by Nizet (1946). The observations were confirmed by Beaven and White (1954) who also demonstrated that benzene was a final product in the reaction of oxyhemoglobin with PHZ. Benzene was also produced when MHB was reacted with PHZ in
presence of oxygen. The amount of benzene produced, in the oxyhemoglobin-PHZ reaction, was however found to be lower when the atmosphere above the reacting system was anaerobic (Beaven and White, 1954). Augusto et al. (1982) explained the partial stoichiometric relationship as follows:

$$6 \text{ PHZ} + 6 \text{ Oxygen} + 1 \text{ heme} \rightarrow 5 \text{ Benzene} + 1 \text{ heme}$$ (modified)

Goldberg et al. (1976) studied the kinetics of benzene formation in both the MHB-oxyhemoglobin and oxyhemoglobin-PHZ systems. They found that addition of catalase almost completely inhibited the reaction in the MHB system, indicating a role for scavengable $\text{H}_2\text{O}_2$ in the oxidative pathway leading to benzene formation. Catalase did not inhibit the corresponding reaction in the oxyhemoglobin-PHZ system. Incubation of carbomonoxyhemoglobin with PHZ under air also does not lead to benzene formation (Goldberg et al., 1976) suggesting the role of bound oxygen in the oxyhemoglobin mediated oxidation of PHZ to benzene.

Rostorfer and Cormier (1957) using a chemiluminescence technique to assay $\text{H}_2\text{O}_2$, provided the initial evidence that $\text{H}_2\text{O}_2$ was produced in reacting oxyhemoglobin-PHZ systems. The production of $\text{H}_2\text{O}_2$ itself in the reaction of oxyhemoglobin with PHZ was more definitively demonstrated by Cohen and Hochstein (1964). Both Rostorfer and Cormier (1957) and Cohen and Hochstein (1964) indicated $\text{H}_2\text{O}_2$ was formed when MHB and MHB-erythrocytes were incubated aerobically with PHZ. In both cases, however the authors pointed out that oxyhemoglobin produced by reduction of MHB followed by reoxygenation could be the actual generator of $\text{H}_2\text{O}_2$.

Goldberg and Stern (1975) were the first to report the
formation of superoxide anion both in oxyhemoglobin-PHZ and MHB-PHZ systems. In support of this Misra and Fridovich (1976) demonstrated that SOD inhibited the rate of reduction of nitroblue tetrazolium (NBT) in oxyhemoglobin-PHZ system. The mechanism of formation of superoxide anion in oxyhemoglobin and MHB system appeared to however differ. In case of MHB-PHZ system the generation of superoxide was a consequence of peroxidase activity of MHB on PHZ in presence of H$_2$O$_2$ (Goldberg et al., 1976) and catalase prevented the formation of superoxide (Goldberg and Stern, 1975). While SCN$^-$ is inhibitory to MHB-PHZ system, superoxide anion production was only partially inhibited in oxyhemoglobin-PHZ system. The SCN$^-$ was suggested to act as substrate for MHB peroxidase and to compete with PHZ (Goldberg et al., 1976). Similar observations were made using catalase which suggested that a pathway other than one involving MHB peroxidase may be operative in oxyhemoglobin-PHZ system. In this system the superoxide anion could be formed via phenyldiazenyl radical (PDAR) formed via loss of an electron from PDA to oxygen (Goldberg et al., 1976). Misra and Fridovich (1976) suggested that the formation of PHZR, a potential reducing agent that reacts with oxygen could be a precursor of superoxide radical.

Goldberg and Stern (1977) first suggested that the PR could play a role in the chemistry of hemoglobin-PHZ systems. Its formation in reacting oxyhemoglobin-PHZ and erythrocyte-PHZ systems was demonstrated by Hill and Thornalley (1981). The most likely immediate precursor of PR is phenyldiazeylen radical (PDAR) formed via loss of an electron from PDA to oxygen (Goldberg et al., 1976; Goldberg and Stern, 1977).

