CHAPTER - 4

Discussion
Exposure of humans to high amounts of fluoride for prolonged periods results in a debilitating condition clinically referred to as 'Fluorosis'. Endemic fluorosis occurs in geographic areas when natural water contain high concentration of fluoride. Shortt et al. (1937) recorded the incidence of fluorosis, dental and skeletal for the first time from India in Nellore district of Andhra Pradesh. Subsequently many pockets of endemic fluorotic areas have been identified in different states of India (Pandit, 1940; Daver, 1945; Siddiqui, 1955; Kashiwal and Solomon, 1959; Singh et al., 1961; Singh and Jolly, 1961, 1970; Jolly et al., 1969; Teotia et al., 1969, 1971; Ramamohana Rao, et al., 1974; Krishnamachari, 1978; Sidhu et al., 1985).

Studies carried out since 1973 have shown that in some parts of this country the appearance of newer manifestation of fluorosis in the form of endemic 'genuvalgum' (Krishnamachari and Kamala Krishnaswamy, 1973, 1974; Krishnamachari, 1974).

The disease is prevalent in endemic forms among communities which habitually consume high fluoride (2 - 16 ppm) containing subsoil water obtained from wells. The population of the village 'Ralla Anantapuram' have been depending solely on five wells, three bore wells
and two open dug wells, for drinking and cooking purpose, whose fluoride content ranged from 7.2-10.7 ppm (Table 1). Hence, majority of the population of the village are exposed since birth to toxic levels of fluoride exposing to the deleterious effects manifesting in dental and skeletal fluorosis. However, the symptoms and severity was less among female members who migrated to this village after their marriage with local residents.

Epidemiological data in this study of the village pertained to duration of residence in the endemic area which leads to fluorosis, age of on-set of pathological conditions, literacy, socio-economic and nutritional status and clinical symptomology.

The earliest evidence of dental fluorosis was observed among children around 6-10 years of age (Table 4) and skeletal fluorosis around 11-20 years of age (Table 6). As age advanced the manifestations of skeletal fluorosis became more evident, restricting physical movements and impedment for walking. The more severe effects are predominated in males (Table 7).

The village is located in semiarid zone with a day temperature ranging from 35-42°C for most part of the year. The population of the village are manual
labourers either working in the fields or in lime-stone kilns. Owing to the hot climatic conditions and their occupation, they consume 4-6 L (equivalent to 36-54mg F/day) of water per day and loss of water is more through sweat. Fluoride with in the range of 0.5 to 4mg/day has been tentatively recommended as safe adequate level for adults (Smith, 1986). Sweat fluoride concentration is lower than that of plasma and is not markedly influenced by fluctuations in plasma fluoride concentration (Henschler et al., 1975) and is considered to be of minor importance as a route for fluoride elimination (WHO, 1984). Excretion of fluoride occurs primarily via the urine which accounts for 90% of the total excretion (Largent, 1961; Hodge et al., 1970; Whitford and Pashley, 1979; Messer, 1984). Fecal excretion accounts for the bulk of the remainder (Hodge et al., 1970; US NAS, 1971; Messer, 1984). Thus the population of the village are exposed to greater risk due to higher retention of fluoride.

Earlier studies have indicated the incidence, and severity of chronic fluoride intoxication are greatly influenced by socio-economic, climatic and nutritional status; being higher in poorer segments of population with nutritional deficiency signs (Galagan et al., 1957;
Showley et al., 1966; Reddy and Srikantia, 1971; Krishnamachari and Kamala Krishnaswamy, 1973, 1974; Krishnamachari, 1978; Parker et al., 1979; Teotia and Teotia, 1984, 1988). Several studies have also identified the influence of staple diet on fluoride toxicity (Pandit et al., 1940; Lakshmaiah and Srikantia, 1977; Krishnamachari, 1978).

