INTRODUCTION
Introduction

"Councils to which time is not called, time will not ratify." The words come from an eminent stateman (E. R. Cutler, 1971), but they are no less relevant to the experience of biologists. Dealing as we do with types of biological organisation that are the products of vast periods of evolutionary history, it is both our privilege and our duty to explore the dimension of time and in doing so to enlarge our understanding of our own organisation, and that of all the biological systems which surround us and interact with us.

The picture that emerges from the research data obtained in favor of one of the omnipresent environmental pollutant, phenylhydrazine, is of the role of hydrazines as a class of compounds widely used in laboratory (to induce anemia and reticulocytosis), industry (as rocket propellants) and human therapy (as antihypertensive and antineoplastic agent). The metabolic activation of hydrazine seems to generate radical agents responsible for several toxic effects, including carcinogenesis (Prough and Moloney, 1985). Many of the applications of hydrazine, especially as rocket propellants and in medicine, give them considerable toxicological importance. Hydrazine is also an agricultural and environmental contaminant frequently found in the herbicide as maleic hydrazide which may be responsible for its toxic effects. Among others the most striking toxic effects of a number of hydrazine derivative appears to be hemolysis and the structural requirements for their hemolytic action have been demonstrated on red blood cells (Flores and Frieden, 1970). For maximum hemolytic activity, hydrazine should be monosubstituted preferably with an aromatic group, such as a phenyl ring, and certain substitution like halogenation, methylation or nitration enhances the toxicity with the potentiation of their hemolytic action.

Hemolysis:

Phenylhydrazine is one of the most extensively studied compounds, and has been identified as a well known and effective hemolytic agent that is routinely used for inducing anemia in experimental laboratory animals (Domfest et al., 1983; Erslev, 1964; Rothberg, ...
1959; Saterborg, 1974). Phenylhydrazine, owing to its hemolytic effects, was at one time used in the treatment of polycythemia vera (Altnow and Carey, 1926; Brown and Giffin, 1926; Owen, 1924). However, because of its potential toxicity it has long been discontinued for the treatment of this disorder. Phenylhydrazine and its derivatives are well known agents that induce deleterious changes of red blood cell properties and lead to hemolysis and/or phagocytosis. It is generally assumed that all of these changes are mediated by formation of superoxide, hydrogen peroxide and radicals derived from phenylhydrazine (Augusto et al., 1982). However, the precise mechanism of destruction of phenylhydrazine - damaged erythrocytes has not been completely elucidated. Erythrocytes altered by phenylhydrazine administration are removed from the circulation by the reticuloendothelial system, primarily within spleen and to a lesser extent within the liver indicating a extravascular mechanism of destruction (Azen and Schilling, 1964; Iyengar and Gupta, 1973; Rifkind, 1966).

Phenylhydrazine has been reported to cause direct lysis of erythrocytes (Beutler et al., 1955; McLissac et al., 1958). This compound has been reported, among other mechanism, to alter the red cell membrane integrity by thiol oxidation (Jacob and Jandl, 1962). Exposure of erythrocytes to oxidative stress may cause oxidation that lead to deleterious changes of properties (Palek and Lux, 1983). The targets for these oxidations may be membrane lipids (Flynn et al., 1983), sulfhydryl groups of membrane proteins (Allen et al., 1978), Fe (II) ions, and sulfhydryl groups of hemoglobin (Dormandy, 1971; Gordon-Smith and White, 1974).

Reaction of phenylhydrazine with oxyhemoglobin leads to formation of methemoglobin, oxidation to hemicrme and other denatured derivatives and causes Heinz body formation (French et al., 1978). Hemoglobin precipitation in the form of Heinz bodies within the red cell causes hemolytic anemia (Beaven and White, 1954; Itano et al., 1977; Peisach et al., 1975). It has been shown that reactive intermediates such as
phenyldiazine and phenyl radicals are formed upon oxidation of phenylhydrazine by oxyhemoglobin. These radicals have all been implicated in its cytotoxicity (Misra and Fridovich, 1976; Hill and Thomalley, 1981).