The brown coloration imparted to blood by PHZ and the brown green color given to erythrocyte suspensions by PHZ and
APHZ has been known since last century. Beaven and White (1954) noted that incubation of oxyhemoglobin with various phenylhydrazines led to alteration of the protein and its precipitation as "green hemoglobin". Because of the likely involvement of this substance in Heinz body formation, a significant amount of research effort has been devoted to understanding the nature of green hemoglobin and the processes leading to its formation. Ontizde Montellano et al. (1981) and Saito and Itano (1981) isolated protoporphyrin IX containing green pigment as the dimethyl ester by treating the product from reaction of oxyhemoglobin and PHZ with acidic methanol under air and used spectroscopic and mass spectroscopic techniques to make structural assignments. The compound was shown to be a N-phenylated porphyrin. The precursor of the N-phenylheme moiety is a globin-stabilized $\sigma$-bonded phenyliron complex (III) which rearranges in the presence of electron acceptors to give N-phenylated porphyrin (Shetlar and Hill, 1985; Beaven and White, 1954).

Several lines of evidence indicate that Hb does not interact chemically with PHZ in absence of oxygen. Beaven and White (1954) noted that benzene is not formed when PHZ is added to Hb under nitrogen, while Ontizde Montellano and Kunze (1981) under similar conditions showed that the chromophore of green hemoglobin was not formed as a product in Hb-PHZ system after reaction with acidified methanol. The latter product is formed in the oxyhemoglobin-PHZ system. Rostorfer and Totter (1956) and Itano and Robinson (1961) provided chemical and spectroscopic evidence for the lack of reaction between PHZ and Hb. Benzene and nitrogen are observed as final products in the reduction of MHB with PHZ in the absence of oxygen (Rostorfer and Totter, 1956).
Several reports on the in vivo and in vitro effects of PHZ on the rbc membrane lipids are available. Jain and Subramanyam (1976) observed intense reticulocyte response in PHZ treated rats that rose to nearly 100% after repeated injections of the drug. The reticulocyte obtained after PHZ treatment of rats were more osmotically fragile compared to those of the untreated animals. Reticulocytosis was accompanied by a progressive increase in total lipids, some phospholipids and neutral lipids. While the PS of the reticulocytes increased the PC content was lowered significantly.

In a subsequent study, Jain and Subramanyam (1978 a) reported that PHZ, under certain conditions that induce 100% reticulocytosis, causes TBA reactivity in circulating cells and more significantly in those obtained from spleen. This was inferred by the authors as evidence of PHZ effects on rbc membrane peroxidation and rapid uptake of the peroxidized cells by the spleen. Jain and Subramanyam (1978 b) also observed that PHZ-induced reticulocytes have shorter life spans than those formed in response to bleeding.

In a subsequent study Jain and Hochstein (1980 a) reported enhanced fluorescence in reticulocyte rich fraction from rats following PHZ administration but could not detect either Heinz bodies or methemoglobin in the cells. In addition decrease in spectrin polypeptides accompanied by formation of large molecular weight polymeric material was also observed. These workers suggested that PHZ localized at the membrane undergoes autooxidation to form species capable of initiating lipid peroxidation and the ensuing membrane alterations form the basis for enhanced sequestration and decreased survival of even reticulocytes in
absence of intracellular damage. Considerable lipid peroxidation also takes place in mature rbc deficient in glucose-6-phosphate dehydrogenase that result in their rapid sequestration. Rice-Evans and Hochstein (1981) demonstrated that treatment of hemoglobin-free erythrocyte ghosts with PHZ results in the peroxidation of membrane lipids and increase in microviscosity of the lipids and decrease in the fluidity of the membrane. Such alterations in the rbc membrane are known to facilitate rapid sequestration of the cells from circulation. Jain and Hochstein (1980 b) demonstrated that the in vitro exposure of rbcs to MDA, an important product of lipid peroxidation, causes the formation of fluorescent chromolipids characteristic of those produced during the peroxidation of membrane phospholipids as well as oligomerization of membrane spectrin. Comparable changes were also observed in membrane of older cell populations isolated from freshly drawn untreated blood, suggesting the role of products of lipid peroxidation in the in vivo rbc senescence and eventual sequestration. The reticulocytes of PHZ-treated animals also show comparable alterations (Hochstein and Jain, 1981). These reticulocytes have shorter half lives in circulation than the normal erythrocytes and reticulocytosis induced by bleeding. The authors suggest that both the young and old cells are effected by PHZ.