Sorghum and bajra based diets have shown to result in higher retention of fluoride than rice based diet (Krishnamachari, 1978). The affected population of the village consume mainly ragi based diet and rice is consumed on rare occasions like feasts.

The variation of the hematological parameters; hemoglobin, erythrocyte population, PCV, MCV and MCH (Tables 9-13) observed in the present study in subjects with fluorosis points to the incidence of hypochromic microcytic anemia. The effect of fluoride on hematological indices in laboratory animals has received considerable attention in the literature (Agate et al., 1970). Ginn and Volker (1944) observed a reduction of hemoglobin in rats which received 50 ppm of fluoride as NaF in water daily for 150 days. Valja Vec (1932) noted a slight reduction in hemoglobin level in rabbits receiving 10 to 30 mg NaF/kg for 151 days. Hirao (1972)
pointed out changes in hemoglobin, erythrocyte population and hematocrit in experimental animals maintained on high fluoride intake. Susheela and Jain (1983) suggested the involvement of adrenal cortex in hematological changes observed in fluoride intoxicated rabbits. However, in humans the variations in hematological parameters appears to be not consistent. Roholm (1937) noted a slight reduction in number of erythrocytes but the hemoglobin level being unaffected. In a study of 55 cases of fluorosis by Singh et al. (1965) revealed, the hemoglobin levels varied from 8 to 15.5 g/100 ml. However, Uslu (1981) did not observe any change in hemoglobin and hematocrit values in human subjects in fluorotic area. The information provided so far by different workers is inadequate to offer a scientific rationale for the wide spectrum of deviations observed both in experimental and human fluorosis.

The most common cause of anemia in underdeveloped and developing countries is dietary deficiency of iron. The serum iron levels and iron-binding capacity (Table 15) did not show any marked variation in fluorotic subjects indicating no defect in the transportation of iron in these patients. The levels of serum iron is a reflection of the body status of iron, which inturn is
balanced by the dietary availability and absorption on the one hand and the rate of utilization for the synthesis of iron porphyrins, mainly hemoglobin. The utilization of iron for heme synthesis requires the participation of copper and zinc. Copper, zinc and iron are the three essential micronutrients involved in various anemic manifestations (Prasad, 1978; Singh and Kanwar, 1981a).

According to Ruliffson et al. (1963) and Wegner et al. (1976) fluoride enhances absorption of iron in animals. In experimentally fluorosed rats Kahl et al. (1973) observed a decrease in 59Fe incorporation in the blood with concomitant 59Fe uptake by bone marrow and liver. Singh and Kanwar (1981a) noted the storage of iron in the bone and liver in mice following fluoride administration implying that the iron uptake is in excess of its utilization and this is explained on the basis of concomitant depletion of copper and zinc from the tissues following fluoride administration. Benard et al. (1958) explained anemia in fluorosed rabbits due to the inhibition of 59Fe and glycine incorporation into protoporphyrin, a precursor of hemoglobin and not due to want of iron.
Inverse correlation between the per cent prevalence of genuvalgum in a village of endemic fluorosis and the concentration of copper in drinking water have been noticed by Krishnamachari (1976).

The levels of copper and zinc were reported to be lower in bone samples of fluorotic patients. Thus it appears that subjects with fluorosis appear to suffer from copper and zinc deficiency rather than iron deficiency in erythropoietic tissues, resulting in defective utilization of iron for hemoglobin synthesis (Singh and Kanwar, 1981a; Singh, 1984). However, the serum copper level was reported to be normal in subjects with endemic genuvalgum, suggestive of homeostatic mechanism operating in the subjects inspite of copper deficiency (Krishnamachari, 1982).

It is also pertinent to note that food grains habitually consumed by humans with fluorosis have higher amounts of molybdenum in them. Sorghum, pearl millet and ragi grown and consumed in endemic areas of fluorosis contained higher amounts of molybdenum than that grown in nonfluorotic areas in India and also rice grown in endemic areas (Deosthale et al., 1977). The population of the village surveyed and the subjects undertaken for the present study consume ragi as staple
diet, a point which should be taken into consideration. Well known relations exist between high molybdenum intake and association with increased excretion of copper (Underwood, 1977).