Hemoglobin-catalysed phenylhydrazine oxidation, a model for Heinz body hemolytic anemias, is known to result in hemoglobin degradation and erythrocyte lysis. The catalytic reaction is terminated by inactivation of the prosthetic heme groups after each heme moiety catalyses the consumption of six oxygen molecules and six phenylhydrazines, and the formation of five benzenes. Analogous reactions are observed with substituted phenylhydrazines and to a lesser degree due to their slower oxidation, with alkylhydrazines. Ortho-substituted phenylhydrazines do not give the N-aryl heme derivatives even though they inactivate the hemoproteins (Augusto et al., 1982). Heinz body formation, autolysis of hemoglobin, interaction of the thiol groups between denatured hemoglobin and a type of red cell spectrin, appears to be the primary events that lead to fragility of the cell membrane and hemolysis.

Thus treatment of erythrocytes with phenylhydrazine results in a variety of hemoglobin denatured products (met-hemoglobin, reversible and irreversible hemichromes, sulphotemoglobin, Heinz bodies) a methemoglobin-phenyldiazine complex, a complex of reactive intermediates of phenylhydrazine and heme, changes in erythrocyte membranes such as spectrin degradation, protein cross-linking and band three clustering, lipid peroxidation as well as alteration of cellular thiol status by glutathione oxidation, depletion of ATP stores and inactivation of enzymes (Ortiz de Montellano and Kerr, 1983). Incubation of red cells with phenylhydrazine for 1 hr. has been found to cause hemolysis of 50% of the cells (Mc Issae, 1958). Experiments performed in vivo demonstrates that erythrocytes although not directly lysed by phenylhydrazine, are rapidly removed from the circulation by macrophages after exposure to this drug. This event occurs primarily in the spleen, although the liver becomes increasingly more important in the sequestration of altered
erythrocytes as the dose of phenylhydrazine is increased (Azen and Schilling, 1964). In this regard phenylhydrazine is a potent oxidant that may cause erythrocyte membrane damage via interaction with thiol group (Jacob and Jandl, 1962). In addition to all these events, interaction of phenylhydrazine with hemoglobin produces generation of acid soluble peptides (Domenico et al., 1989). It has been reported that hemoglobin oxidised by phenylhydrazine is more susceptible to proteolytic degradation by erythrocyte proteinases (Goldberg and Boches, 1982). Protein fragmentations have been observed in the course of irradiation producing highly reactive free radicals such as hydroxyl and superoxide (Wolff et al., 1986). The reaction of phenylhydrazine with oxyhemoglobin is extremely complex, since, in addition to end reaction compounds (nitrogen and benzene) several intermediate products are generated as phenyl diimide, phenylhydrazyl radical, phenyl radical, as well as different by side products such as superoxide, hydrogen peroxide and probably hydroxyl groups (Beaven and White, 1954; Itano, 1970; French et al., 1978; Hill and Thornalley, 1981; Goldberg and Stem, 1975; Cohen and Hochstein, 1964; Sadrzadeh, 1984).

Leucocytosis:

Long-term phenylhydrazine treatment causes pronounced anemia and a concomitant increase in the numbers of circulating leucocytes in Long-Evans rats (Naughton et al., 1990), the leucocytosis is caused mainly by an elevation in mononuclear cells, most notably in the lymphocyte population. Phenylhydrazine has been reported to cause the direct lysis of erythrocytes by non-immune mechanisms (Mc Issaq et al., 1958). However, recent reports indicate that phenylhydrazine can cross-link red cell band three protein (senescent antigen), resulting in binding of autologous immunoglobin G (IgG) (Miszta, 1987).

Enzymatic changes in RBC:

The activity of various enzymes in red blood cells is also significantly altered in phenylhydrazine mediated insult (Scheuch and Rapoport, 1960; Ortiz De Montellano and
Phenylhydrazine significantly influences energy metabolism of red cells. It stimulates aerobic glycolysis and glucose utilization via the oxidative pentose phosphate pathway and provokes alterations of energy state in the cells (Kostic, 1989). Phenylhydrazine may induce oxidative damage to the enzymes of the glycolytic chain in the red blood cells (Scheuch and Rapoport, 1960), but increased lactate and pyruvate formation, unchanged level of 2,3-BPG and stimulation of oxidative pentose phosphate pathway indicate that the whole glycolytic sequence is preserved and the enzyme inactivations occurs within regulatory limits.