In a more recent study Jain (1988) provided further evidence for lipid peroxidation in senescent rbcs separated on the stractan density gradient into three main fractions. While the TBA reactivity of the various fractions was similar there was density dependent increase in phospholipid-MDA adduct and relative lipid fluorescence characteristic of MDA-phospholipid adducts. Age dependent increase in lipid peroxidation also appears to occur in bovine erythrocytes (Bartosz et al., 1982) and
as mentioned earlier in rat erythrocytes (Jain and Hochstein, 1980a). Jain (1988) attributed the enhanced susceptibility to lipid peroxidation of aged erythrocytes to lowered activity of enzymes that detoxify radicals generated by hemoglobin, like the glucose-6-phosphate dehydrogenase and glutathione peroxidase (Clark and Shohet, 1985; Bartosz et al., 1978; Glass and Gershon, 1984). Bates and Winterbourn (1984), in their in vivo studies on rabbits, however, observed that Heinz body formation was maximum just prior to cell destruction while lipid peroxidation was highest when circulating reticulocyte level was highest. These workers, contrary to the observations of Jain and Hochstein (1980b), suggest that lipid peroxidation occurs principally in the immature cells and hemoglobin denaturation presumably contributes more than lipid peroxidation to hemolysis.

The in vitro studies of PHZ on erythrocyte lipid peroxidation are more contradictory. While Jain and Subramanyam (1976) reported that comparable changes occurred in PHZ-treated mature rbc and reticulocytes, no detectable lipid peroxidation products could be observed in rbc treated in vitro with PHZ or APHZ (Goldberg and Stern, 1977; Hill and Thornalley, 1981).

Effect of PHZ on Erythrocyte Membrane Proteins

A significant number of studies are also available on the effects of PHZ and related hydrazines on erythrocyte membrane proteins both in vivo and in vitro. In view of lack of uniformity of the experimental conditions, considerable discrepancies, however, exist in the results that look at times contradictory.

Unequivocal evidence suggesting the formation of MDA in
rbc's treated with the PHZ and related compounds is available (Jain and Hochstein, 1980 b; Jain, 1988). Human erythrocyte exposed to MDA exhibit, in addition to the formation of fluorescent lipids (Jain and Hochstein, 1980 b), significant cross-linking of the spectrin polypeptides bands 1 and 2 and formation of large molecular weight aggregates. Density based separation of cells into various populations followed by their membrane polypeptides analysis revealed loss of bands 1 and 2 in the more dense older cells that also contained the large molecular weight adducts similar to those formed on MDA treatment. Platt and Falcone (1988) reported in more recent studies that comparable alteration in membrane polypeptides are also evident in human erythrocytes with several types of unstable hemoglobins. Jain and Hochstein (1980 a) also observed in an earlier study that reticulocytes formed in response to PHZ treatment in rats show the disappearance of bands 1 and 2 and formation of large protein adducts that failed to enter the gels on polyacrylamide gel electrophoresis performed in presence of SDS.

Reinhart et al. (1986) conducted a study of the in vitro effect of PHZ on rbc membrane proteins. A dose dependent retention of hemoglobin by the membrane, formation of hemoglobin dimers and trimers that were predominantly disulfide linked were observed. Formation of very little if any large molecular weight protein adducts detected in absence of DTT accompanied by the disappearance of bands 1, 2 and 2.1 were evident at higher PHZ concentrations. At very high concentration of PHZ band 6 also disappeared suggesting its complexing with other proteins or elution from the membrane. In addition, considerable smearing of various bands in the gel was markedly decreased on reduction of disulfides. The authors also noted a sharp band at the boundary of band 3 and faint band corresponding to Mr 260,000, the band 1
of spectrin (Reinhart et al., 1986). Earlier studies also indicated a high molecular weight band of Mr 260,000 in the membranes of APHZ-treated glucose-6-phosphate dehydrogenase deficient erythrocytes (Palek et al., 1978) and in the senescent erythrocytes isolated by density fractionation procedures (Snyder et al., 1983). In both these studies the complex was characterized as a spectrin-hemoglobin complex.