Human blood is believed to contain two forms of fluoride namely 'exchangeable' and 'nonexchangeable' (Taves, 1968) or 'ionic' and 'nonionic' (Venkateswarlu et al., 1971). Essentially one form is recognised as inorganic fluoride and the other as covalently bound organic fluoine (Yamamoto et al., 1989).

Estimation of relative distribution of fluorine in plasma and red cells and also the fraction associated with inorganic and organic are varied (Paez et al., 1980; Bourbon et al., 1984; Jacyszyn and Marut, 1986; Yamamoto et al., 1989). Recent study by Jacyszyn and Marut (1986) indicated the mean fluoride concentration in erythrocytes is about six times higher than that in blood serum. However, estimations of Yamamoto et al. (1989) indicated the distribution of total fluorine serum vs clot was 3:2 and the fraction of nonionic fluoride in whole blood appeared to be 74%.

Guy et al. (1976) isolated and characterized the compounds that comprise the major portion of the nonionic fluorine fraction of human serum and found them
to be predominantly perfluoro-fatty acid derivatives containing six to eight carbons. However, the data on change in nonionic (organic) component and their nature is lacking although, the ionic form appears to predominate with higher intake of fluoride (Guy et al., 1976).

Fluorine, ionic or nonionic may associate with plasma membrane and may cause perturbation in structure which leads to enhanced permeability and osmotic swelling. Alternatively, it may penetrate into cellular space and either directly or through its metabolites, alter cellular constitution and metabolism.

The capacity of erythrocyte to survive in the circulation is generally thought to be consequence of factors that affect their mechanical properties. Determinants of cellular deformability include extrinsic properties eg. cell shape, and the surface area/cell volume ratio, and intrinsic properties eg. internal viscosity and membrane mechanical behaviour (LaCelle and Weed, 1971; Corry and Weiselman, 1978; Pfafferott et al., 1982). Among the biological processes likely to affect the mechanical behaviour of the plasma membrane are those which involve the change in lipid composition and peroxidation of endogenous membrane phospholipids.
The observed changes of erythrocyte membrane lipid profile in chronic fluorosis (Table 17), increase in both cholesterol and phospholipids, indicate a compensative mechanism operating in erythrocytes of fluorosed humans to resist the fluidizing effect of fluoride, and also possibly reflect a biochemical change in membrane composition.

Cholesterol, one of the major lipid constituent of the erythrocyte membrane, interacts with different classes of membrane phospholipids and modulates the membrane fluidity and consequently the membrane function (Suhail et al., 1988). Changes in erythrocyte membrane cholesterol level affect cell fragility (Cooper et al., 1975), membrane microviscosity (Borochov et al., 1979) and transport function (Grunze et al., 1980).

Red cell membranes are labile to lipid peroxidation (Hochstein, 1966). Owing to their content of polyunsaturated lipids and to the fact that they are directly exposed to molecular oxygen and involves the generation of free radical intermediates and to produce semistable peroxides (Tappel, 1973).

The observed increase in membrane lipid peroxidation in red cells of subjects with fluorosis
(Table 16) might have resulted in altered membrane lipid composition.

Hepatic microsomal lipid peroxidation was markedly increased in rabbits administered high doses of fluoride (10 and 100 ppm) for sixty days through drinking water (Soni et al., 1983). Chronic fluoride intoxication of rats also have been shown to result in enhanced lipid peroxidation in liver, brain and serum, a high fat diet causing further enhancement and a diet with protein or methionine and vit.E restoring to the original level (Antonyan, 1980).