Alterations of ion-permeability in phenylhydrazine-treated red blood cells have been demonstrated (Shalev et al., 1981; Orringer, 1984). Although their cause is still not known, they might be explained by membrane changes, e.g., by phenylhydrazine-induced lipid peroxidation (Bates and Winterboum, 1984) and changes of membrane phospholipid composition (Arduini et al., 1986).

Heinz body formation:

Phenylhydrazine and acetylhydrazine are typical of a wide range of drugs that react with oxyhemoglobin via a redox mechanism that causes Heinz body formation and red cell hemolysis. In addition to causing irreversible oxidation of hemoglobin to hemichrome and other denatured derivatives, these drugs give rise to superoxide, hydrogen peroxide and drug-free radical intermediates (Cohen and Hochstein, 1964; Misra and Fridovich, 1976; Valenzuela, 1977). Heinz body formation is the most obvious effect of these drugs, and red cells containing Heinz bodies are readily removed from circulation by the spleen (Rifkind, 1965).

Iron release:

Oxidizing agent such as phenylhydrazine induces iron release from iron complexes in erythrocytes. The release of iron is accompanied by methemoglobin formation (Goldberg
et al., 1976). Also it should be considered that H₂O₂ which can easily be formed in erythrocytes challenged with phenylhydrazine and other oxidising agents can release iron from hemoglobin (Gutteridge, 1985; Puppo and Halliwell, 1988). In the case of phenylhydrazine, oxidase and peroxidase reactions of oxyhemoglobin and methemoglobin occurs that lead to formation of both O₂⁻ and phenylhydrazine radicals (Phenylhydrazine and phenyl radicals, Maples et al., 1988). These radicals can denature the hemoglobin (Stern, 1989). Iron is probably released from denatured hemoglobin and can induce lipid peroxidation if the cell is depleted of GSH. Lipid peroxidation is always associated with hemolysis. GSH, in turn, when present in sufficient amount, can prevent lipid peroxidation even in the face of substantial iron release, possibly by regenerating α-tocopherol in the membranes (Reddy et al., 1982). GSH may also have a role in the prevention of iron release by removing H₂O₂, by decreasing autoxidation of redox agents and by interacting with the radicals generated from the drugs.

**Deformity and life span of RBC:**

In another logic drug-induced shortening of erythrocyte life span is generally thought to occur by one of the two mechanisms. Chemicals may associate primarily with plasma membrane and cause a perturbation of structure which leads to enhanced permeability and osmotic swelling, alternatively, they may penetrate intracellular space either directly or through their metabolites, alter cellular constituents and metabolic pattern. Phenylhydrazine is commonly thought to affect erythrocyte survival by the latter mechanism. Thus administration of phenylhydrazine in vivo may lead to the production and accumulation of hydrogen peroxide in amounts above the detoxifying capacity of cellular protective mechanisms (Cohen, 1965). Thus the hemolytic effects of phenylhydrazine, a reducing agent, may be viewed as a consequence of its autoxidation and the subsequent oxidation of the essential sulfhydryl groups of the enzymes and membrane proteins, oxidation of hemoglobin, and the initiation of peroxidation reactions in membrane phospholipids. Such effects would be more pronounced in older cells with a
diminished capacity to detoxify peroxide via the glutathione peroxidase pathway. It is also reported that the reticulocytes produced as a result of phenylhydrazine treatment of animals contain decreased amount of spectrin polypeptides and increased amount of high molecular weight membrane proteins (Jain and Hochstein, 1980). Additionally, the presence of fluorescent chromolipids associated with the peroxidation of membrane phospholipids is enhanced in these cells (Bidlack and Tappel, 1973).

These observations suggest that oxidative cell membrane alterations may be sufficient to explain the decreased survival of both reticulocytes and old cells exposed to phenylhydrazine. Spectrin is a major protein constituent of erythrocyte membranes and is presumably involved as a determinant of cell shape and deformity (Greenquist and Shohet, 1975). A decrease in the spectrin content and increase in high molecular weight protein in the membrane, and the polymerized products of membrane lipid peroxidation might well be expected to alter the rigidity and the life span of affected cells.