More direct evidence suggesting the formation of spectrin and hemoglobin complexes was presented by Sauberman et al. (1983) and Snyder et al. (1983) in human erythrocytes treated with H$_2$O$_2$. Unequivocal evidence supporting the formation of H$_2$O$_2$ in response to PHZ in human rbc is available (Cohen and Hochstein, 1964). The H$_2$O$_2$ exposed cells underwent echinocytosis, exhibited decreased membrane deformability and increased interaction with antiglobulin serum (Snyder et al., 1985). Prior treatment with carbon monoxide but not with the antioxidant BHT totally inhibited these changes suggesting the role of hemoglobin (Snyder et al., 1983). The protein complex formed could not be dissociated by disulfide reduction. Its formation could be prevented by blocking the free SH groups of spectrin but not hemoglobin, with N-ethylmaleimide (Sauberman et al., 1983). In a more recent study Snyder et al. (1988 b) have shown that pretreatment of intact erythrocytes with 0.1-0.2 mM methylmaleimide restricted lipid peroxidation and spectrin-hemoglobin cross-linking resulting from H$_2$O$_2$ treatment. The treatment also decreased the H$_2$O$_2$ induced alterations in cell shape and deformability as well as their recognition by the antiglobulin serum. Methemoglobin formation was, however, not affected. In view of the preferable reactivity of spectrin sulfhydryls, they were suggested to play an important role in hemoglobin oxidation-induced formation of spectrin-hemoglobin complexes and accompanying effects on membrane
properties (Snyder et al., 1988a).

Convincing evidence is available which suggests that cytoplasmic domain of band 3 protein contain binding sites for glyceraldehyde-3-phosphate dehydrogenase, aldolase, phosphofructokinase and hemoglobin (Juliano, 1973). Waugh and Low (1985) demonstrated that denatured hemoglobin binds with high affinity to the cytoplasmic domain of band 3, where it competes with glyceraldehyde-3-phosphate dehydrogenase. More recently Low et al. (1985), using immunoblotting studies, demonstrated that the bound denatured hemoglobin, because of its multiple binding sites, causes the clustering of band 3 albeit only a small fraction of the total band was cross-linked. Band 3 clustering caused either by or PHZ treatment results in a striking increase in the binding of autologous antibodies, that appear to play an important role in the recognition and uptake of damaged and presumably senescent erythrocytes from the circulation (Low et al., 1985).

Normal erythrocytes inhibited low level of proteolysis but exposure to oxidants seems to enhance the process (Goldberg and Boches, 1982). The system appears to preferentially degrade the oxidant damaged proteins (Davies and Goldberg 1987a; 1987b). Since circulating aged erythrocytes seem to contain lowered levels of antioxidant system (Clark and Shohet, 1985), oxidants induced proteinases were believed to be responsible for aging-induced decrease of most membrane polypeptide levels (Yamamoto et al., 1988). The therapeutic agents phenelzine, hydralazine also stimulate proteolysis in rbcs in vitro (Runge-Morris et al., 1988).

Activation of proteolysis in response to oxidative stress in general and hydrazines in particular has also received
considerable attention. Goldberg and Boches (1982) reported, based on their studies with rabbit reticulocytes, that protein oxidized either by PHZ or nitrite were rapidly degraded by ATP-ubiquitin dependent proteolytic system. The involvement of ATP-dependent proteolytic system in the intracellular degradation of puromycin altered hemoglobin and that containing amino acid analogues in reticulocytes was shown in an earlier study (Klemes et al., 1981). Arduni and Stern (1985) reported that PHZ-exposed erythrocytes underwent loss of spectrin network together with lipid packing at the outer leaflet without any gross modification of the membrane fluidity. In a subsequent study however, Fagan et al. (1986) provided evidence that an ATP-independent proteolytic system may be largely responsible for the degradation of PHZ-damaged hemoglobin in rabbit erythrocytes. A high molecular weight alkaline protease was shown to be involved (Fagan et al., 1986; Waxman et al., 1985). Fagan et al. (1986) suggested that the protease may interact with the other ATP-independent proteases like calpain and procalpain.

The calcium-stimulated procalpain-calpain system distributed widely in several animal systems have also been shown to be present in human and other rbcs (Pontremoli and Melloni, 1986, Melloni et al., 1982a; 1982b; 1984). The system has also been implicated in proteolysis associated with oxidative stress. Exposure of glucose-6-phosphate dehydrogenase deficient human erythrocytes to the major toxic component of the Favabean divicine resulted in calcium mobilization, calpain activation and release of an acid protease from the membrane (Morelli et al., 1987). The released acid endoprotease was shown to play an important role in proteolysis of oxidatively damaged proteins (Pontremoli et al., 1979, 1984). Morelli et al. (1987) demonstrated that calpain triggers the destruction of proteins respon-
sible for Heinz body formation in erythrocytes exposed to APHZ.