While several aspects of mechanism of lipid peroxidation are unclear, iron appears to have an extensive role in the catalysis of lipid peroxidation (Aust et al., 1985). Oxygen free radicals are often proposed to be the causative agents of lipid peroxidation, with the hydroxyl radical (·OH) considered to be the ultimate initiator of lipid peroxidation. The ·OH can be formed by a series of reactions involving superoxide (O₂⁻), hydrogen peroxide (H₂O₂) and iron (Miller and Aust, 1989).

\[
\begin{align*}
O_2^- + Fe(III) & \rightarrow O_2 + Fe(II) \quad (1) \\
2O_2^- + 2H^+ & \rightarrow H_2O_2 + O_2 \quad (2) \\
Fe(II) + H_2O_2 & \rightarrow Fe(III) + OH^- + ·OH \quad (3)
\end{align*}
\]
Lipid peroxidation and membrane alterations, initiated by the generation of toxic free radicals either extramembranously (or) with in the membrane itself, may further add to the oxidation of hemoglobin and ferrihemochrome accumulation resulting in susceptibility of RBC to splenic sequestration (Itano et al., 1975, 1976, 1977).

Increased oxidation of glucose via HMP shunt leads to accumulation of NADPH in red cells of fluorotic patients (Table 27). It is well known that NADPH can promote membrane lipid peroxidation of RBC in the presence of cyt P-450 system of liver microsomes (Hochstein and Ernster, 1963; Pfeifer and McCay, 1971; Jain, 1989). Recent studies (Tomoda et al., 1977; Blisard and Mieyal, 1977; Starke et al., 1984) have shown that oxyhemoglobin in RBC can act like Cyt P-450 in the presence of NADPH. Studies of Jain (1989) have shown that in vitro treatment of RBC hemolysate with NADPH can result in the formation of phospholipid malonyldialdehyde (MDA) adduct and TBA reactivity.

Apart from the protective role of GSH and related thiols against lipid peroxidation (Christophersen, 1968, 1969; Little O'Brien, 1968; Burk et al., 1983; Kosower and kosower, 1983; Meister and Anderson, 1983) there are
reports to indicate that thiols can also act as cooxidants (Misra, 1974; Rowley and Halliwell, 1982; Tee et al., 1982). Thiols on autooxidation appear to produce $\text{O}_2$ and in the presence of iron, generate the $\cdot$\text{OH} through an iron-catalysed Heber-Weiss reaction as shown below.

\[
\begin{align*}
\text{RSH} + \text{Fe(III)} & \rightarrow \text{RS} + \text{Fe(II)} \quad (1) \\
\text{Fe(II)} + \text{O}_2 & \rightarrow \text{Fe(III)} + \text{O}_2^- \quad (2) \\
2\text{O}_2^- + 2\text{H}^+ & \rightarrow \text{H}_2\text{O}_2 + \text{O}_2 \quad (3) \\
\text{Fe(II)} + \text{H}_2\text{O}_2 & \rightarrow \text{Fe(III)} + \cdot\text{OH} + \cdot\text{OH} \quad (4)
\end{align*}
\]

The $\cdot$\text{OH} is a strong oxidant capable of initiating lipid peroxidation (Tien et al., 1982). Tien et al. (1982) from their study on microsomes showed that in the presence of high intracellular GSH concentration chelation of cellular iron plays a central role in control of in vivo lipid peroxidation. The increase in GSH concentration in the blood suggests that this mechanism might be responsible for the enhanced lipid peroxidation in the red cells of these patients.

It is suggested that methemoglobin may act as scavenger of reactive intermediates necessary for propagation of chain reactions involved in lipid peroxidation (Trotta et al., 1981). Membrane binding of methemoglobin
derived products was observed in membranes incubated with methemoglobin and t-butyl hydroperoxide (Trotta et al., 1981). The formation of methemoglobin can result either from autooxidation of hemoglobin or may be induced by several chemical agents. The proportion of methemoglobin in the total hemoglobin depends on the balance between methemoglobin formation and its reduction back to ferrous hemoglobin. In red cells exposed to oxidative stress, hemoglobin undergoes conversion to methemoglobin and a variety of degradation products (Peisach et al., 1985). In the red cells methemoglobin reduction occurs by both a NADH and NADPH reductase systems (Agar and Harley, 1972; Board et al., 1977; Huey and Beitnger, 1982; Nikinmaa, 1990). However, NADH dependent reduction system appears to be of importance in vivo in human erythrocytes (Beutler, 1975).