Association with immune system:

Recent studies have provided evidence that immune system gets activated during phenylhydrazine-induced anemia (Domfest et al., 1983). This is characterized by a rapid increase in the serum immunoglobulin titers, B lymphocyte population of the peripheral blood and spleen with alteration in the ratio of T helper to T suppressor cells. A parallelism between the onset or degree of phenylhydrazine-induced anemia and rise in IgG levels, prominent peripheral lymphocytosis and the shift in the lymphocyte subpopulations, have been demonstrated. Further, histologic studies showed the presence of blastogenic lymphoid cells in the lymph nodes and spleens of phenylhydrazine-treated rats (Domfest, 1986). Light microscopic studies demonstrated an enhanced lymphopoiesis in the lymphoid tissues and bone marrows of phenylhydrazine-treated rats and the appearance of atypical and blastic lymphoid cells in the peripheral circulation. It has also been found that chronic phenylhydrazine administration caused an elevation in prostacyclin and prostaglandin E2
levels and promoted a state of transient thrombocytopenia (Domfest et al., 1990). All these events have been associated with immune activation and the generation of drug-induced hemolytic anemia by immune mechanisms. Multiple injections of dexamethasone to rats receiving phenylhydrazine at a dose known to induce anemia suppressed the anemic response of the drug and confirmed that the anemia was associated with immune activation (Domfest et al., 1992).

**Toxic effects on different vertebrates/different organs:**

Phenylhydrazine hydrochloride lyses the erythrocyte nuclear membrane and then develops anemia by damaging the plasma membrane in chickens (Zentgraf et al., 1974). Phenylhydrazine in doses comparable to those used in mammalian systems produces a rapid hemolysis and total anemia in amphibia without jeopardising their survival and normal behaviours (Flores and Frieden, 1968; Frangioni et al., 1972). The effect of phenylhydrazine hydrochloride in causing anemia in fish, Florida gar, and Lata has also been reported (Mcleod et al., 1978; Bhattacharya, 1981).

All homeotherms and poikilothersms thus far studied regenerate erythrocytes and hemoglobin in response to anemia caused by phenylhydrazine-induced hemolysis or by bleeding (Zentgraf and Franke, 1974; Grasso, 1973). Injections of phenylhydrazine increases the number of reticulocytes in different animals (Jain and Subrahmanyam, 1978; Maruyama and Yamada, 1977). It has been found that the effect of phenylhydrazine is prevalent on mature red cells and not on the reticulocytes or on red cell precursors as red cell regeneration occurs along with the breakdown of red cells in the later period of hemolysis (Iyengar and Gupta, 1973). But the reticulocytes formed in animals in response to phenylhydrazine have a shorter life span than formed as a consequence of bleeding (Jain and Hochstein, 1980).

Phenylhydrazine-induced hemolysis and spleen enlargement with concomitant
increases in specific activities of glucocerebrosidase and arylglucosidase in liver and spleen (but not in kidney) have been studied (Hara and Radin, 1979a; Hara and Radin, 1979b). In adult mice suffering from a phenylhydrazine-induced hemolytic anemia, erythropoietic islands have been observed in liver, and within 2 days after beginning of 4 daily injections of phenylhydrazine, erythroid elements appear in the sinusoids and central veins (Ploemacher and Van Soest, 1977).

The structural and biochemical changes in liver, spleen and other organs in phenylhydrazine treated animals have been reported. The appearance of proliferating and non-proliferating CFU-S (colony forming unit stem cell) in bone marrow and spleen respectively of phenylhydrazine-treated mice has been observed (Wright and Lord, 1977). The increased activity of nuclear RNA polymerase of lymphoid and erythroid rich mouse spleen has been found in PHH-induced anemia (Spivak, 1973).