Runge-Morris et al. (1988) made a detailed investigation of proteolysis induced by various hydrazines in the erythrocytes and hemolysates incubated at neutral pH. Among the hydrazines investigated PHZ was most stimulatory followed by methylhydrazine, hydrazine and APHZ was least stimulatory. While the inhibitors of glycolysis restricted proteolysis minimally, those that inhibited electron transport were surprisingly more effective. Evidence was provided by the authors to show that hydrazine free radical, generated as a consequence reaction of hydrazines with oxyhemoglobin, damage protein that become more susceptible to cellular proteolytic enzymes.

Yamamoto et al. (1989) demonstrated that exposure of erythrocytes to PHZ for 24 hours resulted in over 40% activation and solubilization of a membrane associated protease characterized as cathepsin E. Binding of hemoglobin and presumably other membrane proteins onto the membrane preceded the effects on cathepsin E. The binding of hemoglobin appeared to be a prerequisite for the cathepsin activation and solubilization. The activated protease preferentially degraded spectrin polypeptides although significant degradation of band 3 was also observed. Comparable changes in membrane polypeptides were also observed by Yamamoto et al. (1989) in erythrocytes aged either in vivo or in vitro.

In spite of the acid pH range over which the isolated cathepsin E is active, it may play a significant role in proteolysis of the membrane since membrane associated form of the enzyme and that in presence of ATP acts more efficiently at neutral pH (Melloni et al., 1982 and Yamamoto et al., 1989).
Effect of PHZ on ATPase

Calcium-ATPase may be of crucial importance for the survival of the rbcs (Shalev et al., 1981). Calcium-ATPase contains one or more reactive SH groups, oxidation of which leads to complete inhibition of enzyme activity (Sarkadi et al., 1980). This enzyme normally functions to maintain a very steep gradient between external (10^{-3} M) and intracellular (<10^{-5} M) calcium (Schatzman, 1975) and collapse of this gradient is associated with decreased rbc deformability and premature destruction (Clark et al., 1981). Indeed the accumulation of calcium triggered by exposure of nucleated cells to a variety of toxins have been suggested as a final common pathway of cell death (Schanne et al., 1979). In view of this Shalev et al. (1981) hypothesized that oxidants such as PHZ might inactivate erythrocyte calcium-ATPase. Such inhibition would predispose the cell to accumulation of calcium. The resultant elevation of intraerythrocytic calcium might then be important in the genesis of oxidant-induced hemolytic disease.

Calcium-ATPase is readily inhibited by a variety of SH reagents (Sarkadi et al., 1980). PHZ causes inhibition of human rbc calcium-ATPase during in vitro incubations (Shalev et al., 1981). Even more striking inhibition of this enzyme was observed following administration of a small dose of PHZ to mice. This inhibition of enzyme activity was perceptible up to 1 week after PHZ injection, suggesting that the damage to the enzyme, at least to a certain extent, is irreversible. Accompanying this profound inhibition of calcium-ATPase activity, rbc calcium content rose to values 3-4 times than the normal value shortly after PHZ treatment. These increased rbc calcium levels persisted for at least two days (Shalev et al., 1981).
Objectives of the thesis

Populations in the civilized societies are exposed to several hydrazine derivatives that serve as fuels, antioxidants, anticorrosives, pesticides, dyes, explosives and drugs used in the treatment of tuberculosis, hypertension, anti-neoplastic agents and monoamine oxidase inhibitors. In general hydrazines also represent a class of agents recognized for their potential hemolytic and other toxic effects. Phenylhydrazine is one of the most reactive among hydrazine derivatives capable of causing several alterations in the erythrocytes both in vitro and in vivo. A large number of studies have described these effects and explain the phenylhydrazine action on the basis of its ability to react with hemoglobin and form highly reactive radicals. Few studies, however, suggest comparable effects of phenylhydrazine in hemoglobin-free erythrocyte ghosts. The objective of this work was to investigate and compare the effects of phenylhydrazine on intact erythrocytes and hemoglobin-free erythrocyte ghosts. The effects on lipid peroxidation, membrane sulfhydryls and polypeptide were investigated in detail.