The increase in the activity of methemoglobin (NADH dependent) reductase (Table 32) in erythrocytes of fluorotic patients is suggestive of a part of adaptation necessary for the reduction of methemoglobin whose generation might have increased by autooxidation of hemoglobin in these RBCs. However, the inhibition of glycolysis (Table 25) in the erythrocytes of these
patients may not provide sufficient NADH for methemoglobin reduction.

Mammalian erythrocytes are equipped with both nonenzymatic and enzymatic scavenging systems to eliminate oxygen radicals. Antioxidant enzymes i.e. SOD, catalase and GSH-Px are essential to cells in removing $O_2^{-}$ and $H_2O_2$ from the tissues exposed to oxidative stress (Chance et al., 1979). Catalase activity was found to be increased in the erythrocytes of fluorotic patients in the present study (Table 18). Increased catalase activity has been reported under various metabolic and nutritional perturbances (Lee et al., 1981; Burr et al., 1987; Nilanjana et al., 1987; Ji et al., 1988). Catalase is known for its high specificity for substrate $H_2O_2$ (Chance et al., 1979) and linear increase in activity over a wide range of $H_2O_2$ concentration. The organs that possess high catalase content, maintain a low steady state concentration of $H_2O_2$. The increased production of $H_2O_2$ by erythrocytes of subjects suffering with fluorosis might have induced the catalase activity observed in the present investigation.

GSH-Px catalyses the reduction of $H_2O_2$ to $H_2O$ using GSH as the donor substrate (Mills, 1957). GSH-Px is also later found to act on a variety of organic hydro-
peroxides (Little and O'Brien, 1968). Studies with genetically acatalasemic, and cyanomethemoglobin erythrocytes showed the importance of GSH-Px than catalase in the dissipation of \( H_2O_2 \) (Jacob et al., 1965; Sweder Van Asbeck, 1985). Peroxide destroying enzymes such as catalase are not known to decompose lipid hydroperoxides (O'Brien, 1969). GSH-Px appears to be the major mechanism for intracellular decomposition of lipid hydroperoxides (Cohen and Hochstein, 1963; Christophersen, 1966; O'Brien and Little, 1969; Flohe, 1982). The crucial role of preventing membrane peroxidative damage induced by lipid peroxidation has been ascribed to GSH-Px. Parallel increase in GSH-Px activity with catalase in erythrocytes of fluorotic patients in this study (Tables 23 and 18) might be a form of adaptation on the part of the system to counteract the oxidative stress. However, these compensatory changes were not sufficient enough for complete protection against oxidative assault as evident by increased lipid peroxidation.

The erythrocyte membrane is composed of lipid bilayer that contains several integral proteins and a protein lattice that underlies the bilayer and is associated with it through protein-protein and protein-lipid interactions (Haest et al., 1978; Branton et
al., 1981; Williamson et al., 1982; Cohen, 1983; Marchesi, 1983; Bennett, 1985; Chasis and Shohet, 1987). This protein lattice is often referred to as the membrane skeleton; consisting of spectrin, actin, tropomyosin, and proteins 4.1 and 4.9 (Chasis and Shohet, 1987).

Dobretsov et al. (1977) have shown that lipid peroxidation results in an increased phospholipid bilayer rigidity. An increase in the high molecular weight proteins and predominance of components with molecular weights - 93 Kd and 20 Kd (Fig. 6), and in the polymerized products of lipid peroxidation in erythrocytes of fluorotic patients might be expected to alter the rigidity and the life span of affected cells (Dobretsov et al., 1977; Heusinkveld et al., 1977).