Hydrazine sulphate displays either a differential effect on RNA biosynthesis or diminution. In new born mice this drug stimulates DNA biosynthesis in liver and kidney tissues, but inhibits the same in lung tissues (D'Souza, 1975a). But Hydrazine sulphate does not affect RNA biosynthesis in liver and lung of normal adult mice (D'Souza, 1975b). Differential effect on RNA levels in rat tissues has been reported in case of hydrazine (Banks et al., 1967). They have reported an increase in RNA levels of liver tissue but have observed no effect on kidney and ovary of rats treated with hydrazine. Hydrazine sulphate inhibits DNA biosynthesis in all the tissues of new born mice, lung or kidney tissue of adult mice and lung and tumor tissue of tumor bearing mice.

Hepatotoxic effects of hydrazine on glycogen and lipid metabolism have been established both biochemically and histologically. Increased glycogenolytic activity by hydrazine has been found (Fertney et al., 1967). Decrease in glycogen content by hydrazine and its derivatives has been demonstrated (Ray, 1970). In acute and chronic
intoxication by hydrazine hydrate in white rats and rabbits considerable depletion of liver 
glycogen reserves has been observed. Accumulations of lipids are more marked in liver and 
kidney with high dose of hydrazine in animals (Black, 1965). Accumulation of lipid in the 
liver, myocardium, kidney and skeletal muscle after hydrazine treatment has been found in 
monkeys. Serious damage and fatty degeneration of the liver in monkey has been observed 
after hydrazine treatment (Patrick, 1964).

Earlier studies indicate a hypoglycaemic effect of hydrazine on different animals 
(Underhill and Karelitz, Jr., 1923). The fasted dogs injected with hydrazine show a 
hypoglycaemia which continues till death of the animals (Fortney, 1982). Poisoning with 
phenylhydrazine causes a long term hyperglycaemia (Malachovskis, 1964).

Endocrine Association:

Although much is known about the role of hormones in the erythropoiesis in 
vertebrates since decades before, mediation of the hormones either for potentiation or 
counteraction of the hemolytic stress during PHH insult has not yet been studied 
substantially.

Castration of male animals leads to a mild anemia which can be reversed by the 
administration of androgens (Blacher, 1926; Shirakura, 1961). The anemia produced by 
hypophysectomy was also corrected by the administration of androgens (Crafts and 
Meineke, 1957). In contrast to the effect of castration on male animals, castration of female 
animals showed increased erythropoiesis (Steinglass et al., 1941).

When physiological amounts of estrogens were administered to castrated females 
the blood counts declined to values that were normal for females (Vollmer and Gordon, 
1941). The fact that hypophysectomy produces a moderate anemia has been known for 
many years and removal of the posterior and intermediate lobes of the pituitary did not
produce anemia which was, therefore, related to the function of the anterior lobe (Van Dyke et al., 1952). ACTH, growth-hormone, and TSH increased erythropoiesis in normal or hypophysectomized animals (Van Dyke, 1959; Arizzone et al., 1966; Fisher and Crook, 1962).

Ovine prolactin has been found to increase erythropoiesis in normal (Jepson and Lowenstein, 1966), polycythemic and orchidectomized mice (Jepson and Lowenstein, 1964). Thyroidectomy or reduced thyroid function has been found to lead to a moderate anemia which can be corrected by thyroid hormone administration (Hollander et al., 1967).

It was postulated that the anemia following thyroidectomy and the increased erythropoiesis following thyroid administration were due to a general effect on oxygen consumption and the metabolic rate, which in turn regulated the production of erythropoietin (Jacobson et al., 1959). Thyroid hormone appeared to increase erythropoiesis through an effect on erythropoietin production as judged by the lack of response of nephrectomized animals to thyroid hormone (Carnevali et al., 1968). Antiserum to erythropoietin completely blocked the effect of triiodothyronine on increased erythropoiesis (Fisher et al., 1967).

Earlier evidences demonstrated that 17-hydroxycorticosterone or desoxy-corticosterone increased erythropoiesis in the hypophysectomized rat (Fisher and Crook, 1962; Gley and Delor, 1955). Adrenal corticoids appears either to stimulate or suppress erythropoiesis depending on the status of the animal and dosage of hormone administrated.