Amino acid residues potentially involved in cross-linking include histidine, tyrosine, tryptophan, methionine and cysteine, known to undergo photochemical- (or) free radical induced oxidation (Girotti et al., 1979; Koster and Slee, 1983). It seems reasonable to assume that the presence of polymers caused by crosslinking of membrane components subsequent to lipid peroxidation may be a feature which might contribute to the altered physical as well as
biochemical properties of erythrocytes of fluorotic patients.

Human erythrocytes are able to maintain the low sodium and high potassium concentrations in their cells as compared to those in plasma by the function of a membrane bound enzyme Na\textsuperscript{+}-K\textsuperscript{+} activated ATPase (Bernstein, 1954; Chan et al., 1964). The present study indicated that Na\textsuperscript{+}-K\textsuperscript{+}-ATPase activity in erythrocytes of fluorotic patients exhibited significantly low activity than that of controls (Table 34).

Fluoride has been shown to irreversibly inhibit the Na\textsuperscript{+}-K\textsuperscript{+}-ATPase (Yoshida et al., 1968; Robinson, 1975; Robinson et al., 1986). A decrease in renal Na\textsuperscript{+}-K\textsuperscript{+}-ATPase associated with decrease in serum sodium was observed in rats treated with acute toxic dose of fluoride, and angiotensin but not aldosterone effectively counteracted this effect (Suketa and Terui, 1980). The erythrocyte membranes of rabbits ingested with 10 mgNaF/kg body weight for six months were found to exhibit diminished Na\textsuperscript{+}-K\textsuperscript{+}-ATPase activity (Jain and Susheela, 1983). The peroxidation of membrane phospholipids and accumulation of MDA may cause inhibition of membrane Na\textsuperscript{+}-K\textsuperscript{+}-ATPase and the accumulation of intracellular sodium (Pfafferott et al., 1982).
Since 20 to 50% of total cellular ATP produced in erythrocytes is used by plasma membrane Na\(^+\)-K\(^+\)-ATPase (Whittam and Blond, 1965), a diminished availability of ATP due to inhibition of glycolysis in these erythrocytes (Table 25) might have resulted in decrease in erythrocyte membrane Na\(^+\)-K\(^+\)-ATPase activity (Table 34) in fluorotic patients.

Diminution in the activity of Mg\(^{2+}\) and Ca\(^{2+}\) ATPases in the erythrocytes of fluorotic patients (Table 34) is indicative of perturbations in the movement of Mg\(^{2+}\) and Ca\(^{2+}\) across the red cell membrane. Although many membrane components are possible targets for oxidants, Ca\(^{2+}\)-ATPase may be of crucial importance for the survival of red cells. Ca\(^{2+}\)-ATPase contain 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 the very steep gradient between external and intracellular calcium (Schatzmann, 1975) and collapse of this gradient is associated with decreased red cell deformability and premature destruction (Clark et al., 1981). Inhibition of this enzyme may lead to calcium accumulation which in turn, may result in abnormal membrane deformability. Luthra and Kim (1980) have
reported that low calcium produce a marked stimulation of Ca\(^{2+}\)-Mg\(^{2+}\)-ATPase activity of human erythrocytes, while moderate and high calcium concentrations produce progressive inhibition. It appears that the hydroperoxides formed within the cell may selectively inhibit certain enzymes.

Glutathione (GSH); reduced form of the tripeptide \(\gamma\)-glutamyl-cysteinyl-glycine, plays a key role in the detoxification of exogenous as well as endogenous toxicants. It provides protection to erythrocytes non-enzymatically (Awasthi et al., 1989) by acting as a free radical scavenger, a nucleophilic shield, and as a reductant. Enzymatically, GSH participates in detoxification of \(H_2O_2\) and lipid hydroperoxides through mediation of GSH-Px (Mills, 1957; Cohen and Hochstein, 1963) and of electrophilic xenobiotics through the reaction catalysed by GST (Booth et al., 1961; Jakoby, 1978; Awasthi and Singh, 1985).

The level of GSH is regulated by a complex process comprising precursor amino acid transport across cell membranes, intracellular synthesising enzymes, feedback regulation by product formation of intracellular complexes of GSH (Deneke and Fanburg, 1989). In addition another enzymatically catalysed redox mechanism
is available in the cell for rapidly reducing GSSG by GR activity (Deneke and Fanburg, 1989).

The present study has demonstrated a two fold increase in the level of blood GSH in fluorotic patients (Table 19). In elucidating the mechanism of this increase, the activities of glutathione metabolising enzymes in the erythrocytes namely \( \gamma \)-glutamyl-cysteine synthetase, \( \gamma \)-glutamyl transpeptidase, GST, GSH-Px and GR were determined and significant increase in the activities of all these enzymes were observed in the erythrocytes of fluorotic patients (Tables 20-24).

Reports show the correlation between \( \gamma \)-glutamyl-cystein synthetase activity and cellular levels of GSH (Meister and Tate, 1976; Deneke and Fanburg, 1989; Ogino et al., 1989). In some sheep, in which erythrocyte glutathione is genetically lower than control animals, \( \gamma \)-glutamyl-cysteine synthetase exhibits lower activity (Board et al., 1980). Administration of selenium to rats also increase the enzyme activity, with an increase in the hepatic glutathione concentration (Chung and Maines, 1981; Hill and Burk, 1982). This enzyme is inhibited nonallosterically by GSH (Richman and Meister, 1975; Wirth and Thorgeirsson, 1978; Maede, et al., 1982; Meister and Anderson, 1983). Thus the elevation of
γ-glutamyl-cysteine synthetase (Table 20) may stimulate GSH synthesis and in turn the level of GSH in red cells of fluorotic patients.

Primary regulation of precursor availability for GSH synthesis appear to be exercised by two amino acid transport systems, a sodium-independent one that transport cysteine and glutamate and a sodium-dependent system that transports cystine. Transpeptidase activity at the cell surface is responsible for salvaging amino acids for intracellular synthesis of GSH (Deneke and Fanburg, 1989).

The oxidation of GSH by GSH-Px coupled with reduction of GSSG through GR is probably of importance in determining the redox state of glutathione. The GSH/GSSG concentration ratio must be a function of the activities of both these enzymes. As the activities of both these enzymes are high in the erythrocytes of fluorotic patients, it is probable that these enzymes and their activity ratio, are most important for deciding the GSH/GSSG ratio, which in turn influences the thiol/disulphide concentration ratio of other compounds dependent on the redox steady state of the glutathione.
Other features of the coupled oxidation reduction of glutathione through these two enzyme system is the oxidation of NADPH. As glutathione is synthesised in the reduced form the rate of NADPH oxidation by this pathway depends on the rate of GSSG formation, which is probably a function of hydroperoxide concentration (Pinto and Bartley, 1969).

In unstressed situation, 5 to 10% of the total glucose consumption of human red cells is channeled via HMP shunt (Gaetan et al., 1974; Beutler, 1975). The flux is limited by the first enzyme of the pathway G-6-PDH, which, owing to inhibition by high NADPH/NADP ratio, operates at less than 1% of its capacity (Thorburn and Kuchel, 1985). However, under conditions of increased NADPH utilization, the NADPH/NADP ratio decreases and the inhibition of G-6-PDH is released. Such a situation appear to have arisen in erythrocytes of fluorotic patients due to enhanced requirement of NADPH for reduction of GSH. This might have resulted in the observed increase in oxidation of glucose through HMP shunt (Table 27) and increase in G-6-PDH activity (Table 31) in the erythrocytes of fluorotic patients.

Fluoride is known classically to shut down glycolysis by inhibiting enolase in vitro (Warburg and
Christian, 1941; Shearer and Suttie 1970; Messer, 1974; Repaske and Suttie, 1979; Shahed et al., 1979).

Similar inhibition of glycolysis in erythrocytes by fluoride in vitro was observed conforming the earlier reports (Table 26). However, to our knowledge the effect of chronic fluoride toxicity on glycolysis in erythrocytes of human beings has not been carried out. In the present study, there was nearly 70% inhibition of glucose metabolism through glycolysis (Table 25) and diminished activities of PK and LDH (Table 29,30) of erythrocytes of fluorotic patients, suggesting similar situation prevailing under in vivo conditions. However, caution must be exercised in extrapolating the in vitro effects of fluoride to in vivo conditions. The in vivo inhibition of glycolysis suggest that fluoride can inhibit enzymes other than enolase. In rat erythrocytes, Ca$^{2+}$-activated protease is implicated in the regulation of PK (Dahlquist Edberg and Ekman, 1981).

Changes in human erythrocyte glycolytic intermediates have been demonstrated after prolonged in vivo exposure to oxygen under high pressure that suggested inhibition of sulphahydril bearing enzyme glyceraldehyde 3-phosphate dehydrogenase (Mengel et al., 1964; Mengel and Kann, 1966). It is to be asserted that the changes in glycolysis observed in the present study in the erythro-
cytes of fluorotic patients is primarily due to the toxic effects of fluoride (or) to altered lipid peroxidation.

Enzymes like enolase and succinate dehydrogenase requiring divalent cations as cofactors are inhibited by fluoride and the inhibition is enhanced by inorganic phosphate (Wiseman, 1970; Nowak and Maurer, 1981). An enzyme may be inhibited by fluoride either directly (Slater and Bonner, 1952) or through a change in the concentration of such divalent cations as Ca, Mg, Mn etc. in the medium (Colowick and Kaplan, 1955). Results concerning the effect of fluoride on the metabolism of these ions in animals are inconclusive. Leone et al. (1956) found that large dose of NaF given intravenously to dogs slightly reduced the calcium level in blood. Simpson et al. (1980) reported severe hypocalcemia in a patient following ingestion of fatal dose of fluoride. The similarity of fluoride ion to hydroxyl ion (OH) in terms of ionic radius and primary hydration number led Nowak and Maurer (1981) to suggest that F serves as a possible analog of OH⁻ group involved in the gain or loss of water as part of normal reaction mechanism. Inhibition of enolase occurs by the formation of a tightly bound enzyme-metal-F⁻-Pi complex in which the F interacts directly with the metal ion in the active site
and indirectly with the phosphate binding site (Nowak and Maurer, 1981).

The data observed from the present study focussed the toxic effects of chronic consumption of fluoride by human beings on red cell metabolism. The altered red cell membrane lipid and protein composition, lipid peroxidation and ATPase are suggestive of functional impairment of red cell membrane by chronic fluoride toxicity. The rise in GSH status and glutathione metabolising enzymes indicates a form of adaptation on the part of the system to counteract the oxidative stress in the erythrocytes of fluorotic patients. However, the protection provided by GSH, and the scavenging enzymes, viz. GSH-Px and catalase appear to be insufficient to counteract the cellular damage as this protection system is unable to prevent increased lipid peroxidation under this toxic condition.

The inhibition of glycolysis is probably related either to direct effect of fluoride or due to altered enzyme composition or both or lipid peroxidation in red cells of fluorotic patients.

Chronic fluoride ingestion leads to accumulation of fluoride in bone (Neuman and Neuman, 1958; US NAS,
1971; WHO, 1984) an erythropoietic tissue. Membrane damage is most likely to occur in early red cell precursors like reticulocytes, late normoblasts in the bone marrow and erythrocytes which may result in hemolysis and decreased life span of erythrocytes in these patients. The present study highlights the deleterious effects of chronic consumption of toxic dose of fluoride on human erythrocytes.