Role of ACTH on stimulating erythropoiesis is supposed to be mediated via adrenocortical secretion since adrenalectomy abolishes the effect of ACTH (Van Dyke, 1959). Norepinephrine has been found to cause renal hypoxia by constriction of the afferent renal blood vessels which led to a secondary renal ischemia and increased
erythropoietin secretion (Fisher et al., 1968). When the 11-oxygenated cortical steroid, methyl prednisolone acetate (MPA) was administered in large doses to normal mice, it decreased erythropoiesis and inhibited the erythropoietic response to hypoxia (Gordon et al., 1967). This was mainly due to a depression of the marrow response to erythropoietin as demonstrated in MPA-treated plethoric mice which showed a reduced response to the injection of erythropoietin (Gordon et al., 1967).

The hemopoietic populations are organised into a developmental sequence in which multipotent stem cells generate lineage specific committed progenitor cells which in turn produce the dividing and maturing cells in that lineage (Metcalf, 1984). Control of these processes is achieved by the use of a double control system: (1) Local control by specialised stromal cells in the marrow, (2) Control by a group of molecular regulators acting via specific membrane receptors on target cells. The hemopoietic cells require continuous stimulation by appropriate regulators for cell divisions. The marrow stromal cells can produce a number of hemopoietic regulatory factors including interleukin-1 (IL-1), granulocyte-macrophage colony-stimulating factor (GM-CSF), granulocyte colony stimulating factors (G-CSF), macrophage colony stimulating factor (M-CSF), IL-4, L-6, IL-11, IL-9, stem cell factor (SCF), and leukemia inhibitory factor (LIF).

Carnot and Deflandre (1906) had the original idea that blood contains a humoral factor which stimulates erythropoiesis and termed it as "hemopoietine". However as work proceeded it appeared to be involved exclusively in red cell production and "erythropoietin" became the adopted name (White et al., 1960).

The important molecular regulator acting via specific membrane receptors on target cells is erythropoietin (Ep). This hormone is secreted as glycoprotein of molecular weight 34,000, from kidney due to anoxic stimulus. Production of Ep by the rat kidney under hypoxic condition is the result of enhanced mRNA expression rather than release of
preformed erythropoietin (Daynes et al., 1990; Blauer et al., 1991).

The molecular mechanism by which hypoxia or other stimuli such as cobalt induce erythropoietin expression are uncertain but it has been suggested that the oxygen sensor may be a heme-protein (Görgberg et al., 1991). In animals the injection of Ep increases iron incorporation into red cells, induces an elevated level of reticulocytes and mature red cells, and increase the number of erythroid precursors (Spyrk, 1986). In cultures of mouse bone marrow or fetal liver cells erythropoietin stimulates proliferation of mature erythroid progenitors (CFU-E) to form small erythroid colonies.

Matoth et al. (1962) found an increased mitotic index of erythroblasts in vitro, when the cells were treated with plasma from anemic animals or sheep erythropoietin. One of the earliest events to follow an increase in RNA synthesis by marrow cells at the introduction of erythropoietin is an increase in total $^{59}$Fe incorporation in marrow cells (Erslev, 1962). Evidences suggest that delta-aminolevulinic acid synthetase (ALAS) may be a rate-limiting enzyme for heme synthesis in mammalian marrow cells. An increase in ALAS following the administration of erythropoietin could be blocked by actinomycin D showing that a nuclear-mediated event leading to hemoglobin biosynthesis is one of the targets of erythropoietin (Nakao et al., 1968). In some recent studies the role of erythropoietin on the red cell membrane structure has been evaluated (Chaudhuri et al., 1980; Chakraborty et al., 1986; Chattopadhyay et al., 1992).

**Purpose of the present thesis:**

Most of the studies undertaken by the previous workers to demonstrate the toxic effects of PHH, have been carried out using adult animals. No thorough investigation has been made on juvenile (sexually immature) animals to understand whether the juvenile animals respond equally to the toxic effects of PHH like the adults or whether they respond in a different manner. The literature show that very few attempts have been made to
identify the potentiating or protective role of hormones to the toxic effects of PHH. As thyroid hormone appears to be very much tricky in the sense that it affects almost all the metabolic processes in the mammalian vertebrates we were very much tempted to evaluate the role of this hormone altering the toxic effects of PHH, if